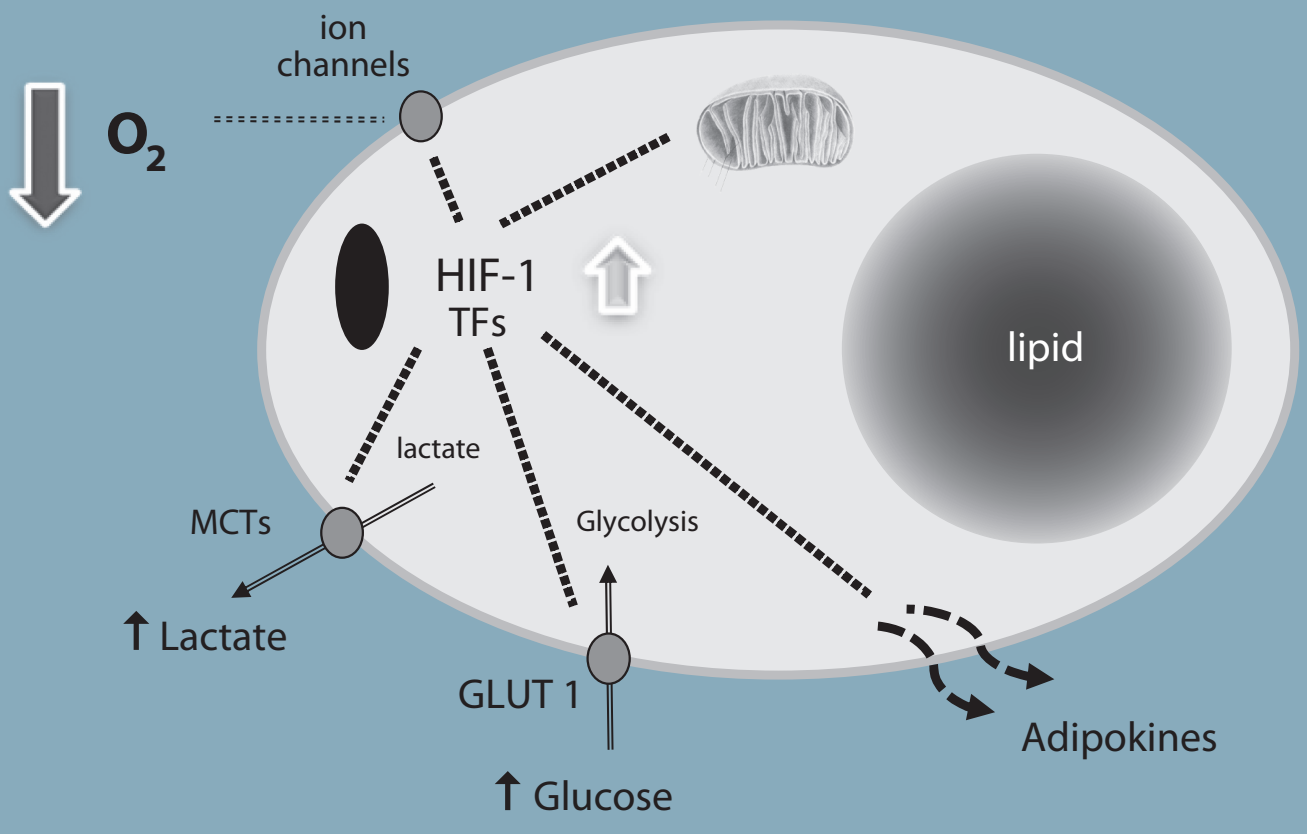
ADIPOBIOLOGY

An International Journal of Adipose Tissue in Health and Disease

Volume 1, 2009



Official Journal of the Bulgarian Society for Cell Biology

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Scope and Purpose

Adipobiology (ISSN 1313-3705) is an official journal of the Bulgarian Society for Cell Biology. The *Journal* is published annually, and includes Reviews, Research Articles, and Dance Round (a form of short, position papers) focused on health- and disease-oriented adipobiology, presented in concise form.

Editorial Policy

Contributors to *Adipobiology* are, in general, invited by the Editors, but idea proposals for Reviews, Research Articles and Dance Round are welcome. Prospective authors should send a brief summary, citing key references, including their own, to the Editors or a member of the Editorial Board. Submission of full-length articles without prior consultation is not prefered. Manuscripts are peer-reviewed by the Editors, Editorial Board members, and/or external experts before final decisions regarding publication are made. All material in *Adipobiology* represents the opinions of the authors and does not reflect opinions of the Bulgarian Society for Cell Biology, the Editors, the Editorial Board, or the institutions with which the authors are affiliated.

Publication of *Adipobiology* is truly a collaborative process. We appreciate the brain-and-heart partnership having with our authors and are committed to further maintaining the excellence of the *Journal*.

Contents

Front cover: Summary of the effects of hypoxia on adipocyte metabolism. GLUT1, factilitative glucose transporter-1; HIF-1, hypoxia-inducible factor 1; MCTs, monocarboxylate transporters; TFs, transcription factors. From *Cellular hypoxia: a key modulator of adipocyte function in obesity?* by Trayhurn *et al* (pages 19-26).

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EDITOR'S INAUGURAL ADDRESS

We have to meet and work together,
and together to believe - cry out, fall down.
Because it was we who suffered for the magic of the greeting.
The great significance of the plain shaking hands.

Hristo Photev (1934-2002)
From *Lithurgy for the dolphins*

Dear Colleagues,

We are pleased to be launching a new international journal, *Adipobiology*, an official journal of the Bulgarian Society for Cell Biology. The Bulgarian Society for Cell Biology aims at promoting, at the national level, the advances of science, research and teaching in the field of cell biology. Doing that in collaboration with other national and international cell biology organizations is our desire. We hope that creative interactions between such a collaboration and our cultural, intellectual and emotional imprints may infiltrate new ideas into adipobiology.

At the evolutionary level, the survival of biological species is mediated by growth, fertility and longevity phenotypes. In other words, humans need food, wine and love to survive. Today, *Homo obesus* (man the obese), like Diogenes (c. 403-323 BC), is increasingly saying *I am a citizen of the world*. Both in relation to human suffering and in financial terms, the costs associated with obesity and its related diseases are enormous. Data from the World Health Organization show that 20 percent of European children are now overweight and that their number increases by 400,000 a year.

In February 2008, Dr Steven Feinstein from Rush University Medical Center, Chicago, IL, USA wrote in the *Preface* of his book *Non-invasive surrogate markers of atherosclerosis* that: "An aging, overweight, sedentary baby boomer population is under siege. Approximately 58 million people worldwide die from cardiovascular diseases each year, nearly 1.2 million from heart attacks and 700,000 from strokes in the United States alone." To these sad numbers, we should disappointingly add nearly 70,000 Bulgarians who die from stroke and myocardial infarction each year in a country with a population of less than 8 million people, as was also signalled in our paper *Homo obesus Bulgaricus* published in volume 6, 2007 of *CV Network Online*, an official forum of the International Academy of Cardiovascular Sciences.

Although the birth of adipoendocrinology may be traced to the 1980s (the identification of lipoprotein lipase and adipsin), the paradigm-shifting discovery of leptin in 1994 was a trigger for further studies on the endocrine and paracrine nature of adipose tissue. Hence *the life of fats* (cf.1), expressing both high IQ (2) and EQ (3), became a fascinating research and clinical challenge.

The present inaugural issue of *Adipobiology* is a product of *work together* of contributors, editors and peer-reviewers highlighting current aspects of adipobiology. The *Journal* is intended to serve as a valuable reference and educational tool, and to cultivate adipocentric thinking about how we can make adipokines and other adipose-derived factors work for the benefits of patients. Sharing the importance of *the joy of doing science* (4), the Editors and the Members of the Editorial Board hope that the reviews, results and hypotheses presented here will foster the interaction between scientists and clinicians. And that they will convey to the reader some of the excitement that ensues from the current progress in adipobiology – both that relating to white adipose tissue and following the recent 'rediscovery' of brown fat (5).

In our selection of authors, we have always been pursuing those brain-and-heart scientists (6) who can contribute state-of-the-science reviews, research articles and hypotheses to the *Journal*. Because we highly value our readers, we would encourage them to give us their feedback. Any ideas that might help make *Adipobiology* even more useful and interesting would be greatly appreciated. The *Journal* will continue the efforts to keep readers' scientific fire for learning and curiosity alive and up-dated – *the great significance of the plain* opening pages.

1. Pond CM. *The Fats of Life.* Cambridge University Press, Cambridge. 1998.
2. Chaldakov GN, Fiore M, Tonchev AB, Hristova MG, Nikolova V, Aloe L. Tissue with high IQ: adipose-derived stem cells in neural regeneration. *Neural Regen Res* 2009; in print.
3. Anghel SI, Wahli W. Fat poetry: a kingdom for PPAR gamma. *Cell Res* 2007; in print.
4. "The joy of doing science and developing scientific understanding greatly exceeds the importance of being first, or even of always being right." In: Anfinsen *et al. Trends Biochem Sci* 1993; 18: 364-365.
5. Frühbeck G, Becerril S, Sáinz N, Garrastachu P, García-Velloso MJ. BAT: a new target for human obesity? *Trends Pharmacol Sci* 2009; 30:387-396.
6. It has been said that "some scientists would rather exchange each other's toothbrushes than their hypotheses and results". We are keeping at a distance from such "toothbrush" scholars, indeed. Because among many –omics sciences we prefer the friendomics.

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HER EXCELLENCY THE CENTENNIAL LIFE

The 100th anniversary of the Nobel laureate Rita Levi-Montalcini

Chou-ju (in Japanese, The longevity is a good thing), a calligraph by Professor Hiroshi Yamamoto, Dean of the Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University, Kanazawa, Japan.

1986, Stockholm. Rita Levi-Montalcini receiving the Nobel prize from King Carl XVI Gustaf of Sweden.

2009, Rome, NGF Symposium. Luigi Aloe (left), Rita Levi-Montalcini and George Chaldakov (right).

The Turin-born Rita Levi-Montalcini won the 1986 Nobel Prize for Medicine for discovering the nerve growth factor (NGF) (photograph left up), a signaling protein that stimulates growth and survival of nerve cells (1-3). Moreover NGF represents the first cell growth factor discovered, thus creating the growth factor paradigm in cell biology.

Recently it was also revealed that NGF is a multipotent molecule that exerts various effects on a large scale of non-neuronal cells as well as cellular functions, thus implicated for the pathogenesis and therapy of not only neurodegenerative diseases, but also cardiometabolic diseases and skin and corneal ulcers (4-10).

Italia celebrated Rita Levi-Montalcini Days (21-23 April 2009), her hundredth birthday being on 22 April 2009. Symbolically, on 21 April 753 BC, Rome, *Citta Eterna*, was founded, that is, 2 762 years ago.

On 21 April 2009 in Rome, Dr Luigi Aloe, the long-standing coworker of Levi-Montalcini, organized an International Symposium held in the Institute of Neurobiology and Molecular Medicine, National Research Council. Invited scientists from many countries including Bulgaria highlighted the recent advance of the NGF's saga that was initiated in the 1950's by Rita Levi-Montalcini at the Washington University in St Louis, MO, USA.

Turning 100 years of age, Rita Levi-Montalcini, who is also serving as a Senator-for-life in Italy, is continuing to work at the Institute of Neurobiology and Molecular Medicine and in the European Brain Research Institute, both placed in Rome, and supported by the Foundation of Rita Levi-Montalcini. In effect, "Her centennial life creates the scientific bridge between two centuries and between two millenia, the pathway followed by many generations", written on the Diploma of the Most Honored Member of the Bulgarian Society for Cell Biology, she received after the lecture of Bulgarian scientist (photograph left down).

"I will present my ongoing results at the next symposium" – Rita Levi-Montalcini said to Dr Luigi Aloe when he informed her about the organization of the Roma-2009 Symposium. Such a positive thinking and feeling may indeed be an important clue for creative longevity. Because *the longevity is a good thing* (see the Caligraphy).

George N. Chaldakov
Editor, *Adipobiology*

1. Levi-Montalcini R. The nerve growth factors 35 years later. *Science* 1987; 237: 1154-1162.
2. Levi-Montalcini R. The saga of the nerve growth factor. *Neuroreport* 1998; 9: R71-83.
3. Levi-Montalcini R. From Turin to Stockholm via St. Louis and Rio de Janeiro. *Science* 2000; 287: 809.
4. Aloe L, Chaldakov GN, editors. Nerve Growth Factor in Health and Disease. *Biomed Rev* 1999; 10: 1-125.
5. Chaldakov GN. The NGF is wider than the neuron. *Arch Ital Biol* 2003; 141: 89-92.
6. Aloe L, Calzá L, editors. NGF and Related Molecules in Health and Disease. *Prog Brain Res* 2004; 146: 1-357.
7. Chaldakov GN, Fiore M, Stankulov IS, Manni L, Hristova MG, Antonelli A, Ghenev PI, Aloe L. Neurotrophin presence in human coronary atherosclerosis and metabolic syndrome: a role for NGF and BDNF in cardivascualr disease? *ProgrBrain Res* 2004; 146: 279-289.
8. Lazarovici P, Marcinkiewicz C, Lelkes PI. Cross talk between the cardiovascular and nervous systems: neurotrophic effects of vascular endothelial growth factor (VEGF) and angiogenic effects of nerve growth factor (NGF) – implications in drug development. *Curr Pharm Des* 2006; 12: 2609-2622.
9. Chaldakov GN, Tonchev AB, Aloe L. NGF and BDNF: from nerves to adipose tissue, from neurokines to metabokines. *Rivista Psichiatr* 2009; 44: 79-87.
10. Chaldakov GN, Tonchev AB, Fiore M, Hristova MG, Pancheva R, Rancic G, Aloe L. Implications for the future of obesity management. In: G Frühbeck, editor. *Peptides in Energy Balance and Obesity*. CAB International 2009; 369-389.

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REVIEW

NOVEL ADIPOCYTE FEATURES DISCOVERED BY ADIPOPROTEOMICS

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Abstract

Obesity and its associated complications will be the most important near-future medical burden in Western-type societies. One hallmark of obesity is the differentiation of preadipocytes into mature fat-loaded adipocytes present in subcutaneous and visceral fat depots. Furthermore, (pre)adipocytes secrete proteins, known as adipokines, with changing profiles during fat accumulation. Adipocytes serve in important function with respect to energy homeostasis, body insulation and organ protection. Adipocyte dysfunction results in the initiation and progression of obesity-associated disorders. Obviously, knowledge of the adipocyte behaviour under different nutritional conditions and the cross-talk of adipocytes with other cells and organs are key issues to develop proper intervention strategies. A full understanding of the adipocyte behaviour requires a systems biology approach with integrated transcriptomics, proteomics and metabolomics data. This review focuses on the contribution of proteomics research in adipocyte biology. Proteome studies on adipocytes exist for almost 30 years but are boosted in the last decade with the enormous technological developments in mass spectrometry technology. The relevance of proteomics technologies in understanding molecular aspects of adipocyte biology is discussed. Recent novel findings and particularly the identification of novel adipokines are highlighted.

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Key words: adipobiology, adipokines, proteomics, 3T3-L1, human models

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Introduction

Adipocytes play an essential role in normal physiology with respect to energy balance, glucose homeostasis, heat regulation and organ protection. Furthermore, adipocytes are involved in the immune response, blood pressure control, haemostasis, bone mass, and thyroid and reproductive function (1,2). A deregulation of the adipocyte function results in metabolic dysfunction of the body and the development of obesity-associated diseases like type 2 diabetes, non-alcoholic steatohepatitis, cardiovascular diseases and cancer (3,4). With the expected increase in the global overweight population (WHO, Fact sheet on obesity and overweight, September 2006, http://www.who.int/topics/obesity/en/), the number of people that suffer from obesity-associated diseases will consequently rise too (5-7). Understanding the molecular events leading to adipocyte dysfunction and the related metabolic complications is thus required to provide targets for appropriate intervention to reduce the upcoming metabolic disease burden. During the last decades genomics technologies provided further insights regarding the molecular events underlying the development of obesity and

related diseases.

Here the contribution of proteomics research with respect to adipocyte biology is highlighted. It is beyond the scope of this review to describe each adipocyte protein that has been identified instead novel biological features of adipocytes discovered by proteomics are discussed.

The proteome: more than just the protein complement of the genome

The molecular events during preadipocyte differentiation have been examined in a broader sense by several large-scale gene analysis studies (8-10). Although transcriptomics data provide important information about transcription rates and mRNA turnover, knowledge about protein expression, post-translational modifications and protein turnover, essential for cellular physiology, cannot be covered by this technology. In addition, the correlation between mRNA expression levels and the expression levels of corresponding protein products is low in mammalian systems (11). For instance, we previously showed that insulin stimulated protein secretion from murine 3T3-L1 adipocytes without increasing the mRNA levels of the corresponding genes (12). Together, additional monitoring of the cellular protein complement, the proteome, is required to further understand the adipocyte behaviour. The importance of this issue is reflected by the overwhelming amount of different proteins that can be produced by a single genome due to alternative splicing and post-translational modifications. In contrast to the genome, the proteome is highly dynamic that differs in time and in response to external factors (13). Furthermore, expression and function of many proteins are modified through interactions with other proteins such as kinases and proteases. In fact, most biological systems are controlled by complex protein-protein interaction networks (14). Consequently, detailed proteome studies may provide additional valuable information about the (patho)physiology of an organism. The dynamic nature of the proteome can be a pitfall for reproducible results, as such, proteome studies require careful experimentation under well-defined conditions.

Proteome analysis strategies

It is currently not possible to analyse the total proteome of an organism at once. This is due to (*i*) extreme complexity of the proteome, (*ii*) the dynamic nature of the proteome, (*iii*) limitations of the analysis techniques to cover the orders of magnitude difference in protein expression levels, and (*iv*) the differences in physical properties of the proteins. Three main strategies that are currently applied in large-scale proteome research are (*i)* gel-based protein separation, either by 1- or 2-dimensional electrophoresis (2-DE), combined with mass spectrometry (15,16), (*ii)* liquid chromatography coupled to tandem mass spectrometry (LC-MSMS) (17,18), and (*iii)* antibody array technology (19,20), see Figure 1.

(Dis)advantages of current proteomics strategies

The 2-DE method is a high-resolution technology enabling the simultaneous visualisation of many different proteins, including splice variants and proteins bearing post-translational modifications (16,21). However, the sensitivity of the 2-DE technique is restricted to relatively high abundant proteins leaving large parts of the proteome obscure. Furthermore, the reproducibility of the 2-DE method heavily depends on experimental conditions. With the introduction of the differential gel electrophoresis (DIGE) technology qualitative and quantitative comparison of two different samples on a single gel became possible by prelabelling of the protein samples with different CyDyes (22,23). This technology is an improvement compared to traditional 2-DE regarding reproducibility, quantitative proteome analyses and statistical confidence of differential protein expression. Still, hydrophobic (membrane) proteins, proteins with low (< 5 kD) or high (> 250 kD) molecular masses and extremely acidic (pI < 3) and basic (pI > 11) proteins are difficult to resolve with 2-DE. Furthermore, many proteins identified among several different studies using 2-DE combined with Maldi-TOF MS appeared to be generally stress-related proteins (24). Considering these proteins as biomarkers for cellular processes other than a stress response should be done with great care. Additional validation studies are required to confirm their specificity.

The LC-MSMS approach is not hampered by the size of proteins and their hydrophobic properties. In combination with stable isotope labelling such as iTRAQ and SILAC LC-MSMS is highly suitable for accurate quantitative proteomics (17,25). Compared to 2-DE, LC-MSMS is a more sensitive method able to cover larger ranges of the proteome and it can be run in an automated fashion (26-28). However, in contrast to 2-DE, LC-MSMS cannot discriminate proteins and their splice variants as the identified peptides are assigned to the same protein. Reliable analysis of post-translational modifications, particularly phosphorylation and glycosylation, requires additional sample preparation due to similar reasons. Furthermore, the tremendous amount of data generated by current MSMS instruments has become a bottleneck in analysis time and requires extensive bioinformatics tools. Despite these limitations, the LC-MSMS technology is currently the most popular approach applied in proteome research.

The antibody array technology is a sensitive and reproducible method to quantitatively measure protein abundances in complex samples. It requires little amounts of sample and allows

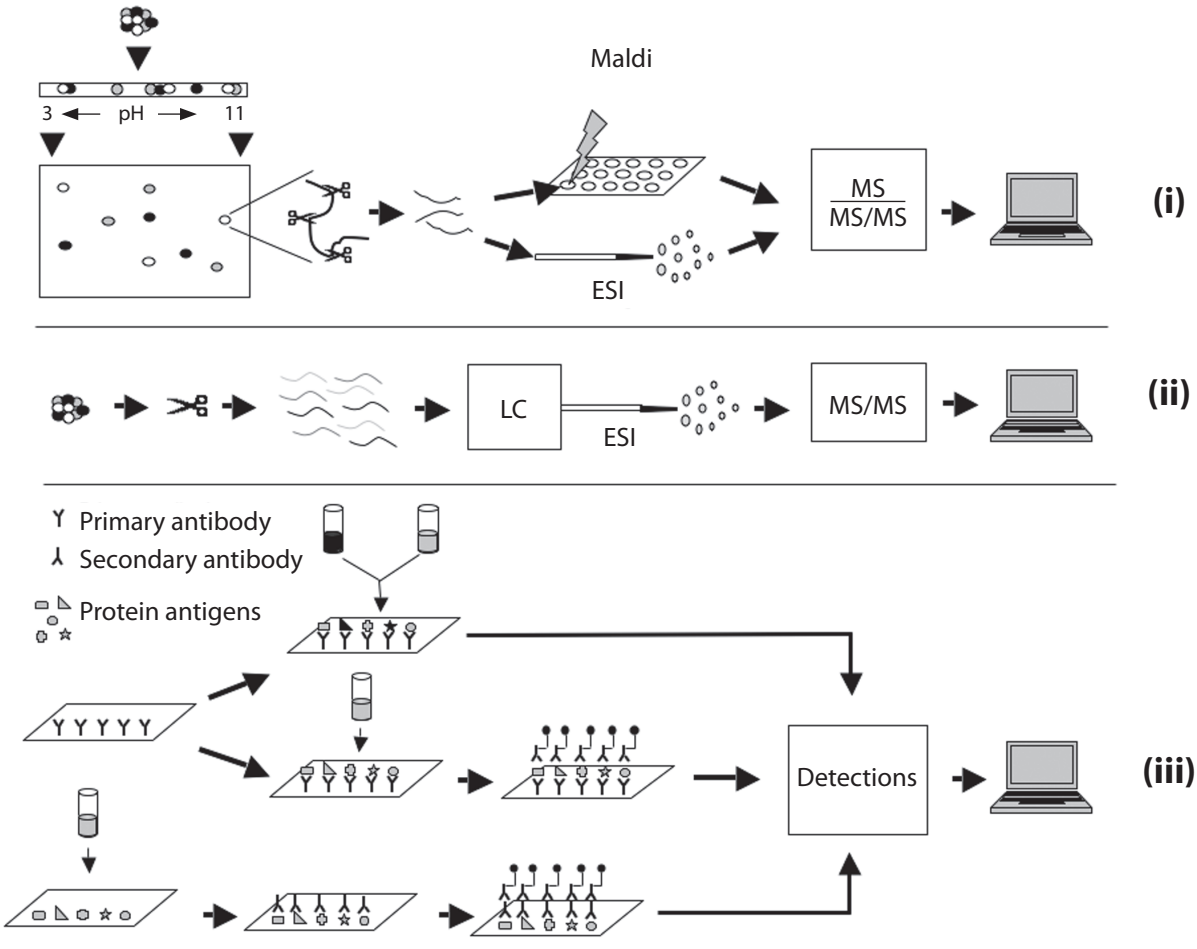

Figure 1. Current strategies in large-scale proteomics studies.

(i) *Proteomics by 2-DE combined with mass spectrometry.* With 2-DE a protein sample is first separated by iso-electric focussing on an immobilized pH gradient (max. pH 3-11) according to the iso-electric points of the proteins. Subsequent separation is based on the molecular mass of the proteins by using sodium dodecyl sulphate polyacrylamide gel electrophoresis. Visualization of the protein spots occurs by staining the gel with silver nitrate, colloidal stains like Coomassie Brilliant Blue or fluorescent dyes. Gels containing different samples are compared by imaging software and differentially expressed protein spots are excised. For identification, proteins are digested (usually by trypsin) and dependent on the type of instrument the peptides can directly be introduced into the mass spectrometer by electrospray ionisation (ESI) or spotted on a target plate for matrix assisted laser desorption ionisation (MALDI) mass spectrometry (see ref. 15 and 16 for details). With the DIGE technology a similar strategy is applied.

(ii) *LC-MSMS-mediated proteomics.* In contrast to the gel-based approach, this procedure starts with digestion of the protein sample. Peptides are subsequently separated by liquid chromatography (LC). Separated peptides are directly introduced into the mass spectrometer by electrospray ionisation (ESI). An alternative to this on-line LC-MSMS approach is an off-line method where the separated peptides are automatically spotted on a Maldi target plate for subsequent MSMS- identification.

(iii) *Antibody array technology.* Three formats of this technology are displayed (see ref. 19 and 20 for details). A commonly used method is pre-labelling of the proteins samples, which are subsequently mixed and applied to glass slides pre-spotted with a set of available antibodies. Detection of the captured fluorescent antigens occurs by laser scanning. Second, unlabelled protein samples can be applied to similar antibody slides after which the captured antigens are detected by a cocktail of labelled secondary antibodies matched to the pre-spotted ones. The third method involves spotting of the protein sample antigens on the slide. Subsequent detection occurs by labelled read-out antibodies.

also detection of post-translational modifications (20). However, current arrays are restricted by the availability of antibodies and cross-reactivity of these. Thus, in contrast to 2-DE and LC-MSMS one can only screen for a selected known part of the proteome and as such the present arrays are less useful as discovery tool. However, with the endorsement of the Human Proteome Organisation (HUPO) antibody program in 2004 (29) together with the ability to generate and validate thousands of antibodies per year, it is anticipated that within the near future antibodies will be available for proteins coded by each human gene locus (30,31). Still, the antibody array is limited in detection of proteins that require harsh solubilisation conditions, such as urea and SDS containing buffers, as these can be detrimental for the antibodies.

Next to common proteins captured by all three technologies, each approach allows identification of specific parts of the proteome. Thus, none of them can currently be marked as "golden standard". Due to the complexity of the proteome it is not expected that one total proteome-covering strategy will emerge in the near future. Therefore, researchers should carefully consider their research interest and choose the proper method(s) to investigate their research questions. As such, different proteomics strategies have been applied with respect to adipocyte biology.

Initial proteome studies on adipocyte biology

Already in 1979 a 2-DE proteome study was conducted on 3T3-L1 differentiation (32). Changes in the biosynthesis of 30 cytoplasmic, 9 non-histone chromosome-associated proteins and 24 membrane proteins were found. Unfortunately, only 1 protein could be tentatively identified as actin. Despite that none of the proteins was sequenced, remaining the identity of the proteins unknown, this study demonstrated for the first time that synthesis of several proteins was altered during 3T3-L1 differentiation. Subsequent studies confirmed these results by showing increased [^{3}H]-leucine incorporation into cellular protein extracts of differentiating 3T3-L1 cells (33) and an increased biosynthesis of glycolytic enzymes in 3T3-F442 cells (34). However, large-scale proteomics studies, including detailed protein identification, on adipocyte biology could only be conducted after the implementation of mass spectrometry in protein research. It lasted until 2001 that the first 2D-maps with identified proteins of mouse adipose tissue were published (35,36).

Proteomics on mouse adipocytes

Initial proteome studies were conducted on mouse 3T3-L1 cells to discriminate and identify differentially expressed proteins during adipocyte differentiation (37-39). Many proteins were identified that had not been described before in 3T3-L1 cells but, dependent on the experimental conditions, each study revealed a different set of proteins next to commonly identified proteins. This demonstrated the power of proteomics studies but at the same time the sensitivity of the 3T3-L1 cellular proteome for different experimental conditions and the reproducibility limitation of the used 2-DE method. However, a commonly observed effect was the alteration of proteins involved in cytoskeletal re-arrangements, which is an important event during 3T3-L1 differentiation (40-42). Furthermore, a proper functioning and remodelling of the cytoskeletal network is required for insulin signalling and GLUT4 translocation in 3T3-L1 adipocytes (43-47). For instance, the actin-based motor protein myosin 5a is a critical factor for anterograde movement of GLUT4 vesicles along the actin network in 3T3-L1 cells upon insulin-mediated stimulation (43).

Next to cytoskeletal arrangements, proteolytic events play a critical role in the initiation of preadipocyte differentiation (48-50). One proteomics study revealed the protease inhibitor α-2-macroglobulin to be involved in adipocyte differentiation (38). Accumulation of intracellular α-2-macroglobulin blocks differentiation while depletion of α-2-macroglobulin by polyclonal antibodies again induced a spontaneous differentiation of 3T3-L1 cells. Similar results were found with freshly isolated mouse preadipocytes. Remarkably, the α-2-macroglobulin protein found in this study was not mouse-derived. Apparently this protein was taken up from the culture medium that was supplemented with bovine serum. α-2-macroglobulin, as protease inhibitor, might regulate this process by proteolysis suppression. Probably, preadipocytes take up this protein to inhibit spontaneous differentiation and upon inactivation of α-2-macropglobulin differentiation is initiated.

Our group investigated proteome changes during 3T3-L1 differentiation followed by starvation (39). We identified four categories of proteins: metabolic enzymes, growth regulatory proteins, proteins with functions in cytoskeleton re-arrangements and protein modifiers. Compared to 3T3-L1 differentiation we observed a non-reciprocal regulation of the glycolytic pathway when mature 3T3-L1adipocytes were subjected to caloric restriction. Several other proteins showed a similar behaviour. This indicates that although caloric restriction induces fat release, it results only in a limited pre-adipocyte protein expression pattern. Apparently, once differentiated, adipocytes do not completely return to their preadipocyte status upon caloric restriction. However, when 3T3-L1 adipocytes were subjected to caloric restriction in the presence with TNF-α, the protein profile closely resembled the preadipocyte expression pattern. Stimulation of PPARγ inhibits TNF-α effects on mature 3T3-L1 cells (51). Thus, we suggested that TNF-α, in addition to caloric

restriction, stimulates mature 3T3-L1 cells to return further to their preadipocyte status by a forced down-regulation of PPARγ.

Organelle proteomics

Several proteomics studies have been conducted on specific cellular fractions isolated from 3T3-L1 cells, (52,53), mice (54,55) and humans (56). Adipocytes store the triglycerides in lipid droplets but the protein composition of adipocyte lipid droplets remained elusive until identified by mass spectrometry (53). Next to proteins also present on lipid droplets of other mammalian systems such as lipid metabolism enzymes and vesicular traffic controlling proteins, a number of adipocyte-specific ones were determined. Several of these were only identified from basal adipocytes such as 17-β-hydroxysteroid dehydrogenase type 7 and CGI-49, and others specifically under lipolytic conditions such as adipophilin, caveolin-1, tubulin and lipid-metabolizing enzymes. Presence of some of these proteins on lipid droplets was confirmed by immunofluorescence (53).

Recently, the nuclear proteome of 3T3-L1 cells was investigated during 5 consecutive days of differentiation using a quantitative five-plex SILAC LC-MSMS method (57). With this novel approach differentially expressed proteins could be identified in a temporal fashion. In the nuclear fraction a total of 581 proteins were identified. Among these were e.g. the T-cell transcriptional regulator THO complex 4 that showed highest expression during mid-stage of differentiation and the chromatin modulator SNF2 α that was down-regulated during the start of differentiation and remained at low expression level during the further differentiation. With this novel proteomics approach, this study contributed to a broader understanding of the transcriptional regulation of adipocyte differentiation.

With respect to other organelles, mitochondrial biogenesis and remodelling during 3T3-L1 differentiation was discovered as a novel property of adipocytes (52). When the same experiment was repeated in the presence of the insulin sensitizing thiazolidinedione (TZD) drug rosiglitazone, again mitochondrial biosynthesis was observed. This was accompanied by increased expression of several mitochondrial proteins involved in fatty acid oxidation in the 3T3-L1 cells (52) as well as in the *ob/ob* mice (54). The correlation between the expression levels of the identified proteins and their corresponding mRNA's was low, which implied a regulation of the mitochondrial proteins at the post-translational level.

Effects of TZD's on the 3T3-L1 proteome

The insulin sensitizers TZD's require adipose tissue for their therapeutic actions (58-60) although they stimulate fat cell differentiation, a known risk factor for insulin resistance. To investigate this paradoxical issue we examined the effect of two TZD's (pioglitazone and rosiglitazone) on the proteome of crude cell lysates of 3T3-L1 cells. We observed a pioglitazone- and rosiglitazone-mediated triglyceride accumulation during 3T3-L1 differentiation and induction of proteins involved in intracellular fatty acid transport, glycerol-3-phosphate synthesis and gluconeogenesis from non-carbohydrate substrates. At the same time, both TZD's induced tricaboxylic acid cycle proteins, the complete fatty acid beta-oxidation pathway and oxidative phosphorylation proteins. Thus, concomitant with increased triglyceride storage the TZD's also increased fatty acid catabolism (61). In a parallel study the effect of rosiglitazone on mature 3T3-L1 adipocytes was investigated with a combined transcriptomics and proteomics study (62). Decreased lipid contents were observed together with a mRNA and protein expression pattern that indicated a switch in metabolism towards lipid catabolism. Furthermore, rosiglitazone reduced adipokine expression both on mRNA and protein level, except for the insulin sensitizer adiponectin and apolipoprotein E (ApoE). Together, these proteomics results added new information to support the view that TZD's exert their therapeutic effects through the fatty acid storage and catabolism capacity of the adipose tissue (63) and through a change in the adipokine profile.

Proteomics on human adipocytes

Despite its value for obesity research the 3T3-L1 model is hampered by its murine nature. A comparison of studies conducted on murine and human adipocytes increasingly showed differences between the two model systems regarding adipogenesis and adipokine secretion (64-67). The value of mouse models to recapitulate human obesity is thus questionable. Detailed comparative proteome studies may reveal how well the mouse model is suited, at least on the proteome level, as a model for human obesity studies. Recently, an in-depth proteome analysis of 3T3-L1 adipocytes was published (68) using subcellular fractionation and high sensitive protein identification by sophisticated mass spectrometry. This strategy revealed 3,287 identified proteins and is currently the largest high confidence proteome map on adipocyte biology. A similar study with subcutaneous and visceral human adipocytes is still awaited but would allow a detailed comparison between the two species but also between the different human fat depots, which enables extensive functional characterization of the adipogenic process.

Proteome studies on human adipogenesis have been conducted with human adult stem cells derived from liposuction aspirates (69) and with mesenchymal stem cells (70,71). The human adult stem cells revealed a relative high percentage (>40%) of identical proteins compared to mouse adipose tissue and 3T3-

L1 cells. In addition, the functional properties of all identified proteins, e.g. cytoskeletal rearrangements, metabolic and redox enzymes and protein processing, were highly similar compared to proteins earlier identified from murine models. These results might be explained by the applied 2-DE method that primarily reveals the high abundant proteins. (Subtle) differences that distinguish human adipocytes from murine adipocytes require in-depth investigation of the proteomes on a larger scale. Ideally this is done by subcellular fractionation of the adipose material and analysis with more sensitive methods like wide-range 2-DE and quantitative LC-MSMS.

Primary human adipocytes can be isolated from fresh adipose tissue but the propagation and manipulation possibilities of these cells are limited which make them less easy to use. Recently, adipose tissue explants have been used (72,73) in proteome studies but these contain next to adipocytes also stromal vascular cells, which make it difficult to determine the origin of the identified proteins. Furthermore, the tissue explants themselves do not allow the examination of the adipocyte differentiation process.

Immortalized human preadipocytes appear a promising model system (74,75) and developments in this field are ongoing. Other interesting cells are preadipocytes derived from a Simpson-Golabi-Behmel syndrome (SGBS) patient (76). SGBS cells can be propagated for at least 30 generations without loosing their differentiation capacity. Differentiated SGBS cells are similar to human subcutaneous adipocytes with respect to morphology and biochemical characteristics but are easier to manipulate and therefore ideal to work with. Currently, the SGBS cells form an important *in vitro* model in the field of adipocyte biology research.

Analysis of adipokine secretion by proteomics

Adipokines play an important role in normal physiology (77), however, disturbances in adipokine profiles coinciding the development of obesity are associated with the onset of metabolic complications (78-82). Proteomics research has had a major impact on the identification of novel adipokines. Started with 3T3-L1 cells, different proteomic approaches have been applied to investigate the change in adipokine profiles during differentiation. Kratchmarova *et al* (83) found 20 different secreted proteins by one-dimensional electrophoresis and LC-MSMS. Wang *et al* (84) identified 41 different proteins by a combination of 2-DE and mass spectrometry. Together, both studies revealed several different proteins that had not been associated before with adipocyte secretion. More importantly, these studies showed for the first time the applicability of proteomics technology on another level of adipocyte biology, that is adipokine expression profiling.

Adipocyte secretion routes

Proteins bearing a N-terminal secretion signal peptide are secreted via the ER-Golgi pathway. However, next to this so-called classical pathway at least 3 additional routes of protein secretion are present in eukaryotic cells (85,86). In a study on adipokine profiles from rat adipose cells 99 proteins out of 183 identified were considered as non-secreted because they lacked a secretion signal peptide (87). However, structural analysis of proteins for signal secretion peptides might lead to an under-representation of adipocyte-derived proteins present in the extracellular space. Our group used a blocking strategy to discriminate between truly secreted proteins and proteins derived from cell leakage (84). This method provided important information about the secretory properties of the identified proteins. For instance, cyclophilin A, which is known to be secreted from vascular smooth muscle cells (88), was identified as a truly adipocyte-secreted protein while the structure of this protein does not indicate secretory motives (84).

We showed that adipocyte secretion is stimulated by insulin but not on the transcriptional level of the secreted proteins. Instead, insulin promoted the transcription of post-translational processing proteins, particularly those that are involved in proteolysis (12). The regulation of processing enzymes can increase protein secretion by shedding of transmembrane proteins, a process termed ectocytosis. This process is another way to bring secretory proteins without secretion motives into the extracellular space (89,90). For example, matrix metalloproteinases (MMP's) are involved in this phenomenon and several subtypes of these proteins have been identified as secreted proteins from adipocytes (12,84,87). Whether these proteins are actively involved in proteolysis of adipocyte-bearing transmembrane proteins and as such stimulate adipokine secretion remains elusive.

Another route for protein secretion by adipocytes is the release of microvesicles. A proteome analysis of microvesicles derived form 3T3-L1 cells revealed many proteins previously identified as adipokine (91). Microvesicles can derive from direct budding from the plasma membrane (92) or from exocytosis of endosome-derived multivesicular bodies known as exosomes (93). Both type of vehicles can be released in the intracellular space and are also found in body fluids. They function in the transfer of proteins and RNA molecules between cells. Furthermore, microvesicles appear to specifically target recipient cells (reviewed in 92,93). With respect to adipokines, this mechanism may function as a targeted communication pathway between the adipose tissue and other tissues like muscle, liver and intestine. In-depth proteome studies of adipocyte-derived microvesicles (94) will be necessary to further understand their functional properties, how they target their recipient cells and how they are related to obesity-related disorders.

Human adipokines identified by proteomics

In the human body the visceral and subcutaneous fat depots are important sources for adipokines present in the circulation. Especially the accumulation of visceral fat and the related increased free fatty acid flux together with a deregulation of visceral adipokine profiles are associated with insulin resistance, endothelial dysfunction and a pro-inflammatory state (80,95). So far, several studies appeared on profiling of human adipokines, each applying a different experimental approach (71-73,96,97). One study investigated mammary fat and its interstitial fluid by a combination of 2-DE, mass spectrometry and antibody array techniques (97). 359 different proteins were identified, with functions in signal transduction, energy metabolism, cellular communication, cell growth and maintenance, and immune response. Although the relation between mammary fat and metabolic disturbances such as insulin resistance is less clear, obese woman are considered to be on a higher risk for developing post-menopausal breast cancer (98). Mammary adipose-derived factors may be involved in this process as they are able to stimulate tumor cell growth.

So far, three studies appeared on adipokine profiles from freshly isolated human subcutaneous and visceral adipose tissue (72,73,96). Secretomes of adipose tissue explants were examined by SDS-PAGE-LC-MSMS (72) and by antibody arrays (73), respectively. The visceral adipose tissue revealed 259 identified proteins from which 108 contained a secretion signal peptide. This left 151 proteins to be secreted via a non-classical pathway or even non-secreted. Indeed, the tissue explants appeared to contain some residual serum and intracellular proteins (72). The secretome from subcutaneous fat was investigated by an array containing antibodies against 120 different cytokines. Sixteen proteins were identified from which 5 were regulated by PPAR agonists (73).

Despite the high significance to investigate human adipokine expression, the above two studies could not discriminate the origin of their identified proteins. As adipose tissue explants mainly consist of adipocytes and stromal vascular cells it remains elusive from which cells the secreted proteins are derived. To resolve questions about adipose tissue-derived proteins in the circulation this is a less severe problem. However, to dissect communication between adipocytes and other cell types this method is less useful. For instance, the question how macrophages are recruited into adipose tissue of obese persons is best studied by using pure cell cultures as secreted factors from differentiated adipocytes showed monocyte chemotactic activities (99).

Secretion profiles from undifferentiated and differentiated primary cultures of human adipose-derived stem cells revealed 81 differentially expressed proteins as identified by a 2-DE approach (96). About 72% of these proteins had already been discovered in previous proteomics studies showing a considerable overlap between rodent and human adipokine profiles. Since this study was conducted with 2-DE, the more subtle differences between the two species remain to be discovered. However, HSP47 (SERPINH1) a serpin family member, was identified as a new human adipocyte-secreted protein.

Adipocyte extracellular matrix

Extracellular matrix (ECM) proteins constitute a major part of the adipocyte secretion profile known today (12,72,83,84,96). Remodelling of the ECM plays an important role in adipocyte differentiation (100,101) and particularly MMP have been demonstrated to be involved in this process *in vitro* (102,103) and *in vivo* (104,105). Previously, we determined a differential expression of several ECM proteins from mature 3T3-L1 cells by a novel stable isotope labelling method (106). Based on these results it was concluded that ECM remodelling is also a functional property of mature 3T3-L1 adipocytes.

Recently it was shown that the ECM of human preadipocytes could be modulated by macrophage-secreted factors and that interstitial fibrosis in obese white adipose tissue coincides with infiltration of inflammatory cells (107,108). This may imply that adipocyte-ECM modulation is associated with the inflammatory state found in the adipose tissue of obese subjects and may be linked to metabolic deregulation. A study by Khan *et al* (109) demonstrated that the absence of the adipocyte ECM protein collagen VI resulted in an improvement of whole body energy homeostasis of *ob/ob* mice together with enlarged adipocyte size. This paradoxal finding was explained by a weakening of the ECM allowing increased triglyceride storage. As such, excess adipose tissue ECM formation during obesity may prevent storage of fat during excess energy intake and may induce plasma glucose and free fatty acid levels.

Together, the adipocyte ECM appears to play an important role in adipose tissue biology. However, the composition of the adipocyte ECM and how it is changed under different inflammatory and nutrient conditions is currently unclear.

Proteomics on human adipose tissue biopsies

So far, a limited number of proteome analyses have been conducted on human subcutaneous and visceral adipose tissue biopsies. These were from low and high-fat oxidizing obese subjects (110), polycystic ovary syndrome subjects (111) and non-diabetic obese subjects (112). All three studies were performed with a 2-DE approach. Claessens *et al* (110) used a subtraction method to discriminate truly adipocyte proteins from contaminating blood-derived proteins and observed an induc-

tion of methylmalonate-semialdehyde dehydrogenase in low-fat oxidizing subjects. This may account for an increased valine catabolism as compensatory mechanism for reduced carbohydrate metabolism.

Upregulation of endoplasmic reticulum stress-related proteins was observed in fat biopsies from non-diabetic obese subjects compared to lean ones (112). Although these proteins are involved in an unfolded protein response, most of these have also been assigned generally stress-related proteins (24). The same is true for the proteins identified by Corton *et al* (111). Together, these studies provided initial insight in proteome changes in adipose tissue from human subjects. In-depth proteome analyses may provide a further understanding of the biology of the adipose tissue with respect to obesity and obesity-related disorders. However, this requires complexity-reducing sample preparation and more sensitive analysis methods.

Conclusion and future directions

Since 2001 proteomics research on adipose biology has evolved and many proteins that had not been associated with adipocytes before have been identified. In addition, by means of proteomics data several novel biological features of adipocytes have been discovered. As indicated, none of the existing proteomics strategies can cover entire proteomes of mammalian systems at once. Thus the coverage of the total proteome of (pre)adipocytes and proteome changes during preadipocyte differentiation requires a combination of current analysis techniques. Subcellular fractionation, wide-scale 2-DE, high sensitive mass spectrometry and high-density antibody arrays will all contribute to a further understanding of adipocyte biology.

Future applications for proteomics research in adipose biology are the further dissection of (*i*) the molecular events during human adipogenesis, (*ii*) the remodelling of the adipocyte ECM, and (*iii*) the link between obesity and obesity-related metabolic complications. Adipocyte-secreted adipokines are supposed to play an important role in obesity-related disorders. However, how the change in adipokine profiles influence neighbour and remote target cells and how they induce metabolic complications remains elusive. A challenging task for proteomics researchers lies ahead.

References

1. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 2006; 444: 847-853.
2. Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* 2005; 184: 285-293.
3. Bluher M. Adipose tissue dysfunction in obesity. *Exp Clin Endocrinol Diabetes* 2009; 117: 241-250.
4. Goossens GH. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. *Physiol Behav* 2008; 94: 206-218.
5. Haslam D. Obesity: a medical history. *Obes Rev* 2007; 8 Suppl 1: 31-36.
6. Haslam D, Sattar N, Lean M. ABC of obesity. Obesity – time to wake up. *BMJ* 2006; 333: 640-642.
7. Haslam DW, James WP. Obesity. *Lancet* 2005; 366: 1197-1209.
8. Guan HP, Li Y, Jensen MV, Newgard CB, Steppan CM, Lazar MA. A futile metabolic cycle activated in adipocytes by antidiabetic agents. *Nat Med* 2002; 8: 1122-1128.
9. Burton GR, Guan Y, Nagarajan R, McGehee RE, Jr. Microarray analysis of gene expression during early adipocyte differentiation. *Gene* 2002; 293: 21-31.
10. Soukas A, Socci ND, Saatkamp BD, Novelli S, Friedman JM. Distinct transcriptional profiles of adipogenesis in vivo and in vitro. *J Biol Chem* 2001; 276: 34167-34174.
11. Pradet-Balade B, Boulme F, Beug H, Mullner EW, Garcia-Sanz JA. Translation control: bridging the gap between genomics and proteomics? *Trends Biochem Sci* 2001; 26: 225-229.
12. Wang P, Keijer J, Bunschoten A, Bouwman F, Renes J, Mariman E. Insulin modulates the secretion of proteins from mature 3T3-L1 adipocytes: a role for transcriptional regulation of processing. *Diabetologia* 2006; 49: 2453-2462.
13. Tyers M, Mann M. From genomics to proteomics. *Nature* 2003; 422: 193-197.
14. Sharan R, Ulitsky I, Shamir R. Network-based prediction of protein function. *Mol Syst Biol* 2007; 3: 88.
15. Gorg A, Drews O, Luck C, Weiland F, Weiss W. 2-DE with IPGs. *Electrophoresis* 2009; 30 (Suppl 1): S122-132.
16. Rabilloud T. Two-dimensional gel electrophoresis in proteomics: old, old fashioned, but it still climbs up the mountains. *Proteomics* 2002; 2: 3-10.
17. Chen G, Pramanik BN. Application of LC/MS to proteomics studies: current status and future prospects. *Drug Discov Today* 2009; 14: 465-471.
18. Domon B, Aebersold R. Mass spectrometry and protein analysis. *Science* 2006; 312: 212-217.
19. Wingren C, Borrebaeck CA. Antibody-based microarrays. *Methods Mol Biol* 2009; 509: 57-84.
20. Kingsmore SF. Multiplexed protein measurement: technologies and applications of protein and antibody arrays. *Nat Rev Drug Discov* 2006; 5: 310-320.
21. Fichmann J, Westermeier R. 2-D protein gel electrophoresis. An overview. *Methods Mol Biol* 1999; 112: 1-7.
22. Marouga R, David S, Hawkins E. The development of the DIGE system: 2D fluorescence difference gel analysis tech-

nology. *Anal Bioanal Chem* 2005; 382: 669-678.
23. Gade D, Thiermann J, Markowsky D, Rabus R. Evaluation of two-dimensional difference gel electrophoresis for protein profiling. Soluble proteins of the marine bacterium Pirellula sp. strain 1. *J Mol Microbiol Biotechnol* 2003; 5: 240-251.
24. Wang P, Bouwman FG, Mariman EC. Generally detected proteins in comparative proteomics – a matter of cellular stress response? *Proteomics* 2009; 9: 2955-2966.
25. Heck AJ, Krijgsveld J. Mass spectrometry-based quantitative proteomics. *Expert Rev Proteomics* 2004; 1: 317-326.
26. Ong SE, Mann M. Mass spectrometry-based proteomics turns quantitative. *Nat Chem Biol* 2005; 1: 252-262.
27. Yates JR, 3rd. Mass spectral analysis in proteomics. *Annu Rev Biophys Biomol Struct* 2004; 33: 297-316.
28. Aebersold R, Mann M. Mass spectrometry-based proteomics. *Nature* 2003; 422: 198-207.
29. Nilsson P, Paavilainen L, Larsson K, Odling J, Sundberg M, Andersson AC, *et al.* Towards a human proteome atlas: high-throughput generation of mono-specific antibodies for tissue profiling. *Proteomics* 2005; 5: 4327-4337.
30. Tegel H, Steen J, Konrad A, Nikdin H, Pettersson K, Stenvall M, *et al.* High-throughput protein production – lessons from scaling up from 10 to 288 recombinant proteins per week. *Biotechnol J* 2009; 4: 51-57.
31. Ponten F, Jirstrom K, Uhlen M. The Human Protein Atlas – a tool for pathology. *J Pathol* 2008; 216: 387-393.
32. Sidhu RS. Two-dimensional electrophoretic analyses of proteins synthesized during differentiation of 3T3-L1 preadipocytes. *J Biol Chem* 1979; 254: 11111-11118.
33. Student AK, Hsu RY, Lane MD. Induction of fatty acid synthetase synthesis in differentiating 3T3-L1 preadipocytes. *J Biol Chem* 1980; 255: 4745-4750.
34. Spiegelman BM, Green H. Control of specific protein biosynthesis during the adipose conversion of 3T3 cells. *J Biol Chem* 1980; 255: 8811-8818.
35. Lanne B, Potthast F, Hoglund A, Brockenhuus von Lowenhielm H, Nystrom AC, Nilsson F, *et al.* Thiourea enhances mapping of the proteome from murine white adipose tissue. *Proteomics* 2001; 1: 819-828.
36. Sanchez JC, Chiappe D, Converset V, Hoogland C, Binz PA, Paesano S, *et al.* The mouse SWISS-2D PAGE database: a tool for proteomics study of diabetes and obesity. *Proteomics* 2001; 1: 136-163.
37. Welsh GI, Griffiths MR, Webster KJ, Page MJ, Tavare JM. Proteome analysis of adipogenesis. *Proteomics* 2004; 4: 1042-1051.
38. Choi KL, Wang Y, Tse CA, Lam KS, Cooper GJ, Xu A. Proteomic analysis of adipocyte differentiation: Evidence that alpha2 macroglobulin is involved in the adipose conversion of 3T3 L1 preadipocytes. *Proteomics* 2004; 4: 1840-1848.
39. Renes J, Bouwman F, Noben JP, Evelo C, Robben J, Mariman E. Protein profiling of 3T3-L1 adipocyte differentiation and (tumor necrosis factor alpha-mediated) starvation. *Cell Mol Life Sci* 2005; 62: 492-503.
40. Kawaguchi N, Sundberg C, Kveiborg M, Moghadaszadeh B, Asmar M, Dietrich N, *et al.* ADAM12 induces actin cytoskeleton and extracellular matrix reorganization during early adipocyte differentiation by regulating beta1 integrin function. *J Cell Sci* 2003; 116: 3893-3904.
41. Lieber JG, Evans RM. Disruption of the vimentin intermediate filament system during adipose conversion of 3T3-L1 cells inhibits lipid droplet accumulation. *J Cell Sci* 1996; 109 (Pt 13): 3047-3058.
42. Rodriguez Fernandez JL, Ben-Ze'ev A. Regulation of fibronectin, integrin and cytoskeleton expression in differentiating adipocytes: inhibition by extracellular matrix and polylysine. *Differentiation* 1989; 42: 65-74.
43. Yoshizaki T, Imamura T, Babendure JL, Lu JC, Sonoda N, Olefsky JM. Myosin 5a is an insulin-stimulated Akt2 (protein kinase Bbeta) substrate modulating GLUT4 vesicle translocation. *Mol Cell Biol* 2007; 27: 5172-5183.
44. Huang J, Imamura T, Babendure JL, Lu JC, Olefsky JM. Disruption of microtubules ablates the specificity of insulin signaling to GLUT4 translocation in 3T3-L1 adipocytes. *J Biol Chem* 2005; 280: 42300-42306.
45. Olson AL, Eyster CA, Duggins QS, Knight JB. Insulin promotes formation of polymerized microtubules by a phosphatidylinositol 3-kinase-independent, actin-dependent pathway in 3T3-L1 adipocytes. *Endocrinology* 2003; 144: 5030-5039.
46. Kanzaki M, Pessin JE. Insulin-stimulated GLUT4 translocation in adipocytes is dependent upon cortical actin remodeling. *J Biol Chem* 2001; 276: 42436-42444.
47. Guilherme A, Emoto M, Buxton JM, Bose S, Sabini R, Theurkauf WE, *et al.* Perinuclear localization and insulin responsiveness of GLUT4 requires cytoskeletal integrity in 3T3-L1 adipocytes. *J Biol Chem* 2000; 275: 38151-38159.
48. Alexander CM, Selvarajan S, Mudgett J, Werb Z. Stromelysin-1 regulates adipogenesis during mammary gland involution. *J Cell Biol* 2001; 152: 693-703.
49. Crandall DL, Busler DE, McHendry-Rinde B, Groeling TM, Kral JG. Autocrine regulation of human preadipocyte migration by plasminogen activator inhibitor-1. *J Clin Endocrinol Metab* 2000; 85: 2609-2614.
50. Patel YM, Lane MD. Role of calpain in adipocyte differentiation. *Proc Natl Acad Sci USA* 1999; 96: 1279-1284.

51. Souza SC, Yamamoto MT, Franciosa MD, Lien P, Greenberg AS. BRL 49653 blocks the lipolytic actions of tumor necrosis factor-alpha: a potential new insulin-sensitizing mechanism for thiazolidinediones. *Diabetes* 1998; 47: 691-695.
52. Wilson-Fritch L, Burkart A, Bell G, Mendelson K, Leszyk J, Nicoloro S, *et al.* Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* 2003; 23: 1085-1094.
53. Brasaemle DL, Dolios G, Shapiro L, Wang R. Proteomic analysis of proteins associated with lipid droplets of basal and lipolytically stimulated 3T3-L1 adipocytes. *J Biol Chem* 2004; 279: 46835-46842.
54. Wilson-Fritch L, Nicoloro S, Chouinard M, Lazar MA, Chui PC, Leszyk J, *et al.* Mitochondrial remodeling in adipose tissue associated with obesity and treatment with rosiglitazone. *J Clin Invest* 2004; 114: 1281-1289.
55. Bluher M, Wilson-Fritch L, Leszyk J, Laustsen PG, Corvera S, Kahn CR. Role of insulin action and cell size on protein expression patterns in adipocytes. *J Biol Chem* 2004; 279: 31902-31909.
56. Aboulaich N, Vainonen JP, Stralfors P, Vener AV. Vectorial proteomics reveal targeting, phosphorylation and specific fragmentation of polymerase I and transcript release factor (PTRF) at the surface of caveolae in human adipocytes. *Biochem J* 2004; 383: 237-248.
57. Molina H, Yang Y, Ruch T, Kim JW, Mortensen P, Otto T, *et al.* Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy. *J Proteome Res* 2009; 8: 48-58.
58. Hallakou S, Doare L, Foufelle F, Kergoat M, Guerre-Millo M, Berthault MF, *et al.* Pioglitazone induces in vivo adipocyte differentiation in the obese Zucker fa/fa rat. *Diabetes* 1997; 46: 1393-1399.
59. de Souza CJ, Eckhardt M, Gagen K, Dong M, Chen W, Laurent D, *et al.* Effects of pioglitazone on adipose tissue remodeling within the setting of obesity and insulin resistance. *Diabetes* 2001; 50: 1863-1871.
60. Oakes ND, Thalen PG, Jacinto SM, Ljung B. Thiazolidinediones increase plasma-adipose tissue FFA exchange capacity and enhance insulin-mediated control of systemic FFA availability. *Diabetes* 2001; 50: 1158-1165.
61. Renes J, van Tilburg J, van Haaften R, Bouwman F, Kodde A, Evelo C, *et al.* Thiazolidinediones regulate expression of proteins involved in triacylglyceride storage and fatty acid oxidation in 3T3-L1 (pre-)adipocytes. *Adipocytes* 2006; 2: 75-91.
62. Wang P, Renes J, Bouwman F, Bunschoten A, Mariman E, Keijer J. Absence of an adipogenic effect of rosiglitazone on mature 3T3-L1 adipocytes: increase of lipid catabolism and reduction of adipokine expression. *Diabetologia* 2007; 50: 654-665.
63. Yki-Jarvinen H. Thiazolidinediones. *N Engl J Med* 2004; 351: 1106-1118.
64. Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, *et al.* The hormone resistin links obesity to diabetes. *Nature* 2001; 409: 307-312.
65. Smith SR, Gawronska-Kozak B, Janderova L, Nguyen T, Murrell A, Stephens JM, *et al.* Agouti expression in human adipose tissue: functional consequences and increased expression in type 2 diabetes. *Diabetes* 2003; 52: 2914-2922.
66. Heilbronn LK, Rood J, Janderova L, Albu JB, Kelley DE, Ravussin E, *et al.* Relationship between serum resistin concentrations and insulin resistance in nonobese, obese, and obese diabetic subjects. *J Clin Endocrinol Metab* 2004; 89: 1844-1848.
67. Arner P. Resistin: yet another adipokine tells us that men are not mice. *Diabetologia* 2005; 48: 2203-2205.
68. Adachi J, Kumar C, Zhang Y, Mann M. In-depth analysis of the adipocyte proteome by mass spectrometry and bioinformatics. *Mol Cell Proteomics* 2007; 6: 1257-1273.
69. DeLany JP, Floyd ZE, Zvonic S, Smith A, Gravois A, Reiners E, *et al.* Proteomic analysis of primary cultures of human adipose-derived stem cells: modulation by adipogenesis. *Mol Cell Proteomics* 2005; 4: 731-740.
70. Lee HK, Lee BH, Park SA, Kim CW. The proteomic analysis of an adipocyte differentiated from human mesenchymal stem cells using two-dimensional gel electrophoresis. *Proteomics* 2006; 6: 1223-1229.
71. Chiellini C, Cochet O, Negroni L, Samson M, Poggi M, Ailhaud G, *et al.* Characterization of human mesenchymal stem cell secretome at early steps of adipocyte and osteoblast differentiation. *BMC Mol Biol* 2008; 9: 26.
72. Alvarez-Llamas G, Szalowska E, de Vries MP, Weening D, Landman K, Hoek A, *et al.* Characterization of the human visceral adipose tissue secretome. *Mol Cell Proteomics* 2007; 6: 589-600.
73. Klimcakova E, Moro C, Mazzucotelli A, Lolmede K, Viguerie N, Galitzky J, *et al.* Profiling of adipokines secreted from human subcutaneous adipose tissue in response to PPAR agonists. *Biochem Biophys Res Commun* 2007; 358: 897-902.
74. Rodriguez AM, Elabd C, Delteil F, Astier J, Vernochet C, Saint-Marc P, *et al.* Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochem Biophys Res Commun* 2004; 315: 255-263.
75. Darimont C, Mace K. Immortalization of human preadipocytes. *Biochimie* 2003; 85: 1231-1233.

