Institute of Endocrinology, Prague

Laboratory of Clinical and Experimental Neuroendocrinology

Charles University, Faculty of Science, Prague

Department of Animal Physiology and Developmental Biology

The role of leptin, resistin and ghrelin in the pathophysiology of anorexia nervosa

Doctoral Thesis

Supervisor: RNDr. Jara Nedvídková, CSc.

Consultant: Doc. RNDr. Stanislav Vybíral, CSc.

Prague 2006

RNDr. Ivana Dostálová

Acknowledgements

It is my great pleasure to thank to my supervisor RNDr. Jara Nedvídková, Csc. for her technical and theoretical support during my master and PhD study and to Doc. RNDr. Stanislav Vybíral, Csc. for his unflagging help and advice, theoretical support, interest on my work and patience.

I would like also thank to Mrs. Diana Riegerová, the laboratory assistant, for her practical help with learning analytical methods and friendly working atmosphere and to nursing staff of the Institute of Endocrinology for their assistance during clinical trials.

My next thank pertains to Doc. MUDr. Hana Papežová, Csc. for granting assistance with enclosing of patients with anorexia nervosa in our study and to all volunteers enrolled in the study for their will to help.

The last, but not least, thank is addressed to my husband Pavel and to my parents for their love, psychical support and especially for their kind assistance with taking care of my one-year-old son Tomášek. I thank with love Tomášek for his smile.

I declare hereby that the present Doctoral Thesis was worked out on my own using the literature listed thereinafter.

06/04/06
Prague 2006

RNDr. Ivana Dostálová

„I would like to point out that eating disorders did not exist in human communities where personality and success would not be the questions of image and figure “

TABLE OF CONTENTS

THE LIST OF ABBREVIATIONS

AN	anorexia nervosa
ACTH	adrenocorticotropic hormone
AgRP	agouti-related protein
BMI	body mass index
CART	cocaine- and amphetamine-regulated transcript
CRH	corticotropine-releasing hormone
DHEA(S)	dehydroepiandrosterone (sulfate)
EPI	epinephrine
FSH	follicle-stimulating hormone
(f; r)T3	(free; reverse) triiodothyronine
(f)T4	(free) thyroxine
GH	growth hormone
GHRH	growth hormone-releasing hormone
GHS-(R)	growth hormone secretagogue-(receptor)
GnRH	gonadotropin-releasing hormone
GPCR	G-protein-coupled receptor
HP-A (-G; -T)	hypothalamus-pituitary-adrenal (-gonadal; -thyroid)
i.c.v.	intracerebroventricular
i.v.	intravenous
IGF-1	insulin-like growth factor-1
IL-6	interleukin-6
LH	luteinizing hormone
mRNA	messenger ribonucleic acid
NE	norepinephrine
NEFA	non-esterified fatty acid
NMU	neuromedin
NPY	neuropeptide Y
Ob-R	leptin receptor
p.o.	per os
POMC	proopiomelanocortin
PPARγ	peroxisome proliferator-activated receptor γ
PRL	prolactin
SNS	sympathetic nervous system
TNF-α	tumor necrosis factor-α
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone
TZDs	thiazolidinediones
WAT	white adipose tissue

1. ABSTRACT

Anorexia nervosa (AN) still remains a highly morbid condition with the highest mortality of any other psychiatric disorder. Beside acute refeeding techniques, no specific interventions have been proven effective in changing the long-term course of AN. Extreme reduction of food intake and hyperactivity characteristic for this starvation status result in severe weight and fat loss together with multiple endocrine perturbations, altered glucose and lipid metabolism as well as in delayed gastric emptying and other gastrointestinal complications.

Loss of adipose tissue is beside disturbances in thermoregulation, lipolysis and lipogenesis associated with altered production and release of adipose tissue-derived proteins, so-called adipocytokines, that play an important role in the autocrine and paracrine regulation of adipose tissue metabolism as well as in the endocrine regulation of metabolism of other peripheral tissues. Furthermore, some of adipocytokines act in the central nervous system to regulate energy balance. Similarly, altered food ingestion in patients with AN could be related to changed production and/or action of gastrointestinal hormones. Thus, both adipose tissue-derived and gastrointestinal hormones could inform central nervous system about acute (food intake) as well as about long-term (fat stores) energy disbalance in peripheral systems.

In the present Thesis, the roles of leptin and resistin, as the representatives of adipocytokines, and ghrelin, the gastrointestinal hormone, in the pathophysiology of AN have been studied. Although alterations in many endocrine systems have been clearly described in patients with AN, the role of these recently characterized proteins in patients with AN is not definitive. The research focused on the role of resistin in human physiology as well as in pathophysiologic conditions, such as malnutrition, brought rather contradictory data. However, the participation of both leptin and ghrelin in the long-term regulation of energy balance as well as in the acute regulation of food intake is well established.

The unique technique of microdialysis allows direct *in vivo* sampling of interstitial fluid from the studied tissue. Moreover, microdialysis is conseptually simple and the principle of the technique could be likened to capillary. The limitation of the procedure is a molecular weight of the tested and/or applicated molecules. That is the practical reason of limited using of *in vivo* microdialysis to measure proteins. Although microdialysis has been used since 1987 in more than 3000 clinical trials mainly in the muscle and adipose tissue and has been described as a suitable technique for *in vivo* measurement of concentrations or dynamic changes in concentrations of glucose, adenosine, glycerol, aminoacids etc., as long as I know, *in vivo* concentrations of adipocytokines have not been explored. Thus, in this Thesis the

unique microdialysis technique has been modified and used for *in vivo* measurement of leptin and resistin levels in the extracellular space of abdominal adipose tissue for the first time.

The present Thesis is divided on abstract, introduction, results, discussion and conclusions. In the introduction part, author is presenting an overview on the experimental field closely connected with the studied questions. The text of introduction also includes the original results of the author and embedded graphs. The result and discussion parts are submitted together in a form of original articles of the author (3 published and 2 submitted for publishing). Finally, the conclusion part summarizes the main results of this Thesis.

2. TARGETS OF THE THESIS

The present Thesis is mainly focused on the alterations of adipocytokines leptin and resistin and gastrointestinal hormone ghrelin in patients with AN. The role of these proteins in the long-term regulation of energy metabolism as well as in the short-term reflection of acute energy disbalance (food intake; a single bout of exercise) is the point of interest. For better understanding of the conditions in the site of production of leptin and resistin, concentrations of these adipocytokines in plasma were compared with those found in the extracellular space of abdominal adipose tissue using *in vivo* microdialysis. Before starting human studies, microdialysis had been tested and modified *in vitro* to be applicable for *in vivo* sampling of leptin and resistin from human subcutaneous adipose tissue. Plasma ghrelin levels were measured under basal conditions and after either a caloric or non-caloric meal of the same volume consumption. Furthermore, the effect of a single bout of exercise on plasma leptin levels was determined. The results obtained in patients with AN were compared with those found in healthy normal-weight women.