76. Wabitsch M, Brenner RE, Melzner I, Braun M, Moller P, Heinze E, *et al.* Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes Relat Metab Disord* 2001; 25: 8-15.
77. Wang P, Mariman E, Renes J, Keijer J. The secretory function of adipocytes in the physiology of white adipose tissue. *J Cell Physiol* 2008; 216: 3-13.
78. Trayhurn P, Wang B, Wood IS. Hypoxia in adipose tissue: a basis for the dysregulation of tissue function in obesity? *Br J Nutr* 2008; 100: 227-235.
79. Trayhurn P, Bing C, Wood IS. Adipose tissue and adipokines – energy regulation from the human perspective. *J Nutr* 2006; 136: 1935S-1939S.
80. Ritchie SA, Connell JM. The link between abdominal obesity, metabolic syndrome and cardiovascular disease. *Nutr Metab Cardiovasc Dis* 2007; 17: 319-326.
81. Trayhurn P. Adipocyte biology. *Obes Rev* 2007; 8 (Suppl 1): 41-44.
82. Antuna-Puente B, Feve B, Fellahi S, Bastard JP. Adipokines: the missing link between insulin resistance and obesity. *Diabetes Metab* 2008; 34: 2-11.
83. Kratchmarova I, Kalume DE, Blagoev B, Scherer PE, Podtelejnikov AV, Molina H, *et al.* A proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics* 2002; 1: 213-222.
84. Wang P, Mariman E, Keijer J, Bouwman F, Noben JP, Robben J, *et al.* Profiling of the secreted proteins during 3T3-L1 adipocyte differentiation leads to the identification of novel adipokines. *Cell Mol Life Sci* 2004; 61: 2405-2417.
85. Nickel W, Rabouille C. Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol* 2009; 10: 148-155.
86. Nickel W. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur J Biochem* 2003; 270: 2109-2119.
87. Chen X, Cushman SW, Pannell LK, Hess S. Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography-MS/MS approach. *J Proteome Res* 2005; 4: 570-577.
88. Jin ZG, Melaragno MG, Liao DF, Yan C, Haendeler J, Suh YA, *et al.* Cyclophilin A is a secreted growth factor induced by oxidative stress. *Circ Res* 2000; 87: 789-796.
89. Huovila AP, Turner AJ, Pelto-Huikko M, Karkkainen I, Ortiz RM. Shedding light on ADAM metalloproteinases. *Trends Biochem Sci* 2005; 30: 413-422.
90. Mullberg J, Althoff K, Jostock T, Rose-John S. The importance of shedding of membrane proteins for cytokine biology. *Eur Cytokine Netw* 2000; 11: 27-38.
91. Aoki N, Jin-no S, Nakagawa Y, Asai N, Arakawa E, Tamura N, *et al.* Identification and characterization of microvesicles secreted by 3T3-L1 adipocytes: redox- and hormone-dependent induction of milk fat globule-epidermal growth factor 8-associated microvesicles. *Endocrinology* 2007; 148: 3850-3862.
92. Cocucci E, Racchetti G, Meldolesi J. Shedding microvesicles: artefacts no more. *Trends Cell Biol* 2009; 19: 43-51.
93. Simons M, Raposo G. Exosomes - vesicular carriers for intercellular communication. *Curr Opin Cell Biol* 2009.
94. Xiao Z, Blonder J, Zhou M, Veenstra TD. Proteomic analysis of extracellular matrix and vesicles. *J Proteomics* 2009; 72: 34-45.
95. Rodriguez A, Catalan V, Gomez-Ambrosi J, Fruhbeck G. Visceral and subcutaneous adiposity: are both potential therapeutic targets for tackling the metabolic syndrome? *Curr Pharm Des* 2007; 13: 2169-2175.
96. Zvonic S, Lefevre M, Kilroy G, Floyd ZE, DeLany JP, Kheterpal I, *et al.* Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. *Mol Cell Proteomics* 2007; 6: 18-28.
97. Celis JE, Moreira JM, Cabezon T, Gromov P, Friis E, Rank F, *et al.* Identification of extracellular and intracellular signaling components of the mammary adipose tissue and its interstitial fluid in high risk breast cancer patients: toward dissecting the molecular circuitry of epithelial-adipocyte stromal cell interactions. *Mol Cell Proteomics* 2005; 4: 492-522.
98. Harvie M, Hooper L, Howell AH. Central obesity and breast cancer risk: a systematic review. *Obes Rev* 2003; 4: 157-173.
99. Han CY, Subramanian S, Chan CK, Omer M, Chiba T, Wight TN, *et al.* Adipocyte-derived serum amyloid A3 and hyaluronan play a role in monocyte recruitment and adhesion. *Diabetes* 2007; 56: 2260-2273.
100. Kubo Y, Kaidzu S, Nakajima I, Takenouchi K, Nakamura F. Organization of extracellular matrix components during differentiation of adipocytes in long-term culture. *In Vitro Cell Dev Biol Anim* 2000; 36: 38-44.
101. Antras J, Hilliou F, Redziniak G, Pairault J. Decreased biosynthesis of actin and cellular fibronectin during adipose conversion of 3T3-F442A cells. Reorganization of the cytoarchitecture and extracellular matrix fibronectin. *Biol Cell* 1989; 66: 247-254.
102. Croissandeau G, Chretien M, Mbikay M. Involvement of matrix metalloproteinases in the adipose conversion of 3T3-L1 preadipocytes. *Biochem J* 2002; 364: 739-746.

103. Bouloumie A, Sengenes C, Portolan G, Galitzky J, Lafontan M. Adipocyte produces matrix metalloproteinases 2 and 9: involvement in adipose differentiation. *Diabetes* 2001; 50: 2080-2086.
104. Chun TH, Hotary KB, Sabeh F, Saltiel AR, Allen ED, Weiss SJ. A pericellular collagenase directs the 3-dimensional development of white adipose tissue. *Cell* 2006; 125: 577-591.
105. Chavey C, Mari B, Monthouel MN, Bonnafous S, Anglard P, Van Obberghen E*, et al.* Matrix metalloproteinases are differentially expressed in adipose tissue during obesity and modulate adipocyte differentiation. *J Biol Chem* 2003; 278: 11888-11896.
106. Bouwman F, Renes J, Mariman E. A combination of protein profiling and isotopomer analysis using matrix-assisted laser desorption/ionization-time of flight mass spectrometry reveals an active metabolism of the extracellular matrix of 3T3-L1 adipocytes. *Proteomics* 2004; 4: 3855-3863.
107. Keophiphath M, Achard V, Henegar C, Rouault C, Clement K, Lacasa D. Macrophage-secreted factors promote a profibrotic phenotype in human preadipocytes. *Mol Endocrinol* 2009; 23: 11-24.
108. Henegar C, Tordjman J, Achard V, Lacasa D, Cremer I, Guerre-Millo M*, et al.* Adipose tissue transcriptomic signature highlights the pathological relevance of extracellular matrix in human obesity. *Genome Biol* 2008; 9: R14.
109. Khan T, Muise ES, Iyengar P, Wang ZV, Chandalia M, Abate N*, et al.* Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. *Mol Cell Biol* 2009; 29: 1575-1591.
110. Claessens M, Saris W, Bouwman F, Evelo C, Hul G, Blaak E*, et al.* Differential valine metabolism in adipose tissue of low and high fat-oxidizing obese subjects. *Proteomics Clin. Appli.* 2007; 1: 1306-1315.
111. Corton M, Botella-Carretero JI, Lopez JA, Camafeita E, San Millan JL, Escobar-Morreale HF*, et al.* Proteomic analysis of human omental adipose tissue in the polycystic ovary syndrome using two-dimensional difference gel electrophoresis and mass spectrometry. *Hum Reprod* 2008; 23: 651-661.
112. Boden G, Duan X, Homko C, Molina EJ, Song W, Perez O*, et al.* Increase in endoplasmic reticulum stress-related proteins and genes in adipose tissue of obese, insulin-resistant individuals. *Diabetes* 2008; 57: 2438-2444.

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REVIEW

CELLULAR HYPOXIA: A KEY MODULATOR OF ADIPOCYTE FUNCTION IN OBESITY?

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Abstract

We have proposed that hypoxia develops in adipose tissue (white) as tissue mass expands in obesity, this leading to the inflammatory response which is considered to underpin the development of obesity-associated diseases. Direct evidence for hypoxia in adipose tissue in obesity has now been obtained in mice and in humans. Studies on adipocytes, both human and murine, in cell culture have shown that the expression and release of several inflammation-related adipokines, such as IL-6, leptin and VEGF are stimulated by low pO_2. The production of adiponectin, which has anti-inflammatory and insulin-sensitising actions, is, however, inhibited. Glucose uptake and the release of lactate are increased in adipocytes by hypoxia, with a corresponding increase in the level of the GLUT1 and MCT1 transporters, consistent with a switch to glycolytic metabolism. In preadipocytes, which do not normally synthesise leptin, low pO_2 leads to induction of the expression and secretion of this key hormone. Recent studies suggest that there are important interactions between hypoxia and specific long-chain fatty acids in the production of inflammation-related adipokines. It is suggested that hypoxia has a pervasive effect on adipocyte physiology and is central to the dysregulation of adipose tissue function that occurs in obesity.

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Introduction

Hypoxia has traditionally been viewed as a phenomenon associated with high altitude and deep sea diving. Animals that live at altitude, or undergo deep dives, have evolved a series of physiological adaptations in order to adjust to the challenge of a lack of oxygen. In the case of altitude, these adaptations may be chronic for those species that live at high elevations. Hypoxia has also been associated with certain pathological conditions, such as wound healing, ischaemic damage and the interior of tumours (1,2). Solid tumours may be extremely hypoxic, and so much so that in their centre there may be minimal O_2 and the local environment can be essentially anoxic (1,2).

Hypoxia is now increasingly understood to be a challenge to which specific cells are exposed in animals that live under conditions of normal environmental O_2 pressure. In a number of tissues, the O_2 tension has been shown to be well below that of arterial blood or of the general level of tissue oxygenation (2). Thus, while the pO_2 of arterial blood is around 104 mmHg and general tissue oxygenation

is 40-50 mmHg, pO_2 values well below this have been reported for tissues such as the brain, spleen and retina (2). In the case of solid tumours, the pO_2 can be as little as 1 mmHg, or even lower.

The possibility that hypoxia occurs in adipose tissue in obesity was first raised by our group in 2004 (3). It was suggested that as adipose tissue mass expands clusters of large adipocytes become distant from the vasculature, resulting in relative hypoxia because of the reduced availability of O_2. The normal diffusion distance of O_2 across tissues is of the order of 100-200 μm, and in some instances the O_2 tension has been reported to be close to zero at 100 μM from the vasculature (2). Since adipocytes are large cells, reaching up to 150 μm, or even 200 μm, in diameter in obesity (4), it is clearly probable that they can become hypoxic. The 'hypoxia hypothesis' proposes that localised O_2-deprivation in large fat cells leads to an inflammatory response in order to increase blood flow and to stimulate angiogenesis (3,5).

In this article, we consider the multiple effects of hypoxia on the function of adipocytes as the dominant and characteristic cell type within adipose tissue. We focus on our studies of human adipocytes since the most extensive investigations so far have been done on the fat cells of man.

White adipose tissue and inflammation

Mature adipocytes amount to about 50% of the total cell content of the major white adipose tissue depots, but this can vary according to site, age, and other factors. The adipocyte is a major secretory cell, releasing not only fatty acids as a fuel for other organs during periods of negative energy balance, but also a multiplicity of other substances. These include lipid moieties such as prostaglandins and endocannabinoids, and a rapidly growing number (up to 100 to date) of protein factors and signals – the adipokines (3,6,7). The various protein factors and signals constitute the '*adipokinome*'. Since several adipokines, notably leptin and adiponectin, are hormones, adipocytes have become recognised as major endocrine cells. The adipokines are highly diverse in terms of function, being involved in appetite and energy balance, vascular haemostasis, blood pressure regulation, angiogenesis, lipid metabolism and insulin sensitivity (3, 6-9).

Adipocytes secrete a number of cytokines, chemokines and acute phase proteins and other proteins related to inflammation and the inflammatory response (3,6-9). Indeed, in obesity there is a state of inflammation in the tissue which is reflected in an increased circulating level of several inflammatory markers, including C-reactive protein, interleukin-6 (IL-6), plasminogen activator inhibitor-1 (PAI-1) and haptoglobin (10,11). The expression and secretion of a number of inflammation-related adipokines is markedly elevated in adipose tissue in obesity; these include IL-6, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP1), PAI-1, visfatin and apelin (3,10,11). However, in contrast to these factors the production and circulating level of the adipocyte-derived hormone adiponectin declines with increased adipose tissue mass (12).

The inflammatory response in adipose tissue in obesity and the major changes in the production of inflammation-related adipokines have been widely linked to the development of several obesity-associated diseases, particularly insulin resistance and the metabolic syndrome (8,9,11,13). Indeed, the link between inflammatory adipokines, insulin resistance and the metabolic syndrome is a crucial concept in obesity. However, the basis for the inflammatory response in adipose tissue as obesity develops has not been clear and the hypoxia hypothesis (3) is a direct attempt to address the issue.

The main pathway by which a low pO_2 is signalled within cells involves the recruitment of hypoxia-sensitive transcription factors. Several such factors have been described, including CREB and NFκB (14). However, the most important pathway is through the HIF-1 (hypoxia-inducible factor 1) transcription factor. HIF-1 consists of 1α and 1β subunits, HIF-1β being constitutively expressed (1,2,15). There are three different forms of the HIF-α subunit, namely -1α, -2α and -3α (forming the corresponding transcription factors, HIF-1, HIF-2 and HIF-3), the breakdown of which occurs in the presence of O_2 (15). The most important appears to be HIF-1α, which is stabilised when O_2 tension is low, enabling the functional transcription factor to be recruited (see Fig. 1), which binds to hypoxia response elements on a number (>70) of genes.

Hypoxia in adipose tissue

The occurrence of hypoxia in white fat depots in obesity has now been directly observed in obese mice. Hypoxia has been shown in mice made obese through the consumption of a high fat diet, and in obese *ob/ob* and KKAy mice (16-18). Two different approaches have been used to examine adipose tissue for hypoxia in obese mice – staining with pimonidazole ('Hypoxyprobe'), a chemical marker of hypoxia, and measurements with an O_2 electrode. While the pimonidazole method is essentially a qualitative procedure (although relative quantitation is possible by western blotting), the studies with an O_2 electrode have indicated that the pO_2 in the adipose tissue of obese mice is around 15 mmHg, as compared with 45-50 mmHg in the lean (17).

The studies showing hypoxia in adipose tissue in obese mice have recently been followed by similar results in humans for the abdominal subcutaneous fat depot (19). An inverse correlation between percent body fat and pO_2 in adipose tissue was demonstrated. However, the degree of hypoxia with increasing fatness was relatively small in the human study and did not lead

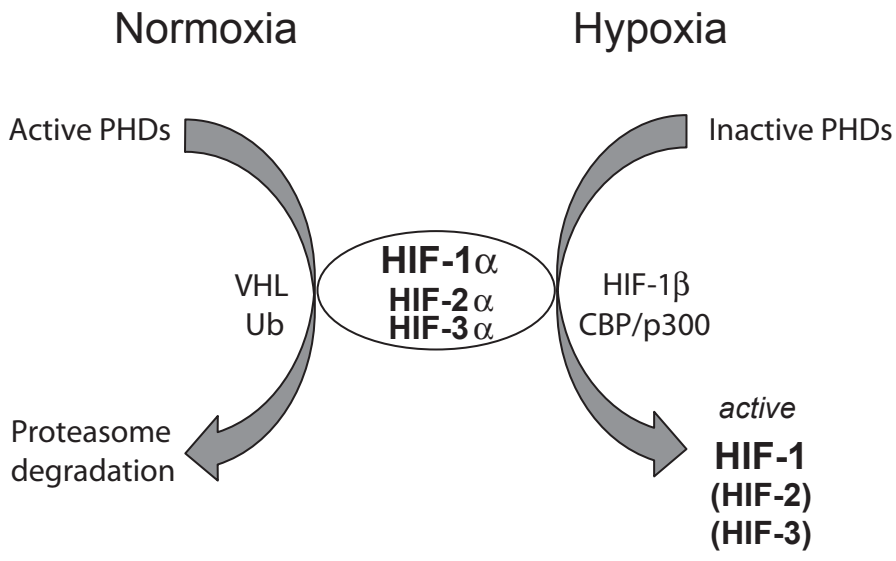

Figure 1. Overview of the recruitment of the HIF-1 transcription factor (and HIF-2 and -3) in response to hypoxia. HIF-1 binds to hypoxia response elements in a number of genes. HIF-1a, hypoxic inducible factor-1a; HIF-2a, hypoxic inducible factor-2a; HIF-3a, hypoxic inducible factor-3a; PHD, propyl hydrogenase domain proteins; VHL, von Hippel Lindau protein; Ub, ubiquitin; HIF-1b, hypoxic inducible factor-1b subunit; CBP/p300, cAMP binding protein binding protein/p300 subunit.

to the activation of the classical target genes of low O_2 tension (19). Further work is clearly needed on humans, and in exploring both in man and in animals whether there is a link between the degree of obesity and the extent of hypoxia.

Analysis of adipose tissue of obese animals has demonstrated that the level of HIF-1α protein is increased (17), indicative of hypoxia. It is important to note, as we have done previously, that HIF-1α mRNA level is not a suitable marker of hypoxia; it is essential to measure HIF-1α protein since the mRNA level is reduced, rather than raised, under low O_2 tension (20). GLUT1 mRNA levels are increased in adipose tissue in obese mice (17), consistent with a state of hypoxia since GLUT1 is well-recognised as a hypoxia-sensitive gene. Lactate levels are also raised, which is suggestive of a switch to glycolytic metabolism, and this is selective to adipose tissue with no increase in muscle lactate (16). Blood flow measurements with radioactively-labelled microspheres show reduced perfusion of adipose tissue in obese mice, and again this is specific to the tissue since the number of microspheres in muscle, kidney, lung and heart were unchanged (16).

The expression of several genes encoding inflammation-related adipokines is increased in adipose tissue in obesity, including IL-6, leptin, PAI-1 and macrophage migration inhibitory factor (MIF) (16,17). On the other hand, the expression of adiponectin, with its anti-inflammatory and insulin-sensitising actions, is reduced. Although these changes in inflammatory adipokine expression are consistent with hypoxia, they are not necessarily a direct response to low O_2 tension and other factors may be involved.

Hypoxia in human adipocytes

In vitro studies using cell culture have been employed to explore the extent to which various adipokine genes are modulated by low O_2 tension. This has been investigated in several studies, including in relation to angiogenesis (21) prior to the development of the hypoxia hypothesis. Although the effects of hypoxia on fat cells have been examined in rodent adipocyte systems (16,17,21,22), such as the 3T3-L1 clonal cell line, studies have also been conducted on human adipocytes (23-25). In our own work on human adipocytes, we have used both adipocytes differentiated from fibroblastic preadipocytes in primary culture (from Zen-Bio) and SGBS (Simpson-Golabi-Behmel Syndrome) cells (26). The latter are a cell strain in which preadipocytes have a high capacity for differentiation into mature adipocytes.

The basic protocol that we have employed in our studies, which is broadly similar to that of other groups, is to take adipocytes at 10-15 days after the induction of differentiation, and to incubate them under either normoxic (21% O_2) or hypoxic (1% O_2) conditions for up to 24/48 h. The level of hypoxia chosen is relatively standard in *in vitro* studies investigating the effects of low O_2 tension, and the measurements in adipose tissue of obese animals suggest that it is equivalent to 2% O_2 (1% O_2 equates to a pO_2 of 7.6 mmHg). Furthermore, it appears that the cellular responses to hypoxia, at least in terms of the recruitment

of HIF-1, occurs at and below 5% O_2 (2). Although most studies of adipocytes in culture have used 1% O_2, there is a need to examine the response to differing levels of O_2 (5% and below) to determine the extent to which the expression of various hypoxia-sensitive genes is modulated by different degrees of hypoxia.

Adipokines

Incubation of human adipocytes under hypoxic conditions upregulates the expression of several inflammation-related adipokines. Thus the mRNA level of leptin, angiopoietin-like protein 4 (Angplt4 – also known as fasting-induced adipose factor) IL-6, MIF, PAI-1 and vascular endothelial growth factor (VEGF) were increased over 24 h (23). In the case of leptin, Angptl4 and VEGF the increases were substantial (>10-fold rise in mRNA). In contrast, adiponectin mRNA was decreased (23). Similar results have been reported for murine fat cells in most cases, including particularly leptin, PAI-1 and adiponectin (16,17,21,22). Importantly, the alterations in gene expression in response to hypoxia are mirrored by parallel changes in the release of the adipokines themselves into the medium of the cultured adipocytes (22,23). Studies with the hypoxia mimetic $CoCl_2$, which leads to the stabilisation of HIF-1α with the recruitment of functional HIF-1, indicate that in most cases the transcription of inflammatory adipokine genes is HIF-1 dependent (23).

The hypoxia-induced changes in adipokine production and release are consistent with the concept that O_2-deprivation underpins the development of inflammation in white adipose tissue in obesity. However, there are no changes in the expression of some key inflammatory adipokines such as TNF-α and MCP-1, at least in human adipocytes (23). Furthermore, subsequent studies have shown that IL-1β expression is reduced rather than increased in these adipocytes by hypoxia (27). Overall, it is evident that some, but by no means all, major inflammation-related genes are modulated in adipocytes by hypoxia.

PCR arrays and microarrays

A 'candidate gene' approach has been the most widely used strategy for examining the effects of hypoxia on gene expression in adipocytes. However, we have also employed PCR arrays for the hypoxia signalling pathway in which the expression of a panel of 85 hypoxia-sensitive genes was probed simultaneously by real-time PCR (28). Application of these arrays to human adipocytes in culture has confirmed the hypoxia-sensitivity of several key genes in fat cells, including GLUT1, leptin, Angptl4 and VEGF. Several other genes were also identified as being sensitive to low O_2 tension in adipocytes, including UCP2 and catalase (28). The expression of one particular gene, MT3 - a member of the metallothionein family - was found to be dramatically induced by hypoxia, a >600-fold increase in MT3 mRNA level being observed over 24 h (28).

The induction of MT3 gene expression in response to hypoxia was rapid (~100-fold increase in mRNA level in 60 min), selective to MT3 rather than other metallothioneins (MT2A mRNA level changed <2-fold) and HIF-1 dependent (28). However, MT3 itself was not detected in the adipocytes, but this may have been a reflection of the lack of sensitivity of the antibodies used to detect the protein. MT3 is suggested to be protective against toxic challenge, and a rapid and substantial induction of MT3 expression has been described in astrocytes in culture where it is suggested that the protein plays a role in the protection of the brain against hypoxic damage (29). A similar protective function against hypoxic stress, including oxidative damage, may also underlie MT3 induction in adipocytes.

In a recent collaborative study, Agilent microarrays were used to screen the effects of hypoxia on global gene expression in human adipocytes (Trayhurn *et al*, unpublished results). These arrays contain 44,000 probes and the expression of >1200 genes was found to be modulated by hypoxia using the stringent criteria of a >2.0-fold difference in mRNA level (at $P<0.01$). Of these, >650 were up-regulated and >600 down-regulated. The genes that were upregulated included two members of the aquaporin (*AQP)* family of water transporters, aquaporin-3 and -5, while among those down-regulated were the peroxisome proliferative activated receptor γ coactivator-1α (*PPARGC1A*) and fatty acid binding protein 5 (*FABP5*). Overall, the full array studies demonstrate that hypoxia has a profound effect on gene expression in human adipocytes. Full analysis of the microarray data is currently underway.

Glucose metabolism

The discussion so far has centred on adipokine production and this has been the main focus of studies on adipocytes in culture. The facilitative glucose transporter, GLUT1, is a hypoxia-sensitive marker gene and is up-regulated, as noted above, in adipose tissue in obesity and in adipocytes in response to low O_2 tension. A study on the effects of hypoxia on the panel of facilitative glucose transporters expressed by human adipocytes found no effects over 24 h on GLUT4, GLUT10 and GLUT12 mRNA level, but GLUT1, GLUT3 and GLUT5 expression was increased (24). GLUT1 protein was also markedly increased (approximately 10-fold), suggesting that the capacity for glucose transport is raised by exposure to low O_2 tension. That this is indeed the case was demonstrated by studies with 2-deoxy-D-glucose, the uptake of which was 3-fold higher in human adipocytes in hypoxia than in normoxia (24). Such a response reflects the reduction in oxidative metabolism in conditions of low pO_2 and the consequent

increased need to generate ATP by glycolysis.

One of the implications of increased glucose utilisation in hypoxia is that the production of lactate would be expected to increase. In a recent study, we have found that lactate release by human adipocytes in cell culture is indeed raised (30). We also found that human adipocytes express three of the proton-linked monocarboxylate transporters (MCTs) responsible for the transport of lactate and similar metabolites across the plasma membrane (31), namely MCT1, MCT2 and MCT4. Both MCT1 and MCT4 are up-regulated by hypoxia in adipocytes, and MCT1, but not MCT4, protein is increased and this is HIF-1 dependent (30). In other cell types, MCT4 protein as well as gene expression has been shown to be augmented by hypoxia (32). Overall, hypoxia in adipocytes at the nutrient level leads to an increase in glucose uptake through the recruitment of additional GLUT1 transporters and this followed by increased lactate production which is removed from the cells by (in all probability) the recruitment of MCT1 transporters.

The fall in ATP production by oxidative metabolism that occurs in hypoxia involves adaptations at the mitochondrial level with improvements in the efficiency of oxidative phosphorylation (33). The main site of O_2 consumption is at complex IV, which comprises cytochrome c oxidase subunit 4 (COX4). However, this is not a maximally efficient process and some leakage can occur at complex III, resulting in the generation of reactive oxygen species. Recent studies in several cell types have shown that under conditions of low pO_2, the mitochondrial protease, LON, is up-regulated and that this leads to the degradation of the COX4-1 subunit (33, 34). This subunit is then replaced through the up-regulation of COX4-2, which increases the efficiency of respiration. We have now found that similar events occur in human adipocytes, there being an up-regulation of COX4-2 and LON expression in response to hypoxia, with an accompanying fall in the expression of COX4-1 (Wang *et al*, unpublished results).

Insulin sensitivity

Although incubation under low O_2 tension for 24 h has no effect on the expression of the insulin sensitive transporter, GLUT4, more prolonged exposure (48 h, or more) leads to a substantial fall in the mRNA level (35). This appears to be a specific effect, and not a result of cell damage, since GLUT1 mRNA level remains elevated and the alterations in GLUT1 and GLUT4 expression are reversed on return to normoxia (35). Such a change in GLUT4 expression clearly has implications for insulin sensitivity in adipose tissue. However, in two recent studies more acute exposure to hypoxia has been shown to lead to insulin resistance in adipocytes independent of any change in GLUT4. Although the basal transport of glucose may be increased in hypoxia, 2-deoxy-D-glucose studies indicate that insulin-stimulated uptake is inhibited (25,36). Furthermore, there are changes in the insulin signalling pathway, with for example a decrease in the phosphorylation of the insulin receptor and of components of the post-receptor signalling cascade (25, 36).

These are important observations which directly link hypoxia in obesity to the associated insulin resistance independently of adipokines. As such, factors that are widely implicated in the modulation of insulin sensitivity, for instance adiponectin and IL-6, may in practise exacerbate insulin resistance rather than being critical to its initiation.

Interactions: hypoxia and fatty acids

Studies to date have essentially considered the effects of hypoxia independent of other influences on adipocyte function. In recent experiments, we have begun to explore the potential interaction between low O_2 tension and other factors which affect adipocytes, and in particular fatty acids. In these experiments, human adipocytes have been incubated with different fatty acids in both normoxia and hypoxia. Initial studies suggest that some long-chain fatty acids can modulate the effects of hypoxia on the expression of certain genes – responses that are both gene and fatty acid selective. For example, while neither hypoxia (1% O_2) nor palmitate (250 μM) alone increase IL-1β mRNA level, together they have a stimulatory effect on the expression of this key inflammatory cytokine (de Oliveira *et al.*, unpublished results). Similarly, palmitate and hypoxia have an additive effect on IL-6 and Angplt4 expression, but this does not occur with oleate (González-Muniesa *et al.*, unpublished results).

Apart from demonstrating the principle of selective interactions between low O_2 tension and other factors that can influence fat cell function, these exploratory studies suggest that the release of fatty acids from adipocytes following the activation of lipolysis could in some cases amplify the effects of hypoxia (and *vice versa*), particularly with respect to the production of inflammation-related factors. This could also be the case with fatty acids derived from the circulation.

Hypoxia and preadipocytes

Several studies have indicated that hypoxia inhibits the differentiation of preadipocytes into adipocytes (37-39). This appears to involve, at least in part, a hypoxia-induced suppression of the expression of the PPARγ nuclear transcription factor which plays a central role in adipocyte differentiation (37). Most studies have focused on murine adipocyte systems, but our own studies have indicated that the differentiation of human preadipocytes is also suppressed by hypoxia (Wang, unpublished results) and PPARγ

gene expression is inhibited (40).

We have also examined the effects of hypoxia on the expression and release of several key adipokines by preadipocytes. The most dramatic observations related to leptin (40). This hormone is not normally synthesised by preadipocytes, its expression and secretion being differentiation-dependent, with leptin mRNA being essentially undetectable. However, incubation of human preadipocytes in hypoxia results in a substantial induction of leptin gene expression. Furthermore, immunoreactive leptin is readily detectable in the medium of the cells, demonstrating that preadipocytes synthesise and secrete the hormone in response to low pO_2. Whether preadipocyte-derived leptin following hypoxia in adipose tissue would make a significant contribution to the circulating level of the hormone is highly problematic. It may, nonetheless, be of importance locally within the tissue through a paracrine role.

Other cells within adipose tissue may also be subject to hypoxia. The most obvious are the macrophages that are now recognised to be recruited into the tissue in obesity and which are considered to contribute to the development of inflammation (41,42). Hypoxia stimulates gene expression in macrophages, leading to an augmentation of the inflammatory response in the cells (17). It is also likely that the endothelial cells within adipose tissue will be subject to hypoxia and the expression of key genes modulated.

Coda

It is evident that hypoxia can have a pervasive effect on adipocyte function (Fig. 2). This ranges from inducing a switch towards glycolytic metabolism for the generation of ATP with the consequent production of excess lactate, to increasing the synthesis of key adipokines such as leptin and VEGF. The induction of insulin resistance, which may occur at several levels, is an important example of how a low O_2 tension can directly lead to cell dysfunction. Although HIF-1α appears to be a key factor in the transmission of the cellular response to hypoxia in adipocytes, it is important to consider the possible role of the HIF-2α and HIF-3α subunits of HIF, as well as that of other transcription factors.

As noted particularly in the case of preadipocytes, hypoxia has the potential to influence the function of the other cell types within white adipose tissue and not just adipocytes. This may in turn lead to amplification, or modulation, of the cross-talk between the different cells in the face of O_2-deprivation. An im-

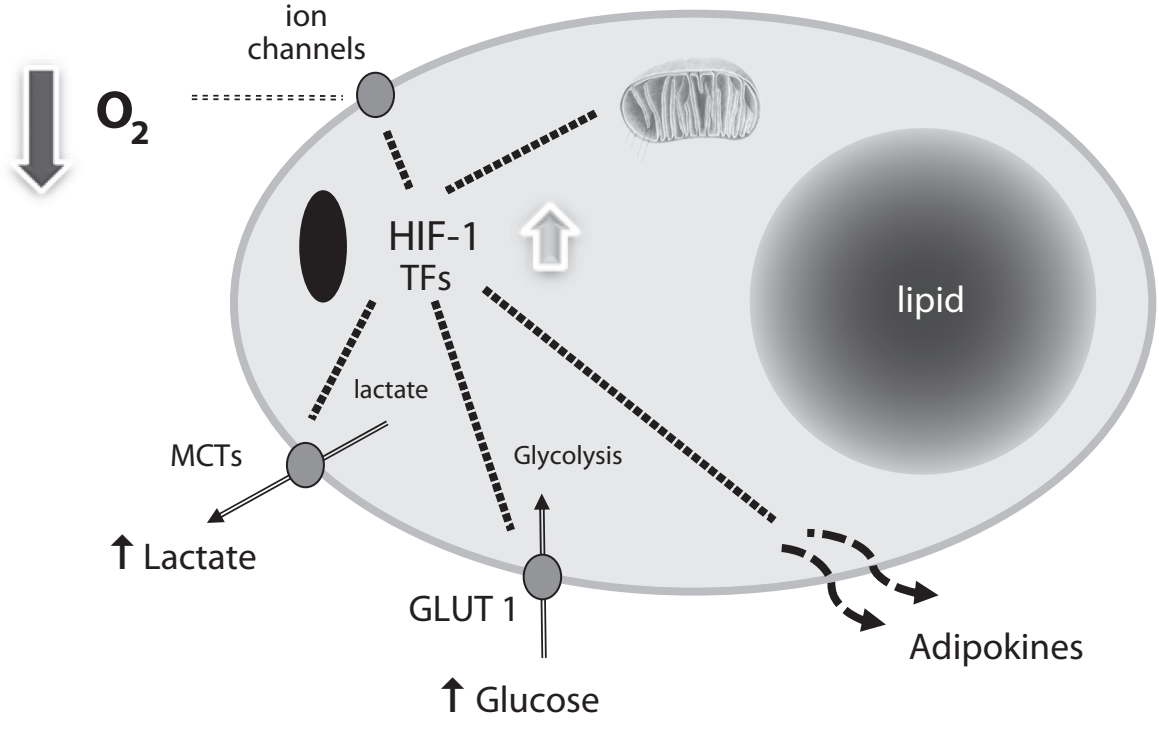

Figure 2. Summary of the effects of hypoxia on adipocyte metabolism. GLUT1, factilitative glucose transporter-1; HIF-1, hypoxia-inducible factor 1; MCTs, monocarboxylate transporters; TFs, transcription factors.

portant issue is the mechanism by which low pO_2 is sensed at the plasma membrane, and this is likely to involve specific hypoxia-sensitive ion channels (43). Indeed, we have recently observed that expression of the KCNA1 (Kv1.1) ion channel is induced in human adipocytes by hypoxia (Wang *et al*, unpublished results); nevertheless, direct modulation of channel activity is of key importance to the acute signalling of low O_2 tension.

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References

1. Semenza GL. Targeting HIF-1 for cancer therapy. *Nat Rev Cancer* 2003; 3: 721-732.
2. Brahimi-Horn MC, Pouysségur J. Oxygen, a source of life and stress. *FEBS Lett* 2007; 581: 3582-3591.
3. Trayhurn P, Wood IS. Adipokines: Inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004; 92: 347-355.
4. Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 2007; 92: 1023-1033.
5. Trayhurn P, Wang B, Wood IS. Hypoxia in adipose tissue: A basis for the dysregulation of tissue function in obesity? *Br J Nutr* 2008; 100: 227-235.
6. Trayhurn P, Beattie JH. Physiological role of adipose tissue: White adipose tissue as an endocrine and secretory organ. *Proc Nutr Soc* 2001; 60: 329-339.
7. Frühbeck G, Salvador J. Role of adipocytokines in metabolism and disease. *Nutr Res* 2004; 24: 803-826.
8. Rajala MW, Scherer PE. The adipocyte – at the crossroads of energy homeostasis, inflammation, and atherosclerosis. *Endocrinology* 2003; 144: 3765-3773.
9. Rosen ED, Spiegelman BM. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 2006; 444: 847-853.
10. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. *J Clin Invest* 2005; 115: 1111-1119.
11. Hotamisligil GS. Inflammation and metabolic disorders. *Nature* 2006; 444: 860-867.
12. Arita Y *et al*. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999; 257: 79-83.
13. Yudkin JS. Adipose tissue, insulin action and vascular disease: Inflammatory signals. *Int J Obesity* 2003; 27 (Suppl 3): S25-S28.
14. Cummins EP, Taylor CT. Hypoxia-responsive transcription factors. *Pflügers Arch Eur J Physiol* 2005; 450: 363-371.
15. Rocha S. Gene regulation under low oxygen: Holding your breath for transcription. *Trends Biochem Sci* 2007; 32: 389-397.
16. Hosogai N *et al*. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 2007; 56: 901-911.
17. Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of *ob/ob* and dietary obese mice. *Am J Physiol Endocrinol Metab* 2007; 293: E1118-E1128.
18. Rausch ME, Weisberg SP, Vardhana P, Tortorielllo DV. Obesity in C57BL/6J mice is characterised by adipose tissue hypoxia and cytotoxic t-cell infiltration. *Int J Obesity* 2008; 32: 451–463.
19. Pasarica M *et al*. Reduced adipose tissue oxygenation in human obesity: Evidence for rarefaction, macrophage chemotaxis, and inflammation without an angiogenic response. *Diabetes* 2009; 58: 718-725.
20. Trayhurn P, Wang B, Wood IS. HIF-1α protein rather than mRNA as a marker of hypoxia in adipose tissue in obesity: Focus on "Inflammation is associated with a decrease of lipogenic factors in omental fat in women," By Poulain-Godefroy et al. *Am J Physiol Regul Integr Comp Physiol* 2008; 295: R1097.
21. Lolmède K, Durand de Saint Front V, Galitzky J, Lafontan M, Bouloumié A. Effects of hypoxia on the expression of proangiogenic factors in differentiated 3T3-F442A adipocytes. *Int J Obesity* 2003; 27: 1187-1195.
22. Chen B *et al*. Hypoxia dysregulates the production of adiponectin and plasminogen activator inhibitor-1 independent of reactive oxygen species in adipocytes. *Biochem Biophys Res Commun* 2006; 341: 549-556.
23. Wang B, Wood IS, Trayhurn P. Dysregulation of the expression and secretion of inflammation-related adipokines by hypoxia in human adipocytes. *Pflügers Archiv Eur J Physiol* 2007; 455: 479-492.
24. Wood IS, Wang B, Lorente-Cebrián S, Trayhurn P. Hypoxia increases expression of selective facilitative glucose transporters (GLUT) and 2-deoxy-D-glucose uptake in human adipocytes. *Biochem Biophys Res Commun* 2007; 361: 468-473.
25. Regazzetti C, Peraldi P, Grémeaux T, Najem-Lendom R,

Ben-Sahra I, Cormont M, *et al*. Hypoxia decreases insulin signaling pathways in adipocytes. *Diabetes* 2009; 58: 95-103.
26. Wabitsch M, Brenner RE, Melzner I, Braun M, Möller P, Heinze E, *et al*. Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes Relat Metab Disord* 2001; 25: 8-15.
27. Wood IS, Wang B, Trayhurn P. Il-33, a recently identified interleukin-1 gene family member, is expressed in human adipocytes. *Biochem Biophys Res Commun* 2009; 384: 105-109.
28. Wang B, Wood IS, Trayhurn P. PCR arrays identify metallothionein-3 as a highly hypoxia-inducible gene in human adipocytes. *Biochem Biophys Res Commun* 2008; 368: 88-93.
29. Tanji K, Irie Y, Uchida Y, Mori F, Satoh K, Mizushima Y, *et al*. Expression of metallothionein-III induced by hypoxia attenuates hypoxia-induced cell death in vitro. *Brain Res* 2003; 976: 125-129.
30. Pérez de Herédia F, Wood IS, Trayhurn P. Hypoxia modulates monocarboxylate transporter (MCT) expression in human adipocytes. *Proc Nutr Soc* 2009; 68: in print.
31. Halestrap A, Meredith D. The SLC16 gene family – from monocarboxylate transporters (MCTS) to aromatic amino acid transporters and beyond. *Pflügers Archiv Eur J Physiol* 2004; 447: 619-628.
32. Ullah MS, Davies AJ, Halestrap AP. The plasma membrane lactate transporter MCT4, but not MCT1, is up-regulated by hypoxia through a HIF-1α-dependent mechanism. *J Biol Chem* 2006; 281: 9030-9037.
33. Semenza GL. Oxygen-dependent regulation of mitochondrial respiration by hypoxia-inducible factor 1. *Biochem J* 2007; 405: 1-9.
34. Fukuda R, Zhang H, Kim JW, Shimoda L, Dang CV, Semenza GL. HIF-1 regulates cytochrome oxidase subunits to optimize efficiency of respiration in hypoxic cells. *Cell* 2007; 129: 111-122.
35. Pérez de Herédia F, Wood IS, Trayhurn P. Acute and chronic hypoxia selectively modulates the expression of glucose transporters (GLUTs) in human adipocytes. *Obesity Facts* 2009; 2 (Suppl 2): 39.
36. Yin J, Gao Z, He Q, Zhou D, Guo Z, Ye J. Role of hypoxia in obesity-induced disorders of glucose and lipid metabolism in adipose tissue. *Am J Physiol Endocrinol Metab* 2009; 296: E333-E342.
37. Yun Z, Maecker HL, Johnson RS, Giaccia AJ. Inhibition of PPAR gamma 2 gene expression by the HIF-1-regulated gene DEC1/stra13: A mechanism for regulation of adipogenesis by hypoxia. *Dev Cell* 2002; 2: 331-341.
38. Zhou S, Lechpammer S, Greenberger JS, Glowacki J. Hypoxia inhibition of adipocytogenesis in human bone marrow stromal cells requires transforming growth factor-β/Smad3 signaling. *J Biol Chem* 2005; 280: 22688-22696.
39. Kim KH, Song MJ, Chung J, Park H, Kim JB. Hypoxia inhibits adipocyte differentiation in a HDAC-independent manner. *Biochem Biophys Res Commun* 2005; 333: 1178-1184.
40. Wang B, Wood IS, Trayhurn P. Hypoxia induces leptin gene expression and secretion in human preadipocytes: Differential effects of hypoxia on adipokine expression by preadipocytes. *J Endocrinol* 2008; 198: 127-134.
41. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW Jr. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003; 112: 1796-1808.
42. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, *et al*. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003; 112: 1821-1830.
43. Lopéz-Barneo J, Pardal R, Ortéga-Saenz P. Cellular mechanism of oxygen sensing. *Annu Rev Physiol* 2001; 63: 259-287.

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REVIEW

ROLE OF LEPTIN IN EARLY METABOLIC PROGRAMMING

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Abstract

Experimental studies in rodents have highlighted the relationship between early postnatal events, undernutrition during pregnancy and/or lactation and the subsequent development of metabolic syndrome, a phenomenon termed developmental programming. However, appearance of metabolic syndrome is dependent not only on prenatal or postnatal predisposition but also on type of nutrition throughout the life cycle. Those experimental findings have been supported by epidemiological data in humans, born to mothers who suffered undernutrition during pregnancy. Leptin is likely involved is such programming and maintaining a critical leptin level during development may allow normal maturation of tissues and pathways involved in metabolic homeostasis, reversing the undesired effects. Leptin disruption during a critical neonatal or prenatal window is sufficient to permanently alter long-term metabolic regulation. Thus, in rodents (in the early postnatal phase), and likely in other species such as primates, and including humans (in the prenatal period), leptin plays a major role in the development of brain circuits which affect future developmental programming of metabolic disease. As postnatal nutritional or therapeutic intervention can ameliorate the consequences of developmental malprogramming, use of leptin as an additive to milk in infant formula which, in contrast to maternal milk, which is devoid of this protein, has been suggested. Alternatively, identification of potential factors elevating leptin levels in maternal milk may also be beneficial. In conclusion, the present data highlight the importance of leptin in the developmental induction of metabolic disease and offer exciting new strategies for therapeutic intervention, by either maternal or neonatal intervention or targeted nutritional manipulation in postnatal life.

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Leptin discovery: hopes and failures

The discovery of leptin, the obese (*ob*) gene product which is not expressed as a functional protein in *ob*/*ob* mice (1), focused the scientific community's attention on its role as an anorexic hormone involved in the negative regulation of food intake. In both wild-type and mutant mice, leptin treatments were found to dramatically reduce body weight by inhibiting food intake and stimulating the depletion of body fat (2-4). However, hopes of using leptin as a therapeutic agent for obesity in humans faded rapidly as it became evident that leptin deficiency was an extremely rare condition in the general population (5). The main source of leptin is white adipose tissue and systemic leptin levels generally reflect concurrent fat mass. As a consequence, hyperleptinemia is found in most cases of obesity. In this context, leptin resistance has been identified as a central feature of the pathogenesis of obesity (6). Leptin activity in the central nervous system (CNS) is dependent on the transport of leptin across the blood-brain barrier (BBB), which is mediated by a saturable receptor-mediated transport system located in the brain microvasculature and choroid plexus (7). Impaired leptin

transport across the BBB has also been proposed as a possible causal mechanism for the development of leptin resistance and obesity (7,8).

Now, over 15 years after its discovery and with over 16,000 leptin-related publications, leptin is known to participate in a wide range of biological functions that include, in addition to its early envisaged function as an adipostat, glucose metabolism, glucocorticoid synthesis, CD4+ T-lymphocyte proliferation, cytokine secretion, phagocytosis, hypothalamic-pituitary-adrenal axis regulation, reproduction, cardiovascular pathology, apoptosis and angiogenesis (9). In short, it is now well-documented that leptin acts like a cytokine hormone with many pleiotropic effects. Furthermore, in recent years, it has become more and more apparent that many of leptin's effects are acquired not only through its central action, but also through its systemic action at the peripheral level.

Postnatal leptin activity in rodents

In the last 5 years, a novel function for leptin has been discovered. In initial experiments carried out by the Auckland group (10,11) and as confirmed by others (12), rats were subjected to maternal undernutrition by providing only 30% of ad-libitum intake to pregnant rat. At weaning, offspring were assigned to either a control diet or a hypercaloric (30% fat) one. Food intake in offspring from undernourished mothers was significantly elevated at an early postnatal age. It increased further with advancing age and was enhanced by the hypercaloric high-fat diet (HFD), accompanied by elevated systolic blood pressure and markedly increased fasting plasma insulin and leptin concentrations. This study (10) was the first to demonstrate that profound adult hyperphagia may be a consequence of fetal programming, a key contributing factor in adult pathophysiology. As undernutrition is characterized by extremely low leptin levels, we investigated the effects of early postnatal leptin treatment of female rat pups on the metabolic phenotype of the adult female offspring. Recombinant rat leptin (2.5 μg/g·day) was injected subcutaneously (sc) from postnatal days 3 to 13. This treatment, followed by exposure to a HFD, resulted in a transient slowing of neonatal weight gain, and normalized caloric intake, locomotor activity, body weight, fat mass, and fasting plasma glucose, insulin, and leptin concentrations in adults, in contrast to the saline-treated offspring of undernourished mothers who developed abnormal levels of all of these features on the HFD (13). This indicated that postnatal programming along with a HFD leading to obesity is reversible. The complete normalization of the programmed phenotype by neonatal leptin treatment implies that leptin reversed the prenatal adaptations resulting from fetal undernutrition.

However, it seems that the postnatal leptin effect may differ between female and male rats. In our recent study with male rat pups (14), the long-term effects of neonatal leptin treatment on body composition and metabolism in male offspring were dependent upon prior developmental programming and postnatal nutrition. Neonatal leptin treatment promoted obesity in male offspring of ad-libitum-fed mothers, particularly when they were given a subsequent HFD, whereas in males born to undernourished mothers, it prevented diet-induced obesity, but only if the animals were fed a standard chow diet. No such protective effect was seen when these male rats were fed a HFD. These findings were in contrast to those found with female pups (13). The results observed for male offspring of mothers who were adequately fed during pregnancy were in agreement with those recently reported by others (15,16). However, as in the latter study only male offspring of normal control dams were treated with leptin, the potential interaction between developmental programming and alterations in neonatal leptin levels was not addressed. In addition, gender-specific differences associated with neonatal leptin treatment were not investigated. Our results were also later confirmed by another group (17-19) who administered leptin orally in physiological doses simulating the natural effects of lactation. They demonstrated that neonate rats treated orally with physiological amounts of leptin during the suckling period are more resistant to age-related increases in body weight in adulthood (17,18) and also more resistant to dietary obesity induced by feeding of a HFD (17). Leptin treatment during lactation prevented excess body-weight gain due to HFD feeding, and these animals had a body weight similar to untreated control animals under a normal-fat diet (17).

To verify the role of leptin in postnatal programming, we treated 2-day-old female rat pups with 10 daily sc injections of a rat leptin antagonist (the higher, more effective dose was 7.5 mg/kg day) which was recently developed in our lab (20). Leptin disruption from day 2 to day 13 led to long-term leptin resistance as evidenced, at 4 months of age, by loss of the treated animals' ability to respond to leptin by reducing food intake and body weight (21). Moreover, the leptin-antagonist-treated animals presented higher susceptibility to diet-induced obesity, as shown by higher body-weight gain when subjected to a HFD, associated with increased adiposity and leptinemia. The susceptibility to obese phenotype was revealed when at the age of 5 months, the rats were transferred from a normocaloric diet to a high-fat, highly palatable diet (Table 1). This observation is reminiscent of that which is usually observed in the case of developmental programming that results from a combination between prenatal undernutrition and postnatal overnutrition. (22). This study clearly demonstrated that in normal rats, independent of adverse fetal programming or genetic de-

Table 1. Effect of rat leptin antagonist (RLA) treatment of neonatal rats on long-term effects tested after 4 and 9 months (m)

Parameter tested	Control[1]	RLA[1]	Significance
Weight after 3 m (g/rat)	250 ± 6	248 ± 5	NS
Leptin after 3 m (ng/ml)	1.3 ± 0.2	1.0 ± 0.1	NS
Δ Food intake in response to leptin after 4 m (%)	–26.2	– 0.0	NM
Δ Weight gain in response to leptin after 4 m (%)	– 4.3 ± 0.8	– 0.0	$p < 0.05$
Weight gain in response to HFD after 9 m (g/rat)	32.9 ± 4.5	49.4 ± 5.0	$p < 0.05$
Abdominal fat in response to HFD after 9 m (g/rat)	29.5 ± 4.4	39.0 ± 4.4	NS
Adiposity in response to HFD after 9 m (%)	8.1 ± 0.5	10.1 ± 0.7	$p = 0.053$
Serum leptin in response to HFD after 9 m (ng/ml)	4.2 ± 0.8	14.4 ± 3.1	$p < 0.05$
Serum insulin in response to HFD after 9 m (ng/ml)	1.2 ± 0.3	1.3 ± 0.3	

[1]The results are presented as mean ± SEM
HFD – high-fat diet, NS – not significant, NM – not measurable

ficiency, leptin disruption during a critical neonatal window is sufficient to permanently alter the long-term metabolic regulation. These results indicated that the postnatal leptin surge, occurring normally in neonatal rodents (23,24), is crucial to ensuring adequate metabolic regulation, and that leptin levels may be a limiting factor during the first 2 weeks of life, at least in rodents, for the onset of normal food-intake regulation. Interestingly, at this stage, leptin regulates metabolic rate, brain circuit formation and hypothalamic neuropeptide expression, but not food intake (25-27).

Involvement of leptin in the development of brain circuits in rodents

The role of the hypothalamus as a main target for leptin action was demonstrated in as early as 1997 in adult animals (28). Later, it was documented that CNS-specific deletion of leptin receptors leads to obesity (29,30). In contrast, transgenic brain-specific restoration of leptin receptors in leptin-receptor-deficient ($Lepr^{db}/Lepr^{db}$) mice reduced obesity (31,32). Within the arcuate nucleus (ARH) located in the third ventricle, leptin acts on two distinct populations of neurons: one is activated by leptin and subsequently promotes weight loss and decreased appetite, expresses α-melanocyte-stimulating hormone (α-MSH; derived from the pro-opiomelanocortin (POMC) precursor and cocaine- and amphetamine-regulated transcript (CART); the other promotes weight gain and is inhibited by leptin, and coexpresses neuropeptide Y (NPY) and *agouti*-related peptide (AgRP) (33). It was documented over 10 years ago that many of the biological effects of leptin are transmitted through projection pathways emanating from these two populations of arcuate neurons to the paraventricular nucleus (PVH) and the lateral hypothalamic area (LHA), as well as to the dorsomedial nucleus (DMH) (33-38). The pioneering study by Bouret and his colleagues (39,40) was the first to demonstrate that leptin acts as a key neurotrophic factor and is cardinal for promoting the maturation of key neural pathways in the hypothalamus, and in particular in the ARH. A series of studies (for the most recent reviews see references 41,42) showed that in mice, leptin is indispensible for the development of the neural circuits responsible for future regulation of food intake in adult animals. It should be noted that those circuits are immature at birth and develop in a time-limited window in the early postnatal phase. Briefly, the innervation of DMH occurs on day 6, that of PVH on days 8-10 and that of LHA on days 10-12. The density of axons from arcuate neurons that innervate other hypothalamic sites involved in the control of energy homeostasis (such as the PVH, DMH, and LHA) is severely reduced in *ob/ob* mouse neonates and remains diminished throughout life (39). Those findings may explain, at least in part, the development of obesity in adult rodents born to dams made hypoleptinemic by maternal undernutrition during pregnancy (43). An interesting observation regarding development of the hypothalamic leptin response was recently made using the intrauterine growth retardation (IUGR) pig model (44-47). We found that in normal-weight piglets leptin receptor expression is mainly localized in the ARH, whereas in female IUGR piglets, it is ~40% lower and localized almost equally to the ARH and PVH (48). Such differences in leptin-receptor distribution may hint at lower leptin sensitivity and in sheep, it has been attributed to lower hypothalamic structural development (49). In conclusion, though leptin does not appear

to regulate food intake or body weight during neonatal life in rodents, its remarkably high plasma levels reported during the postnatal period in both mice, termed "leptin surge" (23,24), and rats (27,50,51) suggests that this metabolically irrelevant surge in fact acts as a developmental signal (24).

Leptin as growth factor

Several reports have indicated that leptin can stimulate cell proliferation in several peripheral organs, such as the kidney (52), pancreas (53) and stomach (54), as well as in osteoblasts (55), breast cancer cells (56), colonic epithelium (57) and gastric mucosa cells (58). In most of these organs and cells, except adipose tissue, leptin also exhibits potent antiapoptotic activity (reviewed in 59). However, such growth-promoting effects may be dependent upon developmental stage, as they are detected mainly when organ maturation is incomplete (such as in IUGR) and they are not always seen in normally developing animals. In recent work, we developed a novel model for studying leptin action using low-birth-weight female piglets taken from the lower third of the litter and weighing between 1.01 and 1.35 kg (48). These IUGR piglets showed a general developmental delay, as evidenced by their reduced body weight and size, as well as diminished growth of almost all organs. Intramuscular leptin administration (0.5 mg/kg day) through postnatal days 2 to 10 induced, within a few days, a rapid increase in the weight and size of IUGR animals and an increase their lean mass to values of control piglets from the upper third of the litter. Analysis of individual organ weights showed an apparent improvement in the growth of organs involved in metabolic regulation, such as the pancreas, liver, and to a minor extent, kidneys. In addition, leptin was able to increase lung weight, which may reflect better maturation of this organ. It should be noted that in normal-body-weight piglets, leptin injections were not effective at increasing organ weight, though overall body weight gain did improve. Leptin treatment also normalized the structural organization of the adipose tissue resulting in a more differentiated stage, and in a 25% lower density of white adipocytes, a lower number of adipocytes, and an apparently equal number of adipocytes in leptin-treated IUGR and control animals. Such changes may affect the susceptibility of IUGR piglets to the development of obesity. In addition, leptin increased brown adipose tissue content, probably leading to improved thermogenic capacity as shown in fetal and newborn sheep (60,61), a factor which is important for neonatal survival.

The role of leptin in prenatal and postnatal human development

Experimental findings in rodents, sheep and pigs raise some fundamental questions: to what extent are these findings relevant to humans and does leptin play any important role? Barker (62,63) focused on the relationship between birth weight and adult disease and suggested a connection between low birth weight and an increased propensity for hypertension, obesity and insulin resistance later in life. Those observations were supported by findings related to the Dutch Famine in the winter of 1944-1945, when reduced maternal caloric intake in late gestation was associated with a higher prevalence of adult obesity occurring in individuals who were of low birth weight at that time. Low birth weight and rapid postnatal weight gain, or catch-up growth, are independent risk factors for the development of obesity and diabetes in adulthood (64,65). Interestingly, individuals with high birth weights (e.g. offspring of mothers with gestational diabetes) exhibit a similar phenotype (64,66,67), indicating that developmental programming may involve a U-shaped relationship between disease prevalence and birth weight. As undernutrition is accompanied by hypoleptinemia, leptin involvement was suggested. Indeed, IUGR children have been shown to have low cord-blood and plasma leptin levels (68), which are associated with rapid postnatal weight gain and a predisposition for developing metabolic syndrome in adulthood (69). Conversely, maternal obesity and/or gestational diabetes results in elevated cord-blood leptin levels and in children being born at increased risk for developing metabolic syndrome (70). Thus a period of relative hypo- or hyperleptinemia may induce maladaptive metabolic changes which contribute to the developmental programming of adult disease (71,72). It seems, therefore, that both the timing and the magnitude of gestational food restriction are critical in determining the obese phenotype. For example, in the Dutch Famine study, the rate of obesity was higher in men exposed in the first half of gestation and lower in men exposed in the last trimester of gestation to maternal undernourishment than in non-exposed men (66). The precise mechanisms responsible for these phenomena may vary with the timing of exposure and are not clear, but there is increasing evidence of intergenerational effects. It has been shown that babies born at both ends of the birth-weight spectrum may develop a phenotype of excess weight gain. In girls, such obesity predisposes them to diabetes in pregnancy, which accelerates a cycle of early diabetes in subsequent generations (73).

The similarity of late phenotypic development in rodents and primates, including humans, occurs despite the fact that hypothalamic neurogenesis in primates occurs in the first quarter of gestation (74,75). The information on human fetal brain architecture, though limited, suggests that early hypothalamic neurogenesis occurs mainly in the ninth and tenth weeks of gestation

(76-80). Furthermore, while the hypothalamic feeding circuits in rodents develop postnatally during the first 2 weeks of life, these circuits appear to develop in utero in primates, including humans. In Japanese macaques, NPY/AgRP fibers are already innervating the PVH in the late second trimester of gestation (81) and in human fetuses, NPY immunoreactive fibers are detected in the ARH and PVH at as early as 21 weeks of gestation (82), showing that development of neural projection in humans occurs later than neurogenesis.

Many epidemiological studies comparing forms of nutrition during lactation are presenting more and more evidence that breastfeeding, but not infant formula, confers protection against obesity later in life. A meta summary of studies on duration of breastfeeding and risk of becoming overweight (83) strongly supports the notion that there is a dose-dependent association between longer duration of breastfeeding and decreased risk of becoming overweight. Though the exact reason for these differences has not yet been elucidated, there is some evidence of leptin involvement. Leptin is present in milk synthesized in the mammary gland (84), and in nursing rats, the leptin in milk has been shown to be transferred from the mother's circulation (85). Leptin concentrations in human milk vary significantly, but there is a positive correlation between leptin concentration in milk and maternal plasma leptin levels and adiposity (86,87), suggesting that the amount of leptin supplied to infants through breast milk depends on the mother's adiposity. Furthermore breast-fed infants have higher plasma leptin values than formula-fed infants (88).

As concluded by Palou and Pico (19), these findings have opened a new area of research on both the use of leptin in the design of more appropriate infant formulas and the identification of potential factors influencing leptin levels in maternal milk, which are aspects of great relevance due to the increasing prevalence of obesity and its associated health complications.

Conclusions

Blockage of leptin actions during a critical period of early life in rodents may have long-term consequences by altering the capacity to respond to leptin in terms of food intake and glucose metabolism during adulthood. This is likely associated with the impaired leptin-dependent neurogenesis and maturation of brain circuits. A similar situation probably exists in other animals as well, such as pigs, sheep, primates and in particular humans, though the timing of the "narrow windows of opportunity" may be temporally different. Milk leptin is also a credible candidate to explain, at least in part, the protective effect of breast feeding on the prevalence of adult obesity and development of metabolic syndrome.

References

1. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; 372: 425–432.
2. Halaas JL, Gajiwala KS, Maffei M, *et al*. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science* 1995; 269: 543–546.
3. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, *et al*. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* 1995; 1: 1155–1161.
4. Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, *et al*. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* 1995; 269: 540–543.
5. Farooqi IS, Jebb SA, Langmack G, Lawrence E, Cheetham CH, Prentice AM, *et al*. Effects of recombinant leptin therapy in a child with congenital leptin deficiency. *N Engl J Med* 1999; 341: 879–884.
6. Zhang Y, Scarpace PJ. The role of leptin in leptin resistance and obesity. *Physiol Behav* 2006; 88: 249–256.
7. Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM. Leptin enters the brain by a saturable system independent of insulin. *Peptides* 1996; 17: 305–311.
8. Banks WA. Blood-brain barrier and energy balance. *Obesity (Silver Spring)* 2006; 14 (Suppl 5): 234S–237S.
9. Gertler A, editor. *Leptin and Leptin Antagonists*. Landes Biosciences, 2009.
10. Vickers MH, Breier BH, Cutfield WS, Hofman PL, Gluckman PD. Fetal origins of hyperphagia, obesity, and hypertension and postnatal amplification by hypercaloric nutrition. *Am J Physiol Endocrinol Metab* 2000; 279: E83–E87.
11. Vickers MH, Breier BH, McCarthy D, Gluckman PD. Sedentary behavior during postnatal life is determined by the prenatal environment and exacerbated by postnatal hypercaloric nutrition. *Am J Physiol Regul Integr Comp Physiol* 2003; 285: R271–R273.
12. Symonds ME, Budge H, Stephenson T, Gardner DS. Experimental evidence for long-term programming effects of early diet. *Adv Exp Med Biol* 2005; 569: 24–32.
13. Vickers MH, Gluckman PD, Coveny AH, Hofman PL, Cutfield WS, Gertler A, *et al*. Neonatal leptin treatment reverses developmental programming. *Endocrinology* 2005; 146: 4211–4216.
14. Vickers MH, Gluckman PD, Coveny AH, Hofman PL, Cutfield WS, Gertler A, *et al*. The effect of neonatal leptin treatment on postnatal weight gain in male rats is dependent on

maternal nutritional status during pregnancy. *Endocrinology* 2008; 149: 1906–1913.

15. Yura S, Itoh H, Sagawa N, Yamamoto H, Masuzaki H, Nakao K, *et al*. Role of premature leptin surge in obesity resulting from intrauterine undernutrition. *Cell Metab* 2005; 1: 371–378.
16. Yura S, Itoh H, Sagawa N, Yamamoto H, Masuzaki H, Nakao K, Kawamura M, *et al*. Neonatal exposure to leptin augments diet-induced obesity in leptin-deficient *ob*/*ob* mice. *Obesity* 2008; 16: 1289–1295.
17. Pico C, Oliver P, Sanchez J, Miralles O, Caimari A, Priego T, *et al*. The intake of physiological doses of leptin during lactation in rats prevents obesity in later life. *Int J Obesity (London)* 2008; 31: 1199–1209.
18. Sanchez J, Priego T, Palou, M., Tobaruela A, Palou A, Pico C. Oral supplementation with physiological doses of leptin during lactation in rats improves insulin sensitivity and affects food preferences later in life. *Endocrinology* 2008; 149: 733–740.
19. Palou A, Pico C. Leptin intake during lactation prevents obesity and affects food intake and food preferences in later life. *Appetite* 2009; 52: 249–252.
20. Salomon G, Niv-Spector L, Gussakovsky EE, Gertler A. Large-scale preparation of biologically active mouse and rat leptins and their L39A/D40A/F41A muteins which act as potent antagonists. *Protein Expr Purif* 2006; 47: 128–136.
21. Attig L, Solomon G, Ferezou J, Abdennebi-Najar L, Taouis M, Gertler A, *et al*. Early postnatal leptin blockage leads to a long-term leptin resistance and susceptibility to diet-induced obesity in rats. *Internat J Obesity* 2008; 32: 1–8.
22. Godfrey KM, Lillycrop KA, Burdge GC, Gluckman PD, Hanson MA. Epigenetic mechanisms and the mismatch concept of the developmental origins of health and disease. *Pediatr Res* 2007; 61: 5R–10R.
23. Devaskar SU, Ollesch C, Rajakumar RA, Rajakumar PA. Developmental changes in ob gene expression and circulating leptin peptide concentrations. *Biochem Biophys Res Commun* 1997; 238: 44–47.
24. Ahima RS, Prabakaran D, Flier JS. Postnatal leptin surge and regulation of circadian rhythm of leptin by feeding. Implications for energy homeostasis and neuroendocrine function. *J Clin Invest* 1998; 101: 1020–1027.
25. Mistry AM, Swick A, Romsos DR. Leptin alters metabolic rates before acquisition of its anorectic effect in developing neonatal mice. *Am J Physiol* 1999; 277: R742–R747.
26. Ahima RS, Hileman SM. Postnatal regulation of hypothalamic neuropeptide expression by leptin: implications for energy balance and body weight regulation. *Regul Pept* 2000; 92: 1–7.
27. Proulx K, Richard D, Walker CD. Leptin regulates appetite-related neuropeptides in the hypothalamus of developing rats without affecting food intake. *Endocrinology* 2002; 143: 4683–4692.
28. Halaas JL, Boozer C, Blair-West J, Fidahusein N, Denton DA, Friedman JM. Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proc Natl Acad Sci USA* 1997; 94: 8878–8883.
29. Cohen P, Zhao C, Cai X, Montez JM, Rohani SC, Feinstein P, *et al*. Selective deletion of leptin receptor in neurons leads to obesity. *J Clin Invest* 2001; 108: 1113–1121.
30. McMinn JE, Liu S-M, Liu H, Dragatsis I, Dietrich P, Ludwig T, *et al*. Neuronal deletion of LepR elicits diabesity in mice without affecting cold tolerance or fertility. *Am J Physiol Endocrinol Metab* 2005; 289: E403–E411.
31. Kowalski TJ, Liu S-M, Leibel RL, Chua SC Jr. Transgenic complementation of leptin-receptor deficiency. I. Rescue of the obesity/diabetes phenotype of LEPR-null mice expressing a LEPR-B transgene. *Diabetes* 2001; 50: 425–435.
32. de Luca C, Kowalski TJ, Zhang Y, Elmquist JK, Lee C, Kilimann MW, *et al*. Complete rescue of obesity, diabetes, and infertility in db/db mice by neuron-specific LEPR-B transgenes. *J Clin Invest* 2005; 115: 3484–3493.
33. Elmquist JK, Coppari R, Balthasar N, Ichinose M, Lowell BB. Identifying hypothalamic pathways controlling food intake, body weight, and glucose homeostasis. *J Comp Neurol* 2005; 493: 63–71.
34. Sawchenko PE. Toward a new neurobiology of energy balance, appetite, and obesity: the anatomists weigh in. *J Comp Neurol* 1998; 402: 435–441.
35. Cone RD, Cowley MA, Butler AA, Fan W, Marks DL, Low MJ. The arcuate nucleus as a conduit for diverse signals relevant to energy homeostasis. *Int J Obes Relat Metab Disord* 2001; 25: S63–S67.
36. Elmquist JK, Ahima RS, Elias CF, Flier JS, Saper CB. Leptin activates distinct projections from the dorsomedial and ventromedial hypothalamic nuclei. *Proc Natl Acad Sci USA* 1998; 95: 741–746.
37. Elias CF, Aschkenasi C, Lee C, Kelly J, Ahima RS, Bjorbæk C, *et al*. Leptin differentially regulates NPY and POMC neurons projecting to the lateral hypothalamic area. *Neuron* 1999; 23: 775–786.
38. Thompson RH, Swanson LW. Organization of inputs to the dorsomedial nucleus of the hypothalamus: a reexamination with Fluorogold and PHAL in the rat. *Brain Res Rev* 1998; 27: 89–118.

39. Bouret SG, Draper SJ, Simerly RB. Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science* 2004; 304: 108–110.
40. Bouret SG, Simerly RB. Minireview: leptin and development of hypothalamic feeding circuits. *Endocrinology* 2004; 145: 2621–2626.
41. Bouret SG, Simerly RB. Development of leptin-sensitive circuits. *J Neuroendocrinol* 2007; 19: 575–582.
42. Bouret SG. Early life origins of obesity: role of hypothalamic programming. *J Pediatric Gastroenterol Nutr* 2009; 48: S31–S38.
43. Ferezou-Viala J, Roy AF, Serougne C, Gripois D, Parquet M, Bailleux V, et al. Long-term consequences of maternal high-fat feeding on hypothalamic leptin sensitivity and diet-induced obesity in the offspring. *Am J Physiol Regul Integr Comp Physiol* 2007; 293: R1056-R1062.
44. Poore KR, Forhead AJ, Gardner DS, Giussani DA, Fowden AL. The effects of birth weight on basal cardiovascular function in pigs at 3 months of age. *J Physiol* 2002; 539: 969–978.
45. Poore KR, Fowden AL. The effect of birth weight on glucose tolerance in pigs at 3 and 12 months of age. *Diabetologia* 2002; 45: 1247–1254.
46. Poore KR, Fowden AL. The effect of birth weight on hypothalamopituitary-adrenal axis function in juvenile and adult pigs. *J Physiol* 2003; 547: 107–116.
47. Poore KR, Fowden AL. The effects of birth weight and postnatal growth patterns on fat depth and plasma leptin concentrations in juvenile and adult pigs. *J Physiol* 2004; 558: 295–304.
48. Attig L, Djiane J, Gertler A, Rampin A, Larcher T, Boukthir S, *et al*. Study of hypothalamic leptin receptor expression in low-birth-weight piglets and effects of leptin supplementation on neonatal growth and development. *Am J Physiol Endocrinol Metab* 2008; 295: 1117-1125.
49. Muhlhausler BS, McMillen IC, Rouzaud G, Findlay PA, Marrocco EM, Rhind SM, *et al*. Appetite regulatory neuropeptides are expressed in the sheep hypothalamus before birth. *J Neuroendocrinol* 2004; 16: 502–507.
50. Smith JT, Waddell BJ. Developmental changes in plasma leptin and hypothalamic leptin receptor expression in the rat: peripubertal changes and the emergence of sex differences. *J Endocrinol* 2003; 176: 313–319.
51. Rayner DV, Dalgliesh GD, Duncan JS, Hardie LJ, Hoggard N, Trayhurn P. Postnatal development of the ob gene system: elevated leptin levels in suckling fa/fa rats. *Am J Physiol Regul Integr Comp Physiol* 1997; 273: R446–R450.
52. Wolf G, Hamann A, Han DC, Helmchen U, Thaiss F, Ziyadeh FN, Stahl RA. Leptin stimulates proliferation and TGF-beta expression in renal glomerular endothelial cells: potential role in glomerulosclerosis. *Kidney Int* 1999; 56: 860–872.
53. Tanabe K, Okuya S, Tanizawa Y, Matsutani A, Oka Y. Leptin induces proliferation of pancreatic beta cell line MIN6 through activation of mitogen-activated protein kinase. *Biochem Biophys Res Commun* 1997; 241: 765–768.
54. Islam MS, Sjöholm A, Emilsson V. Fetal pancreatic islets express functional leptin receptors and leptin stimulates proliferation of fetal islet cells. *Int J Obes Relat Metab Disord* 2000; 24: 1246–1253.
55. Gordeladze JO, Drevon CA, Syversen U, Reseland JE. Leptin stimulates human osteoblastic cell proliferation, de novo collagen synthesis and mineralization: impact on differentiation markers, apoptosis and osteoclastic signaling. *J Cell Biochem* 2002; 85: 825–836.
56. Dieudonne MN, Machinal-Quelin F, Serazin-Leroy V, Leneveu MC, Pecquery R, Giudicelli Y. Leptin mediates a proliferative response in human MCF7 breast cancer cells. *Biochem Biophys Res Commun* 2002; 293: 622–628.
57. Hardwick JC, Van Den Brink GR, Offerhaus GJ, Offerhaus GJ, Van Deventer SJ, Peppelenbosch MP. Leptin is a growth factor for colonic epithelial cells. *Gastroenterology* 2001; 121: 79–90.
58. Schneider R, Bornstein SR, Chrousos GP, Boxberger S, Ehninger G, Breidert M. Leptin mediates a proliferative response in human gastric mucosa cells with functional receptor. *Horm Metab Res* 2001; 33: 1–6.
59. Rayalam S, Della-Ferra MA, Ambati S, Baile CA. Is leptin a pro- or anti-apoptoti agent? In: Gertler A, editor. *Leptin and Leptin Antagonists*. Landes Biosciences, 2009; 43-53.
60. Mostyn A, Bispham J, Pearce S, Evens Y, Raver N, Keisler DH, *et al*. Differential effects of leptin on thermoregulation and uncoupling protein abundance in the neonatal lamb. *FASEB J* 2002; 16: 1438–1440.
61. Yuen BS, Owens PC, Muhlhausler BS, Roberts CT, Symonds ME, Keisler DH, *et al.* Leptin alters the structural and functional characteristics of adipose tissue before birth. *FASEB J* 2003; 17: 1102–1104.
62. Barker DJ. The origins of the developmental origins theory. *J Intern Med* 2007; 261: 412–417.
63. Barker DJ. Obesity and early life. *Obes Rev* 2007; Suppl 1: 45–49.
64. Fernandez-Twinn DS, Ozanne SE. Mechanisms by which poor early growth programs type-2 diabetes, obesity and the metabolic syndrome. *Physiol Behav* 2006; 88: 234–243.
65. Jimenez-Chillaron JC, Hernandez-Valencia M, Lightner A, Faucette RR, Reamer C, Przybyla R, *et al.* Reductions in

caloric intake and early postnatal growth prevent glucose intolerance and obesity associated with low birthweight. *Diabetologia* 2006; 49: 1974–1984.

66. Ravelli GP, Stein ZA, Susser MW. Obesity in young men after famine exposure in utero and early infancy. *N Engl J Med* 1976; 295: 349–353.
67. Ravelli AC, van Der Meulen JH, Osmond C, Barker DJ, Bleker OP. Obesity at the age of 50 y in men and women exposed to famine prenatally. *Am J Clin Nutr* 1999; 70: 811–816.
68. Hauguel-de Mouzon S, Lepercq J, Catalano P. The known and unknown of leptin in pregnancy. *Am J Obstet Gynecol* 2006; 194: 1537–1545.
69. Fonseca VM, Sichieri R, Moreira ME, Moura AS. Early postnatal growth in preterm infants and cord blood leptin. *J Perinatol* 2004; 24: 751–756.
70. King JC. Maternal obesity, metabolism, and pregnancy outcomes. *Annu Rev Nutr* 2006; 26: 271–291.
71. Vickers MH, Krechowec SO, Breier BH. Is later obesity programmed in utero? *Curr Drug Target* 2007; 8: 923–934.
72. Vickers MH. Developmental programming of adult obesity: the role of leptin. *Curr Opin Endocrinol Diabetes Obesity* 2007; 14: 17–22.
73. Levin BE. Metabolic imprinting: critical impact of the perinatal environment on the regulation of energy homeostasis. *Philos Trans R Soc Lond B Biol Sci* 2006; 361: 1107–1121.
74. Keyser A. *Development of the Hypothalamus in Mammals*. Marcel Dekker Inc, 1979.
75. van Eerdenburg FJ, Rakic P. Early neurogenesis in the anterior hypothalamus of the rhesus monkey. *Brain Res Dev Brain Res* 1994; 79: 290–296.
76. Bugnon C, Fellmann D, Bresson JL, Clavequin MC. Immunocytochemical study of the ontogenesis of the CRH-containing neuroglandular system in the human hypothalamus. *CR Acad Sci* 1982; 294: 491–496.
77. Burford GD, Robinson IC. Oxytocin, vasopressin and neurophysins in the hypothalamo-neurohypophysial system of the human fetus. *J Endocrinol* 1982; 95: 403–408.
78. Ackland J, Ratter S, Bourne GL, Rees LH. Characterization of immunoreactive somatostatin in human fetal hypothalamic tissue. *Regul Pept* 1983; 5: 95–101.
79. Mai JK, Lensing-Hohn S, Ende AA, Mai JK, Lensing-Hohn S. Developmental organization of neurophysin neurons in the human brain. *J Comp Neurol* 1997; 385: 477–489.
80. Koutcherov Y, Mai JK, Ashwell KW, Paxinos G. Organization of human hypothalamus in fetal development. *J Comp Neurol* 2002; 446: 301–324.
81. Grayson BE, Allen SE, Billes SK, Williams SM, Smith MS, Grove KL. Prenatal development of hypothalamic neuropeptide systems in the nonhuman primate. *Neuroscience* 2006; 143: 975–986.
82. Koutcherov Y. Organization of human hypothalamus in fetal development. *J Comp Neurol* 2002; 446: 301–324.
83. Harder T, Bergmann R, Kallischnigg G, Plagemann A. Duration of breastfeeding and risk of overweight: a meta-analysis. *Am J Epidemiol* 2005; 162: 397–403.
84. Smith-Kirwin SM, O'Connor DM, De Johnston J, Lancey ED, Hassink SG, Funanage VL. Leptin expression in human mammary epithelial cells and breast milk. *J Clin Endocrinol Metab* 1998; 83: 1810–1813.
85. Casabiell X, Pineiro V, Tome MA, Peino R, Dieguez C, Casanueva FF. Presence of leptin in colostrum and/or breast milk from lactating mothers: a potential role in the regulation of neonatal food intake. *J Clin Endocrinol Metab* 1997; 82: 4270–4273.
86. Houseknecht KL, McGuire MK, Portocarrero CP, McGuire MA, Beerman K. Leptin is present in human milk and is related to maternal plasma leptin concentration and adiposity. *Biochem Biophys Res Commun* 1997; 240: 742–747.
87. Miralles O, Sanchez J, Palou A, Pico C. A physiological role of breast milk leptin in body weight control in developing infants. *Obesity (Silver Spring)* 2006; 14: 1371–1377.
88. Savino F, Nanni GE, Maccario S, Costamagna M, Oggero R, Silvestro L. Breast-fed infants have higher leptin values than formula-fed infants in the first four months of life. *J Pediatric Endocrinol Metab* 2004; 17: 1527–1532.

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REVIEW

EFFECT OF 3-HYDROXY-3-METHYLGLUTARYLCOENZYME A REDUCTASE INHIBITORS (STATINS) ON ADIPOSE TISSUE

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Abstract

Statins are competitive inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, a rate-limiting enzyme in cholesterol synthesis. Statins are widely used in the treatment of hypercholesterolemia and to reduce risk of acute cardiovascular and cerebrovascular events. Statins inhibit synthesis of not only cholesterol but also of non-steroid isoprenoids such as farnesyl- and geranylgeranylpyrophosphate, coenzyme Q (ubiquinone), dolichol, etc., which are involved in multiple cell metabolic and signaling cascades. Adipose tissue may be an important target for statins. Although statins have no effect on body weight and energy balance, they inhibit differentiation of preadipocytes to mature adipocytes and may induce adipocyte apoptosis. Stimulation of lipoprotein lipase in adipose tissue accelerates VLDL metabolism and may contribute to triglyceride-lowering effect of statins. According to some studies, statins reduce insulin sensitivity of adipose tissue and impair glucose metabolism in adipocytes. Statins also inhibit adipose tissue inflammation which plays an important role in obesity-associated pathologies. Finally, statins modulate production of adipokines such as leptin, adiponectin, resistin and visfatin. Currently available data suggest that effects on adipose tissue contribute to both beneficial and adverse consequences of statin therapy.

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Key words: adipokines, atherosclerosis, diabetes mellitus, hyperlipidemia, obesity, statins

Introduction

Statins are competitive inhibitors of 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase, a rate limiting enzyme in cholesterol biosynthesis, which converts HMG-CoA to mevalonate (Fig. 1). Currently available statins may be classified into two groups. Natural statins include lovastatin, which is a fungal metabolite, and its synthetic derivatives, pravastatin and simvastatin. Fluvastatin, atorvastatin and rosuvastatin are fully synthetic compounds with completely different chemical structure. Another synthetic statin, cerivastatin, was withdrawn from the market in 2001 due to many reported cases of fatal rhabdomyolysis. A new synthetic statin, pitavastatin, was introduced in 2003, however, until now is available only in Japan and India.

Statins decrease plasma low-density lipoprotein (LDL) cholesterol by inducing intracellular cholesterol depletion and upregulating hepatic LDL receptors. In addition, statins moderately increase HDL-cholesterol and reduce plasma triglycerides. Many clinical trials have demonstrated that statins effectively prevent acute cardi-

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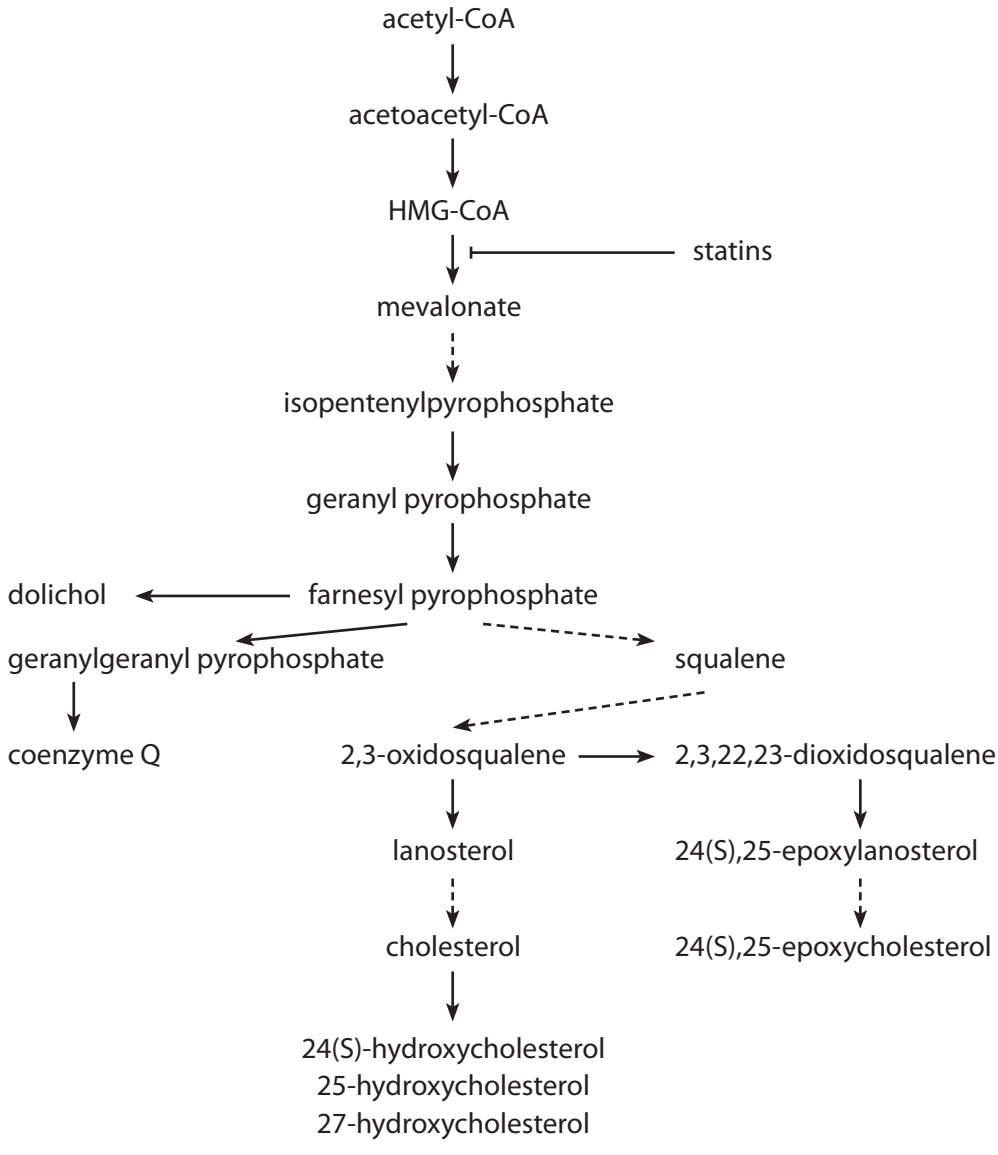

Figure 1. Mevalonate cascade and mechanism of action of statins. Broken arrows abbreviate multiple step reactions. HMG-CoA – 3-hydroxy 3-methylglutarylcoenzyme A.

ovascular events and reduce mortality in primary and secondary prevention of ischemic heart disease (1,2). Initially introduced as cholesterol-lowering drugs, statins posses multiple other lipid-independent or "pleiotropic" atheroprotective activities such as improvement of endothelial function, inhibition of inflammatory reaction, platelet aggregation and thrombosis, and amelioration of oxidative stress. Therefore, beneficial effects of statins are observed not only in patients with hyperlipidemia but also in those with normal cholesterol level. In addition to ischemic heart disease, statins may reduce the risk of ischemic stroke, left ventricular hypertrophy, arrhythmias, Alzheimer's disease, type 2 diabetes mellitus, slow the progression of chronic nephropathies, rheumatoid arthritis and multiple sclerosis, and increase bone mineral density (3-7).

Statins inhibit the rate-limiting step of the mevalonate cascade (Fig. 1); the relevant products of which being not only cholesterol but also many other compounds referred to as nonsteroid isoprenoids. Among them, coenzyme Q (ubiquinone) is an electron carrier in mitochondrial respiratory chain and an important endogenous lipid-soluble antioxidant present in plasma membranes and plasma lipoproteins. Farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP) are posttranslationally attached to various small GTP-binding proteins such as Ras, Rho and Rab which regulate cell growth,

proliferation and survival, intracellular vesicular transport, etc. Dolichol is an essential carrier of carbohydrate moieties used for protein N-glycosylation – the process crucial for transport of membrane-targeted proteins and their biological activities. Depletion of these nonsteroid isoprenoids is responsible for cholesterol-independent pleiotropic activities of statins but also contributes to their adverse effects. Although statins are usually safe and well-tolerated, important complications such as myopathy, hepatotoxicity, polyneuropathy, and gastrointestinal disturbances develop in a small subset of patients.

Lowering plasma cholesterol results mainly from the inhibition of hepatic HMG-CoA reductase, whereas cholesterol-independent effects may be exerted in every cell type. Most currently used statins except pravastatin and rosuvastatin are lipophilic, easily permeate plasma membranes, and affect both hepatic and extrahepatic HMG-CoA reductase. Pravastatin and rosuvastatin are hydrophilic and easily penetrate only into hepatocytes through plasma membrane organic anion transporter. Thus, although they may be as effective as other statins in reducing plasma cholesterol, they much less effectively inhibit mevalonate cascade in extrahepatic cells.

Simvastatin and lovastatin are used as inactive lactones which are *in vivo* enzymatically hydrolyzed to active free acids (8). Simvastatin, lovastatin and atorvastatin are metabolized by cytochrome P450 CY3A4 isoform and their metabolism may be impaired by other substrates or inhibitors of this enzyme (9). Fluvastatin is not metabolized by CYP3A4 but by CYP2C9. Pravastatin and rosuvastatin are the only statins which are in substantial amounts excreted in urine in the unchanged form, although about 10% of administered rosuvastatin is also metabolized by CYP2C9 (10). These hydrophilic statins are metabolized to a much lower degree than other HMG-CoA reductase inhibitors and thus are less prone to interact with other CYP substrates.

Statins are currently used by 25-30 millions people worldwide, mostly by those with recognized cardiovascular diseases or with increased risk of these pathologies. On the other hand, overweight and obesity are important risk factors of hyperlipidemia, atherosclerosis, arterial hypertension and heart failure. In addition, impaired glucose tolerance or type 2 diabetes are frequently observed in overweight/obese subjects and are often accompanied by dyslipidemia. Thus, the large fraction of statin-treated patients have excess of adipose tissue and therefore, the effect of statins on this tissue is clinically significant. However, in comparison to a great body of data about statins accumulated over the last two decades, relatively little is known about their effects on adipose tissue. In this article I review the current knowledge in this field.

Effect of statins on body weight and energy balance

Most studies have shown no effect of statins administered at pharmacological doses on food intake, energy expenditure, body weight and adiposity in animals fed standard diet (11,12). In addition, no gross effect of statins on body weight or adiposity was observed in statin-treated patients. Recently, Araki *et al* (13) have demonstrated that pravastatin (100 mg/kg for 28 days) decreased weight gain and visceral fat accumulation in mice fed high-calorie diet. Moreover, pravastatin increased oxygen consumption and reduced respiratory quotient. These results suggest that statins may prevent the development of obesity by increasing energy expenditure. However, the dose of pravastatin used in this study was higher than in most experimental studies. In addition, these results need to be confirmed for other statins and other models of obesity.

Effect of statins on adipocyte differentiation and survival

Differentiation of preadipocytes to adipocytes is essential for adipose tissue growth and also is a crucial process in the development of obesity. When the amount of triglycerides accumulated per each existing adipocyte reaches the threshold level, novel preadipocytes are recruited to differentiate into mature fat cells and accumulate the surplus of available energy. From this moment, obesity becomes "hyperplastic" (more fat cells) instead of hypertrophic (more triglycerides/cell but unchanged cell number). Hyperplastic obesity is more resistance to treatment since increase in the amount of adipocytes is irreversible. Preadipocyte differentiation is initiated by two transcription factors: peroxisome proliferator-activated receptor-γ (PPAR-γ) and CCAT enhancer-binding protein-α (C/EBP-α), which regulate the expression of adipocyte-specific genes such as enzymes involved in triglyceride storage, leptin, adiponectin etc. On the other hand, preadipocyte factor-1 (Pref-1) is a major inhibitor of preadipocyte differentiation.

Several studies have demonstrated that various statins inhibit preadipocyte differentiation *in vitro*. Nishio *et al* (14) first demonstrated that lovastatin and simvastatin inhibited differentiation of cultured murine 3T3-L1 preadipocytes as evidenced by reduced number of lipid droplets and the amount of triglycerides in statin-treated cells. This effect was ameliorated by mevalonate, farnesyl- or geranylgeranylpyrophosphate but not by squalene or cholesterol. These results indicate that the effect of statins is mediated by depletion of non-steroid isoprenoids. These observations were later confirmed by other authors (15) and also in other cell lines including bone marrow stromal cells (16-18).

Red yeast rice has been used as a natural food colorant and preservative, and as a traditional medicine for improving food digestion and blood circulation in oriental countries. Interestingly, red yeast rice extract, which contains lovastatin, dose-dependently decreased differentiation of 3T3-L1 cells as evidenced by reduced activity of a key enzyme of triglyceride synthesis, glycerol 3-phosphate dehydrogenase (GPDH), decreased triglyceride content, and 30-50% fall in the expression of PPAR-γ, C/EBP-α, adipocyte fatty acid-binding protein-2 (aP2) and leptin (19).

Nicholson *et al* (20) observed that pitavastatin reduced PPAR-γ and increased Pref-1 expression in 3T3-L1 cells while having no effect on PPAR-γ DNA-binding activity. Surprisingly, pitavastatin increased C/EBP-α expression. However, reduction of PPAR-γ and stimulation of Pref-1 were sufficient to inhibit cell differentiation, as evidenced by reduced number of lipid droplets, triglyceride content, fatty acid binding proteins (CD36 and aP2), solute carrier 2A4 (SLC2A4)/glucose transporter GLUT4 expression and adipsin secretion. Interestingly, although these effects of pitavastatin were reproduced by rosuvastatin and simvastatin, they were not prevented by mevalonate or cholesterol, suggesting that anti-adipogenic effect of statins is independent of the inhibition of HMG-CoA reductase. In immortalized murine epididymal preadipocytes, atorvastatin reduced lipid accumulation, C/EBP-α expression and impaired insulin-stimulated lipogenesis (21).

In contrast, Fajas *et al* (22) have shown that treatment of 3T3-L1 cells with either simvastatin or mevastatin increased PPAR-γ expression. This effect was mediated by statin-induced activation of sterol response element-binding protein-1 (SREBP-1) – transcription factor activated by cholesterol depletion. The difference between results of this study and studies mentioned above is most likely associated with culture conditions – authors used cholesterol-free medium which favored statin-induced cholesterol depletion. In cholesterol-replete media statins are unlikely to reduce intracellular cholesterol substantially but reduce non-steroid isoprenoids. In addition, lower statin concentration (0.5 μM vs. 1-10 μM in other studies) was used (22).

Recently, Madsen *et al* (23) have demonstrated that lipophilic simvastatin induces apoptosis of differentiating 3T3-L1 preadipocytes but not of differentiated cells. The effect of simvastatin was prevented by synthetic liver X receptor (LXR) agonists, T0901317 and GW3965. LXR are ligand-activated transcription factors which heterodimerize with the retinoid X receptor and, upon ligand binding, regulate the expression of target genes. LXR are activated by endogenous enzymatically-formed oxygenated cholesterol derivatives (oxysterols) such as 24(S)-, 25- or 27-hydroxycholesterol as well as by 24(S),25-epoxycholesterol, the product of the "shunt pathway" of the mevalonate cascade (Fig. 1). Activated LXR stimulate the expression of genes involved in reverse cholesterol transport, its conversion to bile acids and biliary excretion. In addition, LXR inhibit intestinal cholesterol absorption and cholesterol synthesis. In addition, LXR regulate other processes such as immunity, inflammation, nervous and reproductive system functions. Several studies have demonstrated that statins decrease oxysterol concentrations; especially the level of 24(S),25-epoxycholesterol, and decrease the expression of LXR target genes (24, 25). Madsen *et al*. have demonstrated that proapoptotic effect of simvastatin was not associated with the reduction of either PPAR-γ or SREBP expression or with the inhibition of insulin-like growth factor-1 (IGF-1)-induced activation of prosurvival protein kinase B (PKB)/Akt. In contrast, statin-induced cell death was aggravated by LXRα and LXRβ gene knockouts and was abolished by forced expression of constitutively active LXRα (23). Mauser *et al* (21) have demonstrated that atorvastatin induces apoptosis of differentiating murine epididymal preadipocytes but not of mature adipocytes. This effect resulted from the inhibition of PKB/Akt phosphorylation.

Role of adipose tissue in the effect of statins on plasma lipoproteins

It has been recognized for a long time that plasma cholesterol concentration is proportional to body weight, which suggests the link between adipose tissue and cholesterol metabolism. Indeed, adipose tissue contains more cholesterol than liver, muscle or kidney when expressed on a *per* mg protein basis, and more than all other organs when expressed on a per g of tissue basis. Adipose tissue cholesterol pool constitutes about 25% of the whole-body cholesterol content and may increase up to 50% in obese subjects. However, cholesterol turnover in adipose tissue is relatively slow. The activity of cholesterol biosynthesis pathway in adipocytes is lower than in other tissues and most of cholesterol is provided by plasma lipoproteins. Thus, although lipophilic statins are expected to accumulate in fat cells in substantial amounts, it is unlikely that adipose tissue contributes significantly to statin-induced inhibition of cholesterol synthesis (26). Due to low cholesterol synthesis but high cholesterol demand, especially for a build-up of plasma membrane in rapidly growing adipocyte during triglyceride accumulation, fat cells take-up cholesterol not only from LDL but also from HDL through at least two mechanisms (27): (*i*) scavenger receptor type B1 (SR-B1)-dependent (2/3 of cholesterol uptake), and (*ii*) SR-B1 independent, which requires cholesterol ester transfer protein (CETP), apolipoprotein E and LDL receptor-related protein (LRP). Zhao *et al* (28) have demonstrated that high-cho-

lesterol diet decreases SR-B1 expression in rabbit subcutaneous adipose tissue, whereas atorvastatin administered at 2.5 mg/kg/day for 6 weeks reverses this effect. However, the implications of this effect of atorvastatin for cholesterol balance of adipocytes is unclear.

It is well known that statins reduce not only plasma cholesterol but also triglyceride concentration. The mechanism of the latter effect includes inhibition of hepatic VLDL formation but also enhancement of their clearance. Apart from skeletal muscles, adipose tissue is the most important site of lipoprotein lipase (LPL)-driven VLDL metabolism. The effect of statins on LPL in adipose tissue is controversial. In vitro, pravastatin, simvastatin, atorvastatin and pitavastatin increased LPL expression and activity in 3T3-L1 adipocytes (29,30). In contrast, in vivo studies are not so unambiguous. For instance, atorvastatin and pravastatin increased LPL activity in patients with type 2 diabetes and hypercholesterolemia (31, 32) and simvastatin (but not atorvastatin) had a similar effect in cholesterol-fed rabbits (33). Simvastatin administered at a very high dose (120 mg/kg) for 4 days increased LPL activity in adipose tissue of normal rats. In addition, simvastatin reduced apolipoprotein C-III (the LPL inhibitor) level (34). In contrast, lovastatin (4 mg/kg/day) injected subcutaneously for 13 days had no effect on LPL expression in leptin receptor deficient Zucker fa/fa rats (35). Similarly, atorvastatin given orally for 2 weeks at either 5 or 30 mg/kg/day did not change $mRNA^{LPL}$ level in adipose tissue of fructose-fed rats, a model of hypertriglyceridemia (36). It should be noted that statins reduced plasma triglycerides in both these studies, which indicates that stimulation of adipose tissue LPL is not indispensable for triglyceride-lowering effect of these drugs.

Some studies suggest that statins might affect the balance between triglyceride synthesis and lipolysis in adipose tissue. For example, atorvastatin reduced the expression of acylation-stimulating protein (ASP) and enhanced the expression of hormone-sensitive lipase (HSL) in adipose tissue of fructose-fed rats (36).

Role of adipose tissue in effect of statins on glucose utilization and insulin sensitivity

The effect of statins on glucose metabolism and insulin sensitivity is controversial. Hydrophilic pravastatin has been demonstrated to reduce the incidence of new-onset diabetes by 30% (37). However, several trials have demonstrated worsening of glucose metabolism by simvastatin, atorvastatin and rosuvastatin in patients with pre-existing diabetes (38,39), as well as increase in the rate of onset of new diabetes in non-diabetic patients treated with these drugs (40-44). Takano *et al* (45, 46) have demonstrated that atorvastatin, but not pravastatin or pitavastatin, increases plasma glucose and glycated hemoglobin Hb_{A1c} concentrations in patients with type 2 diabetes. Atorvastatin worsened glucose metabolism in rats with streptozotocin-induced diabetes (47) and in obese, insulin resistant and moderately hyperglycemic NSY mice (39).

Adipose tissue is one of the major sites of glucose disposal and a key target for insulin. Thus, adipose tissue may be the main target for unfavorable effect of statins on glucose metabolism. There are at least three mechanisms through which statins might impair insulin sensitivity of adipocytes. First, as described above, statins inhibit adipocyte differentiation. Differentiated adipocytes are much more insulin-sensitive than non-mature fat cells. Thiazolidinedione derivatives (PPAR-γ agonists), used in the treatment of type 2 diabetes, improve insulin sensitivity partially by stimulating adipocyte differentiation. Nakata *et al* (39) have demonstrated that atorvastatin reduces the expression of SLC2A4/GLUT4, glucose transporter involved in insulin-stimulated glucose uptake, in 3T3-L1 adipocytes, which results from impaired cell differentiation as evidenced by the simultaneous reduction of PPAR-γ and C/EBP-α expression. Simvastatin was 1000 times less potent and pravastatin had no effect at all. Although simvastatin is lipophilic, it is used as an inactive pro-drug (simvastatin lactone) which must be enzymatically hydrolyzed to free acid *in vivo*; this could explain its low potency in cultured adipocytes. In addition, atorvastatin markedly reduced the expression of insulin receptor β-subunit (IR-β). These effects were accompanied by reduced insulin-induced PKB/Akt phosphorylation and glucose uptake. Interestingly, atorvastatin had no effect on SLC2A4/GLUT4 expression in cultured skeletal myocytes indicating that its effect is specific for adipocytes (39). In fully differentiated 3T3-L1 adipocytes the effect of atorvastatin on SLC2A4 expression and insulin-stimulated glucose uptake was still observed but was much less pronounced than in differentiating cells. However, in contrast to immature adipocytes, atorvastatin increased the expression of IR-β and insulin receptor substrate-1 (IRS-1) in fully differentiated adipocytes (39).

Second, insulin stimulates protein farnesyl- and geranylgeranyltransferases (48, 49), and isoprenylated proteins are involved in some aspects of insulin signaling. For example, Rab4 protein is involved in intracellular vesicular transport of SLC2A4/GLUT4 from inactive intracellular pool to the plasma membrane; the key process in insulin-induced glucose uptake. Takaguri *et al* (50) have recently demonstrated that atorvastatin but not pravastatin decreases insulin-induced 2-deoxyglucose uptake by mature 3T3-L1 adipocytes by attenuating insulin-induced translocation of SLC2A4/GLUT4 to the plasma membrane. Atorvastatin had no effect on insulin-induced tyrosine phosphorylation of the IR-β as well as on absolute level of SLC2A4/GLUT4 mRNA and protein, suggesting that impaired translocation of glucose

transporter plays a major role in impairing insulin sensitivity. The effect of atorvastatin was accompanied by the increase in the amount of Rab4 in the cytosolic fraction and decrease in its content in the membrane fraction. Since translocation of Rab4 to the membrane fraction is dependent on its isoprenylation, these data suggest that atorvastatin impairs SLC2A4/GLUT4 translocation secondarily to attenuating isoprenylation of Rab4.

Finally, various statins impaired glycosylation of insulin receptor in 3T3-L1 adipocytes, which resulted in impaired translocation of this receptor to the plasma membrane and accumulation of unglycosylated receptors in endoplasmic reticulum (51). This effect, as well as impairment of insulin-induced glucose uptake, was reproduced by selective inhibitors of protein glycosylation but not by farnesyltransferase inhibitors. Thus, statin-induced dolichol deficiency may impair insulin signaling by interfering with insulin receptor glycosylation.

Statins and adipose tissue inflammation

Recent studies indicate that obesity and the metabolic syndrome are associated with chronic low-grade inflammation of the adipose tissue accompanied by accumulation of macrophages and mast cells which express proinflammatory cytokines such as tumor necrosis factor-α (TNF-α), monocyte chemoattractant protein-1 (MCP-1) or interleukin-6 (IL-6); some of them being synthesized also by adipocytes themselves. Adipose tissue inflammation contributes to insulin resistance and abnormalities of glucose metabolism associated with obesity. Adipose tissue inflammation is driven, at least in part, by activation of lipopolysaccharide (LPS) receptor, Toll-like receptor-4 (TLR-4), by saturated fatty acids, and by hypoxia (see Trayhurn *et al* in this volume of *Adipobiology*). Abe *et al* (52) have demonstrated that pravastatin or pitavastatin administered to leptin-deficient *ob/ob* mice reduced the expression of MCP-1, TNF-α and IL-6 genes in epididymal and subcutaneous adipose tissue. Statins had no effect on body weight as well as on the amount of macrophages in adipose tissue. *In vitro* studies revealed that conditioned medium of cultured LPS-treated macrophages stimulated inflammatory response of adipocytes but this effect was suppressed if macrophages (but not adipocytes) wee pretreated with statins. Indeed, pravastatin and pitavastatin reduced the expression of MCP-1, TNF-α, IL-6 and inducible nitric oxide synthase (iNOS) in LPS-treated macrophages. The TLR-4 receptor triggers two signaling pathways: (*i*) recruitment of Toll/IL-1 receptor (TIR)-domain-containing adaptor protein MyD88, which then activates nuclear factor-κB (NF-κB) and c-Jun N-terminal kinase (JNK), and (*ii*) recruitment of TIR domain-containing adaptor inducing IFN-γ (TRIF), leading to the activation of transcription factor IRF3 which then activates IFN-γ promoter and stimulates its synthesis. Statins inhibited phosphorylation of IRF3, synthesis of IFN-γ, and phosphorylation of its signaling target, STAT1 protein. In contrast, pravastatin or pitavastatin had no effect on MyD88-dependent signaling. Collectively, these data indicate that statins inhibit MyD88-independent TLR-4 signaling in adipose tissue macrophages leading to the attenuation of IFN-γ formation and reduction of proinflammatory response of adipocytes as well as macrophages themselves (52). Treatment with atorvastatin reduced production of C-reactive protein (CRP) (53), IL-6 (54), and TNF-α (55) by adipocytes of cholesterol-fed rabbits. *In vitro*, atorvastatin (54) and cerivastatin (56) decreased IL-6 expression by cultured rabbit and human adipocytes. Finally, simvastatin and pravastatin decreased cytokine-stimulated expression of iNOS in 3T3-L1 adipocytes (57). Reduction of adipose tissue inflammation may contribute to beneficial effects of statins on insulin sensitivity and also to the inhibition of atherogenesis.

Statins and adipokines

Adipokines play an important role in adipose tissue physiology and in obesity-associated complications (58). Herein, I focus on effect of statins on most extensively studied adipokines: leptin, adiponectin, resistin and visfatin.

Leptin

Zhao *et al* (59) have demonstrated that high-cholesterol diet increases plasma leptin concentration in the rabbit more than 2-fold without changing body weight or adiposity. Concomitant treatment with atorvastatin reduced serum leptin and leptin mRNA in subcutaneous adipose tissue simultaneously with decreasing LDL-cholesterol but had no effect on body weight. These data suggest that statins may decrease leptin level. The mechanism of this effect is unclear but may include reduction of either adipose tissue inflammation or oxidative stress because both these conditions stimulate leptin production.

Effect of statins on plasma leptin concentration in humans was addressed in 9 clinical studies (Table 1). In most of them, statins did not change leptin level significantly. One study demonstrated decrease and one increase in leptin. Koh *et al* (63) compared the effect of simvastatin and pravastatin in a crossover study in the same group of hypercholesterolemic patients. They observed that lipophilic simvastatin but not hydrophilic pravastatin increased serum leptin concentration. Although these data suggest that various statins may have divergent effects on leptin level, the overall analysis of data presented in Table 1 indicates that modulation of leptin plays only a minor role in the effect of statins.

Table 1. Effect of statins on plasma leptin concentration in clinical studies

Patients	Number of patients	Treatment	Leptin concentration*	Comments	Ref.
Type 2 diabetes	32	Atorvastatin 40 mg/day 8 weeks	-40%	Placebo-controlled study	60
Non-alcoholic steatohepatitis with hyperlipidemia	31	Atorvastatin 10 mg/day 24 months	No change	No placebo group	61
Overweight with impaired glucose tolerance but not diabetes	30	Simvastatin 20 mg/day 16 weeks	No change	No placebo group	62
Hypercholesterolemia	43	Simvastatin 20 mg/day 8 weeks	+35%	Placebo-controlled study Decrease in insulin sensitivity following simvastatin treatment	63
Hypercholesterolemia	43	Pravastatin 40 mg/day 8 weeks	No change	Placebo-controlled study Increase in insulin sensitivity following pravastatin treatment	63
Healthy non-diabetic volunteers without ischemic heart disease	40	Pravastatin 40 mg/d 12 weeks	No change	Placebo-controlled study	64
Hypercholesterolemia without ischemic heart disease	36	Atorvastatin 10 mg/day 16 weeks	No change	No placebo group, compared to pravastatin-treated group	65
Hypercholesterolemia without ischemic heart disease	36	Pravastatin 10 mg/day 16 weeks	No change	No placebo group, compared to atorvastatin-treated group	65
Type 2 diabetes with hyperlipidemia	29	Atorvastatin 10-40 mg/day 12 weeks	No change	No placebo group	66
Healthy men	24	Simvastatin 10 mg/day 2 weeks	No change	No placebo group, compared to group receiving ezetimibe	67
Hypercholesterolemia	42	Pitavastatin 2 mg/day 12 weeks	No change	No placebo group	68

* Post-treatment vs. pre-treatment percent change of mean or median concentration

Adiponectin

Mauser *et al* (21) have demonstrated that atorvastatin reduced adiponectin expression in differentiated 3T3-L1 adipocytes. In contrast, pravastatin increased adiponectin secretion in the same cell culture, and upregulated adiponectin gene expression and elevated its plasma level in leptin receptor deficient *db/db* mice as well as in high-fat and high-sucrose fed C57BL/6J mice (69). This effect of pravastatin correlated with the improvement of insulin sensitivity and was not accompanied by any changes in body weight. Simvastatin did not change either adiponectin level or insulin sensitivity. Authors suggest that effect on adiponectin may explain differential influence of hydrophilic and lipophilic statins on glucose metabolism (69).

Although leptin is the best characterized adipokine, much more studies addressed the effect of statins on adiponectin in various patient groups. Among them, increase, decrease or no change in adiponectin following statin treatment was noted in 20, 4 and 20 studies, respectively (Table 2). If changes in adiponectin were observed, they were relatively small, rarely exceeding 20-30%. The largest effect was observed for rosuvastatin (73). Authors who observed increase in adiponectin usually suggest that it might contribute to antiatherogenic and antidiabetic

Table 2. Effect of statins on plasma adiponectin concentration in clinical studies

Patients	Number of patients	Treatment	Adiponectin concentration*	Comments	Ref.
Non-alcoholic steatohepatitis with hyperlipidemia	31	Atorvastatin 10 mg/day 24 months	+25%	No placebo group	61
Hypercholesterolemia	43	Pravastatin 40 mg/day 8 weeks	+9%	Placebo-controlled study, Increase in insulin sensitivity during pravastatin treatment	63
Ischemic heart disease with impaired glucose tolerance	20	Pravastatin 20 mg/day 6 months	+35%	Placebo-controlled study Increase in insulin sensitivity during treatment was correlated with increase in adiponectin	70
Type 2 diabetes with hyperlipidemia	64	Pitavastatin 2 mg/day 6 months	+25%	No placebo group	71
Hyperlipidemia with mild hypertension	27	Pravastatin 20 mg/day 6 months	+10%	No placebo group Compared to previous treatment with simvastatin (10 mg/day)	72
Primary hypercholesterolemia	35	Rosuvastatin 10 mg/day 12 weeks	+68%	No placebo group	73
Primary hypercholesterolemia	34	Atorvastatin 10 mg/day 12 weeks	+15%	No placebo group	73
Ischemic heart disease	22	Atorvastatin 10 mg/day 12 weeks	+39%	No placebo group	74
Ischemic heart disease or diabetes or peripheral artery occlusive disease or cerebrovascular disease or a 10-year risk of ischemic heart disease ›20%	102	Atorvastatin 10-80 mg/day 12 weeks	Dose-dependent increase; significant at 40 and 80 mg/day (+25%). Less marked increase in patients with diabetes or metabolic syndrome	No placebo group	75
Hyperlipidemia	72	Pitavastatin 2 mg/day 6 months	+24%	Placebo-controlled study	76
Hypercholesterolemia without ischemic heart disease	36	Atorvastatin 10 mg/day 16 weeks	+7%	No placebo group, compared to pravastatin-treated group	65
Type 2 diabetes	52	Atorvastatin 40 mg/day 8 weeks	Total: no change HMW: +42% MMW: –21% LMW: –23% HMW/total: +25%	Placebo-controlled study	77
Familial combined hyperlipidemia, non-obese patients	22	Atorvastatin 10 mg/day 24 weeks	+13%	No placebo group Compared to fenofibrate-treated group	78
Stable ischemic heart disease with mixed hyperlipidemia	16	Atorvastatin 10 mg/day 4 weeks or 6 months	+25%	Placebo-controlled study	79
Type 2 diabetes	30	Atorvastatin 10 mg/day 12 weeks	+32% (vs. treatment with rosiglitazone alone)	No placebo group Compared to treatment with rosiglitazone alone	80

Patients	Number of patients	Treatment	Adiponectin concentration*	Comments	Ref.
Ischemic heart disease with hypercholesterolemia	115	Pravastatin 10 or 20 mg/day 6 months	+16%	No placebo group	81
Ischemic heart disease; patients undergoing coronary artery bypass grafting (CABG) with LDL-cholesterol >100 mg/dl	32	Pravastatin 10 mg/day 2 months before CABG	+42.3% in serum +59% in visceral adipose tissue Unchanged in subcutaneous adipose tissue +200% (mRNA in visceral adipose tissue)	No follow-up observation, Percent change compared to group receiving no statin (with LDL-cholesterol < 100 mg/dl)	82
Stable ischemic heart disease	16	Pitavastatin 2 mg/day 6 months	+20%	No placebo group	83
Hyperlipidemia with or without diabetes	117	Pitavastatin 2 mg/day 6 months	+25% in diabetic patients No change in non-diabetics	No placebo group	84
Hyperlipidemia with or without type 2 diabetes	75	Pitavastatin 2 mg/day 3 or 6 months	Diabetic patients: +37% (3 months) +64% (6 months) Non-diabetic patients: no change	No placebo group Similar reduction of plasma lipids in diabetic and non-diabetic patients	85
Hypercholesterolemia	43	Simvastatin 20 mg/day 8 weeks	-10%	Placebo-controlled study Decrease in insulin sensitivity by 7% during statin treatment	63
Nondiabetic patients with ischemic heart disease, carotid artery atherosclerosis or leg artery atherosclerosis	43	Simvastatin 40 mg/day 12 weeks	-12%	No placebo group Compared with group treated with pioglitazone	86
Ischemic heart disease - stable angina and normal lipid profile, patients scheduled for coronary angioplasty, treatment started after angioplasty	30	Atorvastatin 10 mg/day 6 months	-20%	Placebo-controlled study	87
Hypercholesterolemia	124	Simvastatin 10-80 mg/day 8 weeks	Dose-dependent decrease (-4 to -10%)	Placebo-controlled study	88
Combined hyperlipidemia	56	Atorvastatin 10 mg/day 8 weeks	No change	Placebo-controlled study	89
Type 1 or type 2 diabetes	77	Atorvastatin 20 mg/day 12 weeks	No change	Placebo-controlled study	90
Overweight with impaired glucose tolerance but not diabetes	30	Simvastatin 20 mg/day 16 weeks	No change	No placebo group	62
Hypertension with hypercholesterolemia	47	Simvastatin 20 mg/day 8 weeks	Non-significant reduction	Placebo-controlled study	91
Type 2 diabetes	53	Simvastatin 20 mg/day 8 weeks	Non-significant reduction	Placebo-controlled study	92

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Patients	Number of patients	Treatment	Adiponectin concentration*	Comments	Ref.
Healthy non-diabetic volunteers without ischemic heart disease	40	Pravastatin 40 mg/day 12 weeks	No change	Placebo-controlled study	64
Non diabetic patients with increased cardiovascular risk (thickened carotid artery intima-media thickness, history of myocardial infarction, proved ischemic heart disease in coronary angiography, unstable angina, cervical or leg artery atherosclerosis, ischemic changes in ECG, stroke, transient ischemic attack, peripheral arterial occlusion)	80	Atorvastatin 40 mg/day 6 months	No change	No placebo group	93
Overweight patients with type 2 diabetes and mixed hyperlipidemia	13	Atorvastatin 10 mg/day 6 weeks	No change	No placebo group	94
Type 2 diabetes with hypertriglyceridemia	194	Atorvastatin 10 or 80 mg/day 6 months	No change	Placebo-controlled study	95
Hyperlipidemia	63	Simvastatin 10 mg/day 6 months	No change	Placebo-controlled study	76
Hypercholesterolemia without ischemic heart disease	36	Pravastatin 10 mg/day 16 weeks	No change	No placebo group	65
Type 2 diabetes with hyperlipidemia	29	Atorvastatin 10-40 mg/day 12 weeks	No change	No placebo group	66
Hypercholesterolemia	32	Atorvastatin 10 mg/day 12 weeks	No change	No placebo group	96
Type 2 diabetes with dyslipidemia	12	Atorvastatin 10 mg/day 8 weeks	No change	No placebo group	97
Kidney transplant recipients	68	Atorvastatin 10 mg/day 12 weeks	No change	No placebo group	98
Hypercholesterolemia	24	Fluvastatin 80 mg/day 12 weeks	No change	No placebo group	99
Non-smoking males with obesity/ metabolic syndrome	15	Simvastatin 80 mg/day 6 weeks	No change following either therapy	No placebo group, cross-over study with simvastatin alone or simvastatin 10 mg/ day+ezetimibe 10 mg/day	100
Healthy men	24	Simvastatin 10 mg/day 2 weeks	No change of total and HMW form	No placebo group, compared to ezetimibe alone or ezetimibe/simvastatin combination	67
Metabolic syndrome	25	Simvastatin 40 mg/day 8 weeks	No change	Placebo-controlled study	101
Hypercholesterolemia	42	Pitavastatin 2 mg/day 12 weeks	No change	No placebo group	68

* Post-treatment vs. pre-treatment percent change of mean or median concentration (if not otherwise stated)
HMW – high-molecular weight adiponectin, MMW – medium-molecular weight adiponectin, LMW – low-molecular weight adiponectin

effect of statins. However, this increase is relatively small e.g. in comparison to PPAR-γ agonists (2-3 fold increase), for which the involvement of adiponectin in insulin-sensitizing effect was demonstrated. In three studies the effect of various statins was directly compared. Koh *et al* (63) observed that pravastatin increased while simvastatin reduced serum adiponectin, which was accompanied by parallel changes in insulin sensitivity. Qu *et al* (73) found that rosuvastatin was much more effective in elevating adiponectin in comparison to atorvastatin, although both drugs similarly reduced LDL-cholesterol. Nomura *et al* (76) found that pitavastatin but not simvastatin slightly elevated adiponectin level, and Ando *et al* (65) observed that atorvastatin but not pravastatin increased adiponectin by 7%. Importantly, only total adiponectin was measured by most authors. Von Eynatten *et al* (77) found that atorvastatin had no effect on total adiponectin but significantly increased high molecular weight (HMW) adiponectin and decreased medium- and low-molecular weight forms. Because HMW adiponectin is a major "beneficial" form of this adipokine, increase in HMW/total adiponectin ratio may markedly improve risk profile of the treated patients. In addition, reciprocal effects on various adiponectin isoforms may explain, at least partially, controversial results of studies in which only total adiponectin was measured. Inami *et al* (84) have found that pitavastatin increases adiponectin level only in diabetic but not in nondiabetic patients with hyperlipidemia. Clearly, baseline profile of risk factors as well as presence or absence of atherosclerosis may affect the effect of statins on adiponectin. Unfortunately, more homogenous patient groups were examined in most studies and the effect in subjects with various risk profiles was not directly compared.

Resistin

Simvastatin inhibited C-reactive protein-induced upregulation of resistin gene expression in human peripheral blood monocytes (102). The effect of simvastatin was reversed by mevalonate and geranylgeranylpyrophosphate but not by farnesylpyrophosphate. Similarly, atorvastatin reduced resistin gene expression in murine 3T3-L1 adipocytes, cultured human preadipocytes and monocyte-macrophages (103, 104).

In most clinical studies, no effect of statins on plasma resistin level was reported (Table 3). Thus, it seems unlikely that reduction of resistin is involved in beneficial effects of statins.

Table 3. Effect of statins on plasma resistin concentration in clinical studies

Patients	Number of patients	Treatment	Resistin concentration*	Comments	Ref.
Type 2 diabetes	32	Atorvastatin 40 mg/day 8 weeks	-40%	Placebo-controlled study	60
Type 1 or type 2 diabetes	77	Atorvastatin 20 mg/day 12 weeks	No change	Placebo-controlled study	90
Overweight patients with type 2 diabetes and mixed hyperlipidemia	13	Atorvastatin 10 mg/day 6 weeks	No change	No placebo group	94
Hypercholesterolemia without ischemic heart disease	36	Atorvastatin 10 mg/day 16 weeks	No change	No placebo group	65
Hypercholesterolemia without ischemic heart disease	36	Pravastatin 10 mg/day 16 weeks	No change	No placebo group	65
Healthy men	24	Simvastatin 10 mg/day 2 weeks	No change	No placebo group, compared to ezetimibe alone or ezetimibe/ simvastatin combination	67
Hypercholesterolemia	32	Atorvastatin 10 mg/day 12 weeks	No change	No placebo group	96
Type 2 diabetes	12	Atorvastatin 10 mg/day 6 months	No change	No placebo group	103
Hypercholesterolemia	42	Pitavastatin 2 mg/day 12 weeks	-11%	No placebo group	68

* Post-treatment vs. pre-treatment percent change of mean or median concentration

Visfatin

Although initially identified as an insulin-sensitizing agent, visfatin is an ambiguous adipokine since it may also induce endothelial dysfunction and promote inflammation thus aggravating atherogenesis. Atorvastatin reduces visfatin gene expression in murine differentiated white adipocytes (21). Until now, the effect of statin therapy on serum visfatin level was examined only in 3 clinical studies. Kostapanos *et al* (105) have demonstrated that rosuvastatin administered at 10 mg/day for 12 weeks reduces serum visfatin by about 10% in patients with primary hyperlipidemia without cardiovascular diseases. In contrast, simvastatin had no effect on visfatin concentration in non-diabetic patients with the metabolic syndrome (106). Similarly, 12-week treatment with atorvastatin did not modify visfatin level in patients with primary hyperlipidemia (107).

Conclusions

Statins have multiple effects in virtually all tissues and adipose tissue is not an exception. Most of currently used statins are lipophilic and thus expected to accumulate in substantial amounts in adipose tissue. Currently available data indicate that although statins have no gross effect on body adiposity, adipose tissue may be the target for both beneficial and adverse effects of these drugs. Statins inhibit adipocyte differentiation, impair insulin signaling in fat cells, inhibit adipose tissue inflammation, and modulate adipokine synthesis and secretion. However, many effects of statins on adipose tissue are controversial, especially their influence on VLDL clearance and adipokine production. Many results were obtained in cultured adipocyte cell lines and thus do not necessarily reflect in vivo situation. More experimental studies are needed to elucidate in more detail effect of statins on adipokines production and the mechanism of these effect, since the results of clinical studies are highly controversial. Due to increasing usage of statins worldwide, elucidating their effects on adipose tissue is important to improve the results of treatment with these drugs.

References

1. Shepherd J, Cobbe SM, Ford I, Isles CG, Lorimer AR, MacFarlane PW, *et al*. Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group. *N Engl J Med* 1995; 333: 1301-1307.
2. Downs JR, Clearfield M, Weis S, Whitney E, Shapiro DR, Beere PA, *et al*. Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study. *JAMA* 1998; 279: 1615-1622.
3. Bersano A, Ballabio E, Lanfranconi S, Mazzucco S, Candelise L, Monaco S. Statins and stroke. *Curr Med Chem*. 2008; 15: 2380-2392.
4. Simko F. Statins: a perspective for left ventricular hypertrophy treatment. *Eur J Clin Invest* 2007; 37: 681-691.
5. Reiss AB, Wirkowski E. Role of HMG-CoA reductase inhibitors in neurological disorders: progress to date. *Drugs* 2007; 67: 2111-2120.
6. Paraskevas KI, Tzovaras AA, Briana DD, Mikhailidis DP. Emerging indications for statins: a pluripotent family of agents with several potential applications. *Curr Pharm Des* 2007; 13: 3622-3636.
7. Adam O, Neuberger HR, Böhm M, Laufs U. Prevention of atrial fibrillation with 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. *Circulation* 2008; 118: 1285-1293.
8. Teiber JF, Draganov DI, La Du BN. Lactonase and lactonizing activities of human serum paraoxonase (PON1) and rabbit serum PON3. *Biochem Pharmacol* 2003; 66: 887-896.
9. Shitara Y, Sugiyama Y. Pharmacokinetic and pharmacodynamic alterations of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors: drug-drug interactions and interindividual differences in transporter and metabolic enzyme functions. *Pharmacol Ther* 2006; 112: 71-105.
10. Windass AS, Lowes S, Wang Y, Brown CD. The contribution of organic anion transporters OAT1 and OAT3 to the renal uptake of rosuvastatin. *J Pharmacol Exp Ther* 2007; 322: 1221-1227.
11. Sandhya VG, Rajamohan T. Comparative evaluation of the hypolipidemic effects of coconut water and lovastatin in rats fed fat-cholesterol enriched diet. *Food Chem Toxicol* 2008; 46: 3586-92.
12. Matafome P, Monteiro P, Nunes E, Louro T, Amaral C, Moedas AR, *et al*. Therapeutic association of atorvastatin and insulin in cardiac ischemia: study in a model of type 2 diabetes with hyperlipidemia. *Pharmacol Res* 2008; 58: 208-214.
13. Araki K, Masaki T, Katsuragi I, Kakuma T, Yoshimatsu H. Effects of pravastatin on obesity, diabetes, and adiponectin in diet-induced obese mice. *Obesity* 2008; 16: 2068-2073.
14. Nishio E, Tomiyama K, Nakata H, Watanabe Y. 3-Hydroxy -3-methylglutaryl coenzyme A reductase inhibitor impairs cell differentiation in cultured adipogenic cells (3T3-L1). *Eur J Pharmacol* 1996; 301: 203-206.
15. Tomiyama K, Nishio E, Watanabe Y. Both wortmannin and simvastatin inhibit the adipogenesis in 3T3-L1 cells during the late phase of differentiation. *Jpn J Pharmacol* 1999; 80: 375-378.
16. Song C, Guo Z, Ma Q, Chen Z, Liu Z, Jia H, *et al*. Simvastatin induces osteoblastic differentiation and inhibits adipo-

cytic differentiation in mouse bone marrow stromal cells. *Biochem Biophys Res Commun* 2003; 308: 458-462.
17. Li X, Cui Q, Kao C, Wang GJ, Balian G. Lovastatin inhibits adipogenic and stimulates osteogenic differentiation by suppressing PPARγ2 and increasing Cbfa1/Runx2 expression in bone marrow mesenchymal cell cultures. *Bone* 2003; 33: 652-659.
18. Liu M, Wang K, Tang T, Dai K, Zhu Z. The effect of simvastatin on the differentiation of marrow stromal cells from aging rats. *Pharmazie* 2009; 64: 43-48.
19. Jeon T, Hwang SG, Hirai S, Matsui T, Yano H, Kawada T, *et al*. Red yeast rice extracts suppress adipogenesis by down -regulating adipogenic transcription factors and gene expression in 3T3-L1 cells. *Life Sci* 2004; 75: 3195-203.
20. Nicholson AC, Hajjar DP, Zhou X, He W, Gotto AM, Han J. Anti-adipogenic action of pitavastatin occurs through the coordinate regulation of PPARγ and Pref-1 expression. *Br J Pharmacol* 2007; 151: 807-815.
21. Mäuser W, Perwitz N, Meier B, Fasshauer M, Klein J. Direct adipotropic actions of atorvastatin: differentiation state-dependent induction of apoptosis, modulation of endocrine function, and inhibition of glucose uptake. *Eur J Pharmacol* 2007; 564: 37-46.
22. Fajas L, Schoonjans K, Gelman L, Kim JB, Najib J, Martin G, *et al*. Regulation of peroxisome proliferator-activated receptor gamma expression by adipocyte differentiation and determination factor 1/sterol regulatory element binding protein 1: implications for adipocyte differentiation and metabolism. *Mol Cell Biol* 1999; 19: 5495-5503.
23. Madsen L, Petersen RK, Steffensen KR, Pedersen LM, Hallenborg P, Ma T, *et al*. Activation of liver X receptors prevents statin-induced death of 3T3-L1 preadipocytes. *J Biol Chem* 2008; 283: 22723-22736.
24. Qiu G, Hill JS. Atorvastatin inhibits ABCA1 expression and cholesterol efflux in THP-1 macrophages by an LXR-dependent pathway. *J Cardiovasc Pharmacol* 2008; 51: 388-395.
25. Bełtowski J. Liver X receptors (LXR) as therapeutic targets in dyslipidemia. *Cardiovasc Ther* 2008; 26: 297-316.
26. Krause BR, Hartman AD. Adipose tissue and cholesterol metabolism. *J Lipid Res* 1984; 25: 97-110.
27. Fazio S, Linton MF. Unique pathway for cholesterol uptake in fat cells. *Arterioscler Thromb Vasc Biol* 2004; 24: 1538-1539.
28. Zhao SP, Wu ZH, Hong SC, Ye HJ, Wu J. Effect of atorvastatin on SR-BI expression and HDL-induced cholesterol efflux in adipocytes of hypercholesterolemic rabbits. *Clin Chim Acta* 2006; 365: 119-124.
29. Saiki A, Murano T, Watanabe F, Oyama T, Miyashita Y, Shirai K. Pitavastatin enhanced lipoprotein lipase expression in 3T3-L1 preadipocytes. *J Atheroscler Thromb* 2005; 12: 163-168.
30. Bey L, Maigret P, Laouenan H, Hamilton MT. Induction of lipoprotein lipase gene expression in 3T3-L1 preadipocytes by atorvastatin, a cholesterol- and triglyceride-lowering drug. *Pharmacology* 2002; 66: 51-56.
31. Endo K, Miyashita Y, Saiki A, Oyama T, Koide N, Ozaki H, *et al*. Atorvastatin and pravastatin elevated pre-heparin lipoprotein lipase mass of type 2 diabetes with hypercholesterolemia. *J Atheroscler Thromb* 2004; 11: 341-347.
32. Schneider JG, von Eynatten M, Parhofer KG, Volkmer JE, Schiekofer S, Hamann A, *et al*. Atorvastatin improves diabetic dyslipidemia and increases lipoprotein lipase activity in vivo. *Atherosclerosis* 2004; 175: 325-331.
33. Verd JC, Peris C, Alegret M, Díaz C, Hernández G, Vázquez M, *et al*. Different effect of simvastatin and atorvastatin on key enzymes involved in VLDL synthesis and catabolism in high fat/cholesterol fed rabbits. *Br J Pharmacol* 1999; 127: 1479-1485.
34. Schoonjans K, Peinado-Onsurbe J, Fruchart JC, Tailleux A, Fiévet C, Auwerx J. 3-Hydroxy-3-methylglutaryl CoA reductase inhibitors reduce serum triglyceride levels through modulation of apolipoprotein C-III and lipoprotein lipase. *FEBS Lett* 1999; 452: 160-164.
35. Kasim SE, LeBoeuf RC, Khilnani S, Tallapaka L, Dayananda D, Jen KL. Mechanisms of triglyceride-lowering effect of an HMG-CoA reductase inhibitor in a hypertriglyceridemic animal model, the Zucker obese rat. *J Lipid Res* 1992; 33: 1-7.
36. Roglans N, Sanguino E, Peris C, Alegret M, Vázquez M, Adzet T, *et al*. Atorvastatin treatment induced peroxisome proliferator-activated receptor alpha expression and decreased plasma nonesterified fatty acids and liver triglyceride in fructose-fed rats. *J Pharmacol Exp Ther* 2002; 302: 232-239.
37. Freeman DJ, Norrie J, Sattar N, Neely RD, Cobbe SM, Ford I, *et al*. Pravastatin and the development of diabetes mellitus: evidence for a protective treatment effect in the West of Scotland Coronary Prevention Study. *Circulation* 2001; 103: 357-362.
38. Sasaki J, Iwashita M, Kono S. Statins: beneficial or adverse for glucose metabolism. *J Atheroscler Thromb* 2006; 13: 123-129.
39. Nakata M, Nagasaka S, Kusaka I, Matsuoka H, Ishibashi S, Yada T. Effects of statins on the adipocyte maturation and expression of glucose transporter 4 (SLC2A4): implications in glycaemic control. *Diabetologia* 2006; 49: 1881-1892.
40. Jula A, Marniemi J, Huupponen R, Virtanen A, Rastas M, Ronnemaa T. Effects of diet and simvastatin on serum lipids, insulin, and antioxidants in hypercholesterolemic men: a randomized controlled trial. *JAMA* 2002; 287: 598-605.
41. Jick SS, Bradbury BD. Statins and newly diagnosed diabetes. *Br J Clin Pharmacol* 2004; 58: 303-309.