The main targets of the Thesis are:

A. *In vitro* experiments

I. To *in vitro* test and modify the microdialysis technique to be applicable for *in vivo* sampling of leptin and resistin from human subcutaneous abdominal adipose tissue.

B. *In vivo* experiments

II. To determine *in vivo* leptin and resistin concentrations in the extracellular space of the abdominal adipose tissue of patients with AN and of healthy age-matched control women and to compare obtained concentrations of leptin and resistin with those measured in plasma of these subjects.

III. To determine plasma ghrelin concentrations under basal conditions and after either a non-caloric fibre or a standard nutritional mixed meal of the same volume consumption and to test the response of plasma ghrelin on food intake, meal volume and meal nutritional value.

IV. To define the effect of a moderate-intensity single bout of exercise (acute energy disbalance) on plasma leptin levels in patients with AN and in healthy age-matched control women.

The studies included in this Thesis have been supported by the Grant Agency of the Czech Republic, No. 303/03/0376 and No. 303/00/1555. The project was done in collaboration with the First Faculty of Medicine, Psychiatric Clinic, Charles University in Prague and with National Institute of Health, Bethesda, USA.

3. INTRODUCTION

3.1. ANOREXIA NERVOSA

3.1.1. Description of the disorder

The diagnostic criteria for AN are summarized in **Table 1**. AN is a severe psychiatric disorder of unclear etiology associated with significant morbidity and mortality. In fact, the illness often leads to chronic ill-health, and patients are 10times more likely to die from complications of the illness than those without it (Hsu 1996). The most striking behavior in individuals with AN is the persistent and willful restriction of intake which can lead to death in 5 - 7% of patients within 10yr of the onset of their disorder. Another distinguishing psychological feature is the irrational fear of becoming fat, frequently in concert with a grossly distorted view of one´s self as overweight. This persists even in the presence of severe emaciation and in the face of life-threatening medical sequelae. The patient often exhibits a phobic response to food, particularly fatty and other calorically dense items. Anorexics develop an obsessive preoccupation with food, eating, dieting, weight, and body shape, and they frequently exhibit ritualistic behaviors involving choosing, preparing, and ingesting meals. Extreme dieting is complicated by other weight-reducing behaviors. Exercise is frequently compulsive and hyperactivity in underweight patients is a curious but often encountered concomitant. Weakness, muscle aches, sleep disturbances, and gastrointestinal complaints, including constipation and postprandial bloating, are common physical findings. The illness is further associated with mood disturbances, as well as alterations of a wide variety of hormonal and metabolic systems.

Currently, many investigators conseptualize these disorders as being due to a combination of cultural-social, psychological, and biological factors. Among various factors associated with increased risk for eating disorders belong female gender, dieting, middle and upper-class social background, personality disorders, family disfunction, constitutional predisposition, profession or pursuit that stresses maintaining a certain body weight etc.

In general, the response of patients with AN to psychoterapeutic or pharmacological treatments has been limited. Basic principles and goals of nutritional interventions and guidelines for clinical treatment of AN are shown in **Table 2** and **Table 3**, respectively.

Table 1. Diagnostic criteria for AN

A. Refusal to mantain body weight at or above a minimal normal weight for age and height (e.g., weight loss leading to maintenance of body weight less than 85% of that expected; or failure to make expected weight gain during period of growth, leading to body weight less than 85% of that expected).

B. Intense fear of gaining weight or becoming fat, even thought underweight.

C. Disturbance in the way in which one's body weight or shape is experienced, undue influence of body weight or shape on self-evaluation, or denial of the seriousness of the current low body weight.

D. In postmenarcheal females, amenorrhea, i.e., the absence of at least three consecutive menstrual cycles. (A woman is considered to have amenorrhea if her periods occur only following hormone, e.g., estrogen administration.)

Specify type:

Restricting type: During the current episode of anorexia nervosa, the person has not regularly engaged in binge-eating or purging behavior (i.e., self-induced vomiting or the misuse of laxatives, diuretics, or enemas).

Binge eating/purging type: During the current episode of anorexia nervosa, the person has regularly engaged in binge-eating or purging behavior (i.e., self-induced vomiting or the misuse of laxatives, diuretics, or enemas).

Source: American Psychiatric Association Diagnostic and Statistical Manual of Disorders, DSM-Q, American Psychiatric Association, Washington, DC, 1994.

Table 2. Basic principles and goals of nutritional intervention in AN

1. Increased energy intake to promote weight restoration, applying a weekly, stepwise increase to achieve the goal weight gain (i.e., 0.5 – 1.0 kg/week).
2. Specific meal plan and dietary guidelines to promote normalization of intake, including some limitations on the number of foods that patients may refuse to eat and accomodating patient preferences that permit nutritional adequacy and weight gain.
3. A new approach to food choices, based on nutrient contributions and other qualities, rather than energy content.
4. Formelly forbidden foods introduced with reassurance and sensitivity to a fear of uncontrollable eating and weight gain.
5. Adequate dietary calcium to permit improved bone mineralization as weight is restored and hormonal abnormalities are corrected.
6. Low-dose daily multiple vitamin with minerals especially if patients are chronically ill.
7. Avoidance of strategies to reduce energy intake and manage hunger (such as overuse of caffeine-containing beverages, chewing gum) or promote energy expenditure (such as excessive exercise).

Source: The management of eating disorders and obesity, edited by DJ Goldstein, MD, PhD, Humana Press Inc. 1999, Totowa, New Jersey.

Table 3. Guidelines for clinical treatment of AN

- Refeeding and weight restoration
- Institute pharmacologic therapy
- Continue therapy for at least 3 month
  - Resumption of menstruation
  - Normalization of caloric needs
  - Remediation of physical complications
  - Remission of pathological eating and body-image distortions
- Revealuate need for continuing pharmacologic therapy

Source: The management of eating disorders and obesity, edited by DJ Goldstein, MD, PhD, Humana Press Inc. 1999, Totowa, New Jersey.

3.1.2. Medical complications

The majority of the medical consequences, including physiologic and metabolic changes, observed in AN are present in starvation states or are a direct result of purging behaviors. With nutritional rehabilitation and the discontinuation of purging behaviors, these abberations can be expected to resolve.

3.1.2.1. Endocrine abnormalities

Endocrine abnormalities are characteristics for AN. Alterations of endocrine systems in AN are summarized in **Table 4**. Among these abnormalities amenorrhea play an important role as an indicator of the loss of body weight, especially of fat mass, under the individual critical limit (Sharp and Freeman 1993). Interestingly, amenorrhea has been described in more than 15% and the disregulation of menstrual cycle in 30% of patients with AN before the weight loss, indicating that psychological factors may play a role (Fries 1977).