42. Heart Protection Study Collaborative Group. MRC/BHF Heart Protection Study of cholesterol lowering with simvastatin in 20,536 high-risk individuals: a randomised placebo-controlled trial. *Lancet* 2002; 360: 7-22.
43. Sever PS, Dahlöf B, Poulter NR, Wedel H, Beevers G, Caulfield M, *et al.* Prevention of coronary and stroke events with atorvastatin in hypertensive patients who have average or lower-than-average cholesterol concentrations, in the Anglo-Scandinavian Cardiac Outcomes Trial – Lipid Lowering Arm (ASCOT-LLA): a multicentre randomised controlled trial. *Lancet* 2003; 361: 1149-1158.
44. Ridker PM, Danielson E, Fonseca FA, Genest J, Gotto AM, Kastelein JJ, *et al*. Rosuvastatin to prevent vascular events in men and women with elevated C-reactive protein. *N Engl J Med* 2008; 359: 2195-2207.
45. Takano T, Yamakawa T, Takahashi M, Kimura M, Okamura A. Influences of statins on glucose tolerance in patients with type 2 diabetes mellitus. *J Atheroscler Thromb* 2006; 13: 95-100.
46. Yamakawa T, Takano T, Tanaka S, Kadonosono K, Terauchi Y. Influence of pitavastatin on glucose tolerance in patients with type 2 diabetes mellitus. *J Atheroscler Thromb* 2008; 15: 269-275.
47. Kanda M, Satoh K, Ichihara K. Effects of atorvastatin and pravastatin on glucose tolerance in diabetic rats mildly induced by streptozotocin. *Biol Pharm Bull* 2003; 26: 1681-1684.
48. Goalstone ML, Wall K, Leitner JW, Kurowski T, Ruderman N, Pan SJ, *et al*. Increased amounts of farnesylated p21Ras in tissues of hyperinsulinaemic animals. *Diabetologia* 1999; 42: 310-316.
49. McCarty MF. Insulin's stimulation of endothelial superoxide generation may reflect up-regulation of isoprenyl transferase activity that promotes rac translocation. *Med Hypotheses* 2002; 58: 472-475.
50. Takaguri A, Satoh K, Itagaki M, Tokumitsu Y, Ichihara K. Effects of atorvastatin and pravastatin on signal transduction related to glucose uptake in 3T3L1 adipocytes. *J Pharmacol Sci* 2008; 107: 80-9.
51. Siddals KW, Marshman E, Westwood M, Gibson JM. Abrogation of insulin-like growth factor-I (IGF-I) and insulin action by mevalonic acid depletion: synergy between protein prenylation and receptor glycosylation pathways. *J Biol Chem* 2004; 279: 38353-38359.
52. Abe M, Matsuda M, Kobayashi H, Miyata Y, Nakayama Y, Komuro R, *et al.* Effects of statins on adipose tissue inflammation: their inhibitory effect on MyD88-independent IRF3/IFN-β pathway in macrophages. *Arterioscler Thromb Vasc Biol* 2008; 28: 871-877.
53. Zhang D, Che D, Zhao S, Sun Y. Effects of atorvastatin on C-reactive protein secretions by adipocytes in hypercholesterolemic rabbits. *J Cardiovasc Pharmacol* 2007; 50: 281-285.
54. Zhao SP, Zhang DQ. Atorvastatin reduces interleukin-6 plasma concentration and adipocyte secretion of hypercholesterolemic rabbits. *Clin Chim Acta* 2003; 336: 103-8.
55. Zhao SP, Wu ZH, Wu J, Hong SC, Deng P. Effect of atorvastatin on tumor necrosis factor alpha serum concentration and mRNA expression of adipose in hypercholesterolemic rabbits. *J Cardiovasc Pharmacol* 2005; 46: 185-189.
56. van Harmelen V, Skurk T, Röhrig K, Hauner H. HMG-CoA reductase inhibitor cerivastatin inhibits interleukin-6 expression and secretion in human adipocytes. *Horm Metab Res* 2003; 35: 466-470.
57. Dobashi K, Araki S, Kubo K, Kawagoe R, Yamamoto Y, Shirahata A. Hydroxymethylglutaryl-CoA reductase inhibitor inhibits induction of nitric oxide synthase in 3T3-L1 preadipocytes. *Life Sci* 2008; 82: 85-90.
58. Chaldakov GN, Fiore M, Tonchev AB, Dimitrov D, Pancheva R, Rancic G, *et al*. *Homo obesus*: a metabotrophin-deficient species. Pharmacology and nutrition insight. *Curr Pharm Des* 2007; 13: 2176-2179.
59. Zhao SP, Wu ZH. Atorvastatin reduces serum leptin concentration in hypercholesterolemic rabbits. *Clin Chim Acta* 2005; 360: 133-140.
60. von Eynatten M, Schneider JG, Hadziselimovic S, Hamann A, Bierhaus A, Nawroth PP, *et al.* Adipocytokines as a novel target for the anti-inflammatory effect of atorvastatin in patients with type 2 diabetes. *Diabetes Care* 2005; 28: 754-755.
61. Hyogo H, Tazuma S, Arihiro K, Iwamoto K, Nabeshima Y, Inoue M, *et al*. Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia. *Metabolism* 2008; 57: 1711-1718.
62. Bulcão C, Giuffrida FM, Ribeiro-Filho FF, Ferreira SR. Are the beneficial cardiovascular effects of simvastatin and metformin also associated with a hormone-dependent mechanism improving insulin sensitivity? *Braz J Med Biol Res* 2007; 40: 229-235.
63. Koh KK, Quon MJ, Han SH, Lee Y, Kim SJ, Park JB, *et al.* Differential metabolic effects of pravastatin and simvastatin in hypercholesterolemic patients. *Atherosclerosis* 2009; 204: 483-490.
64. Gannagé-Yared MH, Azar RR, Amm-Azar M, Khalifé S, Germanos-Haddad M, Neemtallah R, *et al*. Pravastatin does not affect insulin sensitivity and adipocytokines levels in healthy nondiabetic patients. *Metabolism* 2005; 54: 947-951.
65. Ando H, Sugimoto K, Yanagihara H, Tsuruoka S, Saito T, Takamura T, *et al*. Effects of atorvastatin and pravastatin on

glucose tolerance, adipokine levels and inflammatory markers in hypercholesterolaemic patients. *Clin Exp Pharmacol Physiol* 2008; 35: 1012-1017.

66. Chu CH, Lee JK, Lam HC, Lu CC, Sun CC, Wang MC, *et al*. Atorvastatin does not affect insulin sensitivity and the adiponectin or leptin levels in hyperlipidemic Type 2 diabetes. *J Endocrinol Invest* 2008; 31: 42-47.
67. Gouni-Berthold I, Berthold HK, Chamberland JP, Krone W, Mantzoros CS. Short-term treatment with ezetimibe, simvastatin or their combination does not alter circulating adiponectin, resistin or leptin levels in healthy men. *Clin Endocrinol* 2008; 68: 536-541.
68. Ohbayashi H. Pitavastatin improves serum resistin levels in patients with hypercholesterolemia. *J Atheroscler Thromb* 2008; 15: 87-93.
69. Takagi T, Matsuda M, Abe M, Kobayashi H, Fukuhara A, Komuro R, *et al.* Effect of pravastatin on the development of diabetes and adiponectin production. *Atherosclerosis.* 2008; 196: 114-121.
70. Sugiyama S, Fukushima H, Kugiyama K, Maruyoshi H, Kojima S, Funahashi T, *et al*. Pravastatin improved glucose metabolism associated with increasing plasma adiponectin in patients with impaired glucose tolerance and coronary artery disease. *Atherosclerosis* 2007; 194: e43-51.
71. Nomura S, Inami N, Shouzu A, Omoto S, Kimura Y, Takahashi N, *et al*. The effects of pitavastatin, eicosapentaenoic acid and combined therapy on platelet-derived microparticles and adiponectin in hyperlipidemic, diabetic patients. *Platelets* 2009; 20: 16-22.
72. Kai T, Arima S, Taniyama Y, Nakabou M, Kanamasa K. Comparison of the effect of lipophilic and hydrophilic statins on serum adiponectin levels in patients with mild hypertension and dyslipidemia: Kinki Adiponectin Interventional (KAI) Study. *Clin Exp Hypertens* 2008; 30: 530-540.
73. Qu HY, Xiao YW, Jiang GH, Wang ZY, Zhang Y, Zhang M. Effect of atorvastatin versus rosuvastatin on levels of serum lipids, inflammatory markers and adiponectin in patients with hypercholesterolemia. *Pharm Res* 2009; 26: 958-64.
74. Miyagishima K, Hiramitsu S, Kato S, Kato Y, Kitagawa F, Teradaira R, *et al*. Efficacy of atorvastatin therapy in ischaemic heart disease - effects on oxidized low-density lipoprotein and adiponectin. *J Int Med Res* 2007; 35: 534-539.
75. Blanco-Colio LM, Martín-Ventura JL, Gómez-Guerrero C, Masramon X, de Teresa E, Farsang C, *et al*. Adiponectin plasma levels are increased by atorvastatin treatment in subjects at high cardiovascular risk. *Eur J Pharmacol* 2008; 586: 259-265.
76. Nomura S, Shouzu A, Omoto S, Inami N, Shimazu T, Satoh D, *et al*. Effects of pitavastatin on monocyte chemoattractant protein-1 in hyperlipidemic patients. *Blood Coagul Fibrinolysis* 2009; in print.
77. von Eynatten M, Liu D, Bluemm A, Schuster T, Baumann M, Lutz J, *et al*. Changes in adiponectin multimer distribution in response to atorvastatin treatment in patients with type 2 diabetes. *Clin Endocrinol* 2009; 71: 27-32.
78. Arca M, Cambuli VM, Montali A, Sentinelli F, Filippi E, Campagna F, *et al*. Serum adiponectin is decreased in patients with familial combined hyperlipidemia and normolipaemic relatives and is influenced by lipid-lowering treatment. *Nutr Metab Cardiovasc Dis* 2009; in print.
79. Nakamura T, Kodama Y, Takano H, Umetani K, Fujioka D, Saito Y, *et al*. Increase in circulating levels of adiponectin after treatment with statin and fibrate in patients with coronary artery disease and hyperlipidemia. *Atherosclerosis* 2007; 193: 449-451.
80. Chu CS, Lee KT, Lee MY, Su HM, Voon WC, Sheu SH, *et al*. Effects of rosiglitazone alone and in combination with atorvastatin on nontraditional markers of cardiovascular disease in patients with type 2 diabetes mellitus. *Am J Cardiol* 2006; 97: 646-650.
81. Sakamoto K, Sakamoto T, Ogawa H; Kumamoto Joint Research on Hypercholesterolemia Investigators. The effect of 6 months of treatment with pravastatin on serum adiponection concentrations in Japanese patients with coronary artery disease and hypercholesterolemia: a pilot study. *Clin Ther* 2006; 28: 1012-1021.
82. Saito S, Fujiwara T, Matsunaga T, Minagawa K, Fukui K, Fukuda I, *et al*. Increased adiponectin synthesis in the visceral adipose tissue in men with coronary artery disease treated with pravastatin: a role of the attenuation of oxidative stress. *Atherosclerosis* 2008; 199: 378-383.
83. Arao K, Yasu T, Umemoto T, Jinbo S, Ikeda N, Ueda S, *et al*. Effects of pitavastatin on fasting and postprandial endothelial function and blood rheology in patients with stable coronary artery disease. *Circ J* 2009; 73: 1523-1530.
84. Inami N, Nomura S, Shouzu A, Omoto S, Kimura Y, Takahashi N, *et al*. Effects of pitavastatin on adiponectin in patients with hyperlipidemia. *Pathophysiol Haemost Thromb* 2007; 36: 1-8.
85. Nomura S, Shouzu A, Omoto S, Inami N, Tanaka A, Nanba M, *et al*. Correlation between adiponectin and reduction of cell adhesion molecules after pitavastatin treatment in hyperlipidemic patients with type 2 diabetes mellitus. *Thromb Res* 2008; 122: 39-45.
86. Forst T, Pfützner A, Lübben G, Weber M, Marx N, Karagiannis E, *et al*. Effect of simvastatin and/or pioglitazone on insulin resistance, insulin secretion, adiponectin, and proinsulin levels in nondiabetic patients at cardiovascular

risk – the PIOSTAT Study. *Metabolism* 2007; 56: 491-496.
87. Chan KC, Chou HH, Huang CN, Chou MC. Atorvastatin administration after percutaneous coronary intervention in patients with coronary artery disease and normal lipid profiles: impact on plasma adiponectin level. *Clin Cardiol* 2008; 31: 253-258.
88. Koh KK, Quon MJ, Han SH, Lee Y, Ahn JY, Kim SJ, *et al.* Simvastatin improves flow-mediated dilation but reduces adiponectin levels and insulin sensitivity in hypercholesterolemic patients. *Diabetes Care* 2008; 31: 776-782.
89. Koh KK, Quon MJ, Han SH, Chung WJ, Ahn JY, Seo YH, *et al.* Additive beneficial effects of fenofibrate combined with atorvastatin in the treatment of combined hyperlipidemia. *J Am Coll Cardiol* 2005; 45: 1649-53.
90. Shetty GK, Economides PA, Horton ES, Mantzoros CS, Veves A. Circulating adiponectin and resistin levels in relation to metabolic factors, inflammatory markers, and vascular reactivity in diabetic patients and subjects at risk for diabetes. *Diabetes Care* 2004; 27: 2450-2457.
91. Koh KK, Quon MJ, Han SH, Chung WJ, Ahn JY, Seo YH, *et al.* Additive beneficial effects of losartan combined with simvastatin in the treatment of hypercholesterolemic, hypertensive patients. *Circulation* 2004; 110: 3687-3692.
92. Koh KK, Quon MJ, Han SH, Ahn JY, Jin DK, Kim HS, *et al.* Vascular and metabolic effects of combined therapy with ramipril and simvastatin in patients with type 2 diabetes. *Hypertension* 2005; 45: 1088-1093.
93. Forst T, Wilhelm B, Pfützner A, Fuchs W, Lehmann U, Schaper F, *et al.* Investigation of the vascular and pleiotropic effects of atorvastatin and pioglitazone in a population at high cardiovascular risk. *Diab Vasc Dis Res* 2008; 5: 298-303.
94. Otto C, Otto B, Frost RJ, Vogeser M, Pfeiffer AF, Spranger J, *et al.* Short-term therapy with atorvastatin or fenofibrate does not affect plasma ghrelin, resistin or adiponectin levels in type 2 diabetic patients with mixed hyperlipoproteinaemia. *Acta Diabetol* 2007; 44: 65-68.
95. van Hoek M, van Tol A, van Vark-van der Zee LC, Jansen H, Kastelein JJ, Sijbrands EJ, *et al.* Role of plasma adiponectin on the HDL-cholesterol raising effect of atorvastatin in patients with type 2 diabetes. *Curr Med Res Opin* 2009; 25: 93-101.
96. Chu CS, Lee KT, Lee MY, Su HM, Voon WC, Sheu SH, *et al.* Effects of atorvastatin and atorvastatin withdrawal on soluble CD40L and adipocytokines in patients with hypercholesterolaemia. *Acta Cardiol* 2006; 61: 263-269.
97. Yin WH, Jen HL, Chen JW, Lin SJ, Young MS. Differential effects of peroxisome proliferator-activated receptor ligands and sulfonylurea plus statin treatment on plasma concentrations of adipokines in type 2 diabetes with dyslipidemia. *Diabetes Metab* 2006; 32: 229-235.
98. Bayés B, Granada ML, Lauzurica R, Pastor MC, Navarro M, Bonet J, *et al.* Effect of low doses of atorvastatin on adiponectin, glucose homeostasis, and clinical inflammatory markers in kidney transplant recipients. *Transplant Proc* 2005; 37: 3808-3812.
99. Sonmez A, Dogru T, Tasci I, Yilmaz MI, Pinar M, Naharci I, *et al.* The effect of fluvastatin on plasma adiponectin levels in dyslipidaemia. *Clin Endocrinol* 2006; 64: 567-572.
100. Hajer GR, Dallinga-Thie GM, van Vark-van der Zee LC, Olijhoek JK, Visseren FL. Lipid-lowering therapy does not affect the postprandial drop in high density lipoprotein-cholesterol (HDL-c) plasma levels in obese men with metabolic syndrome: a randomized double blind crossover trial. *Clin Endocrinol* 2008; 69: 870-877.
101. Devaraj S, Siegel D, Jialal I. Simvastatin (40 mg/day), adiponectin levels, and insulin sensitivity in subjects with the metabolic syndrome. *Am J Cardiol.* 2007; 100: 1397-1399.
102. Hu WL, Qian SB, Li JJ. Decreased C-reactive protein-induced resistin production in human monocytes by simvastatin. *Cytokine* 2007; 40: 201-206.
103. Ichida Y, Hasegawa G, Fukui M, Obayashi H, Ohta M, Fujinami A, *et al.* Effect of atorvastatin on in vitro expression of resistin in adipocytes and monocytes/macrophages and effect of atorvastatin treatment on serum resistin levels in patients with type 2 diabetes. *Pharmacology* 2006; 76: 34-39.
104. Shyu KG, Chua SK, Wang BW, Kuan P. Mechanism of inhibitory effect of atorvastatin on resistin expression induced by tumor necrosis factor-α in macrophages. *J Biomed Sci* 2009; 16:50.
105. Kostapanos MS, Derdemezis CS, Filippatos TD, Milionis HJ, Kiortsis DN, Tselepis AD, *et al.* Effect of rosuvastatin treatment on plasma visfatin levels in patients with primary hyperlipidemia. *Eur J Pharmacol* 2008; 578: 249-252.
106. Pfützner A, Hanefeld M, Lübben G, Weber MM, Karagiannis E, Köhler C, *et al.* Visfatin: a putative biomarker for metabolic syndrome is not influenced by pioglitazone or simvastatin treatment in nondiabetic patients at cardiovascular risk – results from the PIOSTAT study. *Horm Metab Res* 2007; 39: 764-768.
107. Derdemezis C, Filippatos T, Tselepis A, Mikhailidis D, Elisaf M. Effects of ezetimibe, either alone or in combination with atorvastatin, on serum visfatin levels: a pilot study. *Expert Opin Pharmacother.* 2008; 9: 1829-1837.

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RESEARCH ARTICLE

ADIPOKINE CONCENTRACTIONS ARE SIMILAR IN FEMORAL ARTERY AND CORONARY VENOUS SINUS BLOOD: EVIDENCE AGAINST *IN VIVO* ENDOCRINE SECRETION BY HUMAN EPICARDIAL FAT

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Abstract

Human epicardial adipose tissue expresses and secretes *in vitro* hormones and inflammatory cytokines and chemokines collectively termed adipokines. We hypothesized that human epicardial fat did not secrete adipokines into coronary blood under basal conditions *in vivo*. Adiponectin, leptin, resistin, tumor necrosis factor-α, monocyte chemoattractant protein-1, active plasminogen-activator inhibitor-1, interleukin-1β,-6,-8 and vascular endothelial growth factor were measured simultaneously in femoral arterial (a surrogate for coronary arterial) blood and coronary sinus venous blood from eleven patients, mostly young non-obese women, without known heart disease undergoing cardiac catheterisation for radioablation of supraventricular tachycardia. Mean adipokine concentrations were not significantly different in both vessels. In contrast, free fatty acid levels were significantly higher in femoral arterial than coronary sinus blood in keeping with net uptake of free fatty acids by the myocardium. Femoral artery levels of monocyte chemoattractant protein-1, active plasminogen activator inhibitor-1, leptin and resistin showed positive correlations with BMI in descending order of significance but adiponectin showed no relationship. Values for the other adipokines were below the assay detection limit in several patients negating the use of regression analysis. As opposed to their secretion *in vitro*, the adipokines described above are not secreted into coronary blood by human epicardial adipose tissue under near-normal basal conditions *in vivo* and are more likely released into the interstitium of the myocardium and coronary vessels to function as local paracrine regulators.

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Key words: adipose tissue, adipokines, inflammation, paracrine, gradients

Introduction

The physiological functions of human epicardial adipose tissue (EAT) are not well-defined most likely because this strategically located adipose tissue depot is difficult to access and study, and most of the information about it comes from humans with severe cardiac disease or by inference from animal experiments (1,2). Hypothetically, EAT's functions include lipid storage for myocardial energy use, coronary artery mechanical buffering against arterial wave torsion, coronary artery vasomotion and remodeling, protection of the cardiac and coronary autonomic nerve supply, and secretion of adipokines, a collective definition for white adipose tissue-derived hormones, growth factors, coagulation mediators, and pro-and anti-inflammatory cytokines and chemokines (1-3).

The hormones, adiponectin and leptin, and the cytokines, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), interleukin(IL)-1β and IL-6 are secreted *in vitro* by explants of EAT obtained from patients undergoing coronary artery bypass graft for severe

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coronary atherosclerotic disease (CAD) or heart valve replacements without CAD (4,5). mRNAs for these proteins and resistin, IL-8, active plasminogen-activator inhibitor-1 (aPAI-1), and vascular endothelial growth factor (VEGF) are expressed by EAT sampled intraoperatively (4-7). By definition, the designation of epicardial fat as an endocrine tissue requires that leptin and adiponectin or other adipokines are secreted from EAT into the coronary venous effluent. The results of experiments testing adiponectin handling across the human coronary vascular bed have been controversial. In subjects without angiographic CAD or type 2 diabetes mellitus (T2DM), there was a small (~5%) significant increase in coronary venous sinus (CVS) compared to coronary artery (CA) adiponectin suggesting release of adiponectin from EAT (8). In another report (9), aortic root adiponectin levels were significantly higher (~10-15%) than CVS adiponectin levels in non-diabetic patients without and with CAD suggesting cardiac uptake of this adipokine (9). Secretion of the other adipokines into human coronary blood has not been determined.

The purpose of this study was to measure simultaneous femoral artery (FA) and CVS blood concentrations of adiponectin, leptin, resistin, aPAI-1, MCP-1, TNF-α, IL-1β,-6,-8, VEGF, insulin, glucose, and free fatty acids (FFA) in patients undergoing cardiac catheterization to ablate supraventricular tachycardia (10). We hypothesized that adipokine levels would not be higher in CVS than in FA blood in this group of patients. The rationale for including insulin, glucose and FFA was to use them as comparators of hormone and metabolic substrate metabolism by the heart during the experiment.

Subjects and methods

Patients

Each patient had an established diagnosis of atrioventricular nodal re-entry supraventricular tachycardia (SVT) for which slow pathway radiofrequency catheter ablation (10) was deemed necessary by the cardiologist (EJ). Exclusion criteria were age 16 or under; a left ventricular ejection fraction equal to or less than 50%; evidence of coronary atherosclerosis, cardiomyopathy, chronic valvular heart disease and congestive cardiac failure; past or present cigarette smoking; the presence of acute or chronic pulmonary, hepatic, renal, collagen-vascular, gastrointestinal or neuromuscular disease and T2DM, defined as a fasting blood glucose of 126 or more (11). This study was approved by the local Institutional Review Board. All patients involved gave their informed consent.

Sampling proceedure

In the morning after an overnight fast, patients were given endotracheal general anesthesia in the cardiac catheterization laboratory.Electrocardiographic and hemodynamic monitoring were established. Lactated Ringers solution without glucose was infused via a peripheral vein. Catheters were inserted into a FA for hemodynamic monitoring and into the CVS. The position of the catheter tip in the CVS was confirmed fluoroscopically just before withdrawing blood samples to ensure no mixing of right atrial with CVS blood. Under stable hemodynamic conditions, the first 7-10 ml and 5 ml of blood drawn simultaneously from the FA and CVS catheters respectively were discarded to avoid contamination and 10 ml were drawn from the FA and the CVS over ~30 sec 10 and 5 minutes before the start of atrial and ventricular programmed electrical stimulation. The samples were immediately transferred into heparinised tubes in ice. Plasma was separated at 4C and samples stored at –80C until assayed.

Assays

Glucose was measured by autoanalyser in the hospital laboratory. FFA were measured by Quest Diagnostics, Nichols Institute, San Juan Capistrano, CA, using an *in vitro* enzymatic colorimetric method that recognizes a variety of FFA including palmitic, stearic, arachidonic, oleic, palmitoleic, linolenic and linoleic. Total adiponectin, resistin, TNF-α, MCP-1, IL-1β,-6,-8, aPAI-1, and VEGF were measured by Bioscience Division Laboratories, Millipore Corporation, St Charles, MO using LINCOplex well plate immunoassays with specific antibody-immobilised fluorescent-labelled microsphere beads. Assay sensitivity (MinDC) was 145 pg/ml for adiponectin, 6.7 pg/ml for resistin and 1.3 pg/ml for aPAI-1, and per cent intra-assay and inter-assay variation (% cv) were respectively 3.4 and 13.7 for adiponectin, 2.2 and 17.2 for resistin, and 4.1 and 9.9 for aPAI-1. For each of TNF-α, MCP-1, IL-1β,-6,-8 and VEGF assays, Min DC was 3.2 pg/ml, intra-assay % cv was 5.8-10.5% and inter-assay % cv was 7.0-15.9. Insulin and leptin were measured by double antibody radioimmunassays. For insulin, Min DC was 2μU/ml, intra-assay % cv 3.2 and inter-assay % cv 3.9. For leptin, MinDC was 0.5 ng/ml, intra-assay % cv 5.0 and inter-assay % cv 4.5. Fractionated plasma catecholamines were measured by high pressure liquid chromatography with electrochemical detection (Esoterix Laboratories, Burlington, NC).

Statistical Analysis

FA and CVS concentrations of glucose, FFA, insulin and each adipokine drawn at 10 and at 5 minutes were averaged. For each substance, differences in FA and CVS concentrations were analysed using a 2-tailed Student's t test. A p value of <0.05 was considered to indicate a significant difference.

Results

Patients

The upper part of Table 1 shows the order patients were recruited to the study. In compliance with prespecified selection criteria and by chance, all patients except number 4 were female. Patient 1 (58 yr) and 8 (79 yr) were older, weighed more, had hypertension controlled with one or 2 blood pressure drugs and had normal chest xrays, normal echocardiograms (ECHO), normal ejection fractions (EF) of 60% and normal coronary angiograms.The remaining 9 younger non-obese patients had normal chest xrays, normal ECHO and normal EF (58-65%) and the performance of coronary angiography in them was deemed not to be clinically necessary. Patients 1, 5 and 11 were taking estrogen-containing medications.

Plasma glucose, FFA, insulin and adipokines in FA and CVS blood

Table 1 shows the average of the pre-ablation 10 and 5 min values for glucose, FFA, insulin and each adipokine in FA and CVS blood in each patient and the means for the group. Concentrations of glucose were barely but significantly higher in FA (mean +/-sem,103.3+/-1.7mg/dl) than CVS (102.3+/-1.6 mg/dl),p=0.03. Concentrations of FFA were significantly higher in FA (821+/-99 nmol/ml) than in CVS (703+/-95 nmol/ml), p=0.007. Adiponectin, leptin, resistin, aPAI-1 and MCP-1 were detected in FA and CVS in all subjects and despite variation in values from individual to individual, mean values were not significantly different. However,it should be noted that the p value for the aPAI-1 difference was 0.07 for eleven patients. TNF-α, IL-1β,-6,-8 and VEGF were not detectable in all subjects but for the available number of detected values, the means for FA and CVS blood showed no differences.

As an indicator of sympathetic adrenomedullary activation at the time of blood sampling during the procedure, FA epinephrine and nor-epinephrine concentrations in 8 patients (data not shown) were in the normal range (respectively <100 pg/ml and <400 pg/ml) confirming no catecholamine release under these experimental conditions.

Table 1. Glucose, FFA, insulin and adipokine concentrations in femoral artery (FA) and coronary venous sinus (CVS) blood.

Patient	1		2		3		4		5		6		7		8		9		10		11		Mean ± sem		p
Age/Gender	58	F	36	F	17	F	18	M	47	F	24	F	19	F	79	F	40	F	37	F	26	F	36.5 ± 5.8		
BMI	25.8		19.7		23.9		19.5		21.9		22.6		20.1		40		43.3		40.5		24.1		27.4 ± 2.8		
	FA	CVS	FA	CVS	FA	CVS	FA	CVS	FA	CVS	FA	CVS	FA	CVS	FA	CVS	FA	CVS	FA	CS	FA	CS	FA	CS	
glucose mg/dl	111	110	98	96	97	96	107	104	107	104	101	101	96	97	106	105	112	112	101	101	100	99.5	103.3 ± 1.7	102.3 ± 1.6	0.03
Insulin µU/ml	4.5	4.0	6.5	6.5	7.5	7.0	5.0	7.0	6.0	6.0	16.0	18.0	3.5	3.5	30.0	23.5	34.0	28.0	10.0	9.0	7.5	8.0	11.9 ± 3.2	11.0 ± 2.5	0.31
FFA nmol/ml	675	550	595	740	740	555	455	360	512	410	570	475	765	580	1135	845	975	740	1535	1425	1075	1060	821 ± 99	704 ± 95	0.007
Adiponectin µg /ml	64.3	56.5	12.6	11.7	31.7	28.8	38.0	46.8	20.2	27.4	16.8	15.8	24.1	24.0	18.8	18.8	9.5	8.6	9.9	9.8	14.7	14.7	23.7 ± 4.9	23.9 ± 4.7	.0.88
Leptin ng/ml	10.9	10.9	1.6	1.6	5.8	6.0	1.5	1.5	6.4	6.7	11.6	11.8	2.6	2.7	46.3	41.1	22.3	27.9	11.3	11.2	8.4	8.5	11.8 ± 3.7	11.7 ± 3.9	0.89
Resistin ng/ml	14.8	13.1	17.4	13.4	10.7	11.3	6.8	9.3	8.2	9.8	12.2	16.1	14.4	14.0	21.1	26.0	31.4	29.1	11.7	11.8	13.7	17.2	14.8 ± 2.1	15.6 ± 1.9	0.37
aPAI-1 ng/ml	13.2	13.0	8.7	8.9	14.5	13.2	15.0	21.5	10.8	11.8	5.7	6.8	4.8	5.6	40.3	42.0	28.9	29.6	50.5	53.9	7.4	7.6	18.2 ± 4.6	19.5 ± 4.8	0.07
MCP-1 pg/ml	180	182	137	141	108	109	92	101	196	199	195	189	143	162	375	379	270	280	362	347	166	190.0	202.2 ± 28.7	207.2 ± 27.5	0.15
TN F - α pg/ml	15	14	5	5	234	207	5	5	10	6	ND	ND	ND	ND	ND	ND	7	6	ND	ND	5	5	40.1 ± 32.3	35.4 ± 28.6	0.26
IL-1β pg/ml	160.5	155.0	ND	ND	91.0	73.0	5.5	5.0	18.0	13.5	6.5	5.5	ND	ND	ND	ND	6.5	7.0	ND	ND	ND	ND	48.0 ± 26.4	43.2 ± 24.8	0.15
IL-6 pg/ml	260.0	250.0	9.0	8.0	440.0	386.0	10.0	9.0	254.0	238.0	16.5	8.5	ND	ND	55.5	52.5	30.0	28.0	5.5	4.4	ND	ND	120.1 ± 52.7	109.3 ± 47.8	0.109
IL-8 pg/ml	103.0	93.0	6.0	5.0	162.0	144.0	ND	ND	118.0	107.0	ND	ND	ND	ND	25.5	26.5	4.5	5.5	5.0	6.0	ND	ND	60.5 ± 24.8	55.2 ± 22	0.12
VEGF pg/ml	667	613	14	10	998	943	59	67	543	494	129	75	ND	ND	376	407	ND	ND	277	236	ND	ND	382.9 ± 119.6	355.6 ± 114.1	0.06

ND = values below the assay detection limit (see methods for details)

Regression analysis

Table 2 shows the relationship between BMI and FA concentrations of adipokines, insulin and FFA from 11 patients. In descending order of statistical significance, there were positive correlations between MCP-1, aPAI-1, insulin, FFA, leptin and resistin. Adiponectin and glucose showed no correlation with BMI. Correlations of BMI with the remaining adipokines were not performed because results fell below assay detection limits in several patients invalidating the analysis due to inadequate numbers of data.

Table 2. Regression analysis: femoral artery adipokines, insulin and FFA related to BMI

Substance	n	r	p
MCP-1	11	0.88	0.0003
aPAI-1	11	0.88	0.0003
Insulin	11	0.79	0.003
FFA	11	0.78	0.004
Leptin	11	0.75	0.008
Resistin	11	0.65	0.025

Discussion

In a group of otherwise healthy patients with SVT and no other cardiac abnormalities, we found no significant differences between fasting basal concentrations of adiponectin, leptin, resistin, aPAI-1, MCP-1, TNF-α, IL-1β,-6,-8 and VEGF in FA compared to CVS blood. Given the 7.1% higher aPAI-1 values in CVS than FA (p=0.07), the number of patients examined may not have been sufficient to exclude aPAI-1 release. With this provision, the data supports the hypothesis that these proteins are not secreted into the coronary vein effluent in relatively healthy people and that their physiological function in EAT is probably not endocrine but paracrine whereby they are secreted from EAT directly into the closely apposed myocardium and coronary vessels. The results also suggest that the contribution of EAT to overall adipokine turnover normally is negligible. This does not exclude the possibility that other hormones released from white adipose tissue depots into systemic blood that were not measured in this study such as retinol-binding protein 4 (12), angiotensin II (13) and omentin (14) might be secreted from EAT in endocrine fashion.

The lack of an increase in CVS adipokines relative to FA adipokines observed in this study might be due to the fact that EAT was not in a state of chronic inflammation which is characterized by increased expression of all the adipokines measured except for adiponectin (2,3) and nerve growth factor and brain-derived neurotrophic factor (15) which are decreased. Firstly, most (8 of 11) of the patients were non-obese and likely to have had normal EAT thickness defined by ECHO in a population of healthy women and men of similar BMI (16). In this situation, there would be no stimulus for macrophage infiltration into adipose tissue mediated by adipocyte hypertrophy from weight gain and no generation of inflammatory cytokines by adipocytes and inflammatory cells in the stromal-vascular fractions of EAT (2). It is of interest that we did not observe any step-up in adipokine gradients in 3 patients with stage III obesity but the significance of this finding remains unclear pending further studies. Secondly, at autopsy, macrophage density is low in EAT surrounding normal coronary arteries (17).We infer that this was also the case in our patients who were assumed not to have CAD by virtue of absence of risk factors such as age, smoking, hypertension or diabetes mellitus or by normal coronary angiograms in selected cases. By contrast, in patients with severe CAD, inflammatory adipokines are expressed and secreted in greater amounts from EAT (4,5,7) and therefore the potential exists that one or more adipokines might be released from EAT into coronary blood under pathophysiological circumstances. This issue requires further study.

Regression analysis and correlation of systemic blood adipokines with BMI was not the primary focus of this investigation. Despite this, we noted significant positive correlations between BMI and FA concentrations of leptin and aPAI-1 as previously reported (18,19), and between BMI and resistin. The positive correlation we found between BMI (mean 27.4 kg/m^2) and MCP-1 contrasts with no correlation in a study in which peripheral vein blood was obtained from obese (mean BMI 43.5) otherwise healthy women (20). The difference could be due to different body weights, selection criteria and number of subjects. Total adiponectin did not show the expected inverse relationship with BMI (21), probably because the numbers were too small. In several patients, values for the remaining adipokines were below the assay detection limit likewise reducing the numbers for analysis and limiting any definitive conclusions.

Mean FFA were significantly lower (14.0%) in the CVS than the FA. This difference is an underestimate of myocardial FFA extraction because radioactive tracer-labelled FFA infusions show that while FFA are being taken up, endogenous FFA are simultaneously released from EAT by lipolysis which raises the effluent FFA concentration (22,23).Conclusions about glucose flux across the heart in these experiments cannot be made without the use of radioactive glucose tracers (24).The barely perceptible higher glucose concentration in FA than CS implies glucose turnover at much lower rates compared to FFA, in keeping with the normal myocardial preference for FFA over glucose as en-

ergy substrate in the basal fasting state (25,26). Different results might be observed under non-fasting or hyperglycemic and/or hyperinsulinemic circumstances (24) or under conditions of increased cardiac work and oxygen consumption (25).There was no observed uptake of insulin even though the heart has insulin receptors (27) which mediate insulin removal from circulating blood. Our study has several methodological limitations. Firstly, we assumed that concentrations of adipokines in the FA and the CVA are the same. Ethical constraints did not permit us to place a catheter in the CVA or the aortic root next to the CA for blood sampling. However, sampling of FA blood instead of CVA blood has been used in human heart metabolism experiments during cardiac catheterization (25). Human aortic root and peripheral vein adiponectin concentrations are not significantly different (28) so that sampling the FA closer "upstream" to the coronary orifice would more likely reflect CVA blood than would a peripheral vein sample. These lines of evidence support FA as a surrogate for CVA blood. Secondly, we assumed that blood flow across the coronary vascular bed remained constant during sampling because direct measurements of coronary flow were not made. This was likely to be the case because during the procedure, general hemodynamic parameters were stable and the rate of blood withdrawal from the CVS was slow (24). Thirdly, measurements of arteriovenous differences across the coronary vascular bed are simple representations of net flux and cannot accurately quantitate release or uptake of each substance. For example, we cannot exclude the possibility that adiponectin and leptin may be taken up by the myocardium from the coronary influx at the same time as they are released into the efflux from EAT, resulting in no net change in their transmyocardial values. Lastly, EAT thickness or volumes were not measured but this should not detract from the principle findings of the study. On the contrary, if the effluent concentrations of one or more coronary adipokines had been higher, it would have been essential to quantitate the amount of EAT to explain the out-versus-in differences between them.

Conclusion

We were unable to demonstrate any differences between FA and CVS levels of adiponectin, leptin, resistin, aPAI-1, MCP-1, TNF-α, IL-1β,-6,-8 and VEGF in blood traversing the hearts of subjects under basal conditions. In this context, EAT is not an endocrine organ. Adipokines expressed by human EAT are more likely released into the interstitium of the myocardium and coronary vessels to act as local paracrine regulators *in vivo*.

Acknowledgements

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References

1. Rabkin S. Epicardial fat: Properties, function, and relationship to obesity. *Obesity Rev* 2007;8:253-261.
2. Sacks HS, Fin JN. Human epicardial adipose tissue: a review. *Am Heart J* 2007;153:907-917.
3. Iacobellis G, Barbaro G. The double role of epicardial adipose tissue as a pro- and anti-inflammatory organ. *Horm Metab Res* 2008;40:442-445.
4. Mazurek T, Zhang L, Zalewski A. Mannion JD, Diehl JT, Arafat H, *et al*.Human epicardial adipose tissue is a source of inflammatory mediators.*Circulation* 2003;108:2460-2466.
5. Cheng K-H, Chu L-S, Lee K-T, Lin T-H, Hsieh C-C, Voon W-C, *et al*. Adipoctyokines and proinflammatory mediators from abdominal and epicardial adipose tissue in patients with coronary artery disease. *Int J Obesity* 2007;32:268-274.
6. Baker AR, da Silva NF, Quinn AL, Harte AL, Pagano D, Bonser RS, *et al*. Human epicardial adipose tissue expresses a pathogenic profile of adipocytokines in patients with cardiovascular disease. *Cardiovasc Diabetol* 2006;5:1-7.
7. Kremen J, Dolinkova M, Krajickova D, Blaha J, Anderlova K, Lacinova Z, *et al.* Increased subcutaneous and epicardial adipose tissue production of proinflammatory cytokines in cardiac surgery patients: possible role in post-operative insulin resistance. *J Clin Endocrinol Metab* 2006;91:4620-4627.
8. Date H, Imamura T, Ideguchi T, Kawagoe J, Sumi T, Masuyama H, *et al*. Adiponectin produced in coronary circulation regulates coronary flow reserve in non-diabetic patients with angiographically normal coronary arteries. *Clin Cardiol* 2006;29:211-214.
9. Furuhashi M, Ura N, Moniwa N, Shinshi Y, Kouzu H, Nishihara M, *et al*. Possible impairment of transcardiac utilization of adiponectin in patients with type 2 diabetes. *Diabetes Care* 2004; 27:2217-2221.
10. Lesh MD, Van Hare GF, Epstein LM, Fitzpatrick AP, Scheinman MM, Lee RJ, *et al.* Radiofrequency catheter ablation of atrial arrhythmias. Results and mechanisms. *Circulation* 1994;89:1074-1089.
11. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Follow-up Report on the Diagnosis of Diabetes Mellitus. *Diabetes Care* 2003;26:3160-3167.

12. Graham TC, Yang Q, Blüher M, Hammarstedt A, Ciaraldi TP, Henry RR, *et al.* Retinol-binding protein 4 and insulin resistance in lean, obese and diabetic subjects. *N Eng J Med* 2006;354:2552-2563.
13. Harte A, McTernan P, Chetty R, Coppack S, Katz J, Smith S, *et al.* Insulin-mediated upregulation of the renin angiotensin system in human subcutaneous adipocytes is reduced by rosiglitazone. *Circulation* 2005;111:1954-1961.
14. De Souza Batista CM, Yang RZ, Lee MJ, Glynn NM, Yu DZ, Pray J, *et al*. Omentin plasma levels and gene expression are decreased in obesity. *Diabetes* 2007;56:1655-1661.
15. Manni L, Nikolova V, Vyagova D, Chaldakov GN, Aloe L. Reduced plasma levels of NGF and BDNF in patients with acute coronary syndromes. *Int J Cardiol* 2005;102:169-171.
16. Iacobellis G, Willens HJ, Barbaro G, Sharma AM. Threshold values of high-risk echocardiographic epicardial fat thickness. *Obesity* 2008;16:887-892.
17. Vela D, Buja M, Madjid M, Burke A, Naghavi M, Willerson JT, *et al*. The role of periadventitial fat in atherosclerosis. An adipose subset with potential diagnostic and therapeutic implications. *Arch Pathol Lab Med* 2006;130:108-115.
18. Zhongmin MA, Gingerich RL, Santiago JV, Klein S, Smith CH, Landt M. Radioimmunoassay of leptin in human plasma. *Clin Chem* 1996;42:944-948
19. Kruszynska YT, Yu JG, Olefsky JM, Sobel B. Effects of troglitazone on blood concentrations of plasminogen activator inhibitor 1 in patients with type 2 diabetes and in lean and obese normal subjects. *Diabetes* 2000;49:633-639.
20. Malavazos AE, Ermetici F, Coman C, Corsi MM, Morricone L, Ambrosi B. Influence of epicardial adipose tissue and adipocytokine levels on cardiac abnormalities in visceral obesity. *Int J Cardiol* 2006;121:132-134.
21. Heidemann C, Sun Q, van Dam RM, Meigs JB, Zhang C, Tworoger SS, *et al*. Total and high-molecular weight adiponectin and resistin in relation to the risk for type 2 diabetes in women. *Ann Intern Med* 2008;149:307-316.
22. Wisneski JA, Gertz EW, Nease RA, Mayr M. Myocardial metabolism of free fatty acids. *J Clin Invest* 1987;79:359-366.
23. Nelson RH, Prasad A, Lerman A, Miles JM. Myocardial uptake of circulating triglycerides in non-diabetic patients with heart disease. *Diabetes* 2007;56:527-530.
24. Peterson LR, Herrero P, McGill J, Schechtman KB, Kristieva-Ware Z, Lesniak D, *et al*. Fatty acids and insulin modulate myocardial substrate metabolism in humans with type 1 diabetes. *Diabetes* 2008;57:32-40.
25. Neglia D, De Caterina A, Marraccini P, Natali A, Ciardetti M, Vecoli C, *et al.* Impaired myocardial metabolic reserve and substrate selection flexibility during stress in patients with idiopathic dilated cardiomyopthy. *Am J Physiol Heart Circ Physiol* 2007;293:H3270-H3278.
26. Abel ED. Glucose transport in the heart. *Frontiers Biosci* 2004;9:210-215.
27. Abel ED. Insulin signaling in heart muscle: lessons learned from genetically engineered mouse models. *Curr Hypert Pract Rep* 2004;6:416-423.
28. Takano H, Kodama Y, KittaY, Nakamura T, Obata J, Mende A, *et al.* Transcardiac adiponectin gradient is independently related to endothelial vasomotor function in large and resistance coronary arteries in humans *Am J Physiol Heart Circ Physiol* 2006;291:H2641-H2648.

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RESEARCH ARTICLE

SERUM-BORNE FACTORS IN CANCER PATIENTS WITH ADVANCED CACHEXIA: INFLUENCE ON ADIPOSE CELLS

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Abstract

Background

The clinical syndrome cancer cachexia is recognized by a considerable weight loss being out of proportion to any reduction in energy intake. The underlying mechanisms are not completely known, but the marked weight loss is attributable to depletion of adipose tissue as well as skeletal muscle mass. Enhanced lipolysis in adipocytes, apoptosis of preadipocytes may be important for loss of adipose tissue.

Results

Sera from cachectic cancer patients induced apoptosis in cultured human preadipocytes at a higher rate than sera from non-cachectic cancer patients (control group). There was a tendency towards increased mRNA levels of the pro-apoptotic Bcl-2 gene Bax after incubation of preadipocytes with cachectic sera. Moreover, the mRNA levels of anti-apoptotic Bcl-XL and pro-apoptotic Bcl-XS were increased and decreased, respectively, as compared to incubation with control sera. However, lipolysis was not enhanced in cultured human adipocytes after incubation with sera from cachectic cancer patients as compared to non-cachectic cancer patients.

Methods

Serum samples from cachectic cancer patients (n=8) and non-cachectic cancer patients (n=6) were collected. Human SGBS (Simpson-Golabi-Behmel syndrome) preadipocytes and differentiated adipocytes were incubated in the presence of serum from cachectic and non-cachectic (control) cancer patients. Induction of apoptosis and necrosis was examined by cell staining with Hoechst 342 (HO342) and propidium iodide (PI), respectively. Expression of pro- and anti-apoptotic Bcl-2 genes was measured by quantitative RT-PCR. Lipolysis was monitored by measuring the release of radiolabeled fatty acids.

Conclusion

Our *in vitro* data suggest that apoptosis of preadipocytes can be increased by serum-borne factors in cancer cachexia. Death or survival of preadipocytes may depend on the balance of pro- and anti-apoptotic mediators. Further studies of patients with cancer cachexia will be needed to reveal if the disease involves loss of adipose tissue due to apoptosis of preadipocytes. We could not show that serum-borne factors associated with cachexia have a major impact on lipolysis in cultured human adipocytes.

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Introduction

Cachexia is characterized by marked weight loss in individuals with systemic conditions like cancers or serious infections. The weight loss is reflected as a reduction in both adipose tissue and skeletal muscle mass, and it cannot be explained by anorexia alone because it is not reversed by supplementation of nutrients (1). This suggests that a significant metabolic component is involved (2). Approximately 50 % of late stage diseased cancer patients develop cachexia and the occurrence is especially high among patients with pancreatic and gastric cancers, where about 83-87 % suffers from cachexia (3).

Adipose tissue includes a heterogeneous population of cells like mature white adipocytes, T-cells, dendritic cells, nerve cells and endothelial cells (4). A stem cell population within the adipose stromal compartment can be differentiated *in vitro* toward the adipogenic as well as other lineages (5). Adipose tissues can also communicate with other tissues in the body by production and release of several hormones, termed adipokines, that may act locally or systemically to alter many biological processes like energy expenditure and food intake (6). Dur-

ing starvation, the basal metabolic rate is reduced as the body adapts to conserve energy as well as muscle and adipose tissues (7). In contrast, cancer cachexia is assosiated with enhanced or unchanged energy expenditure and loss of both adipose and muscle tissue (8-11). Two processes may determine adipose tissue mass in cachexia: reduced adipocyte size (hypotrophy) and decreased adipocyte number (hypoplasia).

The hydrolysis of triglycerides from adipose tissue can be regulated by a cAMP-mediated process involving both hormone-sensitive lipase (HSL) and lipid droplet surface proteins like perilipin (12). Hypotrophy of adipocytes seems to arise from an increase in lipolysis, rather than a decrease in lipogenesis (13).

The number of adipocytes present in an organism may be influenced by the adipocyte differentiation process, which generates mature adipocytes from progenitor cells, roughly termed preadipocytes. Depletion of these progenitor cells, occurring by apoptosis or necrosis, may limit the regeneration of adipocytes and result in loss of adipose tissue. The Bcl-2 family is a set of apoptosis-regulatory proteins which act via regulated protein-protein interactions (14,15). The protein family is divided into two groups: the antiapoptotic and proapoptotic family members, based on whether they counteract or promote the apoptotic process. Bcl-2 and Bcl-XL are among the antiapoptotic proteins, preventing mitochondrial release of cytochrome C (16-18), whereas Bax and Bcl-XS are examples of proapoptotic proteins. The different members compete with each other in controlling cytochrome C release from the mitochondria (19). Bax exists in an inactive, cytosolic form that gets inserted into the mitochondrial membrane on a proapoptotic signal and executes its pro-apoptotic activity via release of cytochrome C (20,21). There may be a connection between regulation of expression of certain Bcl-2 family proteins and apoptosis in adipocytes (22,23).

Here, we have investigated the possible role of apoptosis in preadipocytes and lipolysis in mature adipocytes during cancer-induced cachexia.

Results

Clinical data from cancer patients with and without cachexia are presented in Table 1. The patients in the cachectic group had lower BMI and serum albumin than the control group, as expected from the inclusion criteria. In addition, serum concentrations of several adipokines were measured (Table 1). Serum concentrations of interleukin-6 (IL-6) were significantly higher in the cachectic patients compared to the controls (Table 1). Adiponectin levels in serum tended to be elevated, whereas leptin levels tended to be lower, when cachectic patients were compared to control patients (Table 1).

Apoptosis

A pool of precursor preadipocytes can differentiate and replenish the adipose tissue with mature adipocytes (24). This normally maintains the lipid storing capacity of the adipose tissue during the turnover of adipocytes. It is possible that wasting of adipose tissue seen in cachexia involves apoptosis of preadipocytes.

Table 1. Clinical variables and adipokine serum concentrations in advanced cancer patients with and without cachexia (control)

	Cachexia n = 8		Control n = 6		P-value
Age (years)	70	(42 - 78)	69	(49 - 80)	0.95
BMI (kg/m²)	19.0	(15.0 - 20.0)	26.5	(22.0 - 32.0)	< 0.01
Albumin (mg/mL)	32	(25 - 35)	42	(33 - 49)	0.01
Adiponectin (µg/mL)	23.0	(9.0 - 28.6)	12.6	(4.0 - 25.8)	0.11
Resistin (ng/mL)	24.3	(15.9 - 28.1)	32.4	(13.6 - 66.2)	0.40
Leptin (ng/mL)	2.5	(0.0 - 14.0)	13.0	(2.0 - 22.0)	0.09
IL-6 (pg/mL)	29	(4 - 112)	0	(0 - 50)	0.04
TNF-α (pg/mL)	1.75	(0.80 - 3.10)	1.80	(1.10 - 2.70)	0.85

Values are medians (minimum value - maximum value). P-values indicate differences between the groups according to the Mann-Whitney U-test. IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha

To test if serum-borne factors might alter the degree of apoptosis of preadipocytes, we incubated cultured SGBS preadipocytes with sera from cancer patients with or without cachexia. Subsequently, we measured the appearance of apoptosis and necrosis using HO342 and PI, respectively. For validation purposes SGBS preadipocytes were incubated with 100 ng/mL TNF-α, and we could distinguish between viable cells (Fig. 1A), apoptotic cells (Fig. 1B), and necrotic cells (Fig. 1C). SGBS preadipocytes were incubated for 72 h with 10 % serum from the patients. Sera from cachectic patients induced apoptosis in SGBS preadipocytes at a higher rate than sera from control patients (Fig. 1D). The proportions of necrotic cells were similar after incubation with sera from cachectic and non-cachectic patients (Fig. 1E).

To substantiate the involvement of preadipocyte apoptosis in cachexia we measured the mRNA levels by RT-PCR of several apoptosis-regulatory proteins in the Bcl-2 family. Bax and Bcl-XS are proapoptotic proteins whereas Bcl-2 and Bcl-XL are anti-apoptotic proteins. $mRNA^{Bcl\text{-}2}$ was detected in Jurkat cells but not in the SGBS cells (data not shown). In SGBS preadipocytes incubated for 72 h with cachectic sera there was a tendency towards increased $mRNA^{Bax}$ levels as compared with cells incubated with

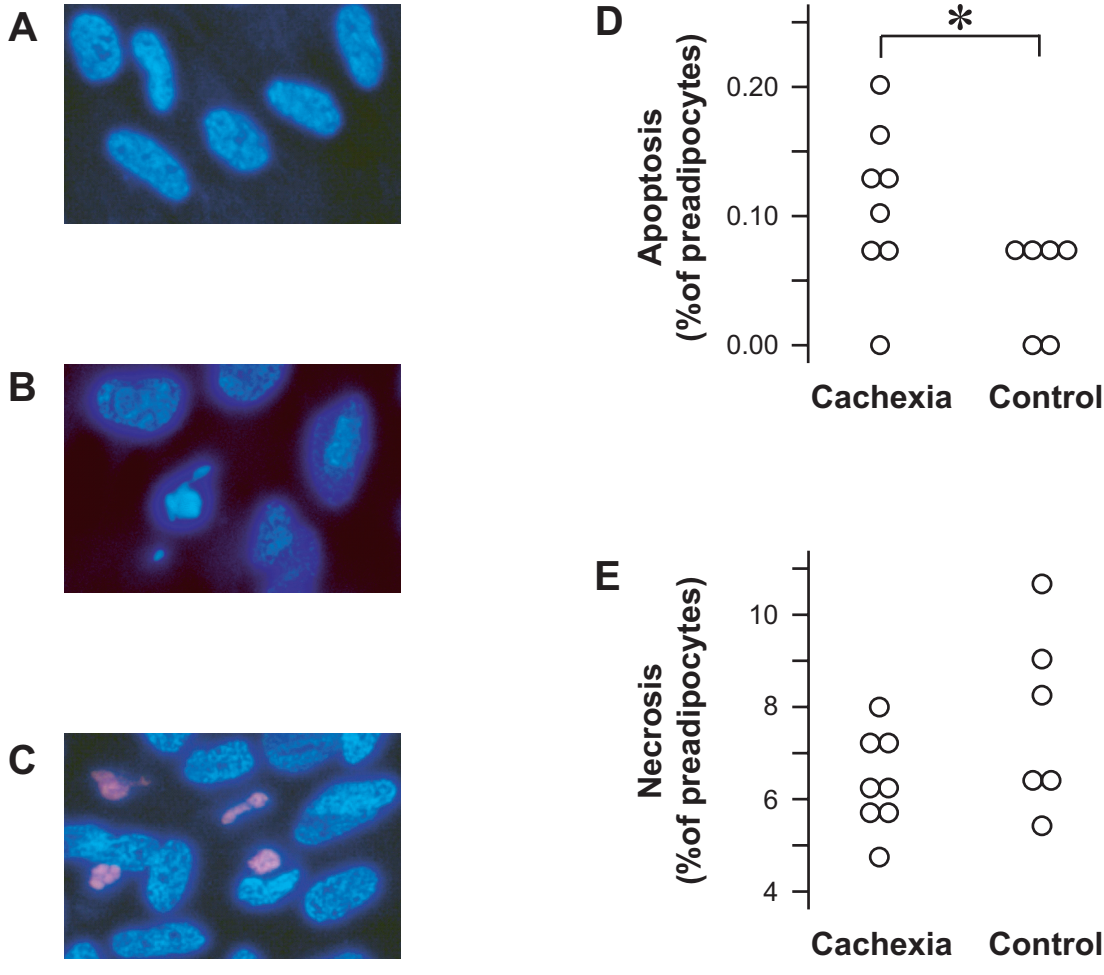

Figure 1. Effect of cachectic patient sera on apoptosis and necrosis in SGBS preadipocytes. Microscopic image of viable (**A**), apoptotic (**B**) and necrotic (**C**) SGBS preadipocytes stained with PI and HO342A after incubation with TNFα (8 h; 100 ng/mL). SGBS preadipocytes were incubated with sera (72 h; 10 %) from patients with cachexia (n=8) or without cachexia (control; n=6) prior to microscopic analysis of apoptosis (**D**) and necrosis (**E**). The charts display the proportion of apoptotic or necrotic cells relative to viable cells; each point represents mean values of two independent cell experiments with serum from one subject.
**P ≤ 0.05 using Mann-Whitney U-test*

control sera (P = 0.16; Fig. 2A). In cells incubated with cachectic sera there was a correlation between $mRNA^{Bax}$ levels and the proportion of apoptotic cells (Fig. 2B). After 72 h incubation with cachectic sera mRNA levels of antiapoptotic Bcl-XL and proapoptotic Bcl-XS were increased and decreased, respectively, as compared to incubation with control sera (Fig. 2D,E).

Prolonged incubation of SGBS preadipocytes with cachectic and control sera (7 days), did not show differences in the proportion of apoptotic and necrotic cells (data not shown). Furthermore, there were no difference in Bax, Bcl-XL and Bcl-XS mRNA levels (data not shown).

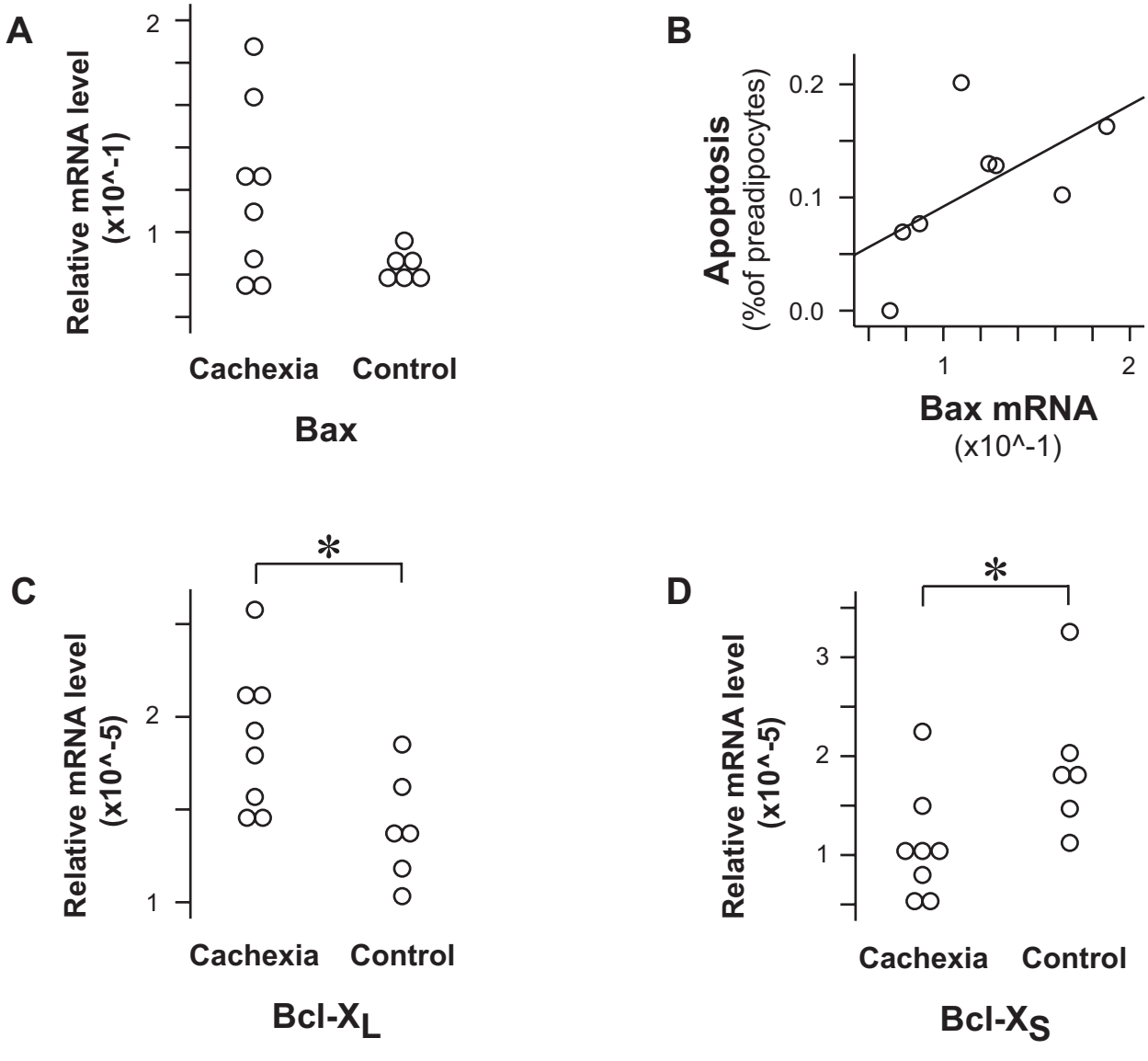

Figure 2. Effect of cachectic patient sera on expression of genes encoding apoptosis regulatory proteins in SGBS preadipocytes. SGBS preadipocytes were incubated in the presence of 10 % sera from cancer patients with cachexia or without cachexia (control) for 72 h. Then, mRNA levels of apoptosis regulatory proteins were measured by quantitative RT-PCR and normalized to the housekeeping gene G3PDH. Relative levels of mRNA encoding the pro-apoptotic protein Bax were calculated (**A**). Bax mRNA levels correlated to the proportion of apoptotic cells (Spearman's rho = 0.07) after SGBS preadipocytes were incubated with sera from patients with cachexia (**B**). Relative levels of mRNA encoding the anti-apoptotic protein Bcl-XL (**C**) and the pro-apoptotic protein Bcl-XS (**D**) were also determined. Each point in the diagram represents the mean value of two separate cell experiments measured in triplicates.