3.1.2.2. Hematological and electrolyte abnormalities

Common abnormalities in hematopoiesis include leukopenia with a relative leukocytosis. With nutritional rehabilitation, the depressed white cell count returns to normal. Metabolic alkalosis is frequently seen in patients who induce vomiting, with associated hypokalemia, hypochloremia and elevated serum bicarbonate. A metabolic acidosis may be encountered in some patients who abuse large quantities of laxatives. Volume depletion associated with dehydration in those who fast or purge can increase aldosterone, leading to potassium excretion from the kidneys, resulting in hypokalemia. In addition, this indirect renal loss of potassium can compound direct loss through self-induced vomiting. The aforementioned

electrolyte disturbancies are often associated with the physical symptoms of lethargy and weakness.

Emaciation and electrolyte disturbancies can be associated with other significant abnormalities, especially in those patients who purge. An electrocardiogram may show flattening or inversion of T-waves, ST-segment depression, and a prolonged QT interval. Severe hypokalemia can lead to serious arrhythmias and the risk of cardiac arrest. The risk of heart failure is associated with the injudicious rapid refeeding of emaciated anorexics and must be avoided during the period of weight restoration.

Table 4. Alterations of plasma hormonal and biochemical parameters in AN

↓	LH (↓ response to GnRH); ↓ FSH
↑/ =	**GH; ↓ IGF-1**
=	**TSH** (but delayed response to TRH)
=	**ACTH**; ↓ reaction on CRH
↓/ =	**PRL**; ↓ response to TRH
↓	T4; ↓ T3; ↑/ = **rT3; = fT4; ↓ fT3**
↑/ =	**serum cortisol** (↓ suppression in dexamethasone test)
↑	cortisol excretion per day
↓	DHEA, DHEAS
↓	**Estradiol** ; ↓ Estron; ↓ progesterone;
↓	Testosterone
↓	**Serotonin**
↓	**Insulin; = glucose; = C-peptide**
↓	**Plasma leptin; ↑ plasma ghrelin; ↓ plasma resistin; ↑ plasma adiponectin**
=	**plasma EPI; plasma NE; plasma glycerol; plasma NEFA**

Source: **Nedvidkova et al. 2003; Dostalova et al. 2005; Dostalova et al. (unpublished results)**; *Hoster D.W.: Eating Disorders: Obesity, anorexia nervosa, and bulimia nervosa. In: Williams Textbook of Endocrinology. Ed. Wilson JD, Foster DW, W.B.Saunders Co., 1992.*

3.1.2.3. Chemistry abnormalities

Laboratory tests reflecting liver function may reveal elevation of serum enzymes, most likely indicating some degree of fatty degeneration. This may be observed in the state of emaciation as well as during the period of refeeding. In younger patients, serum cholesterol levels may be elevated. Carotinemia, which normalizes with weight restoration, is often seen in malnourished anorexics. Parotid gland enlargement and an associated elevation in amylase is often seen in anorexic patients who binge and self-induce vomiting.

3.1.2.4. Gastrointestinal complications

Delay in gastric emptying due to extended caloric restriction may cause a sense of postprandial discomfort and early satiety. Both are frequently observed and contribute to the persistence of restricting behavior in AN. Constipation due to fluid and caloric restriction is common and frequently requires management strategies. In patients who self-induce vomiting, gastritis and esophageal erosions are possible complications. Acute gastric dilation and esophageal rupture are rare medical emergencies and can lead to shock and death in patients who binge.

3.1.2.5. Long-term complications

In those patients whose illness persists for several years or longer, the risk of osteoporosis evolves. The degree to which bone density can be reversed with sustained nutritional rehabilitation is unclear. Also the above mentioned amenorrhea can be counted among chronic abnormalities, because amenorrhea is persisting in almost 50% of patients with AN after weight restoration (Warren and Vande Wielle 1973) leading to inability to become pregnant. Other long-term comlications include skeletal-muscular injuries, such as sprains and fractures, resulting from compulsive physical exertion in a compromised patient, and the persistence of a generally compromised medical state.

3.2. LEPTIN

3.2.1. Leptin physiology

Leptin is a 167 amino acid protein product of the ob gene that was discovered in 1994 through positional cloning in the ob/ob obese mouse, a model of morbid obesity resulting from absence of leptin due to a gene mutation (Zhang et al. 1994). Human as well as murine leptin is predominantly produced by adipocytes (Zhang et al. 1994) with subcutaneous adipose tissue representing the main source (Hube et al. 1996; Van Harmelen et al. 1998), next in the stomach (Mix et al. 2000) and to a minor extent by placenta (Jakimiuk et al. 2003), mammary epithelium (Ishikawa et al. 2004), intestine (Aparicio et al. 2005), skeletal muscle (Solberg et al. 2005), and the brain (Popovic et al. 2001).

Leptin expression is not only chronically regulated by whole body fat mass (Considine et al. 1996), but also acutely by feeding and fasting consistent with its fast-acting role in appetite control (Kolaczynski et al. 1996; Chan et al. 2003; Kmiec et al. 2005). Interestingly, leptin production was shown to be a direct function of adipocyte size (Hamilton et al. 1995; Couillard et al. 2000). In addition, a series of hormones and cytokines were reported to regulate adipocyte leptin production *in vitro* and *in vivo*. Among these, insulin, glucocorticoids, estrogens, and inflammatory cytokines have been shown to induce (Coppack 2001; Machinal-Quelin et al. 2002; Kanu et al. 2003), whereas catecholamines have been shown to inhibit leptin production (Pinkney et al. 1998; Couillard et al. 2002).

Leptin circulates in the serum in a free form or bound to leptin-binding proteins, and the sum of free and bound leptin (i.e., total leptin) is generally accepted standard of measurement. Like other hormones, leptin is secreted in a pulsatile way and has a substantial diurnal variation with an increase of about 50% in the late evening and the decline in early morning hours that might be related to an intrinsic circadian component, meal timing, and the sleep-wake cycle (Sinha et al. 1996; Simon et al. 1998; Elimam and Marcus 2002). Importantly, women have higher leptin concentrations than men even after adjusting for BMI, which may be due to differential body-fat distribution or the effects of sex steroids (Rosenbaum et al. 1996; Saad et al. 1997).

In addition to appetite and body weight regulation, leptin shares with other cytokines a marked pleiotropism and is involved in the regulation of physiological processes as diverse as reproduction (Chehab et al. 1996; Ashworth et al. 2000), hematopoiesis (Gainsford et al. 1996), angiogenesis (Sierra-Honigmann et al. 1998), immmune response (Gainsford et al. 1996), blood pressure control (Fruhbeck 1999), and bone formation (Ducy et al. 2000). The wide spread distribution of leptin receptors furthermore suggests that leptin, in addition to its

effects on the central nervous system, also directly affects peripheral tissues (Tartaglia et al. 1995; Lee et al. 1996; **Fig. 1**).

Reduced leptin concentrations seen in congenital leptin deficiency and lipodystrophy cause hyperphagia, impaired thermogenesis, insulin resistance, hyperlipidemia and hypogonadism, all of which are reversible by leptin treatment (Ahima and Hileman 2000; Flier and Elmquist 2004). In normal humans and rodents, the fall in leptin is an important signal for the switch between fed and fasted states, leading to suppression of thyroid, growth and reproductive hormones, stimulation of appetite and inhibition of thermogenesis and immunity (Ahima 2000).

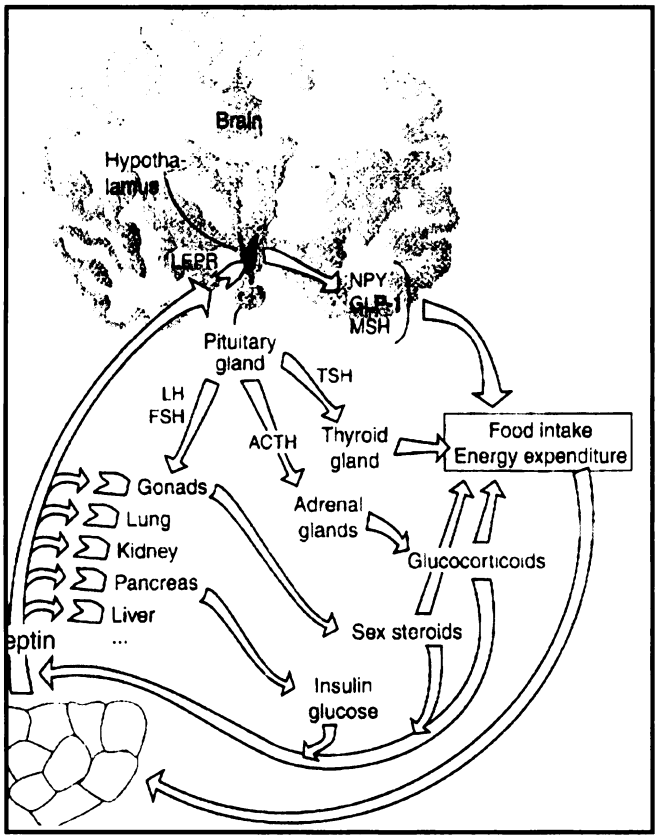
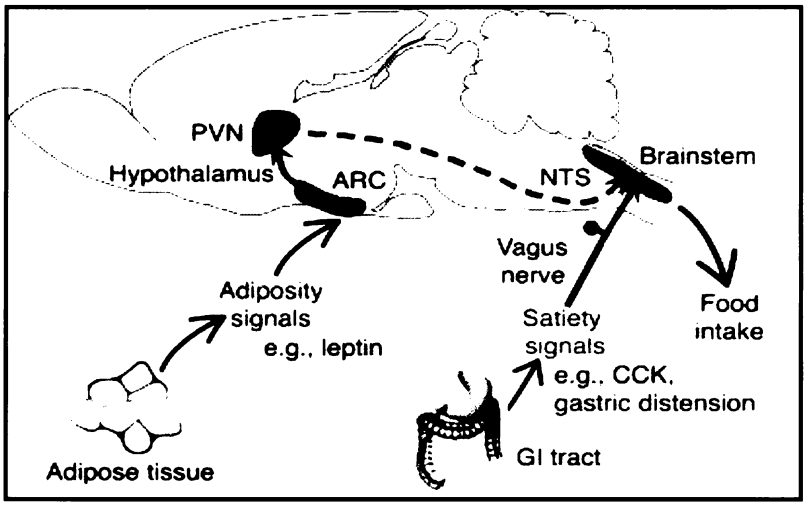


Figure 1. Central and peripheral effects of leptin on the regulation of body weight and metabolic and endocrine parameters. Leptin secretion by adipocytes is influenced by nutritional status, insulin, glucocorticoids and sex steroids (note: Although catecholamines are not shown in the schema, both adrenaline and noradrenaline have been shown to inhibit leptin production by adipocytes). Leptin receptor (LEPR) has been found in brain, mainly in hypothalamus and in choroid plexus, where the food intake is regulated via modulation of many neurotransmiters such as neuropeptide Y (NPY), glucagon-like peptide-1 (GLP-1) and melanocyte-stimulating hormone (MSH). Leptin further takes part in regulation of secretion of thyroid hormones, cortisol and sex steroids. LEPR has been found also in many peripheral tissues including lungs, liver, kidney, gonads and stomach.

Source: Wauters M, Considine SV, Van Gaal LF. Human leptin: from an adipocyte hormone to an endocrine mediator. Eur J Endocrinol 2000 143(3):293-311.

3.2.2. Leptin and energy balance: mechanism of action

Leptin crosses the blood-brain barrier via a saturable possibly leptin receptor-mediated transport mechanism (Banks et al. 1996). Central action of leptin on hypothalamic nuclei results in inhibition of appetite and food intake, reduction of body weight, and increased energy expenditure (Campfield et al. 1995) mainly by repressing neuropeptide Y (NPY)/agouti-related protein (AgRP) neurons and inducing anorexigenic proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART) neurons (Kristensen et al. 1998; Ahima 2000; Cowley et al. 2001). Neurons expressing the leptin receptor in the nucleus tractus solitarius also seem to play a role in regulating energy balance (Schwartz et al. 2000) **(Fig. 2 A, B)**.



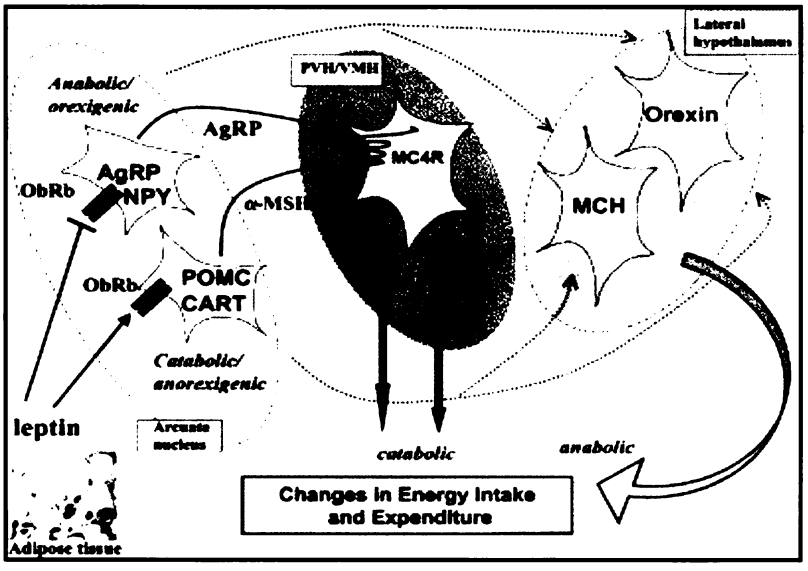


Figure 2A. Adiposity signals such as insulin and leptin circulate in proportion to body fat mass and act on hypothalamic ARC neurons that project to hypothalamic areas such as the lateral hypothalamus (not shown) and nucleus paraventricularis (PVN). In turn, these "second order" neurons project to hindbrain autonomic centers such as the nucleus tractus solitarius (NTS) that process afferent input from satiety signals such as cholecystokinin (CCK). Input from descending, leptin-sensitive hypothalamic projections is integrated in the NTS with vagally mediated input from CCK, such that the timing of meal termination is regulated by changes in body fat content.