**P ≤ 0.05 using Mann-Whitney U-test*

Lipolysis

Mature, lipid-loaded SGBS adipocytes were differentiated in vitro from SGBS preadipocytes. Free fatty acids were released into the culture medium upon induction of lipolysis (Fig. 3A). During cachexia increased lipolytic activity may lead to shrinking of adipocytes and subsequently the adipose tissue as a whole. Sera from cachectic patients did not display increased lipolytic activity but, contrary to our expectations, tended to inhibit lipolysis in the cells (P=0.09; Fig. 3B). In addition, 3T3-L1 cells of murine origin were differentiated into adipocytes and used to assay lipolytic activity. In the 3T3-L1 adipocytes cachectic sera induced lipolysis at a higher rate than sera from control patients (Fig. 3C).

Discussion

Our main finding is that preadipocytes of human origin cultured in the presence of sera from cancer patients with cachexia show increased apoptosis when compared to preadipocytes cultured with sera from control patients. Preadipocytes are considered precursor cells that may differentiate into adipo-

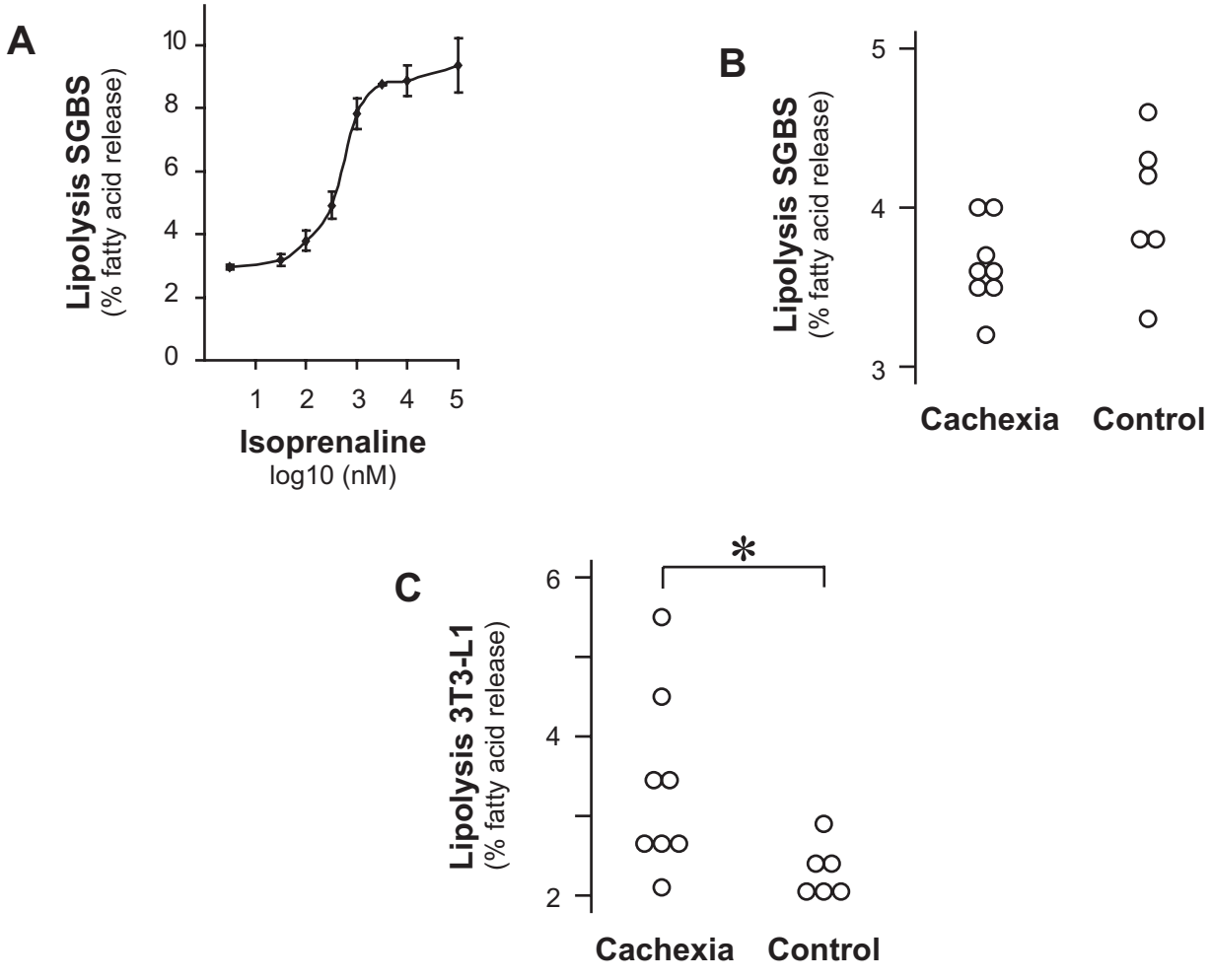

Figure 3. Effect of cachectic patient sera on lipolysis in cultured adipocytes. SGBS preadipocytes were differentiated into adipocytes prior to measurements of lipolysis, estimated as the release of fatty acids into the culture media. Lipolysis was induced in SGBS adipocytes after treatment with various doses of isoprenaline (3-100,000 nM; 4 h) (**A**). Mature SGBS adipocytes were exposed to sera (3 %) from patients with cachexia and without cachexia (control) for 4 h and the lipolytic activity was measured (**B**). The 3T3-L1 cell line of murine origin was differentiated into adipocytes prior to incubation with patient sera (3 %; 4 h) and measurements of lipolysis (**C**).

cytes. Decreased numbers of adipocytes may account for the loss of adipose tissue mass during cachexia. Moreover, a reduction in adipocyte number is hypothesized to occur via preadipocyte and adipocyte apoptosis, and possibly adipocyte dedifferentiation (25). Apoptosis in adipocytes has been observed during cancer cachexia in rabbits and patients (26, 27). Our findings show that also apoptosis of preadipocytes may play a role during cachexia.

Capacities for apoptosis of preadipocytes may vary among different fat depots (28), and SGBS preadipocytes may not reflect the cachectic process in all of them. After treatment with or without cachectic sera, only a small proportion of the SGBS preadipocytes stained with HO342 displayed altered morphology of the nuclear chromatin consistent with apoptosis. Such alterations are associated with late stages of the apoptotic process and it is possible that the methodology used here leads to an underestimation of the number of apoptotic cells.

Whereas apoptosis was enhanced in SGBS preadipocytes incubated with sera from cachectic cancer patients, necrotic cell death was unchanged. Detachment of necrotic cells from the surface of the culture dish may explain this inconsistency. It is also possible that the induction of apoptosis is only starting after 3 days (72 h) of incubation, and that a cumulative effect on the number of necrotic cells occurs at a later time-point. Although sera from the cachectic group tended to increase the number of necrotic preadipocytes after 7 days of incubation, this was still not statistical significant (data not shown). Limitations in our ability to conclude may be due to the relatively small number of patients in our study and variation due to heterogenous types of cancer.

The effect of sera from cachectic cancer patients, i.e. cell death or cell survival, may depend on the balance of pro- and anti-apoptotic mediators. Moreover, the mRNA levels of antiapoptotic Bcl-XL and proapoptotic Bcl-XS were increased and decreased, respectively, as compared to incubation with control sera. This finding was surprising, but may represent a compensatory response. There was a tendency towards increased mRNA levels of proapoptotic Bax after incubation with cachectic sera. Our findings suggest that Bax is a better measure than Bcl-XL for apoptosis in our experimental set-up.

There are several putative mediators of apoptosis which may be present in cachectic sera. Proinflammatory cytokines such as TNF-α and IL-6 have been implicated in adipose atrophy in cachexia (11). Whereas TNF-α induces apoptosis (29), IL-6 has been shown to represent an anti-apoptotic signal (30,31). In accordance with previous studies (32), the cachectic sera used in our present study contained higher levels of IL-6 than the non-cachectic, whereas TNF-α levels were similar. The increased IL-6 levels might represent a compensatory response to increased levels of some unidentified proapoptotic factor.

Also adipokines might affect apoptosis (33). Adiponectin and leptin concentrations tended to be altered in serum from cachectic patients as compared to non-cachectic patients. Because the levels of both these adipokines in serum are influenced by the mass of adipose tissue, we cannot conclude from our study that cachexia per se affects adipokine levels. Further studies of adipokine concentrations in BMI-matched cancer patients are warranted to determine if adipokines may play a role in cancer cachexia.

Conclusion

Factors circulating in the blood of cancer patients with cachexia can increase apoptosis of preadipocytes *in vitro*.

Methods

Origin of serum samples. Serum was collected from patients for use in biological assays monitoring apoptosis and lipolysis in adipocytes. The patients were recruited at Ullevål University Hospital, Oslo. Inclusion criteria were: histologically diagnosed cancer in lung, kidney, stomach, pancreas, colon or rectum. All patients included had metastatic disease determined by clinical and radiological findings. Serum samples were collected from a group of cancer patients suffering from cachexia (n = 8; 4 female, 4 male) and a group of cancer patients without evident cachexia as controls (n = 6; 1 female, 5 male). The cachectic group of patients included cancers originating in different tissues like pancreas (2), colon (1), ventricle (1), kidney (1) and lung (3). The control group included cancers originating in colon (4), lung (1) and rectum (1). The patients included in the cachectic group had an advanced stage of cachexia development. In this study, cachexia was defined was BMI ≤ 20, and loss of body weight > 5 % over the past 6 months, and serum albumin concentrations ≤ 37 mg/mL. Patients in the control group had BMI > 20, no weight loss the previous 6 months, and serum albumin concentrations > 37 mg/mL. Written informed consent was obtained from all the participants. The study was approved by the regional Ethics Committee.

Materials. Dulbecco's modified Eagle's medium/Nutrient Mix F12 (DMEM/Nutrient Mix F12), RPMI-1640, Parker 199, biotin, DL-pantothenate, penicillin/streptomycin, L-glutamin, human apo-transferrin, human insulin, cortisol, triiodothyronine (T3), dexamethasone, 3-isobutyl-1-methylxanthine (IBMIX), phosphate-buffered saline (PBS), propidium iodide (PI), Hoechst 33342 (HO342), HEPES, bovine serum albumin (BSA),

diethyl pyrocarbonate (DEPC), sodium dodecyl sulphate (SDS) and staurosporine were bought from Sigma Chemicals Co (St.Louis, MO, USA). Fetal calf serum (FCS) was from Gibco BRL (Paisley, UK). Rosiglitazone was a gift from GlaxoSmith-Kline (Essex, UK) whereas $NaHCO_3$, NaOH, LiCl and chloroform were from Merck (Darmstadt, Germany). Restriction enzymes (EcoRI), ethylene diaminetetraacetic acid (EDTA), Tris, ethidium bromide, RNase inhibitor, isopropyl-β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were obtained from Promega (Madison, WI, USA). Agarose was bought from Bio Whittaker Molecular Applications (Rockland, ME, USA). Dithiothreitol (DTT) was purchased from Bio-Rad Laboratories (Hercules, CA, USA) Bicinchoninic acid (BCA) Protein Assay Reagent was from Pierce (Rockford, IL, USA). Tumor necrosis factor-alpha (TNF-α) was from R&D Systems Inc. (Minneapolis, MN, USA), whereas Genoprep™ mRNA Beads were obtained from Genovision AS (Oslo, Norway). Omniscript™ Reverse Transcriptase kit was bought from Qiagen GmbH (Hilden, Germany), while TOPO™ Cloning Reaction and Transformation kit was from Invitrogen Corp. (Carlsbad, CA, USA). JETquick mini and maxiprep kits were bought from Genomed GmbH (Bad Oeynhausen, Germany). LightCycler™ Faststart DNA Master SYBR Green I, LightCycler™ Faststart DNA Master Hybridization Probes, LightCycler™ Color Compensation Set, LightCycler™ capillaries and LightCycler™ instrument were supplied from Roche Molecular Biochemicals (Mannheim, Germany). Primers were from Eurogentec (Seraing, Belgium) and labeled hybridization probes were ordered from TIB MOLBIOL (Berlin, Germany).

Cells. The SGBS cells were kindly provided by Professor Martin Wabitsch (34). The cells were derived from the stromal cells fraction of subcutaneous adipose tissue of an infant with Simpson-Golabi-Behmel syndrome. During the differentiation process SGBS cells developed a gene expression pattern similar to that found in differentiating human preadipocytes with a characteristic increase in fat cell-specific mRNAs encoding lipoprotein lipase, glycero-3-phosphate dehydrogenase (GPDH), GLUT4, leptin and others. Differentiated SGBS cells exhibited an increase in glucose uptake upon insulin stimulation and in glycerol release upon catecholamine exposure. SGBS adipocytes were morphologically, biochemically and functionally similar to in vitro differentiated adipocytes from healthy subjects. The SGBS cells were cultured in DMEM/Ham's F12 medium supplemented with 10% heat-inactivated FCS, biotin (8 mg/l), DL-pantothenate (8 mg/l), L-glutamine (2 mmol/l), and streptomycin/penicillin (0.1 mg/ml). SGBS preadipocytes were grown in serum-containing medium in 24-well plates. To induce differentiation to mature adipocytes, confluent preadipocytes were cultured in serum-free basal medium added 20 nmol/l insulin, 0.01 mg/ml apo-transferrin, 0.1 µmol/l cortisol and 200 pmol/l T3 for 14 days. For the first four days of differentiation the medium was supplemented with 25 nmol/l dexamethasone, 500 µmol/l IBMIX and 2 µmol/l rosiglitazone. The medium was changed twice a week. During the experiments cells were incubated with DMEM/Ham's F12 medium supplemented with 10% sterile-filtered patient serum. The cell line Jurkat was purchased from Bio Whittaker (Walkerswille, MD, USA), and was maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, L-glutamine (2 mmol/l), and streptomycin/penicillin (0.1 mg/ml).

Cell staining. For microscopic analysis of cell viability in SGBS preadipocytes, each well (0.5 ml) was first incubated with 3 µl of PI (0.5 mg/ml) in the dark for 30 min. Then 3 µl of HO342 (1 mg/ml) was added and incubation continued for another 30 min in the dark. DNA staining with PI indicates leaky plasma membranes because this dye cannot cross intact cell membranes. HO342 crosses intact membranes and stains DNA in all cells. PI or HO342 associates with DNA and emit red or blue light, respectively, when exposed to ultraviolet light. The blue color becomes more intense when HO342 associates with condensed DNA found in apoptotic cells as compared to normal cells. At least 200 cells per well were counted manually in a Nikon Eclipse TS 100 fluorescence microscope. The cells were photographed with a Nikon Digital Camera DXM 1200.

mRNA isolation. The medium was gently removed from the cells and 100 µl ice-cold RNA lysis/binding buffer (100 mmol/l Tris pH 8.0, 500 mmol/l LiCl, 10 mmol/l EDTA, 1% SDS, 5 mmol/l DTT) was added to each well. The cells were detached by scraping with a pipette tip and the contents of triplicate wells were transferred to an Eppendorf tube and immediately frozen in liquid nitrogen. Cell lysates were stored at –80° C. mRNA was isolated from the cell lysate using Genoprep™ mRNA Beads according to the manufacturer's protocol (Genovision AS, Oslo, Norway). Briefly, 50 µl oligo-dT-beads were prepared, 300 µl frozen cell lysate was defrosted and sonicated for 1-2 s before transfer to the beads and incubation in room temperature for 3-5 min. The beads were washed twice with 250 µl wash solution 1 (10 mmol/l Tris pH 8.0, 150 mmol/l LiCl, 1 mmol/l EDTA, 0.1% SDS), and twice with 250 µl wash solution 2 (10 mmol/l Tris, pH 8.0, 150 mmol/l LiCl, 1 mmol/l EDTA). The mRNA was eluted in 50 µl DEPC water by incubation at 65° C for at least 2 min. The mRNA was stored at –80° C.

Quantitative RT-PCR. The RT reactions were performed using Omniscript™ Reverse Transcriptase kit according to the manufacturer's protocol (Qiagen GmbH, Hilden, Germany). 10 µl of the mRNA was used in each reaction. A LightCycler™ was used in all PCR reactions. It offers kinetic quantification, which is a fast and accurate way for quantification. The SYBR Green I dye, which was used for the genes G3PDH and Bax, binds to minor grooves in double stranded DNA and fluoresces strongly when bound to DNA. In the unbound state the dye has relatively low fluorescence. The intensity of fluorescence from the dye will increase in proportion to the amount of DNA in the PCR. The $mRNA^{Bcl\text{-}X}$ is present as a short and a long form. To distinguish these mRNAs fluorescence resonance energy transfer (FRET) hybridization probes were used. One probe (Bcl-X_{S+L}) was labeled at the 3'-end with fluorescein, which serves as a donor fluorophore. A second probe (Bcl-X_L) was labeled at the 5'-end with LightCycler-Red 640, and a third probe (Bcl-X_S) was labeled with LightCycler-Red 705. Both LightCycler-Red 640 and -705 serve as acceptor dyes. When the donor and acceptor probes recognize adjacent internal sequences in the target gene, the fluorescence signal is generated. The different acceptor probes will fluoresce with different wavelength due to different labeling of the probes, making it possible to quantify both cDNA products in the same reaction. Sequences for primers and probes are described (Table 2). The Bax and G3PDH primers were used at a final concentration of 0.5 µmol/l each, Bcl-X primers 0.75 µmol/l each, and the final concentration of the hybridization probes was 0.3 µmol/l each. The final $MgCl_2$ concentration was optimized to 3 mmol/l for all reactions. 0.2 µl of the cDNA template from the RT-reaction was added for detection of Bax or G3PDH, whereas 0.3 µl was used for Bcl-X detection. The total reaction volume was adjusted to 20 µl using sterile, PCR grade H_2O. A negative control was always included with the samples. PCR programs for G3PDH and Bax quantification were as follows: 95° C, 10 min followed by 45 cycles of 95° C 10 s, 60° C 7 s, 72° C 10 s. PCR program for Bcl-X quantification was: 95° C, 10 min followed by 45 cycles of 95° C 15 s, 55° C 30 s, 72° C 10 s. Due to cross talk between the detection channels of the LightCycler™ instrument when using differently labelled hybridisation probes, a color compensation file had to be created using the LightCycler™ Color Compensation Set. The Bcl-X and Bax genes were cloned to make standard dilution series for quantification of the respective genes on PCR. The TOPO™ Cloning Reaction and Transformation kit was used according

Table 2. Sequences of primers and probes used for RT-PCR

Target mRNA		Sequence
G3PDH	Forward primer	5'-TCATCAACGGGAAGCCCATCACCATCTTC-3'
G3PDH	Reverse primer	5'-GTCTTCTGGTTGGCAGTAATGGCATGGACT-3'
Bcl-2	Forward primer	5'-TGCACCTGACGCCCTTCAC-3'
Bcl-2	Reverse primer	5'-AGACAGCCAGGAGAAATCAAACAG-3'
Bax	Forward primer	5'-ACCAAGAAGCTGAGCGAGTGTC-3'
Bax	Reverse primer	5'-ACAAAGATGGTCACGGTCTGCC-3'
Bcl-X	Forward primer	5'-CGGGCATTCAGTGACCTGAC-3'
Bcl-X	Reverse primer	5'-TCAGGAACCAGCGGTTGAAG-3'
Bcl-X_{S+L}	FRET probe	5'-GACAGCATATCAGAGCTTTGAACA-X-3'
Bcl-X_L	FRET probe	5'-LC Red640-TAGTGAATGAACTCTTCCGGGaAT-p-3'
Bcl-X_S	FRET probe	5'-LC Red705-ATACTTTTGTGGAACTCTATGGGAACA-p-3'

Fluorescence resonance energy transfer (FRET) hybridisation probes; donor fluorophore fluorescein (X); acceptor dyes LightCycler-Red 640 (LCRed640) and LightCycler-Red 705 (LCRed705); phosphate group (p) to block polymerase extension at free 3'-end.

to the manufacturer's protocol (Invitrogen Corp., Carlsbad, CA, USA). 4 µl freshly made PCR product was used in each cloning reaction, and the incubation time was set to 30 min. OneShot™ chemical transformation was utilized, and 50 and 150 µl from each transformation were spread on pre-warmed selective plates (added 40 µl X-Gal and 10 µl IPTG). Plasmid DNA isolation was performed using the JETquick mini- or maxiprep kits according to the manufacturer's protocol (Genomed GmbH, Bad Oeynhausen, Germany). The plasmids were examined by restriction analysis, using EcoRI as restriction enzyme. The DNA contents of the mini- and maxi-preps of the respective genes were measured spectrophotometrically at 260 nm, and standard dilution series were made for PCR.

Lipolysis measurements. During 3 days prior to the experiments SGBS and 3T3-L1 adipocytes were pre-labeled with [1-^{14}C]-D-glucose or [1-^{14}C]-Acetate, respectively. The cells were changed to serum free media 24 h before the incubation with patient sera or isoprenaline. The cells were washed 3 times with preheated PBS, and then given growth media without hormones, but with the addition of fatty acid free BSA (100 µM) and isoprenaline or 3 % serum from cachectic and control patients. After 4 hours incubation the media were removed and the radioactivity released was quantified by counting an aliquot in scintillation fluid using a scintillation counter. Similarly, the cells were lysed and the remaining cell-associated radioactivity was counted. Lipolysis (%) was estimated by calculating (100*released radioactivity/cell-associated radioactivity).

Statistics. The SPSS software version 13.0 for Windows was used for the statistical analysis. Non-parametric methods were used due to their resistance to outliers and skewed data distribution. The differences between groups were tested with the non-parametric Mann-Whitney U-test, and $P \leq 0.05$ was considered statistically significant. Correlations in the separate groups were explored with the Spearman rank correlation coefficient, and $P \leq 0.10$ was considered statistically significant.

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Competing interests

We have no competing interests to declare.

References

1. Evans WK, Makuch R, Clamon GH, Feld R, Weiner RS, Moran E, *et al*. Limited impact of total parenteral nutrition on nutritional status during treatment for small cell lung cancer. *Cancer Res* 1985; 45: 3347-3353.
2. Tisdale MJ. Biology of cachexia. *J Natl Cancer Inst* 1997; 89: 1763-1773.
3. Dewys WD, Begg C, Lavin PT, Band PR, Bennett JM, Bertino JR, *et al*. Prognostic effect of weight loss prior to chemotherapy in cancer patients. Eastern Cooperative Oncology Group. *Am J Med* 1980; 69: 491-497.
4. Cinti S. *The Adipose Organ*. Editrice Kurtis, Milano, Italy, 1999.
5. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, *et al*. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002; 13: 4279-4295.
6. Drevon CA. Fatty acids and expression of adipokines. *Biochim Biophys Acta* 2005; 1740: 287-292.
7. Brennan MF. Uncomplicated starvation versus cancer cachexia. *Cancer Res* 1977; 37: 2359-2364.
8. Moley JF, Aamodt R, Rumble W, Kaye W, Norton JA. Body cell mass in cancer-bearing and anorexic patients. *J Parenter Enteral Nutr* 1987; 11: 219-222.
9. Costa G. Cachexia, the metabolic component of neoplastic diseases. *Cancer Res* 1977; 37: 2327-2335.
10. Tisdale MJ. Wasting in cancer. *J Nutr* 1999; 129: 243S-246S.
11. Bing C, Trayhurn P. Regulation of adipose tissue metabolism in cancer cachexia. *Curr Opin Clin Nutr Metab Care* 2008; 11: 201-207.
12. Londos C, Sztalryd C, Tansey JT, Kimmel AR. Role of PAT proteins in lipid metabolism. *Biochimie* 2005; 87: 45-49.
13. Drott C, Persson H, Lundholm K. Cardiovascular and metabolic response to adrenaline infusion in weight-losing patients with and without cancer. *Clin Physiol* 1989; 9: 427-439.
14. Adams JM, Cory S. The Bcl-2 protein family: arbiters of cell survival. *Science* 1998; 281: 1322-1326.
15. Green DR, Reed JC. Mitochondria and apoptosis. *Science* 1998; 281: 1309-1312.
16. Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* 1997; 91: 627-637.
17. Yang J, Liu X, Bhalla K, Kim CN, Ibrado AM, Cai J, *et al*. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science* 1997; 275: 1129-1132.
18. Kluck RM, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997; 275: 1132-1136.

19. Hengartner MO. The biochemistry of apoptosis. *Nature* 2000; 407: 770-776.
20. Eskes R, Desagher S, Antonsson B, Martinou JC. Bid induces the oligomerization and insertion of Bax into the outer mitochondrial membrane. *Mol Cell Biol* 2000; 20: 929-935.
21. Nomura M, Shimizu S, Ito T, Narita M, Matsuda H, Tsujimoto Y. Apoptotic cytosol facilitates Bax translocation to mitochondria that involves cytosolic factor regulated by Bcl-2. *Cancer Res* 1999; 59: 5542-5548.
22. Navarro P, Valverde AM, Benito M, Lorenzo M. Activated Ha-ras induces apoptosis by association with phosphorylated Bcl-2 in a mitogen-activated protein kinase-independent manner. *J Biol Chem* 1999; 274: 18857-18863.
23. Ishiko O, Sumi T, Yoshida H, Hyun Y, Ogita S. Comparison of expression of apoptosis regulatory proteins in the adipose tissue of tumor-bearing and diet-restricted rabbits. *Int J Mol Med* 2001; 8: 543-547.
24. Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O, *et al.* Dynamics of fat cell turnover in humans. *Nature* 2008; 453: 783-787.
25. Prins JB, O'Rahilly S. Regulation of adipose cell number in man. *Clin Sci (Lond)* 1997; 92: 3-11.
26. Ishiko O, Nishimura S, Yasui T, Sumi T, Hirai K, Honda K, *et al.* Metabolic and morphologic characteristics of adipose tissue associated with the growth of malignant tumors. *Jpn J Cancer Res* 1999; 90: 655-659.
27. Prins JB, Walker NI, Winterford CM, Cameron DP. Human adipocyte apoptosis occurs in malignancy. *Biochem Biophys Res Commun* 1994; 205: 625-630.
28. Tchkonia T, Tchoukalova YD, Giorgadze N, Pirtskhalava T, Karagiannides I, Forse RA, *et al.* Abundance of two human preadipocyte subtypes with distinct capacities for replication, adipogenesis, and apoptosis varies among fat depots. *Am J Physiol Endocrinol Metab* 2005; 288: E267-277.
29. Laster SM, Wood JG, Gooding LR. Tumor necrosis factor can induce both apoptic and necrotic forms of cell lysis. *J Immunol* 1988; 141: 2629-2634.
30. Curnow SJ, Scheel-Toellner D, Jenkinson W, Raza K, Durrani OM, Faint JM, *et al.* Inhibition of T cell apoptosis in the aqueous humor of patients with uveitis by IL-6/soluble IL-6 receptor trans-signaling. *J Immunol* 2004; 173: 5290-5297.
31. Leu CM, Wong FH, Chang C, Huang SF, Hu CP. Interleukin-6 acts as an antiapoptotic factor in human esophageal carcinoma cells through the activation of both STAT3 and mitogen-activated protein kinase pathways. *Oncogene* 2003; 22: 7809-7818.
32. Iwase S, Murakami T, Saito Y, Nakagawa K. Steep elevation of blood interleukin-6 (IL-6) associated only with late stages of cachexia in cancer patients. *Eur Cytokine Netw* 2004; 15: 312-316.
33. Della-Fera MA, Baile CA. Roles for melanocortins and leptin in adipose tissue apoptosis and fat deposition. *Peptides* 2005; 26: 1782-1787.
34. Wabitsch M, Brenner RE, Melzner I, Braun M, Moller P, Heinze E, *et al.* Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. *Int J Obes Relat Metab Disord* 2001; 25: 8-15.

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RESEARCH ARTICLE

COLD EXPOSURE AND ADIPOSE NITRIC OXIDE AND MAST CELLS: INFLUENCE ON AORTA CONTRACTILITY

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Abstract

Both nitric oxide (NO) and mast cells play important roles in adipose and vascular tissue biology. Chronic cold stress decreases the sensitivity of vascular smooth muscle to various contractile agents including norepinephrine (NE). In our previous cold exposure study we found that the contractile response of isolated rat aortas to NE was significantly reduced, and the number of rat aortic adventitial mast cells decreased. Histologically and functionally, white and brown adipose tissue (WAT and BAT) can be distinguished. Beyond its significance in energy store/release and heat production, adipose tissue secretes multiple signaling molecules that have endocrine and paracrine role in the regulation of vascular functions. The aims of the present study were to examine chronic cold exposure-induced alterations in (*i*) the concentration of NO released from selected regions of WAT and BAT in female and male rats, (*ii*) the histochemistry of white and brown adipose mast cells, and (*iii*) whether adipose-derived NO affects the contraction of isolated rat aorta to NE. Twelve females and 12 males Spraque-Dawley rats (150-200 g body weight) were used. The rats were exposed to a cold/freely moving stress for 2 hours each day for 5 consecutive days. At the end of cold exposure, the rats were sacrificed, and samples of thoracic aorta with associated periadventitial adipose tissue (*tunica adiposa*) were obtained. WAT and BAT were isolated from subcutaneous abdominal and interscapular areas, respectively. The concentration of NO was measured by capillary electrophoresis and mast cells were evaluated histochemically. The response of aorta smooth muscles to NE was recorded in the isolated organ bath. To determine whether adipose-derived NO affects aorta contraction to NE, cumulative dose response curves to NE (10^{-8}–10^{-3} M) were obtained with or without isolated WAT/BAT suspended in the organ bath medium. In control animals, a gender-related significant difference in NO production in both WAT and BAT was found, NO levels being significantly higher in female than male rats. Data from the contractile response of isolated aorta to NE suggest that receptor affinity to NE is significantly different between female and male controls. Presence of BAT and WAT (isolated from cold-exposure animals) in the bath changed the response of aorta smooth muscle to NE. Displaying a gender dimorphism, BAT/WAT-derived NO, or other vasorelaxing factors, seem to reduce receptor density and/or affinity to NE. Adipose mast cell histochemistry also showed diversity in respect to subtype, gender, and cold exposure. Altogether, we found (*i*) a gender difference in adipose-released NO and in adipose mast cell histochemistry to cold exposure, and (*ii*) peripheral adipose tissues affect aortic contractile responses to NE likely by a NO-mediated pathway during cold exposure, suggesting that adipose tissue may limit cold-induced excessive vasoconstriction. Our ongoing study aims at the evaluation of whether aortic *tunica adiposa* itself could also contribute to this phenomenon.

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Key words: adipose tissue, aorta, cold, mast cells, nitric oxide, norepinephrine

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Introduction

Adipose tissue has traditionally been recognized as the most important lipid/energy store and heat producer of the body. Moreover, recent studies clearly demonstrate that adipose tissue produces and releases - via endo- and paracrine way - a large number of singnaling proteins, collectively termed adipokines. These exert a variety of local, peripheral, and central effects, including the regulation of cardiovascular functions (1-9).

Generally, two different types of adipocytes are known in mammals: white adipocytes, which store energy as triglycerides and release it according to organism needs, and brown adipocytes, which dissipate energy as heat (4). Although a concept of adipocyte plasticity is recently emerged (4), morphologically and functionally two types, white and brown adipose tissue (WAT and BAT) are described.

Initiated by Soltis and Cassis in 1991 (5), recent progress in cardiovascular adipobiology increasingly demonstrates that periadventitial adipose tissue (*tunica adiposa*) (6) has profound paracrine effect on blood vessel contractility. For instance, adiposa-denuded vessels show reduced contractility to various agents such as norepinephrin (NE), angiotensin II, and phenylephrine, suggestive of a paracrine release of adipose-derived relaxing factor(s) (7-9). Therefore, it should be reasonable to assume that periadventitial adipose tissue, via paracrine way (reviewed in 6) and peripheral WAT and BAT, via endrocrine way, can regulate the arterial tone.

The activity of sympathetic nervous system is increased following cold exposure. This results in generalized vasoconstriction and skeletal muscle shivering to maintain body temperature. However, chronic cold stress, which is one of the stress paradigms having a dramatic effect on sympathetic nerves, has only a moderate pressor effect and decreases the sensitivty of vascular smooth muscles to various contractile agents including NE (10,11) . Hence, there may be a protective mechanism preventing the development of excess vasoconstriction during cold exposure even though sympathetic nerve activity is high.

It has been reported that nitric oxide synthase (NOS) gene expression is significantly higher when body temperature decreased (10). Both WAT and BAT express NOS isoforms (12). "Brown" NO release increases in cold-exposure as well as BAT weight increases in 2-3 fold during cold acclimatization (13-15).

Mast cells are widely distributed in all tissues including adipose tissue (16,17). These are multifunctional cell type, involving in the control of cardiovascular functions, tissue injury and repair, inflammation, thermogenesis, lipid metabolism, and obesity and diabetes (16-22); mast cells are "master cells", to paraphraze Stephen J. Galli (*N Eng J Med* 1993; 328:257-265). Mast cells synthesize and secrete a variety of mediators, including NO (12,15,19,20), which is also accepted as a major determinant of mast cell phenotype (23).

In our previous cold exposure study (11) we found a decrease in the number and degranulation of rat aortic adventitial mast cells. This study also revealed that NE-induced contractile response of isolated rat aorta was significantly reduced.

It is known that there are gender differences in body fat amount and distribution. Men have less body fat and a greater amount of abdominal adipose tissue than women of the same body mass index (24-26). In female rats, it was shown that BAT has higher oxidative and thermogenic capacities (27).

The aims of the present study were to examine chronic cold exposure-induced alterations in (*i*) the concentraction of NO released from selected regions of WAT and BAT in female and male rats, (*ii*) the histochemistry of white and brown adipose mast cells, and (*iii*) whether adipose-derived NO affects the contraction of isolated rat aorta to NE.

Material and methods

In the experiment, 12 females and 12 males Spraque-Dawley rats (150-200g body weight) were used. Rats were divided into four groups: controls (females and males) and cold-stressed (females and males).

In cold stress procedure, the rats were exposed to a cold/freely moving stress for 2 hours (from 8.00 AM to 10.00 AM) each day for 5 consecutive days. Three animals were put in a cage being able to move freely and then placed into cold chamber (+ 4° C). Rats in control groups were kept at room temperature. At the end of cold exposure, the rats were sacrificed by cervical dislocation and samples of thoracic aorta were obtained for measurement of isometric contractile force. Aortas were obtained with their associated periadventitial adipose tissue and cut into ring of approximately 2 mm length. In order to test the effect of NO released from peripheral adipose tissue, that is, WAT- and BAT-derived NO, a bioassay method was applied. Subcutaneous abdominal WAT and interscapular BAT were isolated from both control and cold-exposured female and male rats. For bioassay recording, WAT and BAT, in equal size and weight, was individually anchored into the organ bath medium via silk thread, and cumulative NE response were recorded. White adipose tissue and BAT from male were used for aortas isolated from male and *vice versa*.

Aorta rings with or without isolated BAT/WAT were mounted in organ chambers (Hugo–Sachs 4 container Schuler) filled with Krebs' solution of the following composition (in mM) 118.4 NaCl, 7.4 KCL, 1.2 $MgSO_4$, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$ and 11.7 glucose at 37^{0}C and bubbled with 95%O_2 and 5% CO_2 (pH:7.4). Cumulative dose response curves to NE (10^{-8}-10^{-3} M)

were recorded by Biopac Data Acquisition system.

After mounting, each preparation was equilibrated for 45 min. After equilibration in physiological solution basal tension of the vessel was adjusted to 0.7g and the preparations were stimulated with a 100mM KCL depolarizing solution (11). After wash out and 30 min recovery vessels were exposed to cumulatively added NE (10^{-8}-10^{-3} M).

Determination of NO levels and mast cells histochemistry in adipose tissue

Isolated WAT and BAT samples were seperated for NO measurement and mast cell histochemistry. For mast cells histochemistry, formalin fixed samples were stained with alcian blue/safranin. Mast cells were categorized, as described in our previous reports, as mast cells with no-heparin (blue-stained cells) and mast cells with high heparin (red-stained cells) (11).

For NO measurement, tissue samples, first weighted and then stored in a deep freeze at -80°C until analysis time. Nitric oxide levels were measured by capillary electrophoresis (28). Fused-silica capillary was filled with the background electrolyte consisting of 200 mM lithium chloride, 10 mM borate buffer at pH 8.5. All of the solutions were prepared in a nitrate-free double distilled water. The dilutions of nitrate were made from the stock solutions of 1.06X 10^{-3} M KNO and 4.39X10^{-3} M KBr as internal standard (I.S.). The final concentration of I.S. was always 2.92X10^{-3} M in the calibration and sample solutions. The tissues were homogenized in a 0.5 ml phosphate buffer (PBS) and centrifuged in 5000 RPM for 5 minutes. 100 µl supernatant was taken and 200 µl KBR(ACN) added then centrifuged in 5000 RPM for 5 minutes. 200 µl supernatant was injected. The column was washed and conditioned by rinsing, in turn, 5 min each with 0.1 M NaOH, Besides, 2-min washing with background electrolyte was made between each of the experiments. The detection was made at 214 nm where monochromatic light is absorbed maximum by the related anions. The injection time was 50 ms (corresponds to almost 25 nl) using vacuum injection mode and reversed polarity controlled current of 200 µA corresponds to 12.7 kV was applied.

Statistical analysis

Data are expressed ± S.E.M. Analysis of contractile responses and pD_2 (apparent agonist affinity constant, -log EC_{50}) values were calculated using GraphPad Software V2.04. Statistical differences were evaluated using one way ANOVA followed by the Student Newman-Keuls test. Data of NO concentration of males and females were one-way analysis of variance (ANOVA) followed by Dunnett's. P<0.05 was taken as significant.

Results

Adipose-derived NO

Figure 1 shows NO levels of WAT and BAT of male rats. In control males, NO level of BAT is significantly higher than that of WAT. Cold exposure significantly increased NO levels of WAT and BAT compared to control levels of WAT. Figure 2 shows NO levels of WAT and BAT of female rats. Contrary to male, in control animals the levels of BAT- and WAT-released NO did not display any significant difference. In both types of adipose tissue, NO levels of female rats were not significantly changed

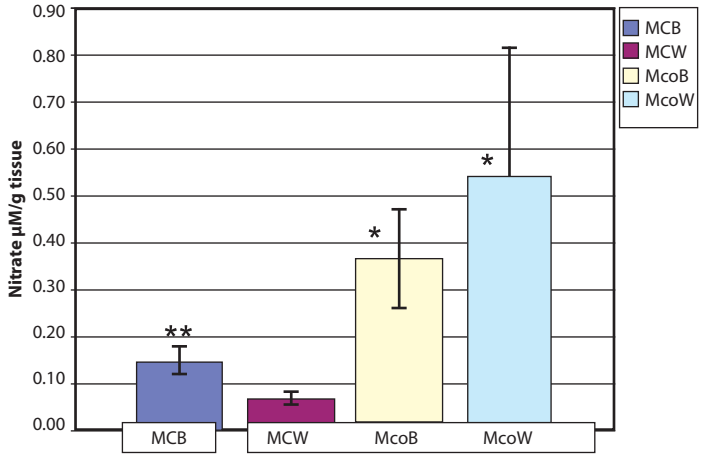

Figure 1. Nitric oxide levels in white and brown adipose tissues of male rats.
Male Control WAT (MCW) - Male Cold WAT (McoW)
Male Control BAT (MCB) - Male Cold BAT (McoB)
*P< 0.001 Significantly different from MCW group
**P<0.001 Significantly different from MCW group

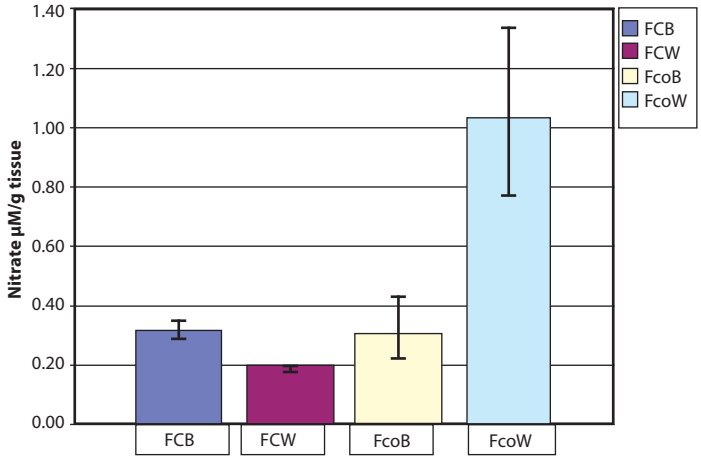

Figure 2. Nitric oxide levels in white and brown adipose tissue of female rats.
Female Control WAT (FCW) - Female Cold WAT (FcoW)
Female Control BAT (FCB) - Female Cold BAT (FcoB)

by cold exposure. Statistical comparison of male and female adipose NO levels are given in Figure 3 A, B. Nitric oxide levels of BAT and WAT of the control animals revealed significant differences between male and female rats. Both WAT and BAT of female animals have higher NO levels than males. Influence of cold lead to significant increase in NO levels of WAT in both sex compared to only male controls.

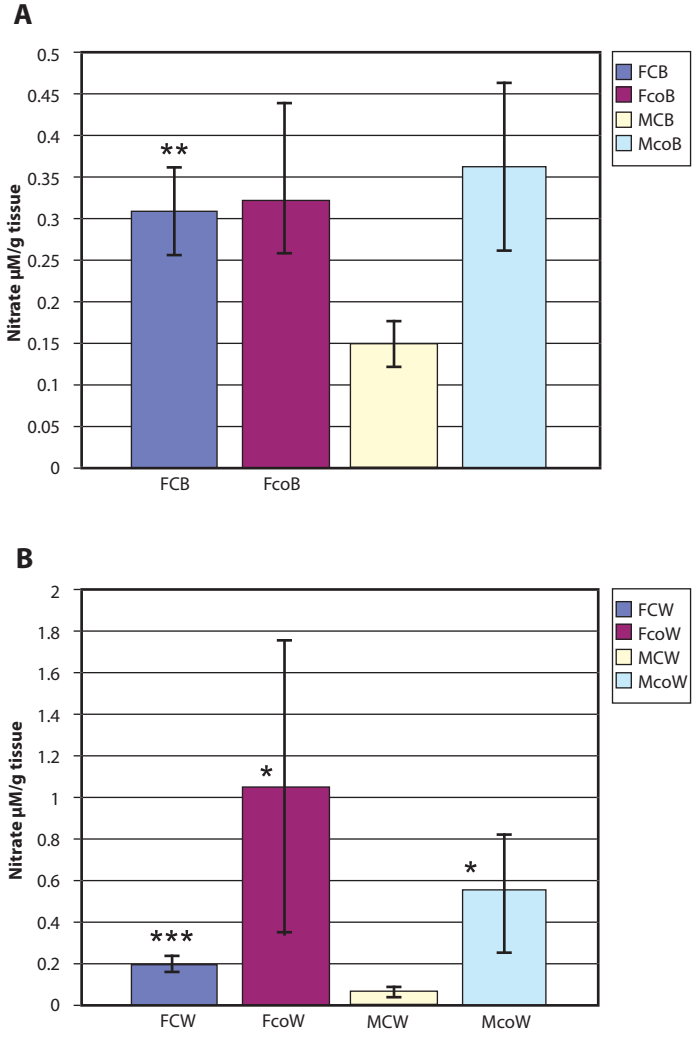

Figure 3 A, B. Comparison of NO levels of brown adipose tissue (A) and white adipose tissue (B) between female and male rats.

Male Control WAT (MCW) - Male Cold WAT (McoW)
Male Control BAT (MCB) - Male Cold BAT (McoB)
Female Control WAT (FCW) - Female Cold WAT (FcoW)
Female Control BAT (FCB)- Female Cold BAT (FcoB)
* P<0.05 Significantly different from MCW
** P<0.01 Significantly different from MCB
**** P<0.01 Significantly different from MCW

Contractile response of aorta

Figure 4 and Table 1 show the concentration-effect curves to NE and pD_2 (apparent agonist affinity constant, -log EC_{50}) values of male and female control rats, respectivelly. The NE dose-response curve for aorta of control male rats shifted to the right, producing a significantly higher pD_2 (-log EC_{50}) value without any significant reduction in the maximum response. The higher pD_2 values indicate reduced sensitivity of aortic smooth mus-

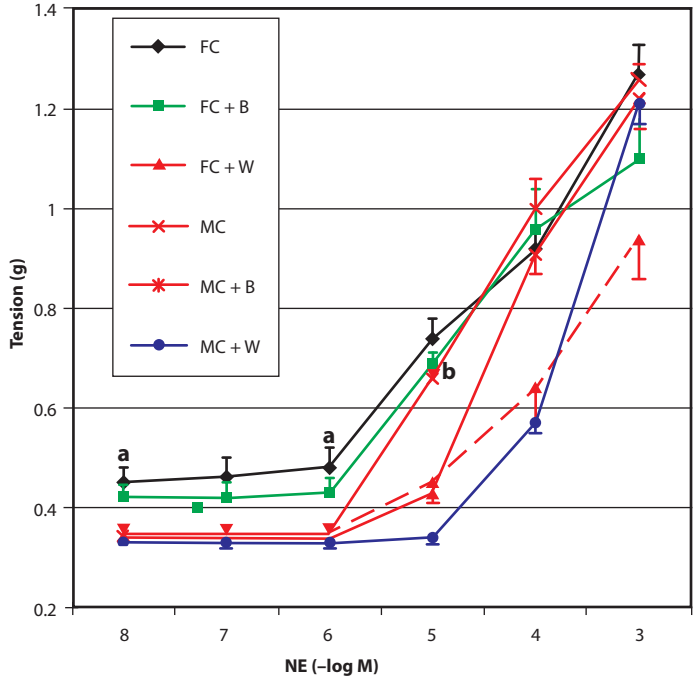

Figure 4. Mean concentration – effect curves to noradrenalin (NE) obtained in aorta rings isolated from male and female controls (MC and FC). +B and + W, with brown adipose and white adipose tissue in the bath medium, respectively.
a: Significantly different from MC, P<0.05
b: Significantly different from MC+W and FC+W, P<0.05

Table 1. Apparent affinity constant (pD2) values (-log EC50) for the effect of NE in the isolated aorta of male and female control rats. +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
** Significantly different from MC group, P<0.05*
*** Significantly different from MC+W group, P<0.05*

	Group	PD_2 ± SEM	n
Female	FC	7.10 ± 0.21*	6
Female	FC+B	6.37 ± 0.19	6
Female	FC+W	6.52 ± 0.20**	6
Male	MC	6.45 ± 0.06	6
Male	MC+B	6.08 ± 0.05	6
Male	MC+W	5.79 ± 0.09	6

cle to NE in male rats. In other words, the sensitivity of aortic smooth muscle of male rats to NE was significantly less than that of female rats. Additionally more sensitivity reduction to NE in the aortic smooth muscle of male rats are observed when WAT, isolated from cold exposured rats, suspended into the bath medium. This response is in correlation with the increased NO levels in WAT isolated from cold-exposed male rats.

Figure 5 and Table 2 show the concentration-effect curves for NE and pD_2 values of aorta vessels isolated from cold exposure and control female rats, respectively. The response to NE (10^{-5}–10^{-3} M concentrations) of aorta of cold exposure female rats decreased but difference is not found statistically significant. The response to NE (10^{-8}–10^{-3} M concentrations) of aorta of cold exposure rats was significantly attenuated by suspending of BAT, isolated from cold exposure female rats, into the bath medium. Presence of BAT in the bath medium significantly increased the pD_2 values. However, NO levels of BAT of the female rats are not significantly changed by cold exposure. It may be suggested that besides NO, other BAT-derived molecule(s) may cause reducing the sensitivity of aortic smooth muscles to NE.

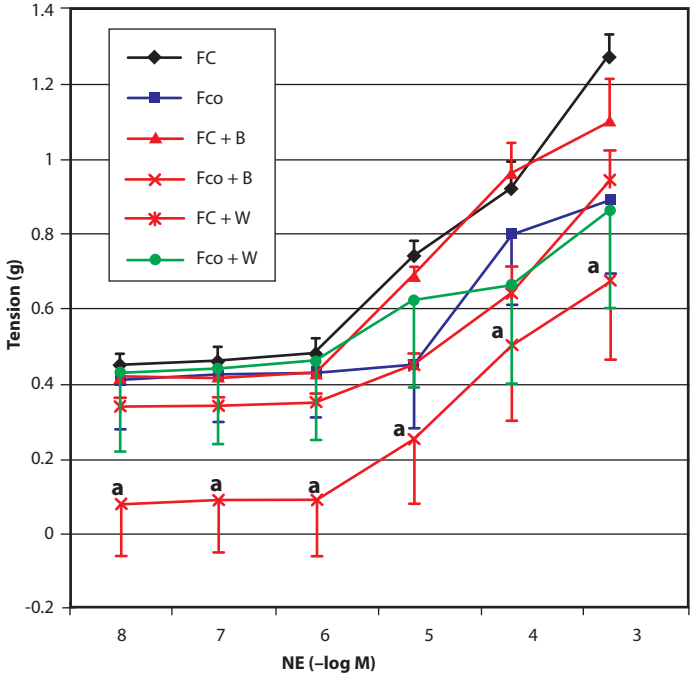

Figure 5. Mean concentration-effect curves to noradrenalin (NE) obtained in aorta rings isolated from control (C) and cold (co)-exposed female rats (F). +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
a: Significantly different from all groups, P< 0.05.

Table 2. Apparent affinity constant (pD2) values (-log EC50) for the effect of NE in the isolated aorta of female rats (F). +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
** Significantly different from FC group, P<0.05*

Group	PD_2 ± SEM	n
FC	7.10 ± 0.21	6
Fco	6.14 ± 0.45	6
FC + B	6.37 ± 0.19	6
Fco + B	4.99 ± 0.47 *	6
FC + W	6.52 ± 0.20	6
Fco +W	5.80 ± 0.68	6

Figure 6 and Table 3 show the concentration-effect curves to

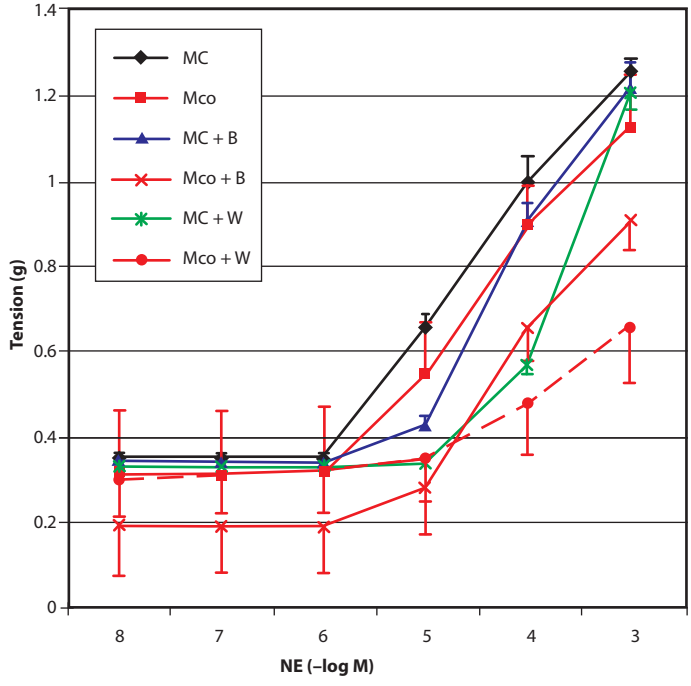

Figure 6. Mean concentration-effect curves to noradrenalin (NE) obtained in aorta rings isolated from control (C) and cold (co)- exposured male rats (M). +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
a: Significantly different from MC and Mco, P< 0.05

Table 3. Apparent affinity constant (pD2) values (-log EC50) for the effect of NE in the isolated aorta of male rats (M). +B and +W, with brown adipose tissues and white adipose in the bath medium, respectively.
** Significantly different from MC, Mco, MC+W groups, P<0.05*

Group	PD_2 ± SEM	n
MC	6.45 ± 0.06	6
Mco	5.63 ± 0.43	6
MC + B	6.08 ± 0.05	6
Mco + B	5.29 ± 0.30	6
MC + W	5.79 ± 0.09	6
Mco +W	4.44 ± 0.61*	6

NE and pD_2 values of aortas isolated from cold exposure and control male rats, respectively. Likewise, in female rats the response to NE for 10^{-5}–10^{-3} M concentrations of the aorta of cold exposured male rats decreased but difference is not found statistically significant. However, when WAT and BAT anchored into the organ bath medium, the contractile response of aortas obtained from cold exposured male rats significantly reduced to all concentration of NE (10^{-8}–10^{-3} M) as compared to the vessels of control and cold-exposed rats. WAT but not BAT causes a significant difference in pD_2 values (Table 3). WAT significantly reduced the sensitivity to NE of aortic smooth muscle isolated from cold exposured male rats. This response is in correlation with increased NO levels of WAT isolated from cold exposured male rats.

Mast cells

We found that BAT displayed more mast cells than WAT in both sex groups. The number of mast cells apprently reduced in both BAT and WAT after cold exposure, possibly resulting from an increased degranulation of these cells. Mast cells stained with alcian blue-safranin also showed different histochemistry according to types of adipose tissues, gender, and cold exposure. While mast cells stained red, blue and mixt in BAT of female rats, which means that mast cells contain heparin together with amines, male rats have only blue-stained mast cells, meaning that heparin can not be detected in the granules of the cells (Fig. 7B). After cold exposure, a few mast cells were detected in WAT and BAT of both sex groups. These mast cells increased heparin content in their granules and stanid with safranin in red (Fig. 7C).

Discussion

In the present study, a gender difference in adipose-released NO is observed. Our *in vitro* bioassay results indicate that aortic contractile response to NE and the effect of WAT- and BAT-derived molecules on the vessels reactivity are also different in male and female rats. Intriguingly, NO, or other vasorelaxing molecules, released from WAT more significantly affect the response of aortas of male rats, whereas molecules released from BAT more significantly affect the response of aortas of female rats. Such a gender as well as white-to-brown adipose difference are difficult to be explained.

We suggest that adipose-derived NO could be a key molecule to rescue the extreme cold-induced vasoconstriction. Nitric oxide also acts as a deactivator agent for NE (29). The NO system, including NOS and its endogenous inhibitor, asymmetric dimethylarginine, is involved in the control of thermogenic function of BAT and lipid metabolism in WAT (4,12,15,17,30-

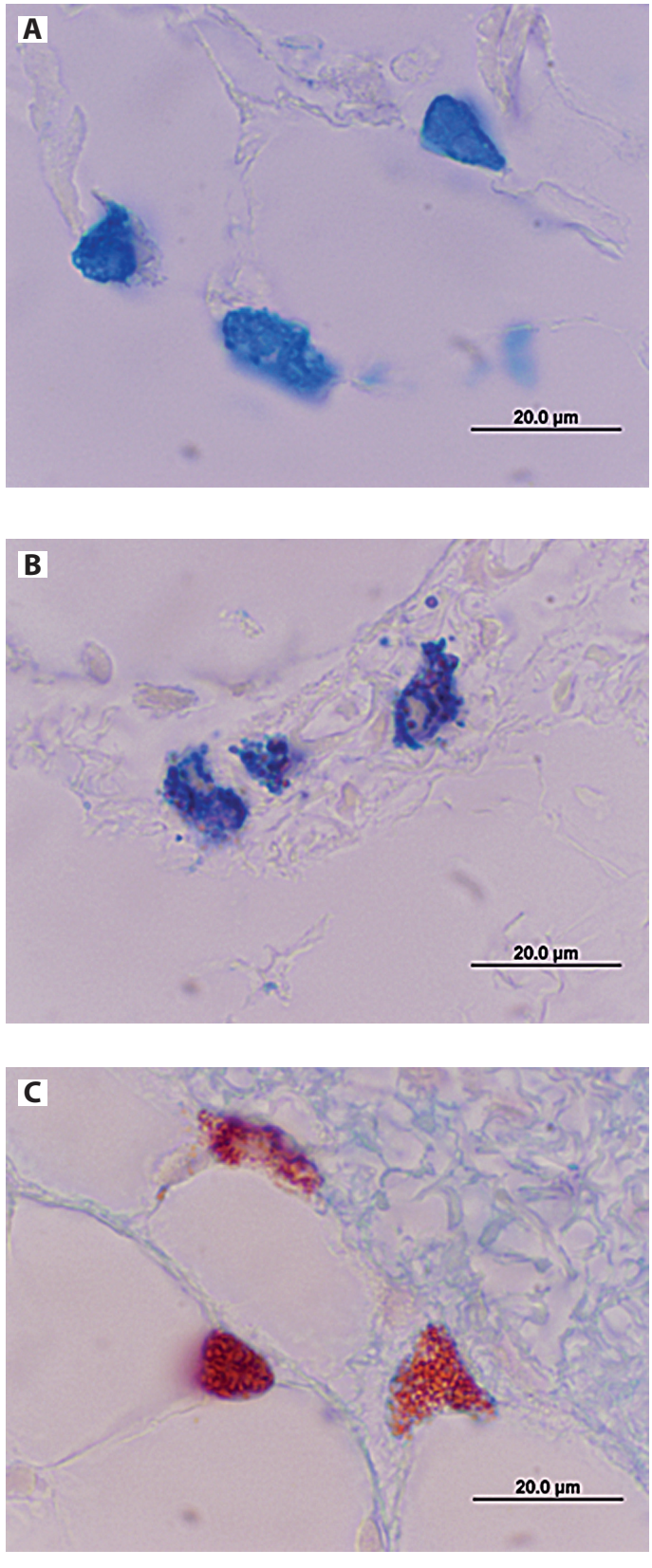

Figure 7. Blue stained mast cells in BAT of male rats (A); Mix stained mast cells in BAT of female rats (B); Red stained mast cells in WAT of cold-exposed male rats (C).

35). Cold exposure generaly diminishes both iNOS immunopositivity and protein levels in intrascapular BAT (35). It was suggested that iNOS may be involved in induction of apoptosis in this tissue. Because of the diminished iNOS activity, intrascapular BAT mass significantly increase in animals acclimated to cold (35). In the present study, cold stress did not significantly change NO levels of BAT isolated both male and female rats possibily by a decreased iNOS activity. On the other hand, cold-induced NO production is remarable in WAT in both sex groups. Further, cold exposure induces a decrease in the number of mast cells as well as changes in their granular content; it is difficult to extrapolate these results to a possible involvement of these cells in adipose tissue-released NO.

Conclusion

A gender difference in NO release and mast cell number and histochemistry in both WAT and BAT to chronic cold exposure of rats is found. Such a difference is also revealed in the contractile response of isolated aortas to NE. We may only speculate that estrogen-induced NO release (36) may somehow be involved in the observed gender difference in our study. It is possible that adipose-released NO may, at least in part, be responsible for the diminished contractile response of aortas to NE during cold exposure; this may limit cold-induced excessive vasoconstriction. Our ongoing study aims at the evaluation of whether aortic periadventitial adipose tissue-derived NO (37,38, cf. 39) and associated mast cells (16) and lympocytes (40) could also influence aortic contractility during cold exposure. In perspective, recently discovered periadventitial adipose tissue-derived vasorelaxants such as adiponectin (41,42), hydrogen sulfide (43,44) and angiotensin 1-7 (45, cf. 46) should be studied in cold exposure experiments. This may also be the case for human BAT (47-49).

References

1. Chaldakov GN, Stankulov IS, Hristova M, Ghenev PI. Adipobiology of disease: adipokines and adipokine-targeted pharmacology. *Curr Pharm Des* 2003;9:1023-1031.
2. Trayhurn P. Endocrine and signalling role of adipose tissue: new perspectives on fat. *Acta Physiol Scand* 2005;184:285-293.
3. Renes J, Rosenow A, Mariman E. Novel adipocyte features discovered by adipoproteomics. *Adipobiology* 2009; 1: 7-18.
4. Cinti S. Transdifferentiation properties of adipocytes in the adipose organ. *Am J Physiol Endocrinol Metab* 2009. doi:10.1152/ajpendo.00183.2009
5. Soltis EE, Cassis LA. Influence of perivascular adipose tissue on rat aortic smooth muscle responsiveness. *Clin Exp Hypertens A* 1991;13:277-296
6. Chaldakov GN. Cardiovascular adipobiology: a novel. Heart-associated adipose tissue in cardivascular disease. *Ser J Exp Clin Res* 2008; 9: 81-88.
7. González MC, Arribas SM, Molero F, Fernández-Alfonso MS. Effect of removal of adventitia on vascular smooth muscle contraction and relaxation *Am J Physiol Heart Circ Physiol* 2001;280:H2876-2881.
8. Gollasch M, Dubrovska G. Paracrine role for periadventitial adipose tissue in the regulation of arterial tone. *Trends Pharmacol Sci* 2004; 25:647-653.
9. Löhn M, Dubrovska G, Lauterbach B, Luft FC, Gollasch M, Sharma AM. Periadventitial fat releases a vascular relaxing factor. *FASEB J* 2002; 16:1057-1063.
10. Cordellini S, Vassilieff VS. Decreased endothelium-dependent vasoconstriction to noradrenaline in acute-stressed rats is potentiated by previous chronic stress: nitric oxide involvement. *Gen Pharmacol* 1998; 30:79-83
11. Tunçel N, Erkasap N, Sahinturk V. The effect of stress and in vivo vasoactive intestinal peptide (VIP) treatment on the response of isolated rat aorta to norepinephrine, angiotensin II and vasopressin, and adventitial mast cells. *Stress* 2000;3:299-308.
12. Canová NK, Lincová D, Kmonícková E, Kameníková L, Farghali H. Nitric oxide production from rat adipocytes is modulated by beta3-adrenergic receptor agonists and is involved in a cyclic AMP-dependent lipolysis in adipocytes. *Nitric Oxide* 2006; 14: 200-211.
13. Han S, Chen X, Yang CL, Vickery L, Wu Y, Naes L, *et al.* Influence of cold stress on neuropeptide Y and sympathetic neurotransmission. *Peptides* 2005; 26:2603-2609.
14. Westfall TC, Yang CL, Chen X, Naes L, Vickery L, Macarthur H, *et al.* Novel mechanism prevents the development of hypertension during chronic cold stress. *Auton Autacoid Pharmacol* 2005; 25:171-177.
15. Saha SK, Kuroshima A. Nitric oxide and thermogenic function of brown adipose tissue in rats. *Jpn J Physiol* 2000; 50:337-342.
16. Chaldakov GN, Tonchev AB, Tuncel N, Atanassova P, Aloe L. Adipose tissue and mast cells. Adipokines as Ying-Yang modulators in inflammation. In: Fantuzzi G, Mazzone T, editors. *Nutrition and Health: Adipose Tissue and Adipokines in Health and Disease*. Humana Press Inc., Tokowa, NJ. 2007; 151-158.
17. Liu J, Divoux A, Sun J, Zhang J, Clément K, Glickman JN, *et al.* Genetic deficiency and pharmacological stabilization of mast cells reduce diet-induced obesity and diabetes in mice. *Nat Med* 2009; July 26: in print.
18. Desautels M, Wollin A, Halvorson I, Muralidhara DV,

Thornhill J.Role of mast cell histamine in brown adipose tissue thermogenic response to VMH stimulation. *Am J Physiol* 1994 266: R831-837.