Figure 2B. Leptin acts directly on arcuate nucleus neurons coexpressing NPY and AgRP, and POMC and CART, via the ObRb form of the leptin receptor expressed on these cells. The former neurons stimulate anabolic and orexigenic effects and are suppressed by leptin, and the latter neurons stimulate catabolic and anorexic actions that promote weight loss, and are activated by leptin. A key downstream target of these neurons are neurons expressing melanocortin 4 receptors (MC4R) that are activated by the POMC product α-MSH, and inhibited by the neuropeptide AgRP. Activation of these neurons promotes catabolism by reducing food intake and increasing energy expenditure. Neurons expressing BDNF in the ventromedial hypothalamus may be downstream of the MC4R neurons. Neurons in the lateral hypothalamus expressing melanin-concentrating hormone (MCH) receive projections from leptin responsive arcuate neurons, and activation of these widely projecting neurons promotes feeding, suppresses energy expenditure, and promotes weight gain.

AgRP, agouti-related protein; ARC, nucleus arcuatus; BDNF; brain-derived neurotrophic factor; CART, cocaine- and amphetamine-regulated transcript; NPY, neuropeptide Y; POMC, proopiomelanocortin.

Source: Morton GJ et al. J Clin Invest 2005 115(3):703-710(A); Flier JS. Cell 2004 116(2):337-350 (B).

3.2.3. Leptin receptor

The leptin receptor (Ob-R) belongs to the cytokine receptor class I family, and contains extracellular ligand-binding, transmembrane and cytoplasmic signaling domains (Myers 2004). Ob-Rs are activated by ligand induced receptor homo- and heterodimerization and utilize janus kinases (JAK) and signal transducers and activators of transcription (STAT) family proteins (Bates and Myers 2003; Myers 2004).

Multiple splice variants of Ob-R mRNAs encoding proteins with identical extracellular domains but different intracellular domains have been detected. Although various Ob-R isoforms (Ob-Ra-e) are derived from alternative splicing of the Lepr transcript, most of the effects of leptin on energy homeostasis and endocrine function occur in the brain through the long form of the receptor (Ob-Rb). Ob-Rb and associated signaling molecules are present in the arcuate nucleus within the hypothalamus, a brain region that is important for body weight regulation, energy homeostasis, autonomic and hormonal regulation (Ahima 2000). Low amounts of Ob-Rb were detected in peripheral tissues including adipose tissue.

Banks et al. (1996) first showed that leptin enters the rodent brain through a specific saturable mechanism distinct from that of insulin. Leptin reaches the brain intact, and it was initially thought that Ob-Rb acted as a „leptin transporter“ (Hileman et al. 2002). In agreement with this notion, the brain transport of i.v. injected leptin is severely attenuated in Koletsky rats lacking all membrane Ob-Rb. Nonetheless, leptin is still present in cerebrospinal fluid of these animals (Banks et al. 2002). There is a close correlation between brain leptin transport and circulating leptin, such that the brain transport is eliminated during fasting in parallel with the rapid fall in leptin (Kastin and Pan 2000). Conversely, refeeding increases brain transport contomitant with rising plasma leptin (Kastin and Pan 2000). These adaptations might involve lipids or other nutritional factors (Kastin and Pan 2000; Banks et al. 2004).

3.2.4. The role of leptin in a short-term regulation of food intake and postprandial satiety

Energy deficits induced by both (52 - 72)-hour fasting (Boden et al. 1996; Weigle et al. 1997) and energy restriction (Keim et al. 1998; Doucet et al. 2000; Weigle et al. 2003) decrease circulating leptin concentrations, whereas 3-day overfeeding increases leptin concentrations (Chin-Chance et al. 2000). The decrease and increase in leptin concentrations observed under conditions of fasting or overfeeding, respectively, are greater than that expected from the minor changes in adipose tissue stores (Boden et al. 1996; Weigle et al.

1997; Chin-Chance et al. 2000). Thus, leptin may also have a role as an acute indicator of energy status, which would promote short-term metabolic and feeding responses prior to the depletion of energy stores (Schwartz et al. 2000).

Studies of leptin responses to food show inconsistent results that may reflect the heterogeneity of postprandial timing of samples, meal content, meal timing and method of meal administration. Postprandial leptin concentrations were found to be dependent on meal macronutrient composition, with high-carbohydrate, low-fat meals producing higher postprandial leptin concentrations as compared with high-fat, low-carbohydrate meals (Havel et al. 1999; Chin-Chance et al. 2000). 24-h circulating leptin levels are also reduced in women consuming high-fat, low-carbohydrate diets compared with those consuming a high-carbohydrate, low-fat diet (Havel et al. 1999). In addition, high-fat feeding for 5 days produces leptin resistance accompanied by increased hypothalamic NPY concentrations in rats (Lin et al. 2001). Regarding other dietary macronutrients, neither protein nor fiber seem to impact circulating leptin concentrations (Heini et al. 1998; Groschl et al. 2003; Knerr et al. 2003).

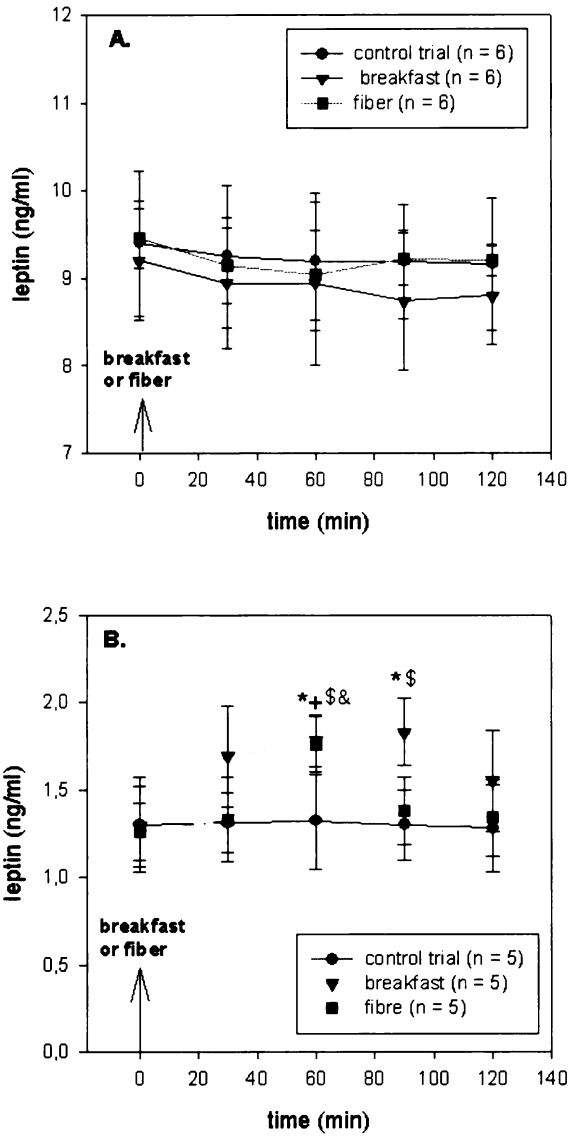


Fig. 3. The effect of either caloric (standardized breakfast; 585 kcal, 32.6 g of fat, 17.6 g of protein, 50.0 g of carbohydrate) or non-caloric (fiber; 0.11 kcal, 4 g of Psyllium in 250 ml of water) meal of the same volume on plasma leptin levels (ng/ml) in healthy control women (C, n = 6; A) and in patients with anorexia nervosa (AN, n = 5; B). Values are means ± SEM. *p < 0.05 breakfast vs. control trial, $^{+}$p < 0.05 fibre vs. control trial, $^{\$}$ resp. $^{\&}$p < 0.05 vs. basal in breakfast resp. fiber.

On the other hand, Romon et al. (1999) suggested that short-term regulation of postprandial satiety and food intake is not influenced by leptin and Korbonits et al. (1997) did not find significant acute change in leptin levels following a 1000 kcal meal when measured

every 20 min for 3 h after food consumption. Moreover, plasma leptin levels in normal human subjects showed no short-term changes after feeding a liquid mixed meal (Drewes et al. 1997). Interestingly, either a standard mixed meal or a dieting fiber of the same volume consumed after 12 h of overnight fasting had no significant influence on plasma leptin concentrations in healthy women when measured in a 30 min intervals for two hours after meal consumption, whereas the same meals significantly increased plasma leptin levels in patients with AN **(Dostalova et al. unpublished results; Fig. 3 A,B)**.

We could conclude that in healthy normal-weight subjects leptin is sensitive to relatively short-term (days) fluctuations in energy balance, but a single meal consumed in usual daily eating time (e.g., breakfast consumed at 08:00 h after overnight fasting) does not alter plasma leptin levels. On contrary, when the daily meal schedule is changed, the 24-h leptin profile is dependent on meal timing (Schoeller et al. 1997). Thus, leptin is, beside its well established role as a factor reflecting body fat stores, probably also factor signaling day-to-day energy disbalance rather than a signal of postprandial satiety. The rapid leptin response to food intake observed in patients with AN could contribute to disability of these patients to gain normal weight, because of abnormally prompt leptin response to a single meal and subsequent signal to stop eating and supports an importance of leptin in the pathophysiology of AN.

3.2.5. Energy deprivation: a low-leptin state

As described earlier, leptin acts as a signal from the periphery to the brain, conveying information about the amount of energy available in adipose tissue or acute changes in energy availability (or both). Starvation elicits changes in several neuroendocrine axes; fall in thyroid hormones to conserve metabolism; increase in stress hormones (cortisol) to mobilise needed energy stores; and rise in GH with a decrease in IGF-1 – i.e., a state of decreased energy expenditure for growth-related processes while enabling GH to increase use of alternative fuels through lipolysis (see **Table 4**). These neuroendocrine alterations have adaptive value by mobilizing needed energy stores and diverting limited resources towards important physiological processes and away from energy-consuming processes that are not essential for immediate survival (e.g., reproductive function). It was postulated that falling leptin concentrations might mediate the neuroendocrine response to fasting (Ahima et al. 1996; Chan et al. 2003). Low leptin concentrations were found to be important in signaling energy deficit to the HP-axes. It is established that leptin has an important role in reproduction and regulation of HP-gonadal (Moschos et al. 2002; Chan et al. 2003) and HP-thyroid (Rosenbaum et al. 2002; Chan et al. 2003) axes. However, its role in regulation of HP-adrenal

(Licinio et al. 1997; Ozata et al. 1999) and HP-GH-IGF-1 axes (LaPaglia et al. 1998) is not definitive.

3.2.6. Leptin in anorexia nervosa

Serum leptin concentrations in women with AN are markedly lower than those of normal-weight women as a result of decreased body weight and fat mass (Audi et al. 1998; Nedvidkova et al. 2000; **Fig. 4; Dostalova et al. 2005**), with a strikingly reduced diurnal variation of leptin concentrations (Balligand et al. 1998; Stoving et al. 1998). Interestingly, concentration of the soluble leptin receptor, which is the main binding protein for leptin in plasma, is raised in these patients (Kratzsch et al. 2002; **Fig. 5; Dostalova et al. 2005**), resulting in an even lower free leptin index and suggesting a role for leptin binding proteins in the regulation of energy homeostasis. Soluble leptin receptor decreases in response to refeeding in some studies (Kratzsch et al. 2002; Misra et al. 2004), but not all (Krizova et al. 2002). Surprisingly, our primary results have shown that *in vivo* leptin concentrations in the extracellular space of abdominal adipose tissue of patients with AN are unchanged in comparison to healthy normal-weight women **(Fig. 4; Dostalova et al. 2005)**. However, the higher than expected local leptin levels in patients with AN could be influenced by the increased number of smaller adipocytes in the vicinity of the microdialysing membrane **(Dostalova et al. 2005)**. Additionally, patients with AN have lower concentrations of leptin in cerebrospinal fluid and a higher ratio of cerebrospinal fluid to plasma leptin than healthy controls (Mantzoros et al. 1997). Dietary treatment for AN results in an increase in serum leptin concentrations as weight recovers (Hebebrand et al. 1997). Concentrations of leptin in cerebrospinal fluid also increase to normal with dietary treatment but this rise occurs before body weight has returned to normal, suggesting a possible mechanism for resistance to weight gain (Mantzoros et al. 1997).

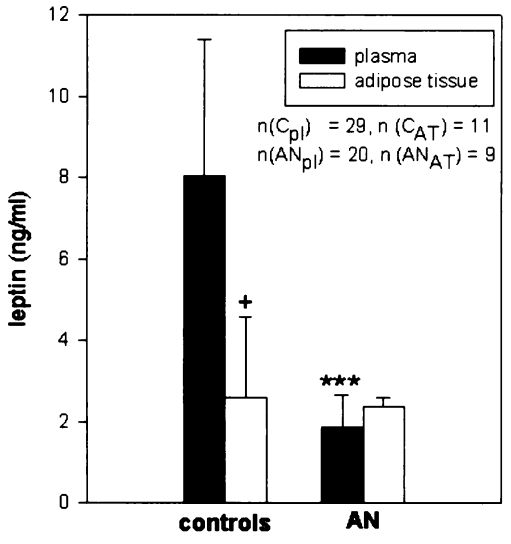


Fig. 4. Leptin levels (ng/ml) in plasma and in extracellular space of abdominal AT in healthy controls (C, n = 29) and in patients with anorexia nervosa (AN, n = 20). Plasma samples were taken at 0800 h and microdialysate samples (n(C) = 11, n(AN) = 9) were collected between 0800 h and 0930 h. Values are means ± SEM. pl = plasma, AT= adipose tissue, ***p < 0.005 vs. controls, +p < 0.05 vs. plasma levels.