19. Forsythe P, Gilchrist M, Kulka M, Befus AD. Mast cells and nitric oxide: control of production, mechanisms of response. *Int Immunopharmacol* 2001; 1:1525-1541.
20. Bidri M, Féger F, Varadaradjalou S, Ben Hamouda N, Guillosson JJ, Arock M. Mast cells as a source and target for nitric oxide. *Int Immunopharmacol* 2001; 1:1543-1558.
21. Dalo NL, Lopez-Ortega AA, Moussatche H. The growth of brown adipose tissue in cold-acclimatized rats after depletion of mast cell histamine by compound 48/80.*Mem Inst Oswaldo Cruz* 1998; 93:215-217.
22. Natanaka K, Tanishita H, Ishibashi-Ueda H, Yamamoto A. Hyperlipidemia in mast cell-deficient W/WV mice. *Biochim Biophys Acta* 1986;878: 440-445.
23. McCauley SD, Gilchrist M, Befus AD. Nitric oxide: a major determinant of mast cell phenotype and function. *Mem Inst Oswaldo Cruz* 2005;100 (Suppl 1):11-14.
24. Rattarasarn C, Leelawattana R, Soonthornpun S, Setasuban W, Thamprasit A. Gender differences of regional abdominal fat distribution and their relationships with insulin sensitivity in healthy and glucose-intolerant Thais. *J Clin Endocrinol Metab* 2004; 89: 6266-6270.
25. Sierra-Johnson J, Johnson BD, Bailey KR, Turner ST. Relationships between insulin sensitivity and measures of body fat in asymptomatic men and women. *Obes Res* 2004;12:2070-7.
26. Onat A, Uğur M, Can G, Yüksel H, Hergenç G. Visceral adipose tissue and body fat mass: Predictive values for and role of gender in cardiometabolic risk among Turks. *Nutrition* 2009; July 23: in print.
27. Justo R, Frontera M, Pujol E, Rodríguez-Cuenca S, Lladó I, García-Palmer FJ, *et al.* Gender-related differences in morphology and thermogenic capacity of brown adipose tissue mitochondrial subpopulations. *Life Sci* 2005 ;76:1147-1158.
28. Tunçel M, Dogrukol-Ak D, Erkasap N. Modified method for the determination of capillary electrophoresis nitric oxide-correlated nitrate in tissue homogenates. *J Chromatogr B Biomed Sci Appl* 2001;751:257-63.
29. Kolo LL, Westfall TC, Macarthur H. Nitric oxide decreases the biological activity of norepinephrine resulting in altered vascular tone in the rat mesenteric arterial bed. *Am J Physiol Heart Circ Physiol* 2004;286:H296-303.
30. Gregory EL. Thermoregulatory aspects of adipose tissue. *Clin Dermatol* 1989; 7:78-92.
31. Cannon B, Nedergaard J. Brown adipose tissue: function and physiological significance. *Physiol Rev* 2004;84:277-359.
32. Spoto B, Parlongo RM, Parlongo G, Sgro' E, Zoccali C. The enzymatic machinery for ADMA synthesis and degradation is fully expressed in human adipocytes. *J Nephrol* 2007; 20: 554-559.
33. Garofalo MA, Kettelhut IC, Roselino JE, Migliorini RH. Effect of acute cold exposure on norepinephrine turnover rates in rat white adipose tissue. *J Auton Nerv Syst* 1996 12; 60:206-208.
34. Gaudiot N, Jaubert AM, Charbonnier E, Sabourault D, Lacasa D, Giudicelli Y, *et al.* Modulation of white adipose tissue lipolysis by nitric oxide. *J Biol Chem* 1998; 273:13475-81.
35. Petrović V, Korać A, Buzadzić B, Korać B. The effects of L-arginine and L-NAME supplementation on redox-regulation and thermogenesis in interscapular brown adipose tissue. *J Exp Biol* 2005; 208:4263-71.
36. Kitamura N, Araya R, Kudoh M, Kishida H, Kimura T, Murayama M, et al. Beneficial effects of estrogen in a mouse model of cerebrovascular insufficiency. *PLoS One* 2009; 4:e5159.
37. Dashwood MR, Dooley A, Shi-Wen X, Abraham DJ, Souza DS. Does periadventitial fat-derived nitric oxide play a role in improved saphenous vein graft patency in patients undergoing coronary artery bypass surgery? *J Vasc Res* 2007; 44:175-181.
38. Malinowski M, Deja MA, Golba KS, Roleder T, Biernat J, Woś S. Perivascular tissue of internal thoracic artery releases potent nitric oxide and prostacyclin-independent anticontractile factor. *Eur J Cardiothorac Surg* 2008; 33:225-231.
39. Gao YJ, Lu C, Su LY, Sharma AM, Lee RM. Modulation of vascular function by perivascular adipose tissue: the role of endothelium and hydrogen peroxide. *Br J Pharmacol* 2007; 151: 323-331.
40. Duffaut C, Zakaroff-Girard A, Bourlier V, Decaunes P, Maumus M, Chiotasso P, *et al.* Interplay between human adipocytes and T lymphocytes in obesity. CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators. *Arterioscler Thromb Vasc Biol* 2009; July 30: in print.
41. Fésüs G, Dubrovska G, Gorzelniak K, Kluge R, Huang Y, Luft FC, *et al.* Adiponectin is a novel humoral vasodilator. *Cardiovasc Res* 2007; 75:719-727.
42. Greenstein AS, Khavandi K, Withers SB, Sonoyama K, Clancy O, Jeziorska M. Local inflammation and hypoxia abolish the protective anticontractile properties of perivascular fat in obese patients. *Circulation* 2009; 119: 1661-1670.
43. Fang L, Zhao J, Chen Y, Ma T, Xu G, Tang C, *et al.* Hydrogen sulfide derived from periadventitial adipose tissue is a vasodilator. *J Hypertens* 2009; July 30: in print.

44. Beltowski J. Hydrogen sulfide (H_2S): the new member of gasotransmitter family. *Biomed Rev* 2007; 18:75-83.
45. Lee RM, Lu C, Su LY, Gao YJ. Endothelium-dependent relaxation factor released by perivascular adipose tissue. *J Hypertens* 2009; 27:782-790.
46. Lee RM, Lu C, Su LY, Werstuck G, Gao YJ. Effects of hyperglycemia on the modulation of vascular function by perivascular adipose tissue. *J Hypertens* 2009; 27: 118-131.
47. van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, *et al*. Cold-activated brown adipose tissue in healthy men. *N Engl J Med* 2009; 360: 1500-1508.
48. Saito M, Okamatsu-Ogura Y, Matsushita M, Watanabe K, Yoneshiro T, Nio-Kobayashi J, *et al*. High incidence of metabolically active brown adipose tissue in healthy adult humans: effects of cold exposure and adiposity. *Diabetes* 2009; 58:1526-1531.
49. Frühbeck G, Becerril S, Sáinz N, Garrastachu P, García-Velloso MJ. BAT: a new target for human obesity? *Trends Pharmacol Sci* 2009; 30:387-396.

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RESEARCH ARTICLE

LEPTIN INCREASES THROMBOXANE A2 FORMATION IN THE RAT

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Abstract

Chronic hyperleptinemia may contribute to various complications of obesity including atherosclerosis, however, the underlying mechanisms are incompletely clear. We examined the effect of leptin on platelet activity by measuring stable metabolites of thromboxane A_2 (TXA_2), TXB_2, 11-dehydro-TXB_2 and 2,3-dinor-TXB_2, in plasma and urine. *In vitro*, leptin stimulated TXB_2 formation by platelet-rich plasma (PRP). *In vivo*, leptin (1 mg/kg ip.) increased urinary excretion of 11-dehydro-TXB_2 and 2,3-dinor-TXB_2. Urinary excretion of these metabolites was also elevated in rats made hyperleptinemic by administration of recombinant leptin (0.5 mg/kg/day) for 8 days. The stimulatory effect of leptin on TXB_2 formation in PRP isolated from hyperleptinemic animals was impaired in comparison to the control group. In rats made obese, hyperleptinemic and hyperinsulinemic/insulin resistant by cafeteria diet administered for 3 months, acute stimulatory effect of leptin on TXB_2 formation by PRP was not impaired. In rats made insulin resistant by fructose feeding for 8 weeks, stimulatory effect of leptin on TXB_2 formation in PRP was augmented in comparison to the control group. Insulin sensitizer, rosiglitazone, decreased insulin level and attenuated the stimulatory effect of leptin on TXB_2 formation in obese and fructose-fed animals. In contrast, rosiglitazone had no effect on insulin level or leptin-induced TXB_2 formation in control rats and rats receiving recombinant leptin for 8 days. These results indicate that: (i) leptin stimulates platelet TXA_2 formation both *in vitro* and *in vivo*, (ii) chronic hyperleptinemia impairs acute stimulatory effect of leptin on platelet activity if insulin sensitivity is normal, (iii) insulin resistance/hyperinsulinemia augments the stimulatory effect of leptin on TXA_2 formation, which results in normal platelet sensitivity to leptin in obesity associated with both hyperleptinemia and hyperinsulinemia, and (iv) PPAR-γ agonists such as rosiglitazone decrease platelet sensitivity to leptin by reducing insulin resistance.

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Key words: platelets, obesity, metabolic syndrome, atherosclerosis, rosiglitazone

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Introduction

Recent studies indicate that an adipose tissue hormone, leptin, is involved in atherogenesis. Plasma leptin concentration is markedly increased in obese humans and in animals with obesity induced by high-calorie diet. In experimental studies leptin has been demonstrated to have many potentially proatherogenic effects. This adipokine induces endothelial dysfunction, oxidative stress, vascular smooth muscle cells hypertrophy and proliferation, stimulates macrophage cholesterol synthesis, and reduces HDL cholesterol level (1). Deficiency of leptin or its receptor reduce atherosclerosis in classic animal models such as apolipoprotein E and LDL receptor knockout mice, whereas administration of exogenous leptin in supraphysiological doses or transgenic hormone overexpression have the opposite effect (2). In addition, many clinical studies indicate the link between high leptin level and atherosclerosis, acute cardiovascular events and ischemic stroke in humans (3).

Platelets play a crucial role in atherosclerosis. Formation of platelet-rich thrombus on the ruptured plaque, with resulting complete or almost complete vessel occlusion, is the ma-

jor mechanism of acute complications of atherosclerosis such as myocardial infarction and ischemic cerebral stroke. In addition, chronic low-grade platelet activation facilitates growth of atherosclerotic plaque. Platelets secrete several mediators which potently stimulate hypertrophy and proliferation of vascular smooth muscle cells including platelet-derived growth factor and thromboxane A_2 (TXA_2). Moreover, activated platelets facilitate leukocyte recruitment to the endothelium by secreting chemotactic and proinflammatory mediators such as a chemokine platelet factor-4, a CD40 ligand, P-selectin, thrombospondin-1 and RANTES (4).

The effect of leptin on platelet function is controversial. Some studies have demonstrated that leptin augments ADP-induced platelet aggregation (5), but other authors observed no effect (6). These studies were performed *in vitro*, and it is well-known that platelet aggregation *in vitro* is at best only a crude estimate of their function in the intact organism (7). Although the positive correlation between serum leptin and urinary excretion of TXA_2 metabolite, 11-dehydro-TXB_2, has been observed in obese women (8), this correlation disappeared after adjustment for anthropometric variables suggesting that it represents the effect of obesity and not necessarily of leptin itself. In addition, resistance to many effects of leptin has been described in obesity. It is unclear if platelets' sensitivity to leptin is preserved or impaired in obesity. If platelets remain sensitive to leptin, chronic hyperleptinemia could contribute to platelets' hyperactivity observed in the metabolic syndrome. However, if platelets become resistant to leptin, the contribution of this adipokine to atherothrombotic complications would be less likely.

To address these issues, in the present study we examined the effect of leptin on platelet function, measured as TXA_2 production, in the rat both *in vitro* and *in vivo*. In addition, we compared the effect of leptin in lean animals and in selected models of the metabolic syndrome. We also tested the effect of rosiglitazone, a PPAR-γ agonist, on leptin-induced TXA2 formation.

Materials and methods

Animals

All studies were performed on adult male Wistar rats weighing 314 ± 8 g. The animals were housed under controlled conditions of temperature (20-22°C), humidity (60-70%), lighting (12 h light/dark cycle) and provided with food and water *ad libitum*. The study protocol was reviewed and approved by the local institutional ethical committee.

Effect of leptin on TXA_2 production *in vitro*

Rats were anesthetized with pentobarbital (50 mg/kg ip.) and blood was withdrawn from the abdominal aorta using sodium citrate as an anticoagulant. Blood was centrifuged at 150×g for 15 min at a room temperature to obtain platelet-rich plasma (PRP). PRP was diluted with Tyrode's buffer (5 mM HEPES, 2 mM $MgCl_2$, 0.1% BSA and 0.1% D-glucose) to a standard concentration of 3×10^8 platelets/ml. To obtain platelet-poor plasma (PPP), PRP was recentrifuged at 1000 × g for 15 min and the supernatant was collected. PRP was incubated with leptin for 10 min and TXB_2 concentration was measured (see below).

Effect of leptin on TXA_2 production *in vivo*

Rats were anesthetized with ethyluretane (1.25 g/kg ip.). Then, a polyethylene catheter was inserted into the urinary bladder for urine collection. Urine was collected for 1 hour and then either leptin (1 mg/kg in 0.5 ml) or an equivolume amount of phosphate-buffered saline (PBS) was injected intraperitoneally. Urine collection was continued for additional 2 hours. After the end of urine collection, animals were euthanized by the lethal dose of pentobarbital.

Induction of hyperleptinemia, dietary-induced obesity and fructose-induced metabolic syndrome

Experimental hyperleptinemia was induced by administration of exogenous recombinant leptin (0.25 mg/kg twice daily sc.) for 8 days as described by us previously (9). Leptin was injected between 7.00 and 8.00 AM and between 7.00 and 8.00 PM. The last dose was given in the morning and plasma for in vitro experiments was obtained 6 hours after this injection. Animals in this group received standard rat chow ad libitum. Obesity was induced by offering the animals a highly palatable "cafeteria diet" for either 1 or 3 months. This diet consisted of standard chow combined 1:1 (wt/wt) with a liquid diet containing equal amounts of sucrose, glucose, whole milk powder and soybean powder suspended in tap water (10). The composition of this diet was similar to standard chow (66% carbohydrates, 20% protein, and 14% fat). In the separate group of animals, fructose was administered in the drinking water at a concentration of 20% for 8 weeks to induce hyperlipidemia and insulin resistance not associated with obesity. In subgroups of control, hyperleptinemic, 3-month obese and fructose-fed animals, insulin sensitizer, rosiglitazone, was administered at 10 mg/kg/day by oral gavage for 8 days before blood collection for acute in vitro experiments.

Measurement of TXA_2 metabolites

TXA_2 is rapidly converted non-enzymatically to its immediate

metabolite, TXB_2, which is further enzymatically metabolized to several derivatives such as 2,3-dinor-TXB_1, 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 (11). Measurement of these terminal metabolites in urine provides a reliable estimate of whole-body platelet-derived TXA_2. In contrast, because TXB_2 has a short half-life (5-7 min), most of this compound found in urine originates from local intrarenal production rather than from systemic sources. On the other hand, concentrations of 2,3-dinor-TXB_2 and 11-dehydro-TXB_2 in plasma are below detection limit of most assays until the sample is concentrated. However, most of TXB_2 found in plasma is synthesized by platelets ex vivo after blood withdrawal. Therefore, we used plasma TXB_2 and urinary 11-dehydro-TXB_2/2,3-dinor-TXB_2 as markers of TXA_2 formation for in vitro and in vivo studies, respectively. TXB_2, 11-dehydro-TXB_2 and 2,3-dinor-TXB_2 were measured by competitive enzyme immunoassay (EIA) methods using Cayman Chemical kits (Cat. #519031, 519501 and 519051, respectively), according to the manufacturer's instruction. The detection limits are 11 pg/ml for TXB_2, 16 pg/ml for 11-dehydro-TXB_2, and 7 pg/ml for 2,3-dinor-TXB_2.

Other assays

Plasma insulin and leptin concentrations were measured by EIA methods using Rat Insulin EIA Kit (SPIbio, Massy, France) and Leptin Enzyme Immunoassay Kit (Cayman Chemical), respectively (10). Lipid profile and plasma glucose were assayed by routine laboratory methods.

Reagents

Recombinant rat leptin was obtained from R&D Systems. Rosiglitazone was purchased from Cayman Chemical. Other reagents were from Sigma-Aldrich.

Statistical analysis

Baseline (without leptin) and leptin-stimulated values of TXA_2 metabolites in plasma and urine of the same animal were compared by repeated-measures ANOVA. Between-group comparisons of TXA_2 metabolites, plasma lipids, glucose, leptin and insulin were done by single-measures ANOVA. $P<0.05$ was considered significant.

Results

Leptin stimulates TXA_2 formation *in vitro*

Leptin increased TXB_2 concentration in PRP isolated from control lean rats in a concentration-dependent manner (Fig. 1). Leptin had no significant effect at physiological concentration (10 ng/ml) as well as at a moderately elevated concentration (50 ng/ml), but significantly increased TXB_2 at 100 ng/ml. Maximal stimulatory effect of leptin was observed at 300 ng/ml (Fig. 1). Addition of COX inhibitor, indomethacin (10 μM) to the blood before isolation of PRP decreased baseline TXB_2 concentration to 243 ± 29 pg/ml ($p<0.001$ vs. sample without indomethacin) and abolished the stimulatory effect of leptin (100 ng/ml leptin + indomethacin: 279 ± 31 pg/ml). In addition, leptin had no significant effect on TXB_2 concentration in platelet-poor plasma (without leptin: 479 ± 57 pg/ml; with 100 ng/ml leptin: 512 ± 53 pg/ml, p=NS).

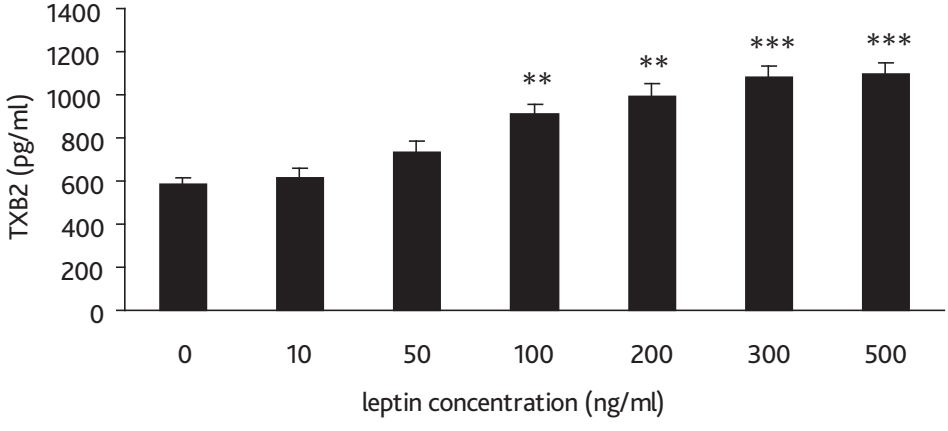

Figure 1. Effect of leptin on thromboxane B_2 concentration in platelet-rich plasma (PRP) of control rats in vitro. PRP was incubated in the presence of various leptin concentrations for 10 min and then TXB_2 was measured. **p<0.01, ***p<0.001 vs. TXB_2 concentration in PRP not treated with leptin.

Leptin stimulates TXA_2 formation *in vivo*

Urinary excretion of 11-dehydro-TXB_2 and 2,3-dinor-TXB_2 increased within 2 hours after a single intraperitoneal leptin injection (Fig. 2). In contrast, leptin had no acute effect on urinary TXB_2. Injection of vehicle (PBS) instead of leptin did not change any TXA_2 metabolite in urine (not shown).

TXA_2 formation is increased in rats receiving leptin for 8 days

Plasma TXB_2 concentration in rats receiving exogenous leptin for 8 days was 612 ± 57 pg/ml and did not differ from control group. However, urinary excretion of 11-dehydro-TXB_2, 2,3-dinor-TXB_2, and TXB_2 was higher in hyperleptinemic than in control animals (Fig. 3). These results indicate that chronic hyperleptinemia increases systemic and intrarenal TXA_2 formation.

Acute effect of leptin on TXA_2 formation is impaired in hyperleptinemic rats

To examine if chronic hyperleptinemia induces resistance of platelets to leptin, we obtained PRP from rats made hyperleptinemic by previous 8-day leptin administration and then studied acute effect of leptin on TXB_2 formation in these samples in vitro (Fig. 4). As can be seen, in hyperleptinemic rats leptin significantly stimulated TXB_2 formation at a concentration no less than 300 ng/ml and did it to a lesser extent than in control animals at both 300 ng/ml and 500 ng/ml. Thus, acute stimulatory effect of leptin on platelet TXA_2 formation is impaired in hyperleptinemic rats.

Effect of leptin on TXA_2 formation in obese and fructose-fed rats

To examine if chronic "endogenous" hyperleptinemia associated with obesity impairs acute effect of leptin on TXA_2 formation, we investigated the effect of leptin on TXB_2 concentration in PRP of rats made obese by high-calorie diet administered for either 1 or 3 months (Fig. 5). Baseline (without exogenous leptin) TXB_2 concentration was slightly but significantly higher in the 3-month but not in the 1-month obese group. After 10-min

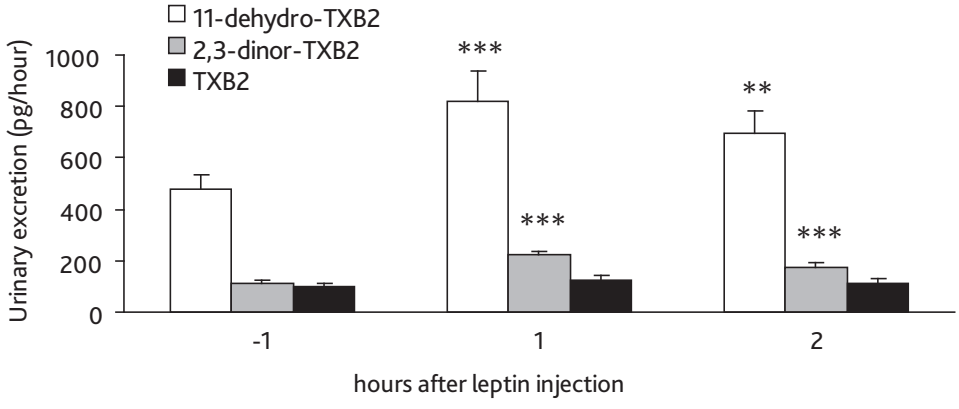

Figure 2. Urinary excretion of TXA_2 metabolites in lean control rats before (-1) and during the first (1) and the second (2) hour after intraperitoneal leptin injection at a dose of 1 mg/kg. **p<0.01, ***p<0.001 vs. pre-injection values.

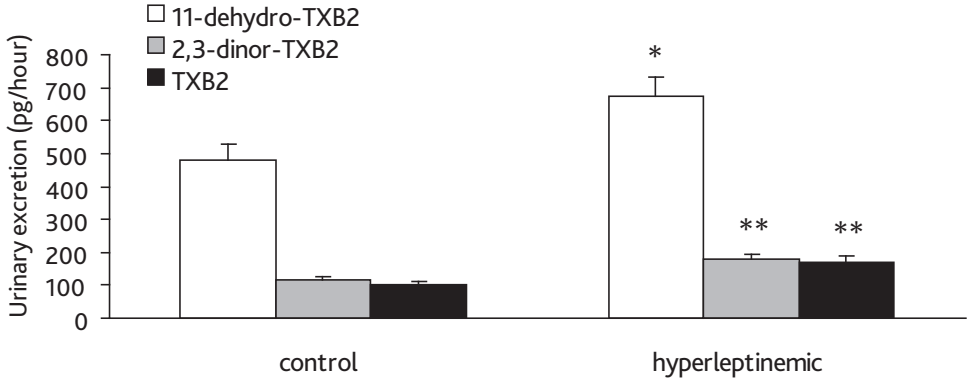

Figure 3. Urinary excretion of TXA_2 metabolites in control rats and in animals receiving exogenous leptin (0.25 mg/kg sc.) for 8 days. Urine was collected for 1 hour in anesthetized animals. Urine collection was started 6 hours after the last leptin injection. *p<0.05, **p<0.01 vs. control group.

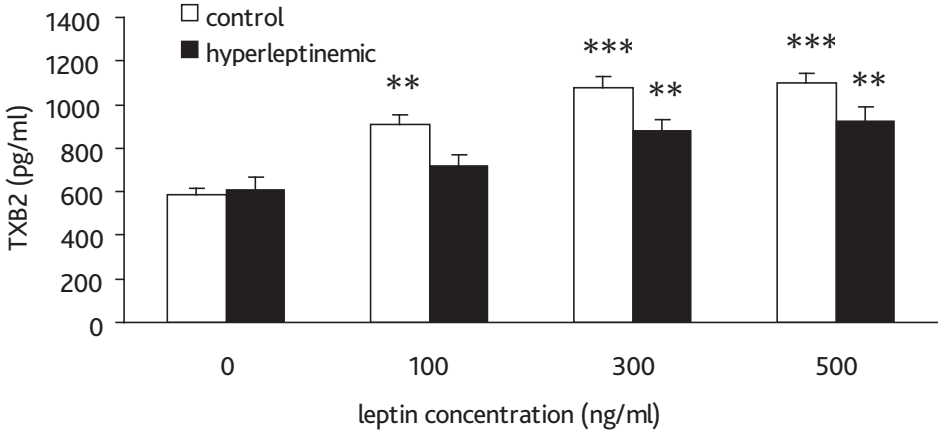

Figure 4. Concentration-dependent effect of leptin on TXB_2 in PRP obtained from control rats and from rats made hyperleptinemic by previous 8-days leptin administration. **p<0.01, ***p<0.001 vs. TXB_2 concentration in PRP not treated with leptin.

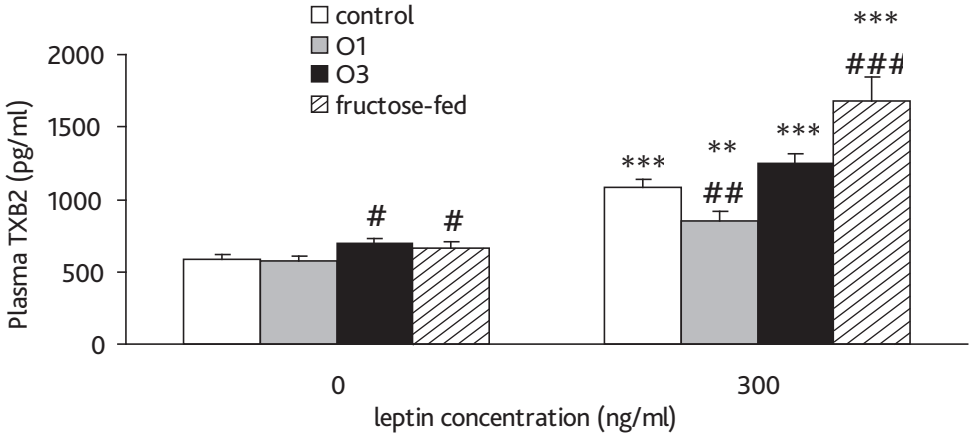

Figure 5. Effect of leptin on TXB_2 formation in PRP of control, 1-month obese (O1), 3-month obese (O3) and fructose-fed rats. **p<0.01, ***p<0.001 vs. TXB_2 concentration in the respective sample not treated with leptin, $^{\#}$p<0.05, $^{\#\#}$p<0.01, $^{\#\#\#}$p<0.001 vs. respective (treated or not treated with leptin) control group.

incubation, leptin (300 ng/ml) increased TXB_2 concentration in PRP of control animals by 84.1% but in 1-month obese group by only 49.6%. Surprisingly, in contrast to short-time obesity, the effect of leptin in 3-month obese group was not impaired in comparison to the control group (stimulation by 80.9%).

Metabolic characteristics of both obese groups is presented in Table 1. As can be seen, plasma leptin is similarly elevated in both obese groups but in contrast to 1-month group, the 3-month group exhibits hyperinsulinemia (a marker of insulin resistance) and dyslipidemia (hypertriglyceridemia and low HDL-cholesterol). Insulin inhibits platelet function and its effect is impaired in insulin resistance states (12). Therefore, we hypothesized that insulin resistance in the 3-month obese group might paradoxically increase the sensitivity of platelets to leptin in comparison to 1-month obese group. To verify this hypothesis, we examined the effect of leptin in fructose-fed rats, which are markedly hyperinsulinemic and hypertriglyceridemic but only slightly hyperleptinemic (Table 1). Leptin (300 ng/ml) increased TXB_2 concentration in PRP of fructose-fed animals to a much greater degree (+153.0%) than in control rats. Thus, fructose-induced metabolic syndrome is associated with enhanced platelet response to leptin. Taken together, these results suggest that in the 3-month obese group resistance of platelets to leptin due to chronic hyperleptinemia is counterbalanced by concomitant hyperinsulinemia/insulin resistance which increases platelets' sensitivity to this adipokine.

Table 1. Characteristics of experimental groups

	Control	Hyperleptinemic	1-month obesity	3-month obesity	Fructose-fed
Body weight (g)	357 ± 5	342 ± 7	429 ± 7***	497 ± 6***	385 ± 9*
Triglycerides (mmol/l)	0.83 ± 0.06	0.74 ± 0.06	0.85 ± 0.05	1.20 ± 0.08***	2.71 ± 0.19***
Total cholesterol (mmol/l)	2.05 ± 0.21	1.97 ± 0.17	1.83 ± 0.11	1.47 ± 0.12*	2.06 ± 0.15
HDL-cholesterol (mmol/l)	1.24 ± 0.07	1.25 ± 0.10	1.19 ± 0.08	0.76 ± 0.08***	1.30 ± 0.12
Plasma glucose (mmol/l)	6.01 ± 0.29	6.09 ± 0.35	6.34 ± 0.43	6.30 ± 0.41	6.178 ± 0.39
Plasma insulin (ng/ml)	2.38 ± 0.24	2.15 ± 0.20	2.58 ± 0.32	3.89 ± 0.46*	4.99 ± 0.61***
Plasma leptin (ng/ml)	4.22 ± 0.41	13.6 ± 1.23***	9.39 ± 0.81***	12.60 ± 1.27***	5.97 ± 0.52*

**p<0.05, **p<0.01, ***p<0.001, compared to control group.*

Rosiglitazone attenuates the effect of leptin on TXA_2 formation in the 3-month obese and fructose-fed rats

To further evaluate the above mentioned hypothesis, we examined the effect of peroxisome proliferator-activated receptor-γ (PPAR-γ) agonist, rosiglitazone (RGZ), which increases insulin sensitivity, on platelet response to leptin in various experimental groups. Rosiglitazone had no effect on plasma leptin, insulin and lipid profile in either control or hyperleptinemic rats. In addition, rosiglitazone did not alter leptin-induced increase in TXB_2 in PRP of these animals (not shown). In the 3-month obese group, rosiglitazone had no significant effect on plasma leptin (without RGZ: 12.60 ± 1.27 ng/ml; with RGZ: 10.21 ± 0.98 ng/ml), but decreased plasma insulin (from 3.89 ± 0.46 to 2.57 ± 0.29 ng/ml, p<0.01) and triglycerides (from 1.20 ± 0.08 to 0.74 ± 0.05 mM, p<0.01). Baseline (without exogenous leptin) TXB_2 concentration was lower in RGZ-treated than in non-treated 3-month obese rats (571 ± 27 vs. 693 ± 39 pg/ml, p<0.05). In addition, leptin (300 mg/ml) increased TXB_2 concentration in RGZ-treated obese rats to 878 ± 87 pg/ml (+53.8%), which is significantly less than in either control or RGZ-untreated obese animals. In the fructose-fed group, RGZ decreased plasma insulin (-54.6%) and triglycerides (-41.7%) and reduced the stimulatory effect of 300 ng/ml leptin on TXB_2 from +153.0% (p<0.01 vs. control group) to +79.4% (p=NS vs. control group).

Discussion

Metabolic syndrome is associated with increased risk of arterial and venous thrombosis (13-15). The pathogenesis of prothrombotic state in the metabolic syndrome is complex and incompletely understood. Increased concentration of some coagulation factors such as fibrinogen, factor VII and tissue factor, deficiency of a major activator of fibrinolysis, tissue plasminogen activator, and excess of fibrinolysis inhibitor, plasminogen activator inhibitor-1 (PAI-1) have been reported in patients with overweight/obesity. In addition, platelet aggregation is augmented in obese animals and humans (16), as evidenced by increased ADP-induced platelet aggregation (6), higher urinary 11-dehydro-TXB_2 excretion (8), plasma P-selectin (17) and soluble CD40 ligand levels (18). Platelets of obese subjects are resistant to inhibitory effects of prostacyclin and adenosine (which elevate intracellular cAMP) and nitric oxide (which elevates intracellular cGMP), as well as to these cyclic nucleotides themselves (19, 20). Moreover, metabolic syndrome is associated with reduced sensitivity of platelets to at least two groups of antiplatelet drugs, acetylsalicylic acid and PY_{12} receptor inhibitors, thienopyridines (21). Increased endothelial cell-platelet interaction has been observed in visceral adipose tissue in murine models of obesity (22).

Several studies have demonstrated that leptin augments ADP-, thrombin- or collagen-induced platelet adhesion and/or aggregation in vitro (5, 23, 24). In addition, exogenous leptin administered to wild-type mice augmented vascular thrombosis induced by vessel wall injury (25, 26). In the present study we examined the effect of leptin on TXA_2 formation both in vitro and in vivo. TXA_2 is one of the most common platelet agonists, and the most widely prescribed antiplatelet drug, acetylsalicylic acid, reduces platelet activity by inhibiting its synthesis. Apart from stimulating platelets, TXA_2 is involved in atherogenesis by inducing vascular smooth muscle cell migration and proliferation (27) and expression of adhesion molecules in endothelial cells (28). To the best of our knowledge, only one previous study

addressed the effect of leptin on TXA_2 formation. In that study, Dellas *et al* (29) have demonstrated that leptin increases TXB_2 generation by isolated human platelets and augments the stimulatory effect of ADP. Our results are consistent with these data. Although the minimal effective leptin concentration (100 ng/ml) is above physiological level (except in cases of extreme obesity), leptin was applied only for 10 min. It is possible that more prolonged incubation with leptin would have resulted in platelet stimulation even at lower concentrations of this adipokine. Increased urinary TXA_2 metabolites in animals receiving leptin for 8 days at doses which raised its level to values observed in obesity (Table 1) is consistent with this hypothesis. In addition, leptin concentration in the blood perfusing adipose tissue may be locally much higher than in systemic circulation. Thus, it is likely that stimulatory effect of leptin on platelets is relevant in pathological conditions.

Obesity is associated with resistance not only to central anorectic but also to some peripheral effects of leptin (30), which partially results from downregulation of leptin receptors and/or postreceptor signaling mechanisms due to chronic hyperleptinemia (31). It is controversial if platelets of obese individuals remain sensitive to leptin or become leptin-resistant. Corsonello *et al* (32) have demonstrated that stimulatory effect of leptin on platelet aggregation is impaired in overweight and obese individuals. In contrast, Corica *et al* (33) found that leptin-induced platelet aggregation was impaired only in morbidly obese but not in overweight subjects, and Dellas *et al* (34) observed normal platelet sensitivity to leptin in morbidly obese patients in comparison to normal-weight controls. Herein we demonstrate that both hyperleptinemia induced in lean rats by administration of exogenous leptin and "endogenous" hyperleptinemia in 1-month obese group are associated with resistance to acute TXA_2-stimulating effect of leptin *in vitro*. Despite this resistance, urinary TXA_2 metabolites are elevated in hyperleptinemic rats. These results indicate that leptin may still contribute to platelet hyperactivity when hormone level is markedly elevated, although the degree of platelet stimulation is undoubtedly lower than would have been if platelets remained leptin-sensitive.

Surprisingly, resistance to leptin was not observed in the 3-month obese group, although endogenous leptin level tended to be higher in these animals than in the 1-month obese group. We hypothesize that despite possible downregulation of leptin signaling, leptin-induced TXA_2 production is augmented by concomitant insulin resistance in these animals. This hypothesis is supported by the following observations: (i) only 3-month obese rats were hyperinsulinemic (and thus presumably insulin resistant), (ii) platelet sensitivity to leptin was augmented in fructose-fed rats which were markedly insulin resistant but relatively normoleptinemic, (iii) rosiglitazone decreased insulin level and platelets' response to leptin in the 3-month obese and fructose-fed but not in either control or hyperleptinemic groups, and (iv) insulin inhibits platelet function and its effect is impaired in the metabolic syndrome (12,19,35). Nevertheless, other explanations cannot be definitely excluded. For example, hyperlipidemia promotes platelet aggregation (36), and both 3-month obese and fructose-fed animals were hyperlipidemic. In addition, rosiglitazone not only reduced insulin level but also improved lipid profile. Whatever the mechanism, the results suggest that PPAR-γ agonists such as rosiglitazone may beneficially modulate platelet function in obesity/metabolic syndrome by reducing their sensitivity to leptin. In addition, variable degree of insulin resistance may determine platelets' sensitivity to leptin in obese subjects and may explain, at least partially, controversial results of previous studies in this field (32-34).

Interestingly, urinary TXB_2 excretion was increased in the hyperleptinemic group. Increased intrarenal TXA_2 production may contribute to the development of arterial hypertension and nephropathy due to its vasoconstricting, antinatriuretic and profibrogenic effects. Increased urinary TXB_2 excretion was observed in many animal models of hypertension such as spontaneously hypertensive rat, Dahl salt-sensitive rat, fructose- or NO synthase inhibitor-induced hypertension as well as in obese Zucker rats which develop severe nephropathy (37-39). Chronic leptin administration induces arterial hypertension (9) and may induce nephropathy (40-41). It remains to be established if excessive intrarenal TXA_2 formation contributes to these leptin-induced complications.

There are several limitations of the present study. First, we measured only TXA_2 formation and did not assess platelet function directly. TXA_2 formation is an established marker of platelet function and allowed us to examine it both in vitro and in vivo in the same experimental model. Second, we examined the effect of leptin alone and did not address its interaction with other platelet agonists. Third, acutely administered leptin increases natriuresis (42,43). It could be suggested that leptin increased urinary 11-dehydro-TXB_2 and 2,3-dinor-TXB_2 secondarily to increasing sodium excretion. This possibility seems, however, unlikely for the following reasons: (i) in contrast to 11-dehydro-TXB_2 and 2,3-dinor-TXB_2, acutely administered leptin had no effect on TXB_2 excretion, (ii) 8-day hyperleptinemia is associated with increased excretion of TXA_2 metabolites whereas natriuresis is reduced in this model (9), and (iii) furosemide increased natriuresis while having no effect on urinary TXA_2 metabolites, whereas COX inhibitor, indomethacin, prevented leptin-induced increase in TXA_2 metabolites but had no effect on leptin-induced increase in natriuresis (unpublished observation).

In conclusion, we have demonstrated that both acutely and chronically administered leptin increases TXA_2 formation in the rat. Chronic hyperleptinemia impairs platelets' response to acutely administered leptin if insulin sensitivity is normal, however, the stimulatory effect of leptin is intact if insulin resistance is concomitantly observed. PPAR-γ agonists may be useful as adjunctive antiplatelet therapy in obesity/metabolic syndrome because they reduce platelets' sensitivity to leptin by ameliorating insulin resistance.

References

1. Beltowski J. Leptin and atherosclerosis. *Atherosclerosis*. 2006; 189: 47-60.
2. Koh KK, Park SM, Quon MJ. Leptin and cardiovascular disease: response to therapeutic interventions. *Circulation* 2008; 117: 3238-3249.
3. Sattar N, Wannamethee G, Sarwar N, Chernova J, Lawlor DA, Kelly A, et al. Leptin and coronary heart disease: prospective study and systematic review. *J Am Coll Cardiol* 2009; 53: 167-175.
4. Jennings LK. Role of platelets in atherothrombosis. *Am J Cardiol* 2009; 103(Suppl):4A-10A.
5. Nakata M, Yada T, Soejima N, Maruyama I. Leptin promotes aggregation of human platelets via the long form of its receptor. *Diabetes* 1999; 48: 426-429.
6. Ozata M, Avcu F, Durmus O, Yilmaz I, Ozdemir IC, Yalcin A. Leptin does not play a major role in platelet aggregation in obesity and leptin deficiency. *Obes Res* 2001; 9: 627-630.
7. FitzGerald GA, Pedersen AK, Patrono C. Analysis of prostacyclin and thromboxane biosynthesis in cardiovascular disease. *Circulation* 1983; 67: 1174-1177.
8. Davì G, Guagnano MT, Ciabattoni G, Basili S, Falco A, Marinopiccoli M, *et al*. Platelet activation in obese women: role of inflammation and oxidant stress. *JAMA* 2002; 288: 2008-2014.
9. Bełtowski J, Jamroz-Wiśniewska A, Wójcicka G, Lowicka E, Wojtak A. Renal antioxidant enzymes and glutathione redox status in leptin-induced hypertension. *Mol Cell Biochem* 2008; 319: 163-174.
10. Bełtowski J, Wójcicka G, Jamroz-Wiśniewska A. Role of nitric oxide and endothelium-derived hyperpolarizing factor (EDHF) in the regulation of blood pressure by leptin in lean and obese rats. *Life Sci* 2006; 79: 63-71.
11. Michelson AD. Methods for the measurement of platelet function. *Am J Cardiol* 2009; 103(3 Suppl): 20A-26A.
12. Trovati M, Mularoni EM, Burzacca S, Ponziani MC, Massucco P, Mattiello L, *et al*. Impaired insulin-induced platelet antiaggregating effect in obesity and in obese NIDDM patients. *Diabetes* 1995; 44: 1318-1322.
13. Mertens I, Van Gaal LF. Obesity, haemostasis and the fibrinolytic system. *Obes Rev* 2002; 3: 85-101.
14. Nieuwdorp M, Stroes ES, Meijers JC, Büller H. Hypercoagulability in the metabolic syndrome. *Curr Opin Pharmacol* 2005; 5: 155-9.
15. Bodary PF. Links between adipose tissue and thrombosis in the mouse. *Arterioscler Thromb Vasc Biol* 2007; 27: 2284-91.
16. Anfossi G, Russo I, Trovati M. Platelet dysfunction in central obesity. *Nutr Metab Cardiovasc Dis* 2009; 19: 440-9.
17. De Pergola G, Pannacciulli N, Coviello M, Scarangella A, Di Roma P, Caringella M, *et al.* sP-selectin plasma levels in obesity: association with insulin resistance and related metabolic and prothrombotic factors. *Nutr Metab Cardiovasc Dis* 2008; 18: 227-232.
18. Angelico F, Alessandri C, Ferro D, Pignatelli P, Del Ben M, Fiorello S, *et al*. Enhanced soluble CD40L in patients with the metabolic syndrome: Relationship with in vivo thrombin generation. *Diabetologia* 2006; 49: 1169-1174.
19. Anfossi G, Russo I, Massucco P, Mattiello L, Doronzo G, De Salve A, et al.. Impaired synthesis and action of antiaggregating cyclic nucleotides in platelets from obese subjects: possible role in platelet hyperactivation in obesity. *Eur J Clin Invest* 2004; 34: 482-489.
20. Russo I, Del Mese P, Doronzo G, De Salve A, Secchi M, Trovati M, *et al.* Platelet resistance to the antiaggregatory cyclic nucleotides in central obesity involves reduced phosphorylation of vasodilator-stimulated phosphoprotein. *Clin Chem* 2007; 53: 1053-1060.
21. Anfossi G, Russo I, Trovati M. Resistance to aspirin and thienopyridines in diabetes mellitus and metabolic syndrome. *Curr Vasc Pharmacol* 2008; 6: 313-328.
22. Nishimura S, Manabe I, Nagasaki M, Seo K, Yamashita H, Hosoya Y, *et al*. In vivo imaging in mice reveals local cell dynamics and inflammation in obese adipose tissue. *J Clin Invest* 2008; 118: 710-721.
23. Corsonello A, Malara A, De Domenico D, Perticone F, Valenti A, Buemi M, et al. Identifying pathways involved in leptin-dependent aggregation of human platelets. *Int J Obes Relat Metab Disord* 2004; 28: 979-984.
24. Elbatarny HS, Maurice DH. Leptin-mediated activation of human platelets: involvement of a leptin receptor and phosphodiesterase 3A-containing cellular signaling complex. *Am J Physiol Endocrinol Metab* 2005; 289: E695-702.
25. Konstantinides S, Schäfer K, Koschnick S, Loskutoff DJ. Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. *J Clin Invest* 2001; 108: 1533-1540.

26. Bodary PF, Gu S, Shen Y, Hasty AH, Buckler JM, Eitzman DT. Recombinant leptin promotes atherosclerosis and thrombosis in apolipoprotein E-deficient mice. *Arterioscler Thromb Vasc Biol* 2005; 25: e119-122.
27. Morinelli TA, Zhang LM, Newman WH, Meier KE. Thromboxane A_2/prostaglandin H_2-stimulated mitogenesis of coronary artery smooth muscle cells involves activation of mitogen-activated protein kinase and S6 kinase. *J Biol Chem* 1994; 269: 5693-5698.
28. Dogné JM, Hanson J, Pratico D. Thromboxane, prostacyclin and isoprostanes: therapeutic targets in atherogenesis. *Trends Pharmacol Sci* 2005; 26: 639-644.
29. Dellas C, Schremmer C, Hasenfuss G, Konstantinides SV, Schäfer K. Leptin signalling and leptin-mediated activation of human platelets: importance of JAK2 and the phospholipases Cγ2 and A2. *Thromb Haemost* 2007; 98: 1063-1071.
30. Martin SS, Qasim A, Reilly MP. Leptin resistance: a possible interface of inflammation and metabolism in obesity-related cardiovascular disease. *J Am Coll Cardiol* 2008; 52: 1201-10.
31. Scarpace PJ, Zhang Y. Leptin resistance: a prediposing factor for diet-induced obesity. *Am J Physiol Regul Integr Comp Physiol* 2009; 296: R493-500.
32. Corsonello A, Perticone F, Malara A, De Domenico D, Loddo S, Buemi M, Ientile R, *et al*. Leptin-dependent platelet aggregation in healthy, overweight and obese subjects. *Int J Obes Relat Metab Disord* 2003; 27: 566-573.
33. Corica F, Corsonello A, Lucchetti M, Mannucci E, Lucchetti M, Bonfiglio C, *et al*. Relationship between metabolic syndrome and platelet responsiveness to leptin in overweight and obese patients. *Int J Obes* 2007; 31: 842-849.
34. Dellas C, Schäfer K, Rohm I, Lankeit M, Ellrott T, Faustin V, *et al*. Absence of leptin resistance in platelets from morbidly obese individuals may contribute to the increased thrombosis risk in obesity. *Thromb Haemost* 2008; 100: 1123-1129.
35. Westerbacka J, Yki-Järvinen H, Turpeinen A, Rissanen A, Vehkavaara S, Syrjälä M, *et al.* Inhibition of platelet-collagen interaction: an in vivo action of insulin abolished by insulin resistance in obesity. *Arterioscler Thromb Vasc Biol* 2002; 22: 167-172.
36. Pedreño J, Hurt-Camejo E, Wiklund O, Badimón L, Masana L. Platelet function in patients with familial hypertriglyceridemia: evidence that platelet reactivity is modulated by apolipoprotein E content of very-low-density lipoprotein particles. *Metabolism* 2000; 49: 942-949.
37. Gomi T, Ikeda T, Sasaki Y, Kosugi T, Shibuya Y, Sakurai J. Protective effect of thromboxane synthetase inhibitor on hypertensive renal damage in Dahl salt-sensitive rats. *Clin Exp Pharmacol Physiol Suppl* 1995; 22: S371-3.
38. Sullivan JC, Sasser JM, Pollock DM, Pollock JS. Sexual dimorphism in renal production of prostanoids in spontaneously hypertensive rats. *Hypertension* 2005; 45: 406-411.
39. Arvola P, Wu X, Kähönen M, Mäkynen H, Riutta A, Mucha I, Solakivi T, Kainulainen H, Pörsti I. Exercise enhances vasorelaxation in experimental obesity associated hypertension. *Cardiovasc Res* 1999; 43: 992-1002.
40. Wolf G, Hamann A, Han DC, Helmchen U, Thaiss F, Ziyadeh FN, Stahl RA. Leptin stimulates proliferation and TGF-β expression in renal glomerular endothelial cells: potential role in glomerulosclerosis. *Kidney Int* 1999; 56: 860-872.
41. Lee MP, Orlov D, Sweeney G. Leptin induces rat glomerular mesangial cell hypertrophy, but does not regulate hyperplasia or apoptosis. *Int J Obes* 2005; 29: 1395-1401.
42. Jackson EK, Li P. Human leptin has natriuretic activity in the rat. *Am J Physiol* 1997; 272: F333-338.
43. Bełtowski J, Wójcicka G, Górny D, Marciniak A. Human leptin administered intraperitoneally stimulates natriuresis and decreases renal medullary Na^+,K^+-ATPase activity in the rat - impaired effect in dietary-induced obesity. *Med Sci Monit* 2002; 8: BR221-229.

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DANCE ROUND

We dance round in a ring and suppose,
But the Secret sits in the middle and knows.
Robert Frost

THE ADIPOSE TISSUE: A NEW MEMBER OF THE DIFFUSE NEUROENDOCRINE SYSTEM?

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Abstract

Adipose tissue is a sophisticated module, consisting of adipocytes and non-adipocyte cellular elements including stromal, vascular, nerve and immune cells. There is at present evidence that sharing of ligands and their receptors constitutes a molecular language of the human's body, which is also the case for adipose tissue and hypothalamus-pituitary gland. Historically, Nikolai Kulchitsky's identification of the enterochromaffin cell in 1897 formed the basis for the subsequent delineation of the diffuse neuroendocrine system (DNES) by Friedrich Feyrter in 1938. In DNES paradigm, the secretion of hormones, neuropeptides and neurotrophic factors is executed by cells disseminated throughout the body, for example, Kulchitsky (enterochromaffin) cells, testicular Leydig cells, and hepatic stellate cells. Here we propose that the adipose tissue might be a new member of DNES. Today (*dnes*, in Bulgarian), adipose tissue is "getting nervous" indeed: (*i*) synthesizes neuropeptides, neurotrophic factors, neurotransmitters, hypothalamic hormones/releasing factors and their receptors, (*ii*) like brain expresses endocannabinoids and amyloid precursor protein and, for steroidogenesis, the enzyme aromatase (P450arom), (*iii*) adipocytes may originate from the neural crest cells, and (*iv*) adipose-derived stem cells may differentiate into neuronal cells. Further molecular profiling of adipose tissue may provide new biological insights on its neuroendocrine potential. Overall this may frame a novel field of study, neuroadipobiology; its development and clinical application may contribute to the improvement of human's health.

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Adipose tissue

In the last 20 years, the physical, mental and economic burden of obesity and related diseases is reaching pandemic proportions. Arguably, we have learned more about the molecular control of food intake and energy homeostasis. It is an intricate feedback system in which food intake and energy expenditure are balanced through brain-adipose, brain-gut, entero-insular and reward circuits.

White and brown adipose tissue (WAT and BAT) are morphological and functional expressions of a dynamic system, consisting of adipocytes and non-adipocyte cellular elements, including stromal, vascular, nerve and immune cells (1). Adipose tissue ("WAT" will be assumed from hereon) also contains cells that have the ability to differentiate into several lineages including neuronal cells. By sending and receiving different types of protein and non-protein signals, adipose tissue communicates via

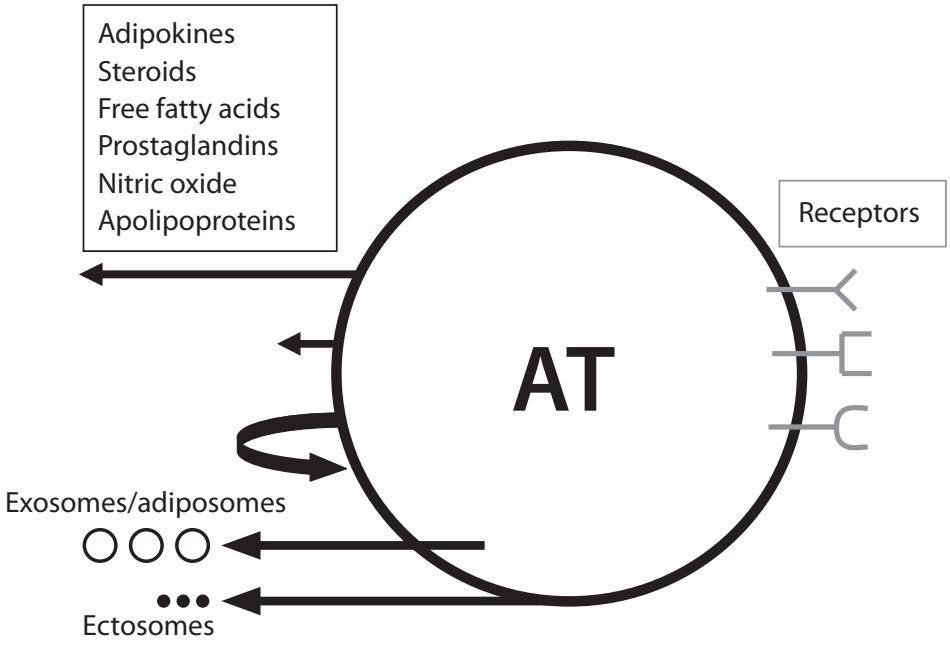

Figure 1. A drawing illustrating both secretory and receptive nature of adipose tissue (AT). At secretory level, AT-derived signaling molecules communicate via multiple pathways such as endocrine (arrows 1,4,5, from up-down), paracrine (arrow 2) and autocrine (arrow 3, curved) as well as via exosomes (multivesicular body-derived microvessels) and ectosomes (plasma membrane-shedding microparticles) (see references 5,89; for exosomes/adiposomes, see 90). At receptive level, AT possesses receptors for various ligands. Modified from 63.

endo- and paracrine way with many organs in the body (Fig. 1). In effect, brain-adipose network plays a pivotal role in the regulation of food intake and energy balance (2) as well as hypothalamic-pituitary cells produce "adipotrophins" (see below). It is increasingly recognized that adipose tissue expresses not only metabolic, but also secretory phenotype, synthesizing and releasing more than 100 signaling proteins designated adipokines (2-5). These are implicated in the regulation of energy, lipid and glucose homeostasis, inflammation, immunity and vascular tone as well as the pathogenesis of cardiometabolic and neurodegenerative diseases.

Neuroendocrinology of adipose tissue

While numerous studies have demonstrated that brain can control adipose tissue functions, it is only now becoming apparent that the control is bidirectional, that is, the adipose tissue can control brain neuroendocrine functions. For instance, (*i*) many neuropeptides and neurotrophic factors and their receptors are shared by the adipose tissue and brain (2-9), (*ii*) the adipokines leptin, adiponectin, resistin and fasting-induced adipose factor (angiopoietin-like protein 4) and their receptors are expressed in the brain (10-15), (*iii*) a subset of adipocytes may originate from the neural crest cells (16), and (*iv*) in cocultures of 3T3-L1 adipocytes with neurons, adipocyte-derived apolipoproteins enhance neuritogenesis and synaptogenesis (17).

Vice versa, adipose tissue produces (*i*) neuropeptide tyrosine (NPY), substance P, calcitonin gene-related protein and other neuropeptides (18-25), and (*ii*) glutamate and gamma-aminobutyric acid (GABA) neurotransmitters, N-methyl-D-aspartate (NMDA) and GABA receptors, and vesicular glutamate transporters (26,27). Moreover, macrophages, mast cells and other immune cells associate with both adipose tissue (3) and pituitary gland (28).

Further, most pituitary hormones and hypothalamic releasing factors, termed "adipotrophins" (29), express their receptors in adipose tissue, creating hypothalamic-pituitary-adipose axis (29,30) as well as some hypothalamic releasing factors are produced by adipose tissue (31,32); recently, pineal-adipose network is also appreciated (see Rančić *et al*' s abstract in this volume of *Adipobiology*). Also, various neurotrophic factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), vascular endothelial growth factor, insulin-like growth factor, and angiopoietin are synthesized and released from adipose tissue (20,25,33-37).

While NGF was first discovered by Rita Levi-Montalcini in 1951 as nerve growth stimulating protein produced in largest amount by the mouse submandibular glands (38), it appears today that the adipose tissue may also be a major biological source of NGF and other neurotrophic factors (reviewed in 39,40).

Noteworthy, semaphorin (Sema3A) and its receptor neuropilin-1 (41), and pantophysin, a protein related to the neuroendocrine-specific synaptophysin (42), are expressed in adipose tissue as well as neural and glial markers in neurally differentiated adipose-derived stromal cells (43-45).

Another neuroendocrine feature of adipose tissue might be its own production of both steroids and endocannabinoids. There is at present clear evidence that adipose tissue, like brain and its aromatase (P450arom) and neurosteroids, produces adiposteroids (see 3,46; the term "adiposteroids" has been introduced by Masuzaki H *et al* in 2004). Endocannabinoids and their receptors, recently extensively studied in food intake control and reward phenomena, are expressed in both hypothalamus/pituitary gland and adipose tissue (47).

Last but not least, it has been recently disclosed a metabolic paradigm for Alzheimer's disease pathogenesis including the role of obesity, cholesterol and adipokines in neurodegeneration (48-50). Also, it is increasingly clear that the hypothalamus is not the only site of leptin action, nor food intake is the only biological effect of leptin. Rather, leptin is a pleiotropic adipokine that supports learning and memory and has neurotrophic activity (14,15,51-53; also Arieh Gertler in this volume of *Adipobiology*; for apelin, a new adipokine, see 54,55). Other neurotrophic factors produced by adipose tissue (20,25,33-37,39,40) may also contribute to neuroprotection in various neuropsychiatric diseases (reviewed in 56).

From enterochromaffin cells to adipose tissue

Historically, Nikolai Konstantinovich Kulchitsky (1856-1925) has identified the enterochromaffin cells found in the crypts of Lieberkuhn of gastrointenstinal mucosa, in 1897. This discovery formed the basis for the subsequent delineation of the diffuse neuroendocrine system (DNES) by Friedrich Feyrter in 1938 (reviewed in 57,58); examples of DNES include Feyrter's Hellen Zellen (clear cells) in pancreas and gut, testicular Leydig cells (59), hepatic stellate cells (Ito cells) (60) and other cells disseminated throughout the body..

Dancing around the accumulating evidence of synthesis and release of multiple neuronal and neuroendrocrine factors and expression of their receptors and various neural markers (Table 1-3), we propose that adipose tissue might be a new member of DNES.

Today (*dnes*, in Bulgarian, Serbian, Polish and Slovak), adipose tissue is "getting nervous" indeed (61). Metaphorically, this talented tissue is increasing dramatically its intelligence quotient (IQ) (62). As well as the gut is considered a second brain (58), the adipose tissue may likely function as a third brain (63). Although "absence of proofs is not proof of absence", further neuroendocrine profiling of adipose tissue is required. It may provide new biological insights on some "newcomers" such as NGF, BDNF, CNTF, nitric oxide (64, also Tunçel *et al* in this vol-

Table 1.
Neuronal and neuroendocrine factors in adipose tissue

Neuropeptides
Agouti protein (2-5)*
Neuropeptide tyrosine (NPY) (20,25)
Calcitonin gene-related peptide (18)
Adrenomedullin (18)
Somatostatin (19)
Insulin-like growth factor (20)
Substance P (21)
Kisspeptin (22)
Neuromedin B (23)
Neurotensin (24)
Mineralocorticoid-releasing factors (31)
Corticotropin-releasing hormone (CRH) (32)
Stresscopin and urocortin (CRH-like peptides) (32)
Apelin (54; cf. 55)
Nesfatin-1 (67)
Neurotrophic factors
Leptin (2-5; cf. 15,51-53)
Apolipoprotein D, E3 (17)
Nerve growth factor (20,25,33,36)
Brain-derived neurotrophic factor (34,35,88)
Angiopoietin-1 (37)
Vascular endothelial growth factor (39)
Ciliary neurotrophic factor (20,39)
Glial cell line-derived neurotrophic factor (39,88)
Steroids (3,46; cf.81-83)
Metallothioneins (65, cf. 66)
Neurotransmitters
Noradrenaline (1)
Glutamate (26)
Gamma-aminobutyric acid (GABA) (26)

* References are indicated in parentheses.

Table 2.
Neuronal and neuroendocrine receptors in adipose tissue

Leptin (ObRb) (4)*
NPY1R, NPY2R, NPY4R (20)
Beta3-adrenergic receptor (25,64)
α2 GABAAR, NR1 NMDAR, GluR2/3 AMPAR** (26,27)
FSH, LH, ACTH, TSH, GH (29,30)
Prolactin, oxytocin, vasopressin (29,30)
p75 neurotrophin receptor (p75NTR) (33,45)
Tropomyosin-related kinase/tyrosine kinase A (Trk A) (43)
Orexin-A, -B (69)
Acetylcholine (muscarinic M3) (70)
Melatonin (84)
Melanocortin-4 receptor (85)

* References are indicated in parentheses.

****** Recent data suggests that glutamate might have neurotrophic effects, while neurotrophins, particularly BDNF, might act as neurotransmitters exerting fast modulating effects on synaptic structure and function (77). AMPAR, alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors of glutamate. Neuronal activation induced via small doses of AMPAR agonists, known as ampakines, has been shown to markedly raise the BDNF levels and lead to cognitive enhancement (78,79).

Table 3.
Neural and neuroendocrine markers in adipose tissue

Semaphorin (Sema3A) (41)*
Neuropilin-1 (41)
Pantophysin (42)
Neuronal nuclear antigen (43)
Nestin (43,44)
Neuron-specific enolase (43,44)
Glial fibrillary acidic protein (43,44)
Vimentin (43)
Stathmin-like 2 (71)
NF70, S100 (72,73)
c-kit (74, cf. 75)
Acetylcholinesterase and choline acetyltransferase (76)
Musashi-1 genes (80)
Amyloid precursor protein/Abeta peptides (86,87)
Beta3 tubulin (88)

* References are indicated in parentheses

ume of *Adipobiology*), metallothioneins (65, cf. 66), neuropeptides B and W and nesfatin-1 (67), and the anti-aging protein klotho (68). Onwards, this may open a novel field of research, neuroadipobiology. Systems biology approach integrating neuroendocrinology, neuroimmunology and neuroadipobiology may indeed contribute to the improvement of human's health and longevity.

Conclusion

In 1999 Albee Messing published in *Hepatology* (volume 29, pp 602-603) Editorial entitled "Nestin in the Liver – Lessons from the Brain". He wrote: "Most neuroscientists manage to get through each day without thinking of the liver even once… but I think that is about to change." This may also be the case for adipose tissue.

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References

1. Cinti S. Transdifferentiation properties of adipocytes in the adipose organ. *Am J Physiol Endocrinol Metab* 2009. doi:10.1152/ajpendo.00183.2009
2. Trayhurn P, Bing C, Wood IS. Adipose tissue and adipokines – energy regulation from the human perspective. *J Nutr* 2006; 136 (7 Suppl): 1935S-1939S.
3. Chaldakov GN, Stankulov IS, Hristova M, Ghenev PI. Adipobiology of disease: adipokines and adipokine-targeted pharmacology. *Curr Pharm Des* 2003; 9:1023-1031.
4. Fantuzzi G, Mazzone T, editors. *Nutrition and Health: Adipose Tissue and Adipokines in Health and Disease*. Humana Press Inc., Tokowa, NJ. 2007.
5. Renes J, Rosenow A, Mariman E. Novel adipocyte features discovered by adipoproteomics. *Adipobiology* 2009; 1: 7-18.
6. Tonchev AB, Boneva NB, Kaplamadzhiev DB, Kikuchi M, Mori Y, Sahara S, *et al*. Expression of neurotrophin receptors by proliferating glia in postischemic hippocampal CA1 sector of adult monkeys. *J Neuroimmunol* 2008; 205:20-24.
7. Wang C, Bomberg E, Billington C, Levine A, Kotz CM. Brain-derived neurotrophic factor in the hypothalamic paraventricular nucleus increases energy expenditure by elevating metabolic rate. *Am J Physiol Regul Integr Comp Physiol* 2007; 293: R992-1002.
8. Coope A, Milanski M, Araújo EP, *et al*. AdipoR1 mediates the anorexigenic and insulin/leptin-like actions of adiponectin in the hypothalamus. *FEBS Lett* 2008; 582: 1471-1476.

9. Stoyanova II, Rutten WLC, le Feber J. Orexin-A and orexin-B during the postnatal development of the rat brain. *Cell Mol Neurobiol* 2009. doi 10.1007/s10571-009-9433-z
10. Brown R, Thompson HJ, Imran SA, Ur E, Wilkinson M. Traumatic brain injury induces adipokine gene expression in rat brain. *Neurosci Lett* 2008; 432: 73-78.
11. Brown R, Imran SA, Belsham DD, Ur E, Wilkinson M. Adipokine gene expression in a novel hypothalamic neuronal cell line: resistin-dependent regulation of fasting-induced adipose factor and SOCS-3. *Neuroendocrinology* 2007; 85:232-241.
12. Brown R, Imran SA, Ur E, Wilkinson M. Valproic acid and CEBPalpha-mediated regulation of adipokine gene expression in hypothalamic neurons and 3T3-L1 adipocytes. *Neuroendocrinology* 2008; 88: 25-34.
13. Brown R, Imran SA, Wilkinson M. Lipopolysaccharide (LPS) stimulates adipokine and socs3 gene expression in mouse brain and pituitary gland in vivo, and in N-1 hypothalamic neurons in vitro. *J Neuroimmunol* 2009; 209: 96-103.
14. Bouret SG, Draper SJ, Simerly RB. Trophic action of leptin on hypothalamic neurons that regulate feeding. *Science* 2004; 304: 108–110.
15. Scott MM, Lachey JL, Sternson SM, Lee CE, Elias CF, Friedman JM, *et al*. Leptin targets in the mouse brain. *J Comp Neurol* 2009; 514: 518-532.
16. Billon N, Monteiro MC, Dani C. Developmental origin of adipocytes: new insights into a pending question. *Biol Cell* 2008; 100:563-575.
17. Kosacka J, Gericke M, Nowicki M, Kacza J, Borlak J, Spanel-Borowski K. Apolipoproteins D and E3 exert neurotrophic and synaptogenic effects in dorsal root ganglion cell cultures. *Neuroscience* 2009; 162:282-291.
18. Gupta P, Harte AL, da Silva NF, *et al*. Expression of calcitonin gene-related peptide, adrenomedullin, and receptor modifying proteins in human adipose tissue and alteration in their expression with menopause status. *Menopause* 2007; 14: 1031-1038.
19. Seboek D, Linscheid P, Zulewski H, *et al*. Somatostatin is expressed and secreted by human adipose tissue upon infection and inflammation. *J Clin Endocrinol Metab* 2004; 89: 4833-4839.
20. Hausman GJ, Barb CR, Dean RG. Patterns of gene expression in pig adipose tissue: Insulin-like growth factor system proteins, neuropeptide Y (NPY), NPY receptors, neurotrophic factors and other secreted factors. *Domest Anim Endocrinol* 2008; 35: 24-34.
21. Karagiannides I, Pothoulakis C. Substance P, obesity, and gut inflammation. *Curr Opin Endocrinol Diabetes Obes* 2009; 16: 47-52.
22. Brown RE, Imran SA, Ur E, Wilkinson M. KiSS-1 mRNA in adipose tissue is regulated by sex hormones and food intake. *Mol Cell Endocrinol* 2008; 281:64-72.
23. Hoggard N, Bashir S, Cruickshank M, Miller JD, Speakman JR. Expression of neuromedin B in adipose tissue and its regulation by changes in energy balance. *J Mol Endocrinol* 2007; 39: 199-210.
24. Koon HW, Kim YS, Xu H, *et al*. Neurotensin induces IL-6 secretion in mouse preadipocytes and adipose tissues during 2,4,6,-trinitrobenzensulphonic acid-induced colitis. *Proc Natl Acad Sci USA* 2009;106: 8766-8771.
25. Mannerås L, Cajander S, Lönn M, Stener-Victorin E. Acupuncture and exercise restore adipose tissue expression of sympathetic markers and improve ovarian morphology in rats with dihydrotestosterone-induced PCOS. *Am J Physiol Regul Integr Comp Physiol* 2009; 296: R1124-1131.
26. Nicolaysen A, Gammelsaeter R, Storm-Mathisen J, Gundersen V, Iversen PO. The components required for amino acid neurotransmitter signaling are present in adipose tissue. *J Lipid Res* 2007; 48: 2123-2132.
27. Kulikov AV, Rzhaninova AA, Goldshtein DV, Boldyrev AA. Expression of NMDA receptors in multipotent stromal cells of human adipose tissue under conditions of retinoic acid-induced differentiation. *Bull Exp Biol Med* 2007; 144: 626-629.
28. Hoek A, Allaerts W, Leenen PJM, Schoemaker J, Drexhage HA. Dendritic cells and macrophages in the pituitary and the gonads. Evidence for their role in the fine regulation of the reproductive endocrine response. *Eur J Endocrinol* 1997; 136:8-24.
29. Schäffler A, Schölmerich J, Buechler C. The role of "adipotrophins" and the clinical importance of a potential hypothalamic-pituitary-adipose axis. *Nat Clin Pract Endocrinol Metab* 2006; 2: 374-383.
30. Schäffler A, Schölmerich J, Buechler C. Hypothesis paper. Brain talks with fat – evidence for a hypothalamic-pituitary-adipose axis? *Neuropeptides* 2005; 39: 363-367.
31. Ehrhart-Bornstein M, Lamounier-Zepter V, Schraven A, *et al*. Human adipocytes secrete mineralocorticoid-releasing factors. *Proc Natl Acad Sci USA* 2003; 100: 14211-14216.
32. Seres J, Bornstein SR, Seres P, *et al*. Corticotropin-releasing hormone system in human adipose tissue. *J Clin Endocrinol Metab* 2004; 89: 965-970.
33. Chaldakov GN, Fiore M, Stankulov IS, *et al*. Neurotrophin presence in human coronary atherosclerosis and metabolic syndrome: a role for NGF and BDNF in cardiovascular disease? *Prog Brain Res* 2004; 146:279-289.
34. Sornelli F, Fiore M, Chaldakov GN, Aloe L. Brain-dervied neurotrophic factor: a new adipokine. *Biomed Rev* 2007; 18: 85-88.
35. Zhao L, Wei X, Ma Z, Feng D, Tu P, Johnstone BH, *et al*. Adipose stromal cells-conditional medium protected glutamate-induced CGNs neuronal death by BDNF. *Neurosci Lett* 2009; 452: 238-240.

36. Hansen-Algenstaedt N, Algenstaedt P, Schaefer C, et al. Neural driven angiogenesis by overexpression of nerve growth factor. *Histochem Cell Biol* 2006; 125: 637-649.
37. Kosacka J, Nowicki M, Kacza J, Borlak J, Engele J, Spanel-Borowski K. Adipocyte-derived angiopoietin-1 supports neurite outgrowth and synaptogenesis of sensory neurons. *J Neurosci Res* 2006; 83: 1160-1169.
38. Levi-Montalcini R. The nerve growth factors 35 years later. *Science* 1987; 237: 1154-1162.
39. Chaldakov GN, Tonchev AB, Fiore M, Hristova MG, Pancheva R, Rancic G, *et al*. Implications for the future of obesity management. In: G Frühbeck, editor. *Peptides in Energy Balance and Obesity*. CAB International 2009; 369-389.
40. Fiore M, Chaldakov GN, Aloe L. Nerve growth factor as a signaling molecule for nerve cells and also for the neuroendocrine-immune systems. *Rev Neurosci* 2009; 20: 133-145.
41. Giordano A, Cesari P, Capparuccia L, Castellucci M, Cinti S. Sema3A and neuropilin-1 expression and distribution in rat white adipose tissue. *J Neurocytol* 2003; 32: 345-352.
42. Brooks CC, Scherer PE, Cleveland K, Whittemore JL, Lodish HF, Cheatham B. Pantophysin is a phosphoprotein component of adipocyte transport vesicles and associates with GLUT4-containing vesicles. *J Biol Chem* 2000; 275: 2029-2036.
43. Dhar S, Yoon ES, Kachgal S, Evans GR. Long-term maintenance of neuronally differentiated human adipose tissue-derived stem cells. *Tissue Eng* 2007; 13: 2625-2632.
44. Gimble JM, Guilak F, Nuttall ME, Sathishkumar S, Vidal M, Bunnell BA. In vitro differentiation potential of mesenchymal stem cells. *Transfus Med Hemother* 2008;35: 228–238.
45. Yamamoto N, Akamatsu H, Hasegawa S, *et al*. Isolation of multipotent stem cells from mouse adipose tissue. *J Dermatol Soc* 2007;48:43-52.
46. Blouin K, Veilleux A, Luu-The V, Tchernof A. Androgen metabolism in adipose tissue: recent advances. *Mol Cell Endocrinol* 2009; 301:97-103.
47. Matias I. The EC system in the adipose tissue and endocrine pancreas. In: J-P Després, V Di Marzo, editors. *Abdominal Obesity and the Endocannabinoid System. From Basic Aspects to Clinical Management of Related Cardiometabolic Risk*. Informa Healthcare USA, Inc.2009; 153-162.
48. Blass JP. A new approach to treating Alzheimer's disease. *Ann NY Acad Sci* 2008; 1147: 122-128.
49. Giordano V, Peluso G, Iannuccelli M, Benatti P, Nicolai R, Calvani M. Systemic and brain metabolic dysfunction as a new paradigm for approaching Alzheimer's dementia. *Neurochem Res* 2007; 32: 555-567.
50. Manning S. Diabetes and dementia: a common link or coincidental coexistence? *Biomed Rev* 2007;18: 59-64.
51. Zhang F, Chen J. Leptin protects hippocampal CA1 neurons against ischemic injury. *J Neurochem* 2008; 107: 578-587.
52. Morrison CD. Leptin signaling in brain: A link between nutrition and cognition? *Biochim Biophys Acta* 2009; 1792: 401-408.
53. Tezapsidis N, Johnston JM, Smith MA, Ashford JW, Casadesus G, Robakis NK, *et al*. Leptin: a novel therapeutic strategy for Alzheimer's disease. *J Alzheimers Dis* 2009; 16: 731-740.
54. Bełtowski J. Apelin and visfatin: unique "beneficial" adipokines upregulated in obesity? *Med Sci Monit* 2006; 12: RA112-119.
55. Reaux A, De Mota N, Skultetyova I, Lenkei Z, El Messari S, Gallatz K, *et al*. Physiological role of a novel neuropeptide, apelin, and its receptor in the rat brain. *J Neurochem* 2001; 77: 1085-1096.
56. Chaldakov GN, Tonchev AB, Aloe L. NGF and BDNF: from nerves to adipose tissue, from neurokines to metabokines. *Rivista Psichiatr* 2009; 44: 79-87.
57. Drozdov I, Modlin IM, Kidd M, Goloubinov VV. From Leningrad to London: the saga of Kulchitsky and the legacy of the enterochromaffin cell. *Neuroendocrinology* 2009; 89: 1-12. 58. Small CJ, Wynne K, Bloom SR. The gut as a second brain. In: G Frühbeck, editor. *Peptides in Energy Balance and Obesity*. CAB International. 2009; 93-113.
59. Davidoff MS, Schulze W, Middendorff R, Holstein AF. The Leydig cell of the human testis - a new member of the diffuse neuroendocrine system. *Cell Tissue Res* 1993; 271: 429-439.
60. Roskams T, Cassiman D, De Vos R, Libbrecht L. Neuroregulation of the neuroendocrine compartment of the liver. *Anat Rec A Discov Mol Cell Evol Biol* 2004;280:910-923.
61. Fliers E, Kreier F, Voshol PJ, Havekes LM, Sauerwein HP, Kalsbeek A, *et al*. White adipose tissue: getting nervous. *J Neuroendocrinol* 2003; 15: 1005-1010.
62. Chaldakov GN, Fiore M, Tonchev AB, Hristova MG, Nikolova V, Aloe L. Tissue with high IQ: adipose-derived stem cells in neural regeneration. *Neural Regen Res* 2009; in print.
63. Chaldakov GN, Fiore M, Tonchev AB, Hristova MG, Rančić G, Aloe L. The adipose tissue as a third brain. *Obesity Metab* 2009; in print.
64. Canová NK, Lincová D, Kmonícková E, Kameníková L, Farghali H. Nitric oxide production from rat adipocytes is modulated by beta3-adrenergic receptor agonists and is involved in a cyclic AMP-dependent lipolysis in adipocytes. *Nitric Oxide* 2006; 14: 200-211.
65. Do MS, Nam SY, Hong SE, Kim KW, Duncan JS, Beattie JH, *et al*. Metallothionein gene expression in human adipose tissue from lean and obese subjects. *Horm Metab Res* 2002; 34:348-351.
66. Pedersen MO, Jensen R, Pedersen DS, Penkowa M, *et al*. Metallothionein-I+II in neuroprotection. *Biofactors* 2009; 35:315-325.

67. Harrold JA, Williams G. Newcomers and supporting actors. In: G Frühbeck, editor. *Peptides in Energy Balance and Obesity*. CAB International. 2009; 61-92.
68. Chihara Y, Rakugi H, Ishikawa K, Ikushima M, Maekawa Y, Ohta J, *et al*. Klotho protein promotes adipocyte differentiation. *Endocrinology* 2006;147:3835-3842.
69. Digby JE, Chen J, Tang JY, Lehnert H, Matthews RN, Randeva HS. Orexin receptor expression in human adipose tissue: effects of orexin-A and orexin-B. *J Endocrinol* 2006; 191: 129-136.
70. Yang TT, Chang CK, Tsao CW, Hsu YM, Hsu CT, Cheng JT. Activation of muscarinic M-3 receptor may decrease glucose uptake and lipolysis in adipose tissue of rats. *Neurosci Lett* 2009; 451: 57-59.
71. Chiellini C, Grenningloh G, Cochet O, Scheideler M, Trajanoski Z, Ailhaud G, *et al*. Stathmin-like 2, a developmentally-associated neuronal marker, is expressed and modulated during osteogenesis of human mesenchymal stem cells. *Biochem Biophys Res Commun* 2008; 374: 64-68.
72. Ning H, Lin G, Lue TF, Lin CS. Neuron-like differentiation of adipose tissue-derived stromal cells and vascular smooth muscle cells. *Differentiation* 2006; 74:510-518.
73. Atanassova P. Immunohistochemical expression of S-100 protein in human embryonal fat cells. *Cells Tissues Organs* 2001; 169:355-360.
74. Bai X, Sadat S, Gehmert S, Alt E, Song YH. VEGF receptor Flk-1 plays an important role in c-kit expression in adipose tissue derived stem cells. *FEBS Lett* 2007; 581:4681-4684.
75. Wrage PC, Tran T, To K, Keefer EW, Ruhn KA, Hong J, *et al*. The neuro-glial properties of adipose-derived adult stromal (ADAS) cells are not regulated by Notch 1 and are not derived from neural crest lineage. *PLoS One* 2008; 3:e1453.
76. Aluigi MG, Coradeghini R, Guida C, Scanarotti C, Bassi AM, Falugi C, *et al.* Pre-adipocytes commitment to neurogenesis 1: preliminary localisation of cholinergic molecules. *Cell Biol Int* 2009; 33:594-601.
77. Georgiev DD, Taniura H, Kambe Y, Yoneda Y. Crosstalk between brain-derived neurotrophic factor and N-methyl-D-aspartate receptor signaling in neurons. *Biomed Rev* 2008; 19:17-27.
78. Lynch G. Glutamate-based therapeutic approaches: ampakines. *Curr Opin Pharmacol* 2006; 6: 82-88.
79. Lynch G, Rex CS, Chen LY, Gall CM. The substrate of memory: Defects, treatments, and enhancement. *Eur J Pharmacol* 2008; 585: 2-13.
80. Nagase T, Matsumoto D, Nagase M, *et al*. Neurospheres from human adipose tissue transplanted into cultured mouse embryos can contribute to craniofacial morphogenesis: a preliminary report. *J Craniofac Surg* 2007; 18:49-53.
81. Zhang Y, Nadeau M, Faucher F, *et al*. Progesterone metabolism in adipose cells. *Mol Cell Endocrinol* 2009; 298:76-83.
82. Cekic M, Sayeed I, Stein DG. Combination treatment with progesterone and vitamin D hormone may be more effective than monotherapy for nervous system injury and disease. *Front Neuroendocrinol* 2009; 30:158-172.
83. Sayeed I, Stein DG. Progesterone as a neuroprotective factor in traumatic and ischemic brain injury. *Prog Brain Res* 2009; 175:219-237.
84. Torres-Farfan C, Valenzuela FJ, Mondaca M, Valenzuela GJ, Krause B, Herrera EA, *et al*. Evidence of a role for melatonin in fetal sheep physiology: direct actions of melatonin on fetal cerebral artery, brown adipose tissue and adrenal gland. *J Physiol* 2008; 586:4017-4027.
85. Bartness TJ, Kay Song C, Shi H, Bowers RR, Foster MT. Brain-adipose tissue cross talk. *Proc Nutr Soc* 2005; 64:53-64.
86. Lee YH, Tharp WG, Maple RL, Nair S, Permana PA, Pratley RE. Amyloid precursor protein expression is upregulated in adipocytes in obesity. *Obesity (Silver Spring)* 2008; 16:1493-1500.
87. Lee YH, Martin JM, Maple RL, Tharp WG, Pratley RE. Plasma amyloid-beta peptide levels correlate with adipocyte amyloid precursor protein gene expression in obese individuals. *Neuroendocrinology* 2009; August 12: in print.
88. Ohta Y, Takenaga M, Tokura Y, Hamaguchi A, Matsumoto T, Kano K, *et al*. Mature adipocyte-derived cells, dedifferentiated fat cells (DFAT), promoted functional recovery from spinal cord injury-induced motor dysfunction in rats. *Cell Transplant* 2008;17:877-886.
89. Deng ZB, Poliakov A, Hardy RW, Clements R, Liu C, Liu Y, *et al*. Adipose tissue exosome-like vesicles mediate activation of macrophage-induced insulin resistance. *Diabetes* 2009; 12 August: in print.
90. Aoki N, Jin-no S, Nakagawa Y, Asai N, Arakawa E, Tamura N, *et al*. Identification and characterization of microvesicles secreted by 3T3-L1 adipocytes: redox- and hormone-dependent induction of milk fat globule-epidermal growth factor 8-associated microvesicles. *Endocrinology* 2007;148:3850-3862.

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DELINEATING ADIPOSE-DERIVED SIGNALS FROM NOISE IN THE PATHOGENESIS OF OBESITY AND RELATED CARDIOMETABOLIC DISEASES

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Several different definitions of *noise* can be found consulting a dictionary. One of the most simple and straightforward defines *noise* as a "sound that is loud, unpleasant, unexpected, or undesired". Some other descriptive characteristics conventionally used in the definition of *noise* are "continuous or repeated, loud and confused sounds". From a technical point of view, *noise* represents "irregular fluctuations that accompany a transmitted signal but are not part of it and tend to obscure it". Depending on the specific scientific speciality or background slight differences in meaning are added or emphasized. In physics, for example, *noise* is viewed as random fluctuations that obscure or do not contain meaningful data or other information, while in computer science *noise* is viewed as irrelevant or meaningless data. Undoubtedly, *noise* reduces the accuracy and repeatability of measurements and introduces distortion in signals. In a biological context *noise* can be the result of diverse circumstances originating an undesirable variation that distorts, obscures or reduces the clarity of a signal under physiological or pathological circumstances. In this sense, contradictory data or controversial results may be viewed as *noise* under given experimental and pathophysiological conditions merely reflecting the "biological complexity". Disentangling the neuroendocrine systems which regulate energy homeostasis and adiposity has been a long-standing challenge in pathophysiology, with obesity as an increasingly important public health problem. Adipose tissue is no longer considered a passive bystander in body weight regulation since it actively secretes a large number of hormones, growth factors, enzymes, cytokines, complement factors and matrix proteins, at the same time as expressing receptors for most of these elements, which influence fuel storage, mobilization and utilization at both central and peripheral sites. Thus, an extensive cross-talk at a local and systemic level in response to specific external stimuli or metabolic changes underpins the multifunctional characteristics of adipose tissue. In addition to the already well-known adipokines, such as interleukins, tumor necrosis factor-alpha, leptin, resistin, and adiponectin, attention has been more recently devoted to "newcomers" to the adipobiology and adipopharmacology fields, which include retinol-binding protein 4, vascular endothelial growth factor, visfatin, vaspin, lipocalin-2, and nerve growth factor among others (1-3). Physiologic, pharmacologic, genomic, proteomic and metabolomic methodologies provide extremely valuable information at a level not previously attainable to help unlock the molecular basis of obesity. However, the information regarding some of these adipose-derived factors is not always homogeneous. When are the fluctuations that we detect from *in vivo* or whole-body measurements *noise*, or the signature of complex cell-wide processing? The potential causes or sources of *noise* regarding concrete adipokines and adipose-derived factors in the context of their true contribution and relevance to adipobiology and their relation to the pathogenesis of obesity and related cardiometabolic diseases will be analysed.

1. Chaldakov GN, Stankulov IS, Hristova M, Ghenev PI. Adipobiology of disease: adipokines and adipokine-targeted pharmacology. *Curr Pharm Des* 2003;9:1023-1031.
2. Trayhurn P. Adipocyte biology. *Obes Rev* 2007; 8 (Suppl 1): 41-44.
3. Catalán V, Gómez-Ambrosi J, Rodríguez A, Salvador J, Frühbeck G. Adipokines in the treatment of diabetes mellitus and obesity. *Expert Opin Pharmacother* 2009;10:239-254.

SITE-SPECIFIC PROPERTIES AND PARACRINE INTERACTIONS IN MAMMALIAN ADIPOSE TISSUE: IMPLICATIONS FOR CROHN'S DESEASE AND HIV INFECTION

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Mammalian adipose tissue is partitioned into a few large and numerous small depots, many with site-specific properties that equip them for paracrine interactions with the immune system, blood vessels (vasocrine interactions with perivascular/periadventitial adipocytes), heart and probably muscle (1). Most adipocyte-derived messengers operate in a paracrine and/or autocrine manner, including adiponectin (2), resistin (3), zinc α2-glycoprotein (4), and chemerin (5). Adipocytes around lymph nodes (and omental milky spots) are specialized for paracrine interactions with lymphoid cells. As well as secreting and binding to cytokines, perinodal adipocytes selectively accumulate fatty acids, especially essential precursors for eicosanoids and docosanoids and release them in response to local lipolytic signals. Dendritic cells and lymph node lymphoid cells obtain many, possibly all, the fatty acids found in structural lipids from adjacent perinodal adipocytes (6). In rats, chronic, mild stimulation alters the lipid composition of adipicytes and dendritic cells near the inflamed site except after many weeks on diets enriched with fish oil. The compositions of adipose triacylglycerol fatty acids near the stimulation site change to counteract imbalances in dietary lipids (7). Site-specific differences in the composition of lipids taken up from contiguous adipocytes create further dendritic cell diversity. By supplying appropriate fatty acids to lymphoid cells, perinodal adipocytes intervene between the diet and immune system nutrition, partially emancipating it from fluctuations in the abundance and composition of dietary lipids. Paracrine control of lipolysis by lymphoid cells reduces competition with other tissues, thus enabling fever and other energetically expensive defences against pathogens to act simultaneously with immune responses, and unrelated functions such as lactation and exercise. Prolonged immune stimulation induces the formation of more adipocytes in perinodal adipose tissue around adjacent lymph nodes; this effect reverses very slowly, and may contribute to mesenteric and omental hypertrophy in chronic inflammatory states such as HIV-infection (8) and in smokers. Perinodal adipose tissue is abnormal in Crohn's disease, leading to insufficiency of the fatty acid precursors of eicosanoids and docosanoids in mesenteric lymph nodes, even when they are plentiful in the diet and in adipose tissue (9). Adipose tissues around lymph nodes, in the omentum and probably 'yellow' bone marrow are specialized and the tissues function together. Adipose tissue partitioned into numerous depots many specialised for paracrine interactions, is a fundamental feature of mammals, essential for multiple simultaneous responses to pathogens and more efficient utilization of lipids in immune responses, starvation, rapid thermogenesis and lactation. Such 'upgrading' of adipose tissue occurred early in the evolution of mammals from reptiles, that lack such active management of stored lipids (10). Pharmacologists should harness the special properties of perinodal adipose tissue to reach drug targets in the immune system, and should consider drug modulation from active lipid management in perinodal adipocytes.

1. Pond CM. *Curr Immunol Rev* 2009;5:150-160.
2. Dietze-Schroeder D *et al. Diabetes* 2005;54:2003-2011.
3. Iacobellis G *et al. Horm Metab Res* 2008;40: 442-445.
4. Bing C *et al. Proc Natl Acad Sci USA* 2004;101: 2500-2505.
5. Roh SG *et al. Biochem Biophys Res Comm* 2007;362: 1013-1018.
6. Pond CM *et al. Brit J Nutr* 2003;89: 375-382.
7. Mattacks CA *et al. Lymph Res Biol* 2004;2: 107-129.
8. Sadler D *et al. J Anat Lond* 2005;207: 761-789.
9. Westcott EDA *et al. Inflamm Bowel Dis* 2005;11: 820-827.
10. Pond CM. *J Exp Zool* 2003;295A: 99-110.

DEVELOPMENT OF LEPTIN ANTAGONISTS AND THEIR EVENTUAL USE AS MEDICAL MODALITIES

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Leptin is a pleotropic hormone that acts both centrally and peripherally. Whereas leptin exhibits positive effects on several physiological functions, such as regulation of energy metabolism, reproductive function and immune response, negative actions, such as enhancement of undesired immune responses in autoimmune diseases, tumorigenesis, elevated blood pressure and cardiovascular pathologies have also been documented (1). Using a sensitive Hydrophobic Cluster Analysis of leptin's and leptin receptor's (LEPR's) sequences, we identified hydrophobic stretches in leptin's A-B loop (amino acids 39-42) that are predicted to affect the site III and to interact with the (LEPR's) immunoglobulin domain. To verify this hypothesis we prepared and purified to homogeneity several alanine muteins of amino acids 39-42 in human, ovine, rat and mouse leptins. CD analyses revealed that those mutations hardly affect the secondary structure. All muteins acted as true antagonists, i.e. they bound LEPR with an affinity similar to the wild-type hormone, had no agonistic activity and specifically inhibited leptin action in several leptin-responsive *in vitro* bioassays. To prolong and enhance the *in vivo* action of leptin antagonists we increased in molecular mass and hydrodynamic volume of leptin antagonists by pegylation, attaching covalently 20 kDa polyethylene glycol (PEG) through antagonist's alpha amino group. The *in vivo* half-life of mono-pegylated (with 20 kDa PEG) leptin antagonist was increased respectively by 13-fold (to 22.7 hours). Administration of the pegylated antagonist in mice produced a rapid, significant and fully reversible weight gain, due to enhanced appetite and increased food consumption. Resulting fat was confined mainly to the mesenteric region with sparing of the liver. In contrast injection of pegylated leptin (PEG-LEP) had opposite effect. The mechanism of severe central leptin deficiency resulted mainly from inhibition of leptin transport across the blood-brain barrier and limited accumulation of pegylated mouse leptin antagonist (PEG-MLA) in the hypothalamic region. Those findings were evidenced by follow-up after the distribution of radio-labeled PEG-MLA or MLA and by Alexa Fluor® 680-conjugated PEG-MLA and MLA in the body. The latter was continuously tracked using the IVIS *in vivo* imaging system. Pegylated and non-pegylated leptin antagonists were also tested *in vivo* as blockers of experimental leptin-enhanced mice models of T-cell dependent and non-dependent acute hepatitis, induced by Cocanavalin A, Pseudomonas exotoxin or LPS + galactosamine and in mice model of chronic liver fibrosis induced by thioacetamide. In all experiments leptin antagonists have shown anti-inflammatory and anti-fibrotic activity and improved survival. Additional experiments to enhance the effectiveness of leptin antagonists by increasing their affinity toward LEPRs are carried out using random mutagenesis followed by selection of high-affinity mutants by yeast surface display method. In conclusion we introduce a novel compound that induces central and peripheral leptin deficiency. This compound is useful in exploring the role of leptin in metabolic and immune processes and could eventually serve as a therapeutic for the treatment of cachexia in wasting diseases such as cancer and AIDS and in leptin-enhanced autoimmune diseases.

1. Gertler A, editor. *Leptin and Leptin Antagonists*. Landes Bioscience, Austin, Texas, USA. 2009.

LEPTIN AS AN ENDOGENOUS CARDIAC HYPERTROPHIC FACTOR: STUDIES INTO MECHANISMS

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Once considered to be solely derived from adipose tissue it is now apparent that leptin can be produced by a multiplicity of tissues, including the heart. Leptin's actions are mediated *via* a family of receptors which have been identified in many peripheral tissues including cardiomyocytes suggesting that leptin functions in a paracrine or autocrine manner. The pathophysiological impact of leptin on the cardiovascular system represents an area of active investigation. Leptin has now been extensively shown to induce cardiomyocyte hypertrophy and blocking of leptin receptors reduces hypertrophy and heart failure in rats subjected to myocardial infarction (reviewed in 1). Leptin may also mediate the prohypertrophic actions of other factors including angiotensin II and endothelin-1 (2). The underlying mechanisms of leptin-induced cardiomyocyte hypertrophy remain to be fully elucidated. We have previously shown that leptin-induced cardiomyocyte hypertrophy is associated with rapid activation of the mitogen-activated protein kinase system including p38 and p44/42 as early as 5 minutes after leptin addition (3). However, hypertrophy was inhibited only by the p38 inhibitor SB203580 but not by the p44/42 inhibitor PD98059. Recent work from our laboratory has shown that a key feature of leptin-induced hypertrophy is significant activation of the RhoA/ROCK pathway and an increase in phosphorylation of the actin binding protein cofilin as well an increase in polymerization of actin, as reflected by a decrease in the G/F-actin ratio (4). Although both p38 and ERK phosphorylation were increased these effects were associated with selective p38 but not ERK translocation into nuclei suggesting that p38 translocation represents a critical mechanism underlying leptin-induced cardiac hypertrophy (5). Moreover, this finding helps to explain why p38 inhibition, but not inhibition of ERK, prevents leptin-induced hypertrophy. Pharmacological inhibition of RhoA/ROCK or disruption of actin filaments blocked leptin-induced cardiomyocyte hypertrophy which was associated with a complete abrogation of leptin-induced nuclear translocation of p38. Another potential target for leptin action in cardiac cells is the phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) pathway. The PI3K/Akt axis regulates many important and diverse cellular processes including cell survival, proliferation, growth, and actin filament remodeling. Indeed, it has been shown that PI3K and Akt are upregulated in response to pressure-overload-induced cardiac hypertrophy. A downstream target of the PI3K/Akt pathway is the mammalian target of rapamycin (mTOR). Inhibition of mTOR by rapamycin attenuates cardiomyocyte hypertrophy induced by different growth factors indicating that mTOR is an important player in cardiomyocyte hypertrophy. Recently, we have identified a critical role for PI3K/mTOR/$p70^{S6K}$ in leptin-induced RhoA activation resulting in cardiomyocyte hypertrophy associated with activation of the transcriptional factor GATA-4 (unpublished). Thus, leptin represents an endogenous cardiac hypertrophic factor the effects of which are mediated by mTOR-dependent RhoA activation resulting in nuclear p38 import and GATA-4 activation.

1. Karmazyn M, Purdham DM, Rajapurohitam V, Zeidan A. *Trends Cardiovasc Med* 2007; 17:206-211
2. Rajapurohitam V, Javadov S, Purdham DM, Kirshenbaum LA, Karmazyn M. *J Mol Cell Cardiol* 2006; 41:265-274.
3. Rajapurohitam V, Gan XT, Kirshenbaum LA, Karmazyn M. *Circ Res*. 2003; 93:277-279.
4. Zeidan A, Javadov S, Karmazyn M. *Cardiovasc Res* 2006; 72:101-111.
5. Zeidan A, Javadov S, Chakrabarti S, Karmazyn M. *Cardiovasc Res* 2008; 77:64-72.

LEPTIN INCREASES PLATELET ACTIVITY IN THE RAT: *IN VITRO* AND *IN VIVO* STUDIES

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Chronically elevated leptin may contribute to various complications of obesity including atherosclerosis (1); however, the underlying mechanisms are incompletely clear. Some of the previous studies have demonstrated that leptin stimulates platelet aggregation, the results of them being controversial. We examined the effect of leptin on platelet activity by measuring stable metabolites of thromboxane A_2 (TxA_2), TxB_2, 11-dehydro-TxB_2 and 2,3-dinor-TxB_2 in plasma and urine. *In vitro*, leptin stimulated TxB_2 formation by platelet-rich plasma (PRP). *In vivo*, leptin (1 mg/kg ip) increased urinary excretion of 11-dehydro-TxB_2 and 2,3-dinor-TxB_2. Urinary 11-dehydro-TxB_2 excretion was also elevated in rats made hyperleptinemic by administration of recombinant leptin (0.5 mg/kg/day) for 8 days. The stimulatory effect of leptin on TxB_2 formation in PRP isolated from hyperleptinemic animals was impaired in comparison to the control group. Acute stimulatory effect of leptin on TXB_2 formation *in vitro* was also impaired in rats made obese and hyperleptinemic by feeding highly palatable "cafeteria" diet for 1-month. In contrast, acute effect of leptin on TXB_2 production was not impaired in animals fed highly palatable diet for 3 months, which were not only obese and hyperleptinemic but also hyperinsulinemic and insulin-resistant. In rats fed a high-dose fructose for 8 weeks (hyperinsulinemia/insulin resistance but normal body weight and leptin level), stimulatory effect of leptin on TXB_2 formation in PRP *in vitro* was augmented in comparison to the control group. Insulin sensitizer, rosiglitazone (10 mg/kg for 8 days), reduced insulin level and decreased the stimulatory effect of leptin on TXB_2 formation in PRP *in vitro* in the 3-month obese and fructose-fed animals but not in either control rats and rats receiving recombinant leptin for 8 days. These results indicate that: (*i*) leptin stimulates platelet TXA_2 formation both *in vitro* and *in vivo*, (*ii*) chronic hyperleptinemia impairs acute stimulatory effect of leptin on platelet activity if insulin sensitivity is normal, (*iii*) insulin resistance/hyperinsulinemia augments the stimulatory effect of leptin on platelet TXA_2 formation, which results in normal platelet sensitivity to leptin in obesity associated with both hyperleptinemia and hyperinsulinemia, (*iv*) PPAR-γ agonists such as rosiglitazone reduce platelet sensitivity to leptin by reducing insulin resistance.

1. Beltowski J. Leptin and atherosclerosis. *Atherosclerosis*. 2006; 189: 47-60.

DIET-INDUCED ALTERATIONS IN LEPTIN EXPRESSION CAN OCCUR INDEPENDENTLY OF CALORIC INTAKE

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Dietary flaxseed has been shown to inhibit cardiac arrhythmias, reduce atherosclerosis and improve vascular function. Flaxseed is an excellent source of the omega-3 fatty acid, alpha linolenic acid (ALA), which is stored in adipose tissue. It is possible that a portion of the beneficial cardiovascular effects achieved through dietary flaxseed may occur through an action on the adipose tissue. We investigated the effects of dietary flaxseed both with and without an atherogenic diet to determine the extent of adipose involvement in the cardiovascular benefits of ALA. Rabbits were fed isocaloric diets: a regular (RG) diet, or a regular diet with added 0.5% cholesterol (CH), or 10% ground flaxseed (FX), or both (CF) for 8 weeks. Plasma was taken before and after dietary treatment for analysis of plasma cholesterol and triglycerides, as well as fatty acid composition. Multiple sources of visceral adipose were assessed by RT-PCR to determine leptin and adiponectin expression. Dietary supplementation with cholesterol significantly increased plasma cholesterol levels. The ingestion of flaxseed with cholesterol supplementation did not reduce the elevated plasma cholesterol levels. Plasma triglycerides were unaffected by either flaxseed or cholesterol treatment. Consumption of flaxseed significantly increased plasma and adipose levels of ALA, and the addition of cholesterol to the diet increased the levels of ALA in both tissues even further. Leptin protein and mRNA expression were lower in animals fed a high cholesterol diet and were elevated in animals fed the cholesterol + flaxseed, despite an isocaloric diet. Changes in leptin were strongly correlated with adipose ALA levels (r^2=0.782) and inversely correlated with atherosclerosis (r^2=0.597). These effects on leptin were specific, as adiponectin expression was not significantly affected by either cholesterol or flaxseed supplementation. The findings suggest that the cardiovascular effects associated with long-term flaxseed consumption may be related to the metabolic effects of leptin. Our data emphasize the important role of adipose tissue in cardiovascular disease and identify for the first time that leptin is influenced not only by the caloric content of the diet but by the lipid composition of the diet as well.

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PATHOPHYSIOLOGICAL AND PHARMACOLOGICAL IMPORTANCE OF PLACENTAL LEPTIN

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The field of physiology and pathology has been profoundly altered by the recent progress in adipobiology, the main message of this "ongoing story" being the discovery that white adipose tissue is a potent endo- and paracrine organ. Leptin, a pioneering member of the adipokine family of multifunctional proteins, is an *ob/ob* gene 16 kD product discovered almost 15 years ago as a key antiobesity factor (1). Leptin mediates its effects by binding and activation of the leptin receptor encoded by the *db* gene. Today its is know that leptin is also produced by extra-adipose sources such as stomach epithelial cells, placenta, skeletal and heart muscles, and brain. Further, leptin is present in both endometrium and blastocyst and its receptor is also found in the embryo and in the endometrium, thus the leptin system may be an important factor in the mother-embryo crosstalk (2). Placental leptin plays a key role also in fetal intrauterine and extrauterine growth and development. Leptin exerts autocrine effects such as invasion by trophoblasts, placental growth, and angiogenesis as well as paracrine effects including influence on fetal growth during normal or complicated pregnancy and labor. Epidemiological and experimental studies have highlighted a relationship between the periconceptual, fetal and early infant phases of life and the subsequent development of adult obesity and type 2 diabetes mellitus, a process referred to as developmental programming (3). Serum levels of leptin vary dramatically during intrauterine and early postnatal life, with 5-10 fold increase in leptin occurring between postnatal days 4 and 10 in female mice, the so-called "leptin surge"; close association between increased fetal leptin and enlarged adipose depot makes leptin a potential biomarker of prenatal obesity (3). Breast milk also contains significant amounts of leptin, which may contribute to circulating levels in the neonate. Armamentarium of future generations of doctors will probably include (adipo)pharmacologically active substances which affect leptin receptors, to possibly prevent developmental malprogramming and recurrent abortions, and influence on gestational diabetes and preeclampsia.

1. Friedman JM. Leptin at 14 yr of age: an ongoing story. *Am J Clin Nutr* 2009; 89: 973S-979S.
2. Cervero A, Simon S. Involvement of leptin in the endometrial function. In: A Gertler, editor. *Leptin and Leptin Antagonists*. Landes Bioscience, Austin, Texas, USA. 2009; 108-115.
3. Vickers MH, Krechowec SO, Gluckman PD, Breier BH. The role of leptin during early life in imprinting later metabolic responses. In: A Gertler, editor. *Leptin and Leptin Antagonists*. Landes Bioscience, Austin, Texas, USA. 2009; 141-162.

LEPTIN-INDUCED CHANGES IN ERYTHROCYTE DEFORMABILITY AFTER *IN VITRO* OXIDATIVE STRESS

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There are indications now that leptin, the *ob* gene product, actively participates not only in metabolic regulations but also in the control of cardiovascular functions (1). Alterations in the cell membranes may underlie some pathogenic mechanisms in cardiovascular disease (2). Recent studies reveal that leptin induces oxidative stress which in turn may decreases erythrocyte deformability, the ability of erythrocytes to change shape. Leptin has specific binding sites in most of the tissues as well as erythrocytes. The present study was planned for investigating the hypothesis that leptin can affect the rheological behavior of erythrocytes, focusing on changes in their deformability. Human erythrocytes were incubated with various concentrations of leptin *in vitro*. As the plasma leptin level of the subjects may affect the results, the subjects are grouped in two according to their body mass index (BMI) values, as normal (BMI<25) and overweight/obese (BMI≥25) (cf. 3). 10% suspensions of erythrocytes were prepared in isotonic phosphate buffer. In order to induce oxidative stress erythrocyte suspensions were exposed to sodium azide (100 μM, 10 min) combined with hydrogen peroxide (20 mM, 10 min). Suspensions were incubated for 10 min with concentrations of 2.5 ng/ml, 25 ng/ml, 250 ng/ml and 2.5 μg/ml leptin before the oxidative stress protocol. Erythrocyte deformability was measured by Laser-assisted Optical Rotational Cell Analyzer (LORCA) at the shear rate of 30 Pa at 37°C and results were expressed as Elongation Index (EI). Oxidative stress protocol decreased the deformability of erythrocytes significantly in both groups studied. The decrease in the normal BMI group was 7.5 ±1.4% and the decrease in the overweight/obese group was 7.1 ±1.2%. Pretreatment of erythrocytes with leptin before the oxidative stress protocol further decreased erythrocyte deformability in the normal BMI group. Leptin-induced significant decrease in erythrocyte deformability was found to be 9.5±1.9% (∗), 9.1 ±1.5% (∗), 9.3 ±1.3% (∗) and 9.9 ±2.1% (∗) for 2.5 ng/ml, 25 ng/ml, 250 ng/ml and 2.5 μg/ml leptin, respectively (∗, $p<0.05$). However, in the overweight/obese group the pretreatment of erythrocytes with leptin did not change the erythrocyte deformability compared to oxidative stress. As a result *in vitro* pretreatment of erythrocytes with leptin before the oxidative stress protocol causes distinct results according to the BMI of the subjects. Pretreatment of erythrocytes with leptin before the oxidative stress protocol in the normal BMI group further deteriorates erythrocyte deformability, but in the overweight/obese group leptin has no further effect. This result indicates a possible decrease in the number and/or downregulation of leptin receptors in the erythrocytes of overweight/obese group.

1. Beltowski J. Leptin and atherosclerosis. *Atherosclerosis*. 2006; 189: 47-60.
2. Tsuda K, Nishio I. Leptin and membrane fluidity of erythrocytes in essential hypertension. An electron paramagnetic resonance investigation. *Am J Hypertens* 2004; 17:375-379.
3. Sharma AM, Kushner RF. A proposed clinical staging system for obesity. *Int J Obesity* 2009; doi:10.1038/ijo.2009.2

ADIPOPROTEOMICS, A VALUABLE TECHNOLOGY FOR FASCINATING FAT

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Adipocytes are important for normal functioning of the human body. The most striking examples are lipodystrophic persons who suffer from metabolic complications. However, adipocyte dysfunctioning, accompanied by adipocyte hypertrophy and disregulated adipokine profiles, is associated with the initiation and progression of obesity-related diseases. Knowledge of the adipocyte behaviour under different nutritional conditions and the crosstalk of adipocytes with other cells and organs is essential to understand the relation between obesity and obesity-related disorders. A full understanding of the adipocyte behaviour requires a systems biology approach with integrated transcriptomics, proteomics and metabolomics data. Adipoproteomics exists for already 30 years but is boosted during the last decade with the enormous technological developments in mass spectrometry technology. Currently 3 major proteomics strategies are applied: (*i*) gel-based protein separation combined with mass spectrometry, (*ii*) liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS), and (*iii*) antibody array technology. However, none of them is able to cover the entire proteome at once due to the complexity of the proteome and technical limitations. Still, proteomics technologies played an important role in the further understanding of the molecular aspects of adipocyte differentiation and particularly adipokine profiling. Novel biological features of adipocytes have been discovered, including: mitochondrial biosynthesis during adipocyte differentiation (1), non-reciprocal regulation of glycolysis by adipocyte starvation (2), thiazolidinedione-induced fatty acid catabolism during adipocyte differentiation (3) and in mature adipocytes (4), transcriptional regulation (5), identification of novel adipokines (6-9) and a novel mechanism of adipokine secretion (10) have been discovered by proteomics investigations. Since none of the current existing proteomics approaches can be applied for total proteome coverage, proteome investigations require a combination of current analysis techniques and subcellular fractionation to reduce sample complexity. Future applications for proteomics research in adipose biology are the further dissection of (*i*) the molecular events during human adipogenesis, (*ii*) the remodelling of the adipocyte extracellular matrix, and (*iii*) the link between obesity and obesity-related metabolic complications. Adipokines are supposed to play an important role in obesity-related disorders. However, how the change in adipokine profiles influence neighbour and remote target cells and how they induce metabolic complications remains elusive. A challenging task for adipoproteomics lies ahead.

1. Wilson-Fritch L, Burkart A, Bell G, *et al*. Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer rosiglitazone. *Mol Cell Biol* 2003; 23:1085-1094.
2. Renes J, Bouwman F, Noben JP, Evelo C, *et al.* Protein profiling of 3T3-L1 adipocyte differentiation and (tumor necrosis factor alpha-mediated) starvation. *Cell Mol Life Sci* 2005; 62:492-503.
3. Renes J, van Tilburg J, van Haaften R, *et al*. Thiazolidinediones regulate expression of proteins involved in triacylglyceride storage and fatty acid oxidation in 3T3-L1 (pre-)adipocytes. *Adipocytes* 2006; 2:75-91.
4. Wang P, Renes J, Bouwman F, Bunschoten A, *et al*. Absence of an adipogenic effect of rosiglitazone on mature 3T3-L1 adipocytes: increase of lipid catabolism and reduction of adipokine expression. *Diabetologia* 2007; 50:654-665.
5. Molina H, Yang Y, Ruch T, *et al*. Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy. *J Proteome Res* 2009; 8:48-58.
6. Kratchmarova I, Kalume DE, Blagoev B, *et al*. proteomic approach for identification of secreted proteins during the differentiation of 3T3-L1 preadipocytes to adipocytes. *Mol Cell Proteomics* 2002; 1:213-222.
7. Wang P, Mariman E, Keijer J, *et al*. Profiling of the secreted proteins during 3T3-L1 adipocyte differentiation leads to the identification of novel adipokines. *Cell Mol Life Sci* 2004; 61:2405-2417.
8. Alvarez-Llamas G, Szalowska E, de Vries MP, *et al*. Characterization of the human visceral adipose tissue secretome. *Mol Cell Proteomics* 2007; 6:589-600.
9. Zvonic S, Lefevre M, Kilroy G, *et al*. Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. *Mol Cell Proteomics* 2007; 6:18-28.
10. Wang P, Keijer J, Bunschoten A, *et al*. Insulin modulates the secretion of proteins from mature 3T3-L1 adipocytes: a role for transcriptional regulation of processing. *Diabetologia* 2006; 49:2453-2462.

ADIPOKINE CONCENTRACTIONS ARE SIMILAR IN FEMORAL ARTERY AND CORONARY VENOUS SINUS BLOOD: EVIDENCE AGAINST *IN VIVO* ENDOCRINE SECRETION BY HUMAN EPICARDIAL FAT

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Today, epicardial adipose tissue is increasingly implicated in the pathogenesis of cardiovascular disease. Human epicardial adipose tissue expresses and secretes *in vitro* growth factors and inflammatory cytokines and chemokines collectively termed adipokines. We hypothesized that human epicardial fat did not secrete adipokines into coronary blood under basal conditions *in vivo*. Adiponectin, leptin, resistin, tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), active plasminogen-activator inhibitor-1 (aPAI-1), interleukin-1beta,-6,-8 (IL-1β,-6,-8), and vascular endothelial growth factor (VEGF), as well as insulin and free fatty acids (FFA), were measured simultaneously in femoral arterial (FA) (a surrogate for coronary arterial) blood and coronary sinus (CS) venous blood from eleven patients (10 women), mean age 36.5 +/- 5.8 yr, range 17-79, BMI 27.4 +/- 2.8 kg/m^2, range 19.7-43.3, without known heart disease undergoing cardiac catheterisation for radioablation of supraventricular tachycardia under general anesthesia. The position of the catheter tip in the CS was confirmed fluoroscopically just before withdrawing blood samples to ensure no mixing of right atrial with CS blood. Under stable hemodynamic conditions, blood samples were taken simultaneously from the CS and FA over ~30 sec 10 and 5 minutes before the start of atrial and ventricular programmed electrical stimulation. Free fatty acids were measured by Quest Diagnostics, Nichols Institute, San Juan Capistrano, CA, using an in vitro enzymatic colorimetric method that recognizes a variety of FFA including palmitic, stearic, arachidonic, oleic, palmitoleic, linolenic and linoleic. Total adiponectin, resistin, TNF-α, MCP-1, IL-1β,-6,-8, aPAI-1, and VEGF were measured by Bioscience Division Laboratories, Millipore Corporation, St Charles, MO using LINCOplex well plate immunoassays with specific antibody-immobilised fluorescent-labelled microsphere beads. Insulin and leptin were measured by double antibody radioimmunassays. Mean adipokine concentrations were not significantly different in both vessels. In contrast, FFA levels were significantly higher in FA than CS blood in keeping with net uptake of FFA by the myocardium. Femoral artery levels of MCP-1, aPAI-1, insulin, FFA, leptin and resistin showed positive correlations with BMI in descending order of significance but adiponectin showed no relationship. Values for the other adipokines were below the assay detection limit in several patients negating the use of regression analysis. We conclude that, as opposed to their secretion *in vitro*, the adipokines described above are not secreted into coronary blood by human epicardial adipose tissue under near-normal basal conditions *in vivo*. Hypothetically, they might be released into the interstitium of the myocardium and coronary vessels to function as local paracrine regulators. Also, it is possible that epicardial adipokines could be secreted into the coronary circulation under pathological conditions such as coronary atherosclerosis.

THE ROLE OF RESISTIN AND INTERLEUKIN-6 IN THE METABOLIC SYNDROME

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The metabolic syndrome is a complex of anthropometric and cardiometabolic symptoms clustered in one individual. According to the criteria of National Cholesterol Education Program – Adult Treatment Panel III, a person to be defined as having the metabolic syndrome, must have any three of five characteristics: abdominal obesity, hypertension, raised plasma levels of triglycerides and glucose, and reduced high density lipoprotein-cholesterol. Adipose tissue plays an essential role in the regulation of cardiometabolic homeostasis in health and disease (1,2). Adipokines, the major signaling proteins secreted by adipose tissue, are increasingly implicated in the pathogenesis of obesity and related disorders, including the metabolic syndrome (2). Although numerous studies associate the adipokines resistin and interleukin (IL)-6 with insulin resistance and inflammation, their role in the pathogenesis of metabolic syndrome is not yet clear (3). In the present study, anthropometric and metabolic variables as well as plasma levels of IL-6 and resistin were analysed in 86 subjects (age 35-65 years) divided in two groups: healthy subjects (controls) and metabolic syndrome patients. Anthropometric and metabolic analyses of 86 subjects (age 35-65 years) were made. In metabolic syndrome patients the follwoing variables were found significantly higher ($p < 0.001$) compared with control subjects: mean systolic and diastolic blood pressure ($p < 0.001$), fasting plasma glucose ($p < 0.05$) and insulin ($p < 0.05$) levels and plasma levels of total cholesterol ($p < 0.05$), low density lipoprotein-cholesterol ($p < 0.05$), very low density lipoprotein-cholesterol ($p < 0.001$), triglycerides ($p < 0.001$) and uric acid, whereas the plasma levels of resistin and IL-6 were not statistically significant ($p > 0.05$) compared with each others. However the IL-6 levels were found correlated with waist circumference.

1. Peral B, Camafeita E, Fernández-Real JM, López JA. Tackling the human adipose tissue proteome to gain insight into obesity and related pathologies. *Expert Rev Proteomics* 2009; 6:353-361.
2. Maury E, Brichard SM. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. *Mol Cell Endocrinol* 2009 August 11; in print.
3. Won JC, Park CY, Lee WY, Lee ES, Oh SW, Park SW. Association of plasma levels of resistin with subcutaneous fat mass and markers of inflammation but not with metabolic determinants or insulin resistance. *J Korean Med Sci* 2009; 24:695-700.

ADIPOCYTE HYPOXIA: A KEY MODULATOR OF ADIPOSE TISSUE FUNCTION IN OBESITY?

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Following the discovery of leptin in 1994, white adipose tissue has become recognised as a major endocrine and signalling organ. The tissue secretes a multiplicity of protein factors - the adipokines - which are involved in a wide range of physiological and metabolic functions through extensive cross-talk both locally within the tissue and distally with other organs. Many adipokines are linked specifically to immunity and the inflammatory response, and the expansion of adipose tissue mass in obesity leads to a state of inflammation within the tissue. This inflammation is considered pivotal in the development of obesity-associated diseases, particularly insulin resistance and the metabolic syndrome; however, the basis for the initiation of the inflammatory response is unknown. We have recently proposed that inflammation in adipose tissue reflects a response to local hypoxia as tissue mass expands in obesity, large adipocytes becoming oxygen-deprived as their distance from the vasculature increases. Direct evidence for hypoxia in adipose tissue in obesity has now been obtained in mice and humans. Studies on adipocytes, both human and murine, in cell culture have shown that the expression and secretion of several key inflammation-related adipokines, including interleukin-6 (IL-6), leptin, macrophage migration inhibitory factor (MIF), angiopoietin-like protein 4 (fasting induced adipose factor) (Angplt4/FIAF) and vascular endothelial growth factor (VEGF) are stimulated by low pO_2. The production of adiponectin, which has anti-inflammatory and insulin-sensitising actions, is, on the other hand, inhibited. Adipokines are not the only proteins in adipocytes whose synthesis is modulated by hypoxia. Expression of the facilitative glucose transporters GLUT1, GLUT3 and GLUT5 is increased, and there is a substantial rise in GLUT1 protein. This is linked to a hypoxia-stimulated increase in glucose uptake, and correspondingly the release of lactate is increased, consistent with a switch to glycolytic metabolism. Synthesis of the monocarboxylate transporter-1 (MCT1), is stimulated by hypoxia, providing a mechanism for the rise in lactate release from hypoxic adipocytes. Recent studies suggest that there are important interactions between hypoxia and other factors that influence adipocyte function, such as selective long-chain fatty acids, in the production of inflammation-related adipokines. For example, while neither hypoxia nor palmitate (250 μM) alone increase $mRNA^{IL\text{-}1\beta}$ level, together they have a stimulatory effect on the expression of this key inflammatory cytokine; similarly, palmitate and hypoxia have an additive effect on IL-6 and Angplt4 expression, but this does not occur with oleate. In summary, it is suggested that hypoxia has a pervasive effect on adipocyte physiology and is central to the dysregulation of adipose tissue function that occurs in obesity.

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MATERNAL NUTRITION DURING PREGNANCY AND ADIPOSITY IN THE OFFSPRING

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Recent observations from epidemiological data and animal experiments suggest that *in utero* nutrition plays an important role in determining the health of an individual in later life. A number of studies show that maternal diet that is low in protein, caloric restricted or high in fat, influences the onset of obesity, dyslipidemia, insulin resistance and hypertension in the offspring. It is also increasingly evident that maternal obesity has adverse outcomes in offspring that extend into adulthood, causing a higher incidence of obesity and cardiovascular disease. A typical North American diet is rich in dietary fats, a fact that has been linked to the increased prevalence of obesity and cardiovascular disease. Both the quantity and quality of fat play an important role in determining the outcome of cardiovascular disease, where a diet rich in saturated fatty acids (SFA) is known to increase the risk of cardiovascular disease while polyunsaturated fats (PUFA) are suggested to be beneficial. Studies have shown that consumption of a high-fat diet before and during pregnancy leads to obesity in the adult offspring of that pregnancy. Obese dams have been shown to have higher levels of leptin both in serum and in milk during lactation than the corresponding nonobese dams (1). The offspring of obese dams were found to be less sensitive to insulin at weaning than offspring of nonobese dams. Further, the consumption of a high fat diet by the offspring of obese mothers show an additive effects on the increase in body weight and adiposity, which could be due to an increase in food intake or decreased energy expenditure. Our recent observations have shown that a continuous exposure to high fat diets, whether rich in SFA or *n-6* PUFA, during prenatal and postnatal time period, were associated with higher body weight in the offspring. Our findings further show that the maternal diet has a dramatic effect on the plasma lipid profile and endothelial function of the offspring. PUFA diets used for our studies were especially rich in linoleic acid, which has previously been shown to enhance adiposity in humans and in rodent models. Others have also suggested that an increased intake of linoleic acid, especially from breast milk during early postnatal development, contributes to an increased incidence of childhood obesity in humans. This notion would be considered relevant to the perspective of looking at the diets of North American population where *n-6* PUFA has been shown to constitute as much as 85% of the total dietary intake of PUFA. Thus, it is possible that high-fat diets enriched with *n-6* PUFA consumed by mothers during pregnancy and lactation lead to an increased risk of obesity in their children who consume high-fat postnatal diets rich in *n*-6 PUFA. The discussion of the present lecture will be focused on current issues and controversies of the importance of maternal diet in the onset of obesity and cardiovascular disease in the offspring. This research is supported by NSERC.

1. Vickers MH, Krechowec SO, Gluckman PD, Breier BH. The role of leptin during early life in imprinting later metabolic responses. In: A Gertler, editor. *Leptin and Leptin Antagonists*. Landes Bioscience, Austin, Texas, USA. 2009; 141-162.

A PERSPECTIVE ON THE IMPACT OF OBESITY, DIABETES AND LIFESTYLE ON CARDIOMETABOLIC HEALTH

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Overweight and obesity are serious public health challenges that affect millions of people in developed and developing countries. *Obesity is not a choice* and requires the same health-care resources and paradigms as treating other chronic deseases (1). Obesity is a major modifiable risk factor that contributes heavily to the onset and development of hypertension, atherosclerosis, type 2 diabetes mellitus, and the metabolic syndrome, collectively termed cardiometabolic diseases (CMD), lifestyle-related diseases or quality of life (QOL)-related diseases. During the last 25 years, the incidence of these diseases have escalated tremendously in Canada. Countless studies have documented that poor dietary habits and sedentary lifestyle contribute heavily to the development of CMD. It has been estimated that over 4 million Canadians have high blood pressure, a comorbid condition that doubles or triples the risk of atherosclerosis or stroke and increases the risk of kidney disease. According to the Heart and Stroke Foundation of Canada, cardiovascular diseases caused 36% of deaths in 2001 and were responsible for 18% of the total hospital costs in Canada. Mediterranean-type food culture, as well as foods high in fibers and low in glycemic load, are associated with a decreased prevalence of metabolic syndrome and type 2 diabetes mellitus and improved serum lipid concentrations. Dietary modifications involving decreased intake of saturated and trans-fats, less carbohydrate, increased ingestion of fresh fruits and vegetables have proven useful in limiting the progression of CMD. Premature cardiovascular morbidity and mortality is preventable through exercise, healthy dietary habits, maintaining a healthy weight, and not smoking. Nutritional interventions and lifestyle modifications, such as regular physical activity (about 30 min/day), restriction of caloric and sodium intake, smoking cessation and moderate alcohol consumption have been positively linked with the reduction of obesity and related CMD. In addition, exercise has highly beneficial effects in lowering blood pressure, decreasing blood coagulation, improving fibrinolytic capacity and plasma lipid profiles, and helping in vascular remodelling. Results of a large international, case-controlled INTERHEART study show that about 80% of cardiovascular disease-related mortality and morbidity is preventable by healthy lifestyle. Contrary to what was previously believed, heredity or the genetic makeup of a person does not play a major role in causing CMD. Given the scope and prevalence of CMD, a population health approach - 'prevention is better than cure'- would be the most appropriate model to adopt to deal with CMD-related mortality and morbidity. Such a preventive strategy would contribute greatly in reducing not only the hospital, drug, and physician service costs, but also employee absenteeism. Recent advance of nutritional science (2) may contribute to the development of healthy food culture. The focus of the present communication is to evaluate the influence of obesity, diabetes and lifestyle on cardiometabolic health, aiming at the improvement of QOL.

1. Sharma AM. Obesity is not a choice. *Obesity Rev* 2009; 10:371-372.
2. Mariman E. Nutrigenomics and nutrigenetics: the "omics" revolution of nutritional science. *Biotechnol Appl Biochem* 2006; 44 (Pt 3): 119-128.