The rise in leptin with dietary therapy correlates substantially with increasing gonadotropins until gonadotropins peak, indicating that increasing leptin in response to weight gain could activate the HPG axis (Holtkamp et al. 2003). Although patients with AN can recover weight with therapy, this weight recovery is not always associated with resumption of menstruation (Audi et al. 1998). An increase in free leptin index, however, is associated with resumption of menstrual function but not with weight recovery alone (Misra et al. 2004). Several observational studies have proposed a critical threshold level for leptin of roughly 2 ng/ml that might be necessary for an increase in LH and thus menstruation to resume (Audi et al. 1998; Holtkamp et al. 2003). Patients with AN using oral estrogen-progesterone contraceptives manifest significantly higher plasma leptin levels than those of the same BMI and body fat content without this treatment **(Dostalova et al. unpublished results)**.

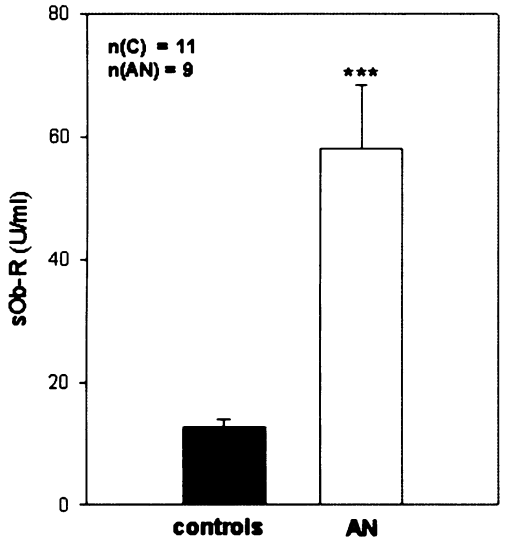


Fig. 5. Soluble leptin receptor in plasma (sOb-R, U/ml) in healthy control women (C, n = 11) and in patients with anorexia nervosa (AN, n = 9). Plasma samples were taken at 0800 h.
Values are means ± SEM. ***p < 0.005 vs. controls.

Blunted leptin production is not involved in AN etiology, but may be responsible or at least contribute to the genesis of certain complications associated with the disease (Brichard et al. 2003). Firstly, low leptin levels may contribute to amenorrhea of AN, even though amenorrhea may occur before substantial weight loss. Leptin plays a major role in reproduction as assessed by the failure of puberta maturation in humans with total leptin deficiency or insensitivity and its reversal by leptin treatment (Cunningham et al. 1999). Administration of recombinant leptin restores puberty and fertility in ob/ob mice and results in the onset of puberty at an appropriate developmental age in human congenital leptin deficiency (Ahima et al. 1997). Secondly, leptin stimulates hematopoesis at the cell stem level as shown by increased lymphopoiesis, myelopoiesis and erythropoesis and leptin also stimulates immune functions (Brichard et al. 2003). Patients with AN frequently suffer from mild anemia and moderate leukopenia accompanied by defective *in vitro* granulopoesis and cytokine production dysregulation which lead to severe infections. Low leptin might exacerbate these hematological and infectious complications. Thirdly, over 90% of patients with AN exhibit osteopenia, which may persist despite weight recovery. Fall of leptin during

starvation is accompanied by reductions in plasma osteocalcin, a marker of osteoblastic activity and hence bone formation rate, and the leptin administration to starved mice prevents this reduction in plasma osteocalcin (Goldstone et al. 2002). Low leptin may contribute to reduce plasma osteocalcin and bone formation rate in patients with AN.

3.2.7. The effect of acute exercise on plasma leptin levels

If leptin is reflective of energy balance, it is conceivable that an increase in energy expenditure, i.e., physical activity, may also modulate plasma leptin. The relationship between exercise and energy balance is complex and may be influenced by many factors, many of those altered in AN. The relative concentrations of the hormones and metabolites that seem to upregulate (cortisol, insulin, glucose) or downregulate (epinephrine) plasma leptin levels change rapidly during and after exercise, depending on exercise intensity, duration and the fitness level of the subjects. How these hormones may interact to prevent the decline in leptin under some conditions and not others remains to be show. Accordingly, recent data have shown that leptin concentration is a significant correlate of resting energy expenditure in men, after statistical adjusment for fat free mass and fat mass (Doucet et al. 2000).

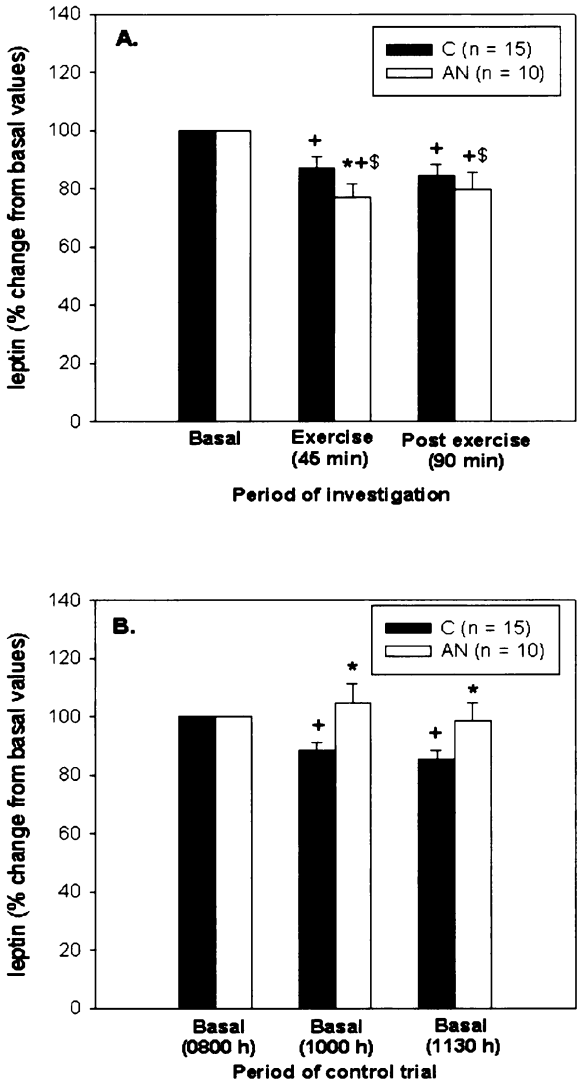


Fig. 6. Changes in plasma leptin levels (%) under the effect of a bout of exercise (2 $W.kg^{-1}$ lean body mass, 45 min; **A**) and during the control trial (**B**) in healthy controls (C, n = 15) and in patients with anorexia nervosa (AN, n = 10). Plasma leptin levels were measured at rest (0800 h), immediately after the end of the exercise bout (1000 h) and after 90 min of recovery (1130 h). Values are expressed as means ± SEM. $^{+}p < 0.05$ vs. basal levels, $^{*}p < 0.05$ vs. C group, $^{\$}p < 0.05$ vs. control trial.