IMMUNE SYSTEM RESPONSE TO STRESS: OBESITY AS A NOVEL MODULATOR

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Studies on the functional association between adipose tissue and the immune system have been "upregulating" since 1994, when leptin secreted from white adipocytes was discovered as an *ob* gene product (1). Onwards, increasing evidences have been demonstrating that adipose tissue via its endo- and paracrine mediators, particularly adipokines, could modulate immune functions, and a special attention addressed to lymph node-associated (perinodular) adipose tissue (2). Stress is an everyday experience that has many impacts on health. Stress response although uniform regardless of the stressor, is modulated by accompanying conditions of the individual, gender and menstrual cycle phase being among the important ones (3). Research into the evaluation of immune functions in *Homo obesus* and experimental animals indicate that the excess adiposity is associated with impaired immune responses. On this background it is reasonable to expect stress-related immune system responses to change in obese individuals. The aim of this study was to investigate the difference in the distribution of peripheral lymphocytes in obese and lean men and women exposed to acute mental stress. Stroop colour-word interference (3) and cold pressor tests were applied to young healthy volunteers (25-35 years of age) of obese [body mass index (BMI)> 25kg/m^2] men (n=9) and women (n=8) and lean (BMI <25kg/m^2) men (n=10) and women (n=12). Heart rate, blood pressure and body temperature were recorded all through the test and afterwards, until the baseline levels were achieved. In the blood samples obtained before and after the tests, we performed plasma cortisol and leptin measurements and lymphocyte subtyping. Our results revealed an activation of sympathetic nervous system accompanied by a decrease in helper T (CD4+)/suppressor T (CD8+) cell ratio in obese men and women in comparison with lean counterparts ($p<0.05$). The decrease in this ratio was in correlation with plasma leptin levels ($r=0.791$). These preliminary results support the previous data about the immunomodulatory effect of obesity, acute stress exerting a stronger immune depressive effect in men than women (4). The gender difference observed in lean individuals in favor of women was preserved for obese individuals. The exact mechanism responsible for alterations in the immune system of obese patients is unknown, but deserves further studies to clarify (5).

1. Friedman JM. Leptin at 14 yr of age: an ongoing study. *Am J Clin Nutr* 2009; 89:973S-979S.
2. Pond CM. Adipose tissue and the immune system. *Prostagl Leukotr Essent Fatty Acids* 2005; 73: 17-30.
3. Pehlivanoglu B, Durmazlar N, Balkanci ZD. Computer adapted Stroop colour-word conflict test as a laboratory stress model. *Erciyes Med J* 2005; 27:58-63.
4. Pehlivanoglu B, Balkanci ZD, Ridvanagaoglu AY, Durmazlar N, Ozturk G, Erbas D, *et al.* Impact of stress, gender and menstrual cycle on immune system: possible role of nitric oxide. *Arch Physiol Biochem* 2001; 109: 383-387.
5. Duffaut C, Zakaroff-Girard A, Bourlier V, Decaunes P, Maumus M, Chiotasso P, *et al.* Interplay between human adipocytes and T lymphocytes in obesity. CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators. *Arterioscler Thromb Vasc Biol* 2009; July 30: in print.

HYPERLEPTINEMIA, PARAOXONASE 1 AND PROTEIN HOMOCYSTEINYLATION: IMPLICATIONS FOR PROATHEROGENIC EFFECT OF THE METABOLIC SYNDROME

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Recent studies have demonstrated that chronic hyperleptinemia may contribute to various complications, including atherosclerosis (1), in subjects with the metabolic syndrome; however, the underlying mechanisms are incompletely clear. Previously, we have demonstrated that experimental hyperleptinemia induced in lean rats by administration of exogenous leptin induces deficiency of paraoxonase 1 (PON1), an antioxidant and atheroprotective enzyme synthesized in the liver and contained in plasma high-density lipoproteins (2). In the present study we examined the effect of leptin on PON1 activity toward one of its physiological substrates, homocysteine thiolactone (HTL), the cyclic thioester of homocysteine which binds to ε-NH_2 groups of protein lysine residues (protein homocysteinylation) (3) thus modifying their biological activity. Hyperleptinemia induced in adult rats by the administration of recombinant leptin (0.5 mg/kg/day for 8 days) decreased plasma PON1 activity toward HTL by 41.0% and increased the amount of HTL bound to plasma proteins by 92.9%, although plasma total homocysteine did not change. The amount of HTL bound to isolated plasma fibrinogen was also increased (by 62.0%) following leptin treatment, which could result in impaired fibrinolysis. Leptin had no effect on PON1 activity and protein homocysteinylation in the liver, but markedly reduced PON1 and enhanced protein homocysteinylation in the kidney and aortic wall. Effect of leptin on PON1 and protein homocysteinylation in plasma was prevented by simultaneous administration of synthetic liver X receptor (LXR) agonist, T0901317, previously demonstrated to reduce atherosclerosis in experimental models. However, T0901317 did not prevent the effect of leptin on aortic and renal protein homocysteinylation. We conclude that: (*i*) chronically elevated leptin impairs HTL metabolism by PON1 which results in enhanced protein homocysteinylation in plasma, vascular wall and the kidney, (*ii*) hyperleptinemia may contribute to atherogenesis and other complications of obesity such as renal damage by augmenting protein homocysteinylation, and (*iii*) T0901317 may be useful in ameliorating protein homocysteinylation in PON1-deficiency states.

1. Beltowski J. Leptin and atherosclerosis. *Atherosclerosis*. 2006; 189: 47-60.
2. Bełtowski J, Wójcicka G, Jamroz A. Leptin decreases plasma paraoxonase 1 (PON1) activity and induces oxidative stress: the possible novel mechanism for proatherogenic effect of chronic hyperleptinemia. *Athersoclerosis* 2003; 170: 21-29.
3. Protein homocysteinylation: a new mechanism of atherogenesis? *Postepy Hig Med Dosw (Online)* 2005; 59:392-404.

METABOLIC SYNDROME AND NERVE GROWTH FACTOR: EFFECTS OF METFORMIN AND NONSTEROIDAL ANTI-INFLAMMATORY DRUG TREATMENT

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Among other cardiometabolic risks, the metabolic syndrome (MSyn) also embodies type 2 diabetes features. It is associated with chronic mild inflammation, suggestive of potential benefits of anti-inflammatory treatment (1,2), and pancreatic-β cell dysfunction. Nerve growth factor (NGF) is a signaling protein discovered for its prosurvival role on neuronal cells, but recent studies reveal that this neurotrophin also exerts various non-neuronal effects including stimulation of insulin secretion by pancreatic-β cells, and these cells synthesize and release NGF (3). Furthermore, the neurotrophins NGF and brain-derived neurotrophic factor (BDNF) are implicated in the pathogenesis of cardiometabolic diseases including MSyn (4). Metformin is a widely used antidiabetic drug, which, in addition to its classical effects on glucose and lipid metabolism, inhibits low-grade inflammation (5). While no available data exists about the effect of metformin on circulating levels of NGF and BDNF, there is evidence indicating that nonsteroidal anti-inflammatory drugs (NSAID) influence the production of these neurotrophins (6,7). The aim of this study was to examine the effect of treatment with metformin alone and in combination with NSAID on plasma levels of NGF and BDNF in patients with mild (n=10) and advanced (n=10) MSyn; selected by the criteria of NCEP-ATP III. All patients with mild MSyn received metformin 850 mg twice daily, whereas advanced MSyn patients received either metformin alone (in the same dosage) (n=4) or in combination with aspirin (500 mg daily) and diclac (diclofenac sodium) (150 mg daily) (n=6). Plasma NGF and BDNF levels were measured by enzyme-linked immunosorbent assay before and after five-month drug treatment. Metabolic/inflammatory variables including plasma levels of lipids, glucose and C-reactive protein as well as BMI and waist circumference were also measured. We found that plasma NGF levels were significantly higher in early and lower in advanced MSyn patients, as compared with healthy subjects (n=10). However, NGF levels were decreased after metformin administration to patients in both groups. By contrast, after metformin-aspirin-diclac treatment, NGF levels were significantly higher, suggesting a stimulatory effect of NSAID on NGF production; no significant alterations in plasma BDNF levels were found. This issue requires further study.

1. Festa A, D'Agostino R Jr, Howard G, Mykkanen L, Tracy RP, Haffner SM. Chronic subclinical inflammation as part of the insulin resistance syndrome: the insulin resistance atherosclerosis study. *Circulation* 2000; 102: 42-47.
2. Thompson MP, Trayhurn P. Effect of salicylate on the expression of adipokines and glucose transporters in human adipocytes is modulated by hypoxia. *Horm Metab Res* 2009; 41:649-652.
3. Larrieta ME, Vital P, Mendoza-Rodriguez A, Cerbón M, Hiriart M. Nerve growth factor increases in pancreatic beta cells after streptozotocin-induced damage in rats. *Exp Biol Med (Maywood)* 2006; 231: 396-402.
4. Chaldakov GN, Tonchev AB, Fiore M, Hristova MG, Pancheva R, Rancic G, *et al*. Implications for the future of obesity management. In: G Frühbeck, editor. *Peptides in Energy Balance and Obesity*. CAB International 2009; 369-389.
5. Bulcão C, Ribeiro-Filho FF, Sanudo A, Ferreira SGR. Effects of simvastatin and metformin on inflammation and insulin resistance in individulas with mild metabolic syndrome. *Am J Cardiovasc Drugs* 2007; 7:219-224.
6. Pomponi M, Di Gioia A, Bria P, Pomponi MF. Fatty aspirin: a new perspective in the prevention of dementia of Alzheimer's type? *Curr Alzheimer Res* 2008; 5: 422-431.
7. Zhao X, Rebeck GW, Hoe HS, Andrews PM. Tarenflurbil protection from cytotoxicity is associated with an upregulation of neurotrophins. *J Alzheimers Dis* 2008; 15:397-407.

THE EFFECTS OF EXERCISE ON SERUM BRAIN-DERIVED NEUROTROPHIC FACTOR

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Brain-derived neurotrophic factor (BDNF) is a member of the protein family of neurotrophins. In addition to its neurotrophic and synaptotrophic actions, such as promotion of the growth and survival of neurons and the learning and memory, respectively, BDNF may play important roles in the regulation of food intake and energy homeostasis, also glucose and lipid metabolism. Exercise is well known to have many health benefits and BDNF may be one of the key factors which mediate these benefits. Serum BDNF has been shown to increase following acute exercise. On the other hand, there were great differences in the BDNF response to exercise between individuals. Therefore, we compared the serum BDNF response to acute exercise between the trained and the untrained to clarify the effects of regular exercise on it. Eight trained and 8 untrained females participated in this study. All participants performed three different intensity exercise test. At first, they performed maximal exercise test to determine their maximal oxygen uptake (VO2max) (High intensity). Then 30 min of cycle ergometer exercise were performed at constant load of 60% (moderate intensity) and 40% (low intensity) of their VO2max. In each exercise, blood samples were taken at baseline and immediate, 30 and 60min after exercise. The serum BDNF level was measured using an enzyme-linked immunoassay (ELISA) kit (Promega, Madison, WI). Serum BDNF concentration was significantly increased immediately after high intensity exercise test in the both groups. While BDNF level in the untrained returned to the baseline level during recovery phase, BDNF level in the trained decreased below the baseline level. Serum BDNF concentration in the both groups increased immediately after moderate intensity exercise and returned to the baseline level during recovery phase. No difference in BDNF response was seen between the groups. Low intensity exercise didn't change the BDNF concentration in the both groups. We found that moderate to acute exercise at moderate to high intensity increase serum BDNF. Acute exercise has been shown to enhance the expression of both BDNF and $mRNA^{BDNF}$ related to lipid oxidation in skeletal muscle cells (1, for exercise-lipid oxidation link, see Jakovljevic *et al* in this volume of *Adipobiology*). The elevation in circulating BDNF may contribute to an exercise-induced improvement of glucose and lipid metabolism, although the action of serum BDNF is still unknown. In addition there was a difference between the groups in the BDNF response during recovery phase, implying that the trained may have an ability to utilize circulating BDNF rapidly. This issue requires further evaluation.

1. Matthews VB, Aström MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek O, *et al*. Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. *Diabetology* 2009; 52:1409-1418.

METABOLIC SYNDROME AND CARDIAC AUTONOMIC NEUROPATHY IN TYPE 2 DIABETIC PATIENTS

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The heart is abundantly innervated and the nervous system precisely controls the function of heart in health and disease (1,2). Cardiac autonomic neuropathy (CAN) is a serious and common complication of both diabetes mellitus and the metabolic syndrome. Despite its relationship to an increased risk of cardiovascular mortality (1), the significance of diabetic autonomic neuropathy has not been fully appreciated. Components of the metabolic syndrome are considered responsible for autonomic nervous dysfunction in type 2 diabetes patients. However, the relation between CAN and metabolic syndrome in non-diabetic patients is at present unclear. The purpose of this study was to evaluate and compare the cardiac autonomic nervous activity and circadian blood pressure in patients with metabolic syndrome only and in diabetic patients with metabolic syndrome. Fourteen diabetic patients, age 55.36 (±4.63) years, and 9 non-diabetic subjects with metabolic syndrome, age 57.63 (±7.55) years, were recruited for the study. Ten control subjects, age 54.38 (±5.38) years, were investigated. Cardiac autonomic nervous activity was studied by 24-hours ECG recording. Heart rate variability (HRV) analysis was performed in time and frequency domains: SDNN, RMSSD, very low frequence (VLF), low frequency (LF) and high frequency (HF), LF/HF ratio was calculated. Circadian blood pressure estimated as the day-night time ratio in systolic and diastolic blood pressure average. Variables including anthropometric (hight, weight, calculated BMI, waist circumference) and metabolic (glucose, cholesterol, HDL-cholesterol, LDL-holesterol, tryglicerides) were measured in all the subjects investigated. Significant differences between diabetic patients and patients with metabolic syndrome only were observed in triglycerides (2.13 vs 3.25, p=0.03), HbA1c (7.20 vs 6.10, p=0.04) and BMI (27.77 vs 28.59). VLFln was significantly lower in diabetic patients than in metabolic syndrome group (p=0.01), only. HFln and LF/HFln significantly differ in type 2 diabetes than in controls (3.98 vs 6.72, p=0.001 and 1.36 vs 0.99, p=0.001). Metabolic syndrome group showed significantly lower values of HFln (4.85 vs 6.72, p=0.002) and higher LF/HFln (1.14 vs 0.99, p=0.007) than controls. Ambulatory blood pressure of the patients showed sifnicantly difference in day-night ratio of systolic blood pressure (1.11 vs 1.16, p <0.05). Glucose level was independently associated with HRV indices in type 2 diabetes. In conclusion, we established the presence of disturbed HRV in diabetic patients, with short duration of disease. These patients also showed blunted nocturnal fall in blood pressure, as other phenomenon of autonomic dysfunction. The other group, with metabolic syndrome only, disturbed HRV was also established. We concluded that the improvement of metabolic parameters could prevent or reduce CAN in patients with metabolic syndrome as well as in type 2 diabetes mellitus patients. Thus knowledge of early symptoms of CAN can encourage patient and physician to improve metabolic control. Despite the clinical importance of CAN, the mechanisms underlying the control of this disorder remain poorly understood. Studies targeting the pathogenic involvement of heart disease-related biomolecules (2) including adipokines might shed additional important light into metabolic syndrome/diabetes-associated CAN.

1. Cabezas-Cerrato J, Hermida RC, Cabezas-Agricola JM, Ayala DE. Cardiac autonomic neuropathy, estimated cardiovascular risk, and circadian blood pressure pattern in diabetes mellitus. *Chronobiol Int* 2009; 26:942-957.
2. Ieda M, Fukuda K. New aspects for the treatment of cardiac diseases based on the diversity of functional controls on cardiac muscles: the regulatory mechanisms of cardiac innervation and their critical roles in cardiac performance. *J Pharmacol Sci* 2009; 109:348-353.

METHYLENETETRAHYDROFOLATE REDUCTASE C677T POLYMORPHISM IN METABOLIC SYNDROME

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Methylenetetrahydrofolate reductase (MTHFR) is one of the key enzymes in homocysteine metabolism, even mildly hyperhomocysteinemia being an independent risk factor for cardiovascular diseases. Adipocentrically, a recent work revealed that homocysteine is secreted by adipose tissue, that is, this metabolite may be considered a new "adipokine" (1) expressing proatherogenic and other toxic effects (2). The MTHFR gene has been mapped to chromosomal region 1p36.3. MTHFR C677T polymorphism results from transition at the nucleotide position 677 in DNA, leading to the substitution of alanine (C) to valine (T) residue at position 226 in the protein; this results in a decreased basal activity of the enzyme by 50%. Recent studies demonstrated a significant association between low serum folate and increased homocysteine levels in cases with MTHFR genotype as well as the significant decreasing of homocysteinemia concentration by moderate daily folic acid intake. The metabolic syndrome is defined according to the National Cholesterol Education Program-Adult Treatment Panel III (NCEP-ATP III) guidelines by the presence of at least 3 of the following factors: waist circumference more than 102 cm. in men and more than 88 cm. in women, triglyceride level more than 150 mg/dl., HDL-cholesterol<= 40 in men, <=50 in women, blood pressure>= 130/85, fasting glucose>= 110 mg/dl. Homozygous MTHFR genotype for T allele may be associated with metabolic syndrome and predisposes to a higher risk for insulin resistance. We aimed to assess the association between this genotype and metabolic syndrome by performing a case-control transversal study including 40 patients, 20 with metabolic syndrome (group 1) and 20 control sex and age-matched cases (group 2). Homozygous genotype was found in 15 patients from group 1 and in 7 cases of group 2, the results being statistically significant. Heterozygous genotype was found equally (in 3 cases of each group). There was no relationship between MTHFR C677T polymorphism and age and sex. In conclusion, the homozygous genotype MTHFR C677T may associate with the metabolic syndrome (cf. 3).

1. Riederer M, Erwa W, Zimmermann R, Frank S, Zechner R. Adipose tissue as a source of nicotinamide N-methyltransferase and homocysteine. *Atherosclerosis* 2009; 204: 412-417.
2. Perła-Kaján J, Twardowski T, Jakubowski H. Mechanisms of homocysteine toxicity in humans. *Amino Acids* 2007; 32:561-572.
3. Pena AS, Belobrajdic DP, Wiltshire E, Gent R, Hirte C, Couper J. Adiponectin relates to smooth muscle function and folate in obese children. *Int J Pediatr Obes* 2009; 15:1-7.

THE ROLE OF HOMOCYSTEINE AND FOLIC ACID IN CORONARY ARTERY DISEASE

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Homocysteine has emerged as a possible risk marker for atherothrombosis, certain neurological diseases and cancer. However, its impact on coronary artery disease (CAD) remains unclear due to the relative lack of clinical trials documenting benefits of treatment such as a reduction of homocysteine values and a decrease in cardiovascular events. In order to investigate the role of homocysteine and folic acid in coronary circulation and coronary artery disease the following basic and clinic investigations were performed: (*i*) evaluation the effects of L-homocysteine thiolactone hydrochloride (100 μM) on rat coronary flow and oxidative stress markers (nitrite outflow, superoxide anion production, index of lipid peroxidation/TBARS production), (*ii*) evaluation the effects of folic acid (100 μM) on rat coronary flow and oxidative stress markers, and mechanisms of folic acid-induced effects (in the presence of certain inhibitors - L-NAME, indomethacin, ketoconazole, ouabain, methylene blue), (*iii*) evaluation the effects of acute D,L-homocysteine thiolactone (5.5 mmol/kg b.w.) and folic acid (0.011 μM/kg b.w.) treatment on acetylcholinesterase (ACh) activity of the brain, heart tissue and the blood of a rat, (*iv*) evaluation the impact of homocysteine in the observational trial consisting of 259 patients of both gender undergoing diagnostic coronary angiography, and (*v*) evaluation the benefits of short-term usage of folic acid concerning endothelial function, carotid wall thickness and myocardial perfusion in pilot study (15 CAD patients, 10 were treated with folic acid 5 mg/daily *per os* for 6 months, 5 received placebo). It has been clearly demonstrated that homocysteine did not change coronary flow but increased oxidative stress in rat coronary circulation. On the contrary, folic acid increased rat coronary flow, decreased nitrite ouflow and decreased superoxide anion production. Surprisingly, both of them decreased ACh activity in rat heart tissue (cf. 1). In CAD patients it has been demonstrated that: (*i*) plasma tHcy values in CAD patients are increased compared to those of the control group (12.04+/-4.54 pmol/l vs 10.92+/-4.37 pmol/l, p=0.036), (*ii*) of the total study population undergoing coronary angiography, 36.16% show a plasma homocysteine concentration that is higher than 15 pmol/l, (*iii*) plasma tHcy levels more frequently exceed 15 pmol/l in the CAD patients than in the control group (23.46% vs 12.70%, respectively), (*iv*) older patients more frequently have plasma homocysteine values higher than 15 pmol/l (p=0.0001) proving that plasma tHcy and age are positively correlated (r=0.341, p=0.0001), (*v*) plasma tHcy concentration in patients with increased uric acid levels more often exceeds 15 pmol/l (p=0.034) demonstrating a positive correlation between plasma tHcy and uric acid (r=0.244, p=0.021), and (*vi*) there is no evidence allowing the conclusion that any correlation exists between plasma tHcy and gender, weight, cholesterol, triglyceride, glucose or C-reactive protein. Treatment with moderate doses of folic acid significantly decreased homocysteine level by 34%; endothelium function was improved by 27% under the treatment, while carotid structure and myocardial perfusion did not show any significant improvement.

1. Rosas-Ballina M, Tracey KJ. Cholinergic control of inflammation. *J Intern Med* 2009; 265:663-679.

INFLAMMATORY MARKERS AND CARDIOVASCULAR PARAMETERS IN THE PREDICTION OF MORTALITY IN HEMODIALYSIS PATIENTS

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The prevalence of cardiovascular disease and mortality rate is high in hemodialysis patients. This study aimed to evaluate the predictive value of inflammatory markers in those patients. Forty two patients (age 55.1 ± 10 years) from our unit were examined. We measured serum level of albumin, lipids, C-reactive protein (CRP), interleukin-6 (IL-6), tumor-necrosis factor-alpha (TNF-α) and erythrocyte phospholipid fatty acid composition, and prospectively followed up these patients until May 2007 (36 months) to determine the incidence and causes of death. Nutritional status was assessed by anthropometric measurements and bioelectric impedance. Cardiovascular parameters were examined by echosonography. Patients with cardiovascular disease had a higher mortality rate than those without heart disease. Serum levels of the inflammatory markers IL-6 (p=0.001) and CRP (p=0.001) were significantly higher in patients who died during the follow-up period as compared with those who survived. Patients with malnutrition had a higher mortality rate than those with normal food intake (p=0.001). The erythrocyte phospholipid polyunsaturated fatty acids were lower among patients who died than those who survived (p=0.04). By Kaplan-Meier survival analysis, elevated levels of serum CRP were a significant predictor of mortality. Differences in survival between patients with elevated vs low serum CRP levels (higher vs lower median CRP levels) were compared using the log rank test. Eight patients died in the group with CRP levels higher than the median, while one fatal event occured in the group with CRP equal to or below the median levels of CRP (p=0.04). In conclusion, among the inflammatory markers studied, serum level of CRP is the most significant predictor of mortality in our hemodialysis patients. The patients who are malnourished and have a high CRP level tend to be at higher risk of mortality in this 36 months follow-up investigation.

NERVE GROWTH FACTOR, PANCREATIC BETA CELLS, ADIPOSE TISSUE AND DIABETES MELLITUS

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Considered as a major risk factor for the development of metabolic syndrome and other cardiometabolic diseases, obesity is a huge medico-social burden of our time. Susceptibility to develop obesity depends on genetics and lifestyle, including dietary habits. Ten years ago our own results strongly suggested that pancreatic beta cells secrete nerve growth factor (NGF) and express its receptor, tyrosine-kinase A (TrkA), and that these findings might be implicated in the pathogenesis of diabetes mellitus (1,2). It was recently reported that adipose tissue may also produce both NGF and brain-derived neurotrophic factor (3). We analyzed metabolic and morphofunctional effects of changing a single parameter in the diet on pancreatic islets and peripancreatic adipose tissue. We developed a model of metabolic syndrome in 8 weeks old Wistar adult male rats, by feeding them during 8 or 24 weeks with a standard chow diet and water (control group) or a 20 % sucrose solution in the drinking water (MSR). After treatment, total body weight, adipose tissue weight, plasma glucose, and arterial pressure were measured. Plasma insulin level was assessed with an ultrasensitive rat insulin ELISA system. In 24 hour culture media of peripancreatic adipose tissue, NGF, interleukin-6 (IL-6), IL-10 and tumor necrosis factor-alpha (TNF-α) were also measured with ELISA. After two months of treatment, body weight was 20% higher in MSR than in the control group; this was mainly due to the increase in abdominal adipose tissue. At the end of six months of treatment, MSR preserved body weight difference, by a 2.2-fold increase in abdominal adiposity. At both stages, arterial pressure was higher than in the controls. We have previously observed that at two months MSR showed hyperinsulinemia. Interestingly, after 6 months, insulin plasma level decreased by 31% in comparison to controls, while glucose concentration tended to increase in MSR, without statistical significance. Preliminary analysis of adipokine concentrations in the culture media of peripancreatic adipose tissue showed an increased released of IL-10, IL-6, TNF-α and NGF after the six month treatment, whereas after the two month treatment, only IL-10 and TNF-α secretion increased. These results highlight the participation of a high sucrose diet as an important risk factor, independently of the genetic background, in the development of metabolic syndrome, impair pancreatic beta cell function and increase adipokine secretion. Our results suggest that in a long term sucrose intake, beta cell number increases; however, insulin secretion in the new cells can be impaired. These alterations may lead to the development of type 2 diabetes mellitus.

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1. Rosenbaum T, Vidaltamayo R, Sánchez-Soto MC, Zentella A, Hiriart M. Pancreatic beta cells synthesize and secrete nerve growth factor. *Proc Natl Acad Sci USA* 1998; 95: 7784-7788.
2. Larrieta ME, Vital P, Mendoza-Rodriguez A, Cerbón M, Hiriart M. Nerve growth factor increases in pancreatic beta cells after streptozotocin-induced damage in rats. *Exp Biol Med (Maywood)* 2006; 231: 396-402.
3. Sornelli F, Fiore M, Chaldakov GN, Aloe L. Adipose tissue-derived nerve growth factor and brain-derived neurotrophic factor: results from experimental stress and diabetes. *Gen Physiol Biophys* 2009; 28:179-183.

OXIDATIVE STRESS IN HEALTH AND DISEASE: FOCUS ON LIPID PEROXIDATION

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Oxidative stress is a state of disturbed balance between (*i*) the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and (*ii*) the antioxidative defense of human body. Increased consumption of oxygen during exercise could be the cause of oxidative stress. The aim of this study was to establish the oxidative status of elite karate athletes in precompetiton and coompetition stage, in the state of rest and after the load, monitoring the parameters of the oxidative stress and components of antioxidative defense in the training process. The group of 30 male, elite karate athletes in training from 16 to 30 ages is included by prospective study of prevalence. Examination were conducted in the precompetition and competition stage, in the state of rest condition and after the loading. Furthermore, parameters of oxidative stress (NO, TBARS, superoxide and H_2O_2) and antioxidant enzymes activities including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase (GR) were measured in the blood plasma and erythrocytes of young active soccer players (from 4 to 9 years of training) and correlated to the functional heart morphology and conductivity parameters. The players were divided in two subgroups according to the age: teen (14 and 15 years old) and premature (16 and 17 years old). There were no differences between the levels of antioxidant enzymes and parameters of oxidative stress in experimental groups. However, measured parameters correlate between them, and correlations are age-specific. In premature group significant negative correlation between SOD and CAT or GPS-Px, and positive between CAT and GSH-Px, and superoxide and H_2O_2 concentrations were calculated. In teen group, significant positive correlations between CAT and GSH-Px, and SOD and the H_2O_2 concentration were found. Finally, in order to examine role of oxidative stress in fetal distress development, we tested 46 mothers and newborn children whose oxidative status was evaluated immediately after the birth by tracking the concentration of superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2) and TBARS in blood plasma, as well as the activity of enzymes of the first line of antioxidative protection - SOD and CAT in erythrocytes. The results of our research show that there is statistically significant difference in values of all parameters in children with and without distress and between mothers of newborn children with or without distress, while statistically significant difference in O_2^- values was not noticed between mothers from the control group and from the examined group. In future studies targeted oxidative stress and peroxidation reactions, a link between exercise-induced myokine expression and lipid peroxidation (1) as well as ROS production by adipose tissue (2) should also be evaluated.

1. Matthews VB, Aström MB, Chan MH, Bruce CR, Krabbe KS, Prelovsek O, *et al*. Brain-derived neurotrophic factor is produced by skeletal muscle cells in response to contraction and enhances fat oxidation via activation of AMP-activated protein kinase. *Diabetology* 2009;52:1409-1418.
2. Shimomura I, Funahashi T, Matsuzawa Y. Metabolic syndrome, adiponectin and fat ROS. *Biomed Rev* 2006; 17:1-10.

MECHANSIMS FOR THE DEVELOPMENT OF DIABETIC ANGIOPATHY AND ITS PREVENTION: ENEMIES AND FRIENDS WITHIN

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As is diabetes itself, diabetic angiopathy is caused by multiple environmental and genetic factors. Screens with endothelial cells and pericytes in culture revealed advanced glycation end-products (AGE) as the major environmental account for the vascular cell changes characteristic of diabetes, and the receptor for AGE (RAGE) as the major cellular factor that responds to AGE (*Biomed Rev* 2000; 11: 19). We created RAGE gene-manipulated animals, and demonstrated that RAGE overexpression accelerates (*J Clin Invest* 2001; 108: 261), but RAGE deficiency ameliorates (*Diabetes* 2006; 55: 2510), the development of diabetic nephropathy. Accordingly, the AGE-RAGE system should be regarded as a prophylactic and therapeutic target in the treatment of this disease. Low molecular-weight heparin was found to be a RAGE antagonist and to be capable of not only preventing but also reversing diabetic glomerulosclerosis (*Diabetes* 2006; 55: 2510). In collaboration with the group of Professor Kobayashi, Osaka Pharmaceutical University, we determined the three dimensional structure of human RAGE protein (*Biochemistry* 2008; 47: 12299), and have conducted virtual and wet screens for RAGE antagonists. Further, through an analysis of polysomal RNA from human vascular cells we have identified a novel splice variant coding for a decoy form of RAGE proteins and termed it an endogenous secretory RAGE (esRAGE) (*Biochem J* 2003; 370: 1097). Endogenous secretory RAGE was able to neutralize AGE actions on vascular endothelial cells, and analyses with an anti-esRAGE antibody revealed a negative correlation between circulating esRAGE and the occurrence and severity of diabetic vascular complications, suggesting protective roles of this decoy receptor against RAGE-related disease.

RELATIONSHIP OF PLASMA PHOSPHOLIPIDS FATTY ACIDS AND THE HOMEOSTASIS MODEL OF INSULIN RESISTANCE IN TYPE 2 DIABETIC PATIENTS: A PILOT STUDY

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Omega-3 fatty acids have proven beneficial effects in patients with dyslipidemia, type 2 diabetes mellitus, and cardiovascular diseases (1). However, exact relationship between polyunsaturated fatty acids (PUFA) and insulin resistance in type 2 diabetes is not full established. We investigated relationship between serum concentration of saturated fatty acids and unsaturated fatty acids and homeostasis model assessment of insulin resistance (HOMA-IR) (2) in type 2 diabetes. We studied 8 consecutive type 2 diabetes patients, 4 females and 4 males; mean age 55.36 (±4.63) years, without serious comorbidity. Blood test for metabolic variables: glucose, HbA1c, triglycerides, cholesterol, HDL-C, and insulin, was performed after 12 hours of overnight fasting. Plasma fatty acids phospholipids profile was determined by GC chromatograph. Plasma phospholipids profile has shown high percentage of stearic acid, oleic acid and linoleic acid. Medium percentage of palmitoleic acid and arahidonic acid, as well as low levels of others, particularly omega-3 fatty acids, were presented. We have shown significant positive association between 5-8-11 eicosatrienoic acid (3) and HOMA-IR (r=.814, p=.01), only. The ratio of n-6 PUFA/n-3 PUFA correlated with HOMA-IR almost significantly (r=.559, p=.09). In conclusion, the results of this study demonstrate that some PUFA as well as the ratio of n-6 PUFA/n-3 PUFA positively correlate with HOMA-IR, indicating that higher intake of these fatty acids aggravates insulin resistance.

1. Anderson BM, Ma DWL. Are all n-3 polyunsaturated fatty acids created equal? *Lipids Health Dis* 2009, 8:33.
2. Morrison JA, Glueck CJ, Horn PS, Schreiber GB, Wang P. Homeostasis model assessment of insulin resistance and body mass index interactions at ages 9 to 10 years predict metabolic syndrome risk factor aggregate score at ages 18 to 19 years: a 10-year prospective study of black and white girls. *Metabolism* 2009; 58:290-295.
3. Macrophage lipid droplets: Inert? Eicosa' not! Lipid droplets are sites of eicosanoid production, implicated in atherosclerosis. In: *Lipidomics Gateway*, 22 July 2009. doi:10.1038/lipidmaps.2009.17

SERUM-BORNE FACTORS IN CANCER PATIENTS WITH ADVANCED CACHEXIA: INFLUENCE ON ADIPOSE CELLS

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The clinical syndrome cancer cachexia is recognized by a considerable weight loss being out of proportion to any reduction in energy intake. This suggests that a significant metabolic component is involved, including adipose tissue (1). The underlying mechanisms are not completely known although the marked weight loss is attributable to depletion of adipose tissue as well as skeletal muscle mass. Two processes may determine adipose tissue mass in cachexia: reduced adipocyte size (hypotrophy) and decreased adipocyte number (hypoplasia). Enhanced lipolysis in adipocytes, apoptosis of preadipocytes may be important for loss of adipose tissue. Serum samples from cachectic cancer patients (n = 8) and non-cachectic cancer patients (n = 6) were collected. Human SGBS (Simpson-Golabi-Behmel syndrome) preadipocytes and differentiated adipocytes were incubated in the presence of serum from cachectic and non-cachectic (control) cancer patients. Induction of apoptosis and necrosis was examined by cell staining with Hoechst 342 (HO342) and propidium iodide (PI), respectively. Expression of pro- and anti-apoptotic Bcl-2 genes was measured by quantitative RT-PCR. Lipolysis was monitored by measuring the release of radiolabeled fatty acids. Sera from cachectic cancer patients induced apoptosis in cultured human preadipocytes at a higher rate than sera from non-cachectic cancer patients (control group). There was a tendency towards increased mRNA levels of the pro-apoptotic Bcl-2 gene Bax after incubation of preadipocytes with cachectic sera. Moreover, the mRNA levels of anti-apoptotic Bcl-XL and pro-apoptotic Bcl-XS were increased and decreased, respectively, as compared to incubation with control sera. However, lipolysis was not enhanced in cultured human adipocytes after incubation with sera from cachectic cancer patients as compared to non-cachectic cancer patients. Our present *in vitro* data suggest that apoptosis of preadipocytes can be enhanced by serum-borne factors in cancer cachexia. We could not show that serum-borne factors associated with cachexia have a major impact on lipolysis in cultured human adipocytes. Death or survival of preadipocytes may depend on the balance between pro- and anti-apoptotic mediators. Adipokines might also affect apoptosis (2). Adiponectin and leptin concentrations tended to be altered in serum from cachectic patients as compared to non-cachectic patients. Further studies of patients with cancer cachexia will be needed to reveal if the disease involves loss of adipose tissue due to apoptosis of preadipocytes. Measurement of adipokine concentrations in BMI-matched cancer patients are warranted to determine if adipokines may play a role in cancer cachexia.

1. Bing C, Trayhurn P. Regulation of adipose tissue metabolism in cancer cachexia. *Curr Opin Clin Nutr Metab Care* 2008; 11: 201-207.
2. Della-Fera MA, Baile CA. Roles for melanocortins and leptin in adipose tissue apoptosis and fat deposition. *Peptides* 2005; 26: 1782-1787.

ADIPOSE TISSUE AROMATASE AND BREAST CANCER: A VIEW OF (ADIPO)PHARMACOLOGIST

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Recent progress of adipobiology provides evidence for a surprising variety of diseases besides obesity and related disorders which associate with adipose tissue paracrine and endocrine activity expressed in various loci of the human body, including breast, endometrium and ovaries. Within these tissues, fibroblasts are the major site of estradiol synthesis mediated by the cytochrome P450 enzyme complex designated aromatase (P450arom) encoded by the CYP19 gene. Epithelial-stromal interactions play key roles for aromatase expression and estrogen production in breast cancer tissue. Upregulated aromatase expression in breast fibroblasts increases the tissue concentration of estradiol, which then activates a large number of carcinogenic genes via estrogen receptor-alpha (ERalpha) in malignant epithelial cells. A model for breast cancer development that focuses on the interaction among locally synthesized estrogens, growth factors and adipokines is increasing appreciated nowadays (1). Note that the peripheral conversion rates of androstenedione to estrone and the adipose tissue aromatase activity and $mRNA^{P450arom}$ levels all increased by a similar factor (two- to four fold) when women in their 20s were compared with those in their 60s. This striking correlation was highly suggestive that the primary site of peripheral estrogen formation in women is the adipose tissue. The fibroblast-to-adipocyte ratio in breast-associated adipose tissue displays large variations from one region to another within the same breast or from one individual to another. These variations in the distribution of fibroblasts determine local estrogen biosynthesis in the breast; cancer develops preferentially in anatomical sites expressing the highest aromatase activity. Aromatase gene expression in adipofibroblasts is influences by various adipokines including TNF-α, leptin, adiponectin and hepatocyte growth factor (1). Aromatase activity in the breast is an attractive resolution to the paradox that breast cancer increases with age, although overall estrogen levels decline. At therapy levels, a Hamlet-like question "To block or not to block estrogen's synthesis and action" remains not completely answered (2). With other words: inhibition of estrogen *production* by aromatase inhibitors (e.g. letrozole) and/or inhibition of estrogen *action* by antiestrogens (e.g. tamoxifen)? A promising approach appears to be the development of *selective aromatase modulators* that target the aberrant overexpression of aromatase in malignant breast epithelial cells and surrounding fibroblasts, while sparing other sites of estrogen action such as bone. The inhibition of epithelial and fibroblast *proliferation* by targeting NGF receptors (3) and adipose tissue-associated mast cells (4) may also be with therapeutic potentials in breast cancer. Further studies on adipobiology and adipopharmacology of cancerogenesis may provide important insights into potential links between obesity and several types of cancer, including breast cancer and its pharmacotherapy.

1. Vona-Davis L, Rose DP. Adipokines as endocrine, paracrine, and autocrine factors in breast cancer risk and progression. *Endocr Relat Cancer* 2007; 14:189-206.
2. The BIG 1-98 Collaborative Group. Letrozole therapy alone or in sequence with tamoxifen in women with breast cancer. *N Engl J Med* 2009; 361:766-776.
3. Naderi A, Hughes-Davies L. Nerve growth factor/nuclear factor-kappaB pathway as a therapeutic target in breast cancer. *J Cancer Res Clin Oncol* 2009;135:211-216.
4. Chaldakov GN, Stankulov IS, Hristova M, Ghenev PI. Adipobiology of disease: adipokines and adipokine-targeted pharmacology. *Curr Pharm Des* 2003; 9:1023-1031.

MOLECULAR MECHANISMS INVOLVED IN REGULATION OF ADIPOGENESIS IN MESENCHYMAL STEM CELLS AND TUMOR-ASSOCIATED FIBROBLASTS

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The purpose of our study was to investigate the molecular mechanisms of adipogenesis regulation in different differentiation stages, both in mesenchymal stem cells (MSC) as well as in tumor-associated fibroblasts (TAF), considering their potential to transform into cells exhibiting intracytoplasmic lipid vacuoles. Normal human MSC were obtained from bone marrow of 8 healthy orthopedics patients undergoing hip replacement surgery. Approximately 10 ml of bone marrow were placed in culture plates, and the fibroblastic-like, plastic adherent fraction, was isolated following multiple passages, and used in our experiments. The MSC were further cultured and expanded in alpha-minimum essential medium, supplemented with 10% fetal calf serum and 2% Penicillin/Streptomycin mixture. Human TAF were isolated using both the explant and collagenase type IV-S by *Clostridium histolyticum* methods. Breast cancer surgical pieces of approximately 5 cm^2 were obtained form 8 female patients, with the histopathological diagnosis of infiltrative ductal mammary carcinoma, and isolated TAF were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum and 2% Penicillin/Streptomycin solution, and grown at 37°C, in humid atmosphere containing 5% CO_2. Starting with passage 2 for MSC and passage 4 for TAF, 20,000 cell/cm^2 were seeded in appropriate culture flasks for histochemistry, immunohistochemistry and molecular evaluation. Nonhematopoietic stem cell medium for generation of adipocytes was used for differentiation, supplemented with 1% Penicillin/Streptomycin. In days 3, 5, 7, and 21 following addition of differentiation media, total RNA extraction was performed and RT-PCR and qRT-PCR methods were used for quantifying expression of peroxisome proliferator-activated receptor gamma (PPARγ), CCAAT/enhancer binding protein (C/EBP) alpha (C/EBPα) transcription factor, and lipoprotein lipase (LPL). Histochemical analysis, using Oil Red O staining, revealed the presence of lipid vacuoles in TAF even after 3 days of induction, while MSC exhibited this property only after 14 days of culture in differentiation medium. Immunohistochemical staining using fatty acid binding protein 4 (FABP4) antibody revealed that both types of cells expressed this marker after 3 weeks culture in appropriate differentiation medium. We found that both MSC and TAF can differentiate towards the adipocytic lineage in variable proportion (30-50%), but FABP4 expression can be detected only in mature adipocytes, 21 days after induction. PPARγ is a molecular marker that is present even in early passages of both MSC and TAF, as well in preadipocytes, starting with day 3, its expression increasing in mature adipocytes and decreasing with passage number in MSC and TAF. C/EBPα and LPL are not present in undifferentiated MSC and TAF, and expression of these markers could be juxtaposed on mature adipocytes development. Altogether, we may conclude that some molecular markers, considered characteristic for adipogenic differentiation, are phenotypically present in MSC and TAF, being upregulated during the differentiation process and downregulated with passage number if no induction factor is present within their environment. TAF also exhibiting the same characteristics as MCS could be an indicator of their common origin and development, thus contributing to accumulation of tumor associated-adipocytes. This could be a promising pathway for targeting specific molecules involved in early adipocytic differentiation, thus preventing accumulation of mature adipocytes and development of obesity, while the tumor-associated adipocytes must be further investigated for determining their role in tumor progression.

STAY THIN: A FIGHT AGAINST OREXINS AND GHRELIN

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Normal-weight man keeps a constant balance between food intake and energy expenditure. When the intake is greater that the expenditure, the excess calories are stored in adipose tissue, producing *Homo obesus*. It has been recognized for decades that the hypothalamus plays a pivotal role in the control of feeding behavior and energy homeostasis, the orexigenic and anorexigenic neurons working collaboratively there. It was shown that orexigenic pathways involve neuropeptide tyrosine (NPY), melanin concentrating hormone, orexin A, *agouti*-related peptide, and endocannabinoids, while the anorexigenic pathways utilize proopiomelanocortin and melanocortin system, cocaine and amphetamine regulated transcript, corticotrophin releasing hormone, and brain-derived neurotrophic factor and ciliary neurotrophic factor. Several peripheral signals, like cholecystokinin, peptide YY, incretins and various "newcomers" such as ghrelin, obestatin, neuropeptides B and W (1) are predominantly associated with the gastrointestinal tract, while the source of other appetite-satiation regulating factors is the white adipose tissue. Today, adipose tissue is recognized as a key (neuro)endocrine organ of the body, producing a large number of signaling proteins (adipokines), some of them being involved in the control of eating behavior and energy homeostasis. Foremost among them is the *ob* gene-encoded adipokine leptin (from the Greek *leptos*, meaning thin). In the present *Stay leptos* lecture we attempt to highlight the molecular control of appetite-satiation and energy homeostasis, emphasizing on the role of the newly discovered orexigenic peptides orexin A, orexin B and ghrelin. Fighting against them may hopefully help humans staying both healthy and handsome, and keeping them protected from eating disorders as well as body dysmorphic disorders such as bigorexia (reverse anorexia) (2). However, "being thin does not automatically mean you are not fat", quoting Jimmy Bell's paradigm of TOFI (Thin Outside, Fat Inside) (3). This issue requires special attention.

1. Harrold JA, Williams G. Newcomers and supporting actors. In: G Frühbeck, editor. *Peptides in Energy Balance and Obesity*. CAB International. 2009; 61-92.
2. Pope HG Jr, Phillips KA, Olivardia R. *The Adonis Complex: The Secret Crisis of Male Body Obsession*. The Free Press, New York. 2000.
3. Rancîć G, Petkoviç A, Sekulovic-Stefanovoć L, Bojaniç V, Ghenev PI. Adipotopography: TOFI *versus* TOTI, or a hidden *Homo obesus*.[abstract]. *Biomed Rev* 2007; 18:120-121.

CLINICAL AND EXPERIMENTAL STUDY OF ADIPOSE TISSUE BEHAVIOUR AFTER AUTOLOGOUS FAT GRAFTING

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The reconstruction of soft tissue defects has become a routine procedure in plastic and reconstructive surgery. Autologous soft tissues are used for correction following extensive deep burns and tumor resections. Adipose tissue taken by liposuction is used for cosmetic and reparation purposes in lipofilling of lips, wrinkles and some tissue defects. Autologus fat grafting however remains an unresolved problem since adequate implant materials are still controversial. It is well known that this therapy has problems of absorption and subsequent volume loss of the implanted adipose tissues. The few histological examinations available are conducted on animals and human volunteers, showing the histological aspects of filler complications (Zimmenman, Clerici, 2004) and perivascular inflammation (Clark *et al*, 1989; Morgan, 1995). However, little is known about the histological appearance of the implanted adipose tissue as it is very difficult to obtain a real view of its morphology and to follow the behavior of the injected filler *in situ*. Studies on *in vitro* culture might elucidate the problem. In the present work we present the results of clinical and experimental studies of autologous fat grafting which compare adipose tissue transplant behavior after two different techniques of purifying: centrifugation at 3400 rpm for 3 min and serum lavage without centrifugation. Clinical evaluation was performed under standardized conditions for lipofilling on a series of 51 female patients. Experimentally, two culture systems: *(i)* in diffusion chambers with viteline membranes, and *(ii)* floating tissue cultures *in vitro* were designed to evaluate the behavior of autologus fat after lipofilling; with them we tried mimic the processes *in vivo*. Survival, structure, and proliferation of the implanted adipose tissue *in vitro* were examined by classical histologic hematoxylin-eosin staining, histochemical Sudan III lipids' demonstration and immunohistochemistry for leptin as a marker of preadipocytes, and cyclin D1 as a marker for cell proliferation. The diffusion chamber cultures revealed that the morphology of *in vitro* adipose cells resembled that *in vivo*: unilocular adipocytes and a small amount of multilocular cells among them. In the cultures of non-centrifugated adipose tissue some small fragments of connective tissue with multilocular cell appearance and positive expression for leptin and cyclin D1 were observed; these were referreed to as preadipocytes. The cytological study of the lamellas in the floating adipose tissue cultures provided a direct evidence of three-dimensional tissue coherence and cell-cell contact in a tissue context, which was in strong contrast to conventional 2-D monolayer cultures. The processes of cell migration, proliferation and adipocyte differentiation were followed: cells with irregular, elongated or roundish shape, central nucleus and tiny lipid droplets proliferated, and migrated from the primary fragment and crept along the fibers of the polyester lamellas; these were accepted as preadipocytes which gradually acquired morphology and biological features of mature adipocytes. The result of culture systems investigating centrifuged and noncentrifuged adipose tissue used for lipofilling revealed that the main differences were presence of a greater amount of preadipocytes in the noncentrifuged adipose tissue cultures and more distinctly expressed cell proliferation. The postoperative clinical results favored the serum lavage purifying technique. In conclusion our data suggest that with transplantation of noncentrifuged adipose tissue more active preadipocytes are applied which could possibly lead to better potential chances of survival and even *de novo* development of adipose tissue.

PRENATAL EXPOSURE TO ETHANOL BUT NOT RED WINE CAUSES AGE-RELATED DEFICITS IN RODENTS: IMPLICATION OF NERVE GROWTH FACTOR AND BRAIN-DERIVED NEUROTROPHIC FACTOR

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Neurotrophic factors, particularly the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), are well known regulators of neuronal development, survival and plasticity, and increasingly implicated as key players in learning and memory in adult and aged life. Decreased neurotrophic activity may therefore be involved in the pathogenesis of alcohol-related neurodevelopmental disorders. However, recent studies implicate region-specific upregulation of BDNF and associated signaling pathways in anxiety and addiction after ethanol exposure (1). Several lines of evidences have raised the possibility that neurotrophins are abnormally regulated in the central nervous system of animal models of chronic ethanol assumption. Nerve growth factor and BDNF are broadly expressed in the mammalian brain. The aim of this study was to investigate the effect of chronic prenatal exposure to alcohol and to red wine in aged-related biological markers and aged-related behavioral responses. Pregnant mice were exposed to ethanol or red wine (both at 11% vol) during pregnancy up to pup weaning. Control groups were exposed to isocaloric sucrose or water. At different time points of mouse life animals were tested for behavioral abilities and for biochemical and molecular analyses. We found that in adult and aged mice ethanol, *but not red wine*, causes memory deficits, reduced presence of NGF and BDNF in the cortex and hippocampus, and down-expression of choline acetyltransferase reactivity in forebrain neurons. Since recent evidences have proposed that red wine may have beneficial action in neurodegenerative and cardiometabolic diseases due to the presence of compounds with strong anti-oxidant and anti-inflammatory properties, like resveratrol (2), our findings are consistent with prior studies that the presence of such polyphenols in the red wine can protect and/or reduce long-term brain damage induced by alcohol consumption. Whether adipose tissue-derived NGF and BDNF (3) may also be involved in such ethanol/red wine experiments, remains to be presented at the 3rd International Smposium on Adipobiology and Adipopharmacology. Whatsoever, Aristophanes's (450-385 BC) conclusion will certainly stay alive forever: "Quickly, bring me a beaker of wine, so that I may wet my mind and say something clever."

1. Davis MI. Ethanol-BDNF interactions: still more questions than answers. *Pharmacol Ther* 2008; 118: 36-57.
2. Nikolova V. Resveratrol: A crossroad of enology and biomedicine. *Biomed Rev* 2007; 18: 89-101.
3. Sornelli F, Fiore M, Chaldakov GN, Aloe L. Adipose tissue-derived nerve growth factor and brain-derived neurotrophic factor: results from experimental stress and diabetes. *Gen Physiol Biophys* 2009; 28:179-183.

EFFECTS OF ACUTE AND CHRONIC ALCOHOL INTAKE ON CORTICAL EXCITABILITY

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Ethanol acts altering GABAergic and glutamatergic neurotransmission, preventing synaptic activation and affecting neuronal plasticity. In humans, the mechanisms underlying short-term changes in cortical plasticity can be investigated with repetitive transcranial magnetic stimulation (rTMS), involving glutamatergic neurotransmission. We studied whether 5Hz rTMS would disclose changes in cortical plasticity. Ten stimuli-5Hz-rTMS trains were applied over the primary motor cortex in 10 healthy subjects before and after acute ethanol intake and in 13 patients with chronic ethanol abuse, but with negative blood ethanol levels at the time of enrolment (range 6-30 years; 13.3 drink/day). The motor evoked potential (MEP) amplitude and the cortical silent period (CSP) duration during the course of rTMS trains were measured. Short-interval intracortical inhibition (3ms) and intracortical facilitation (10ms) were studied by paired-pulse TMS in 4 healthy subjects and 4 patients. In all subjects neurological and electroneurographic examination yielded negative findings. In healthy subjects before and after acute ethanol intake, 5Hz-rTMS increased the MEP size and CSP duration during rTMS. The first CSP in the train was longer after than before ethanol intake. In patients 5Hz-rTMS failed to produce the normal MEP facilitation but left the CSP increase unchanged. Ethanol intake alters cortical excitability and short-term plasticity of primary motor cortex as tested by the MEP size facilitation and CSP lengthening after 5Hz-rTMS. Acute alcohol acts on GABAergic neurotransmission whereas chronic alcohol alters glutamatergic dependent mechanisms of short-term cortical plasticity. This finding suggests that rTMS is a tool for investigating ethanol effects on cortical plasticity in humans.

COLD EXPOSURE AND ADIPOSE NITRIC OXIDE AND MAST CELLS: INFLUENCE ON AORTA CONTRACTILITY

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Both nitric oxide (NO) and mast cells play important roles in adipose and vascular tissue biology. Chronic cold stress decreases the sensitivity of vascular smooth muscle to various contractile agents including norepinephrine (NE). In our previous cold stress study we found that the contractile response of isolated rat aortas to NE was significantly reduced, and the number of rat aortic adventitial mast cells decreased (1). Histologically and functionally, white and brown adipose tissue (WAT and BAT) can be distinguished. Beyond its significance in energy store/release and heat production, adipose tissue secretes multiple signaling molecules that have endocrine and paracrine role in the regulation of vascular functions. The aims of the present study were to examine chronic cold stress-induced alterations in (*i*) the concentration of NO released from selected regions of WAT and BAT in female and male rats, (*ii*) the histochemistry of white and brown adipose mast cells, and (*iii*) whether adipose-derived NO affects the contraction of isolated rat aorta to NE. Twelve females and 12 males Spraque-Dawley rats (150-200 g b.w.) were used. The rats were exposed to a cold/freely moving stress for 2 hrs each day for 5 consecutive days. At the end of cold exposure, the rats were sacrificed, and samples of thoracic aorta with associated periadventitial adipose tissue (*tunica adiposa*) were obtained. WAT and BAT were isolated from subcutaneous abdominal and interscapular areas, respectively. The concentration of NO was measured by capillary electrophoresis and mast cells were evaluated histochemically. The response of aorta to NE was recorded in the isolated organ bath. To determine whether adipose-derived NO affects aorta contraction to NE, cumulative dose response curves to NE (10^{-8}–10^{-3} M) were obtained with or without isolated WAT/BAT suspended in the organ bath medium. In control animals, a gender-related significant difference in NO production in both WAT and BAT was found, NO levels being significantly higher in female than male rats. Data from the contractile response of isolated aorta to NE suggest that receptor affinity to NE is significantly different between female and male controls. Presence of BAT and WAT (isolated from cold-exposure animals) in the bath changed the response of aorta to NE. Displaying a gender dimorphism, BAT/WAT-derived NO, or other vasorelaxing molecules, seem to reduce receptor density and affinity to NE. Adipose mast cell histochemistry also showed diversity in respect to subtype, gender, and cold exposure. Altogether, we found (*i*) a gender difference in adipose-released NO and in adipose mast cell histochemistry to cold stress, and (*ii*) peripheral adipose tissues affect aortic contractile responses to NE likely by a NO-mediated pathway during cold exposure, suggesting that adipose tissue may limit cold stress-induced excessive vasoconstriction. Our ongoing studies aim at the evaluation of whether aortic periadventitial adipose tissue-derived NO (2) could also contribute to this phenomenon. Or we should learn from "stressomes" of *Bacillus subtilis* (3)?

1. Tunçel N, Erkasap N, Sahinturk V. The effect of stress and in vivo vasoactive intestinal peptide (VIP) treatment on the response of isolated rat aorta to norepinephrine, angiotensin II and vasopressin, and adventitial mast cells. *Stress* 2000; 3:299-308.
2. Malinowski M, Deja MA, Golba KS, Roleder T, Biernat J, Woś S. Perivascular tissue of internal thoracic artery releases potent nitric oxide and prostacyclin-independent anticontractile factor. *Eur J Cardiothorac Surg* 2008; 33:225-231.
3. Marles-Wright J, Grant T, Delumeau O, van Duinen G, Firbank SJ, Lewis PJ, *et al.* Molecular architecture of the "stressosome," a signal integration and transduction hub. *Science* 2008; 322:92-96.

CHRONOBIOLOGY OF ADIPOSE TISSUE: PINEAL-ADIPOSE NETWORK

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Today, accumulated evidence shows that energy homeostasis is achieved through integrative actions involving the brain-adipose, brain-gut, entero-insular and reward circuits. Chronobiology (chronomics) is a field of research that examines periodic phenomena in living organisms and their adaptation to light-dark and other cyclic events. These are known as circadian (about 24 hours) and circannual (about one year) rhythms orchestrating by the master biological clocks (intracellular, transcriptional mechanisms) located in the hypothalamus, particularly suprachiasmic nucleus, and the pineal gland; numerous peripheral clocks are placed in almost all tissues and cells. Circadian clocks enable the cells to timely and appropriately respond to environmental stimuli. Recent studies disclose new aspects of chronobiology of various diseases. Obesity and related cardiometabolic diseases may also be viewed as biological clock-disruption (chronodegenerative) disorders (1). Obesity represents a major and growing disease burden and, arguably, studies in the field of adipobiology have enjoyed impressive growth in the last decade. Moreover, it was recognized that hypothalamic-pituitary-adipose axis plays a pivotal role in the regulation of eating behavior and energy balance (2). Accordingly, various neuropeptides, neurotrophic factors and hypothalamic hormones/releasing factors as well as leptin, adiponectin and other adipoknes are shared by the brain and adipose tissue, suggestive for neuroendocrine potentials of adipose tissue (presented at the 2nd ISAA). Here we forward a PAN hypothesis describing that pineal-adipose network that may be involved in the control of food intake and energy homeostasis by regulating the expression and/or activity of enzymes involved in lipid, glucose and protein metabolism. (3,4) as well as the pathogenesis of obesity and related diseases (1). Note that in some species, like Siberian and Syrian hamsters, weight gain in preparation for winter is accompanied by a range of acute metabolic changes similar to the long-term changes seen in type 2 diabetes in humans. The pineal gland hormone melatonin (N-acetyl 5-methoxytryptamine) plays a key role in the regulation of biological rhythms; specific MT1 and MT2 receptors are located in suprachiasmatic nuclei as well as peripheral including adipose tissues. Future studies on (*i*) the chronomics of adipokines and other adipose-derived molecules (5,6), and (*ii*) the expression of adipose clock genes are required; overall, chronotherapeutic approach in obesity and related diseases may be appreciated.

1. Garaulet M, Madrid JA. Chronobiology, genetics and metabolic syndrome. *Curr Opin Lipidol* 2009; 20: 127-134.
2. Schäffler A, Schölmerich J, Buechler C. The role of "adipotrophins" and the clinical importance of a potential hypothalamic-pituitary-adipose axis. *Nat Clin Pract Endocrinol Metab* 2006; 2: 374-383.
3. Froy O.The relationship between nutrition and circadian rhythms in mammals. *Front Neuroendocrinol* 2007; 28:61-71.
4. Fonseca-Alaniz MH, Takada J, Alonso-Vale MI, Lima FB. The adipose tissue as a regulatory center of the metabolism. *Arq Bras Endocrinol Metabol* 2006; 50:216-229.
5. Yu YH, Zhu H. Chronological changes in metabolism and functions of cultured adipocytes: a hypothesis for cell aging in mature adipocytes. *Am J Physiol Endocrinol Metab* 2004; 286: E402-410.
6. Löckinger A, Köberle D, König PS, Saria A, Herold M, Cornélissen G, *et al*. Neuropeptide chronomics in clinically healthy young adults: circaoctohoran and circadian patterns. *Peptides* 2004; 25: 533-542.

THE ADIPOSE TISSUE AS A THIRD BRAIN

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The most momentous changes that have occurred in the field of adipobiology have been the discovery of leptin, adipocyte-specific secretory protein, in the end of 1994. Onwards, research on endocrine and paracrine activity of adipose tissue has been enjoying explosive growth. Adipose tissue is a dynamic system, consisting of adipocytes and non-adipocyte cellular elements including stromal, vascular, nerve and immune cells, which synthesize and release more than 100 signaling proteins designated adipokines. There is at present evidence that the sharing of ligands and their receptors constitutes a molecular language of the human's body, which is also the case for adipose tissue and brain. Recently we propose that the adipose tissue might be a new member of the diffuse neuroendocrine system (DNES). Today (*dnes*, in Bulgarian), adipose tissue is increasingly "getting nervous": (*i*) it expresses neuropeptide tyrosine (NPY), substance P, calcitonin gene-related protein, kisspeptin and other neuropeptides, NGF, BDNF, CNTF and other neurotrophic factors, and hypothalamic hormones/releasing factors collectively termed "adipotrophins" (1), also (*ii*) glutamate and gamma-aminobutyric acid (GABA) neurotransmitters, N-methyl-D-aspartate (NMDA) and GABA receptors, and vesicular glutamate transporters, (*iii*) various neural and glial markers such as nestin, neuron-specific enolase, neural cell adhesion molecules, semaphorin (Sema3A), neuropilin-1, and glial fibrillary acidic protein are expressed in neurally differentiated adipose-derived stem cells, (*iv*) in addition to its effect on food intake and energy homeostasis, leptin is a pleiotropic adipokine that supports learning and memory and has neurotrophic activity (2), (*v*) adipose tissue like brain produces endocannabinoids and amyloid precursor protein, and expresses the key estrogenic enzyme aromatase (P450arom), (*vi*) adipocytes may originate from the neural crest cells, and (*vii*) adipose-derived stem cells may differentiate into neuronal cells, thus implicated in regenerative medicine. Metaphorically, adipose tissue is increasing dramatically its intelligence quotient (IQ). Accordingly, we have proposed that this fascinating tissue might function as a third brain (3), as well as the gut is commonly considered a second brain, and lymphocytes a circulating brain (4). We suggest that the "third brain" may input, process, and output information as the real ("first") brain does. Further molecular profiling of adipose tissue may provide new biological insights on its "brain" potential. Altogether, this may open a novel field of study, neuroadipobiology. A systems and translational adipobiology integrating neural sciences, such as neuroendocrinology, neuroimmunology and neuroadipobiology, may contribute to the improvement of human's health.

1. Schäffler A, Schölmerich J, Buechler C. The role of "adipotrophins" and the clinical importance of a potential hypothalamic-pituitary-adipose axis. *Nat Clin Pract Endocrinol Metab* 2006; 2: 374-383.
2. Gertler A. Role of leptin in early metabolic programming. *Adipobiology* 2009; 1: 27-34.
3. Chaldakov GN, Fiore M, Tonchev AT, Hristova MG, Rancîč G, Aloe L. The adipose tissue as a third brain. *Obesity Metab* 2009; in print.
4. Blalock JE, Smith EM. Conceptual development of the immune system as a sixth sense. *Brain Behav Immun* 2007; 21: 23-33.

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The introduction should briefly indicate the background of the topic, and explain the objectives of the paper.

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References

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