Recent studies focused on the effect of acute exercise on plasma leptin levels brought rather contradictory results with some showed no immediate effect on

leptin levels while another showed a decline in leptin levels. Concretely, the results of Racette et al. (1997) demonstrated that a short bout of moderate intensity exercise does not acutely affect abdominal adipose tissue leptin production or circulating leptin in sedentary healthy humans. Perusse et al. (1997) concluded that there are no meaningful acute (single bout of exercise) or chronic (exercise training) effects of exercise, independent of the amount of body fat, on leptin levels in human, despite large interindividual difference in response to exercise found in this study. Moreover, 45 min of moderate-intensity cycle ergometer exercise did not alter plasma leptin levels in healthy normal-weight women, when measured immediately after and 90 min after an exercise bout (**Fig. 6 A,B; Dostalova, Bartak et al. subm. to press**).

On the other hand, plasma leptin levels in healthy men declined in response to 3-h ergometer exercise to the same level as seen with 3-h epinephrine infusion, whereas leptin mRNA expression was unaffected (Keller et al. 2005). Keller et al. (2005) further obtained indirect evidence from the epinephrine infusion study to suggest that exercise effect on leptin was mediated by epinephrine or epinephrine-induced changes in NEFA, insulin, or glucose concentrations. Furthermore, Jurimae and Jurimae (2005) found that leptin is sensitive to relatively short term (30 min) intense exercise when all major muscles are involved. The study of Elias et al. (2000) has shown that the acute exhaustive exercise in male volunteers leads to decline in plasma leptin levels.

We could conclude that plasma leptin concentrations in healthy normal-weight subjects are unaltered by short duration (60 min or less), non-exhaustive exercise, but may be affected by short-duration exhaustive exercise. Furthermore, it is of great importance of using a resting session when evaluating the effects of exercise on serum leptin to control for fasting- and/or circadian rhythm-induced leptin reductions (Zafeiridis et al. 2003; **Dostalova, Bartak et al. subm. to press**). Among other important factors influencing the leptin response to acute exercise belong age, sex, body composition (BMI, body fat), training status and previous diet of the studied subjects and alterations of hormones affected by exercise having influence on leptin levels. Next, it is possible of delayed (24 - 48 h) leptin responses after physical activity (Van Aggel-Leijssen et al. 1999; Essig et al. 2000; Olive and Miller 2001). Moreover, it was postulated by Hickey and Calsbeek (2001) that in order for exercise to alter serum leptin, a threshold of energy deficit must be achieved. Finally, it was also hypothesized that circulating leptin may be modulated by glucostatic factors at the level of the adipocyte.

Whatever the mechanism is, the decrease in leptin after exercise seems to be a natural part of the mechanism for upregulating energy intake when expenditure increases, long before the fat mass is reduced. All the above mentioned factors important in leptin response to exercise

could be or directly are altered in hyperactive patients with AN. Our group has previously found that AN is associated with markedly increased basal as well as exercise-stimulated activity of SNS, especially NE levels, and exercise-induced lipolysis in subcutaneous adipose tissue (Bartak et al. 2004). The importance of SNS in the decreased plasma leptin levels associated with fasting is supported by numerous studies (Couillard et al. 2002). Thus, there are several reasons why leptin response to exercise may have become a question of interest in AN.

Although it is well established that underweight patients with AN have markedly decreased plasma leptin levels (Nedvidkova et al. 2000; **Dostalova et al. 2005**), the effect of a single bout of moderate-intensity exercise on plasma leptin levels in these patients is less known. A single bout of moderate intensity exercise significantly decreased plasma leptin levels in patients with AN, but not in healthy women, when control trial leptin levels were taken into the account (**Fig. 6 A,B; Bartak, Dostalova et al. 2003; Dostalova, Bartak et al. subm. to press**). Rapid exercise-induced leptin decline in patients with AN could be a part of chronic adaptation to energy deficit in these patients with many factors influencing this abnormally sensitive and prompt response to energy expenditure. However, our results did not confirm that either NE or insulin are the salient factors responsible for the different response of leptin to exercise in AN (**Dostalova, Bartak et al. subm. to press**). To our knowledge, there is no other study examining the effect of moderate-intensity single bout of exercise on leptin in AN.

3.3. GHRELIN

3.3.1. The discovery of ghrelin

The discovery of ghrelin has often been referred to as a classical example of reverse pharmacology, since synthetic ghrelin analogues (growth hormone secretagogues, GHS) were described first, while the natural receptor (GHS-R1a), its endogenous ligand (ghrelin) and the physiological role of ghrelin in energy balance regulation have been discovered stepwise over the last 25 years (Howard et al. 1996; Bowers 2001).

GHS are small synthetic molecules that stimulate growth hormone (GH) release from the pituitary. GHS act through a specific G-protein-coupled receptor (GPCR), named GHS-receptor, which is distinct from the receptor that binds GH-releasing hormone (Howard et al. 1996). This receptor was for some time an example of an "orphan GPCR", that is a GPCR with no known natural ligand. Employing the „reverse pharmacology" paradigm with a stable cell line expressing GHS-R, scientists searched for the endogenous ligand of GHS-R.

The receptor most closely related to GHS-R is the motilin receptor (Feighner et al. 1999), the human forms of these receptors share 52% aminoacid homology. Two receptors for neuromedin U (NMU-R1 and -R2), a neuropeptide that promotes smooth muscle contraction and supresses food intake, are also homologous to GHS-R (Hosoya et al. 2000; Kojima et al. 2000). Because motilin and NMU are found mainly in gastrointestinal organs, it was speculated that the endogenous ligand for GHS-R may be another gastrointestinal peptide. This speculation was confirmed finally by Kojima et al. (1999) by the isolation of ghrelin from stomach tissue.

3.3.2. Ghrelin physiology

The major form of active human ghrelin is, like rat ghrelin, a 28-amino acid peptide with an octanoyl modification at its third aminoacid, serine (Kojima et al. 1999). However, several minor forms of ghrelin peptides were isolated during the course of purification (Hosoda et al. 2003). The fatty acid (n-octanoyl) side chain at serine 3, a biochemical feature which is essential for ghrelin's bioactivity, signifies this gastrointestinal peptide hormone as an endogenous factor unique in mammalian biology (Hosoda et al. 2000).

Ghrelin is predominantly produced by the stomach (Kojima et al. 1999; Date et al. 2000), whereas substantially lower amounts are derived from bowel (Date et al. 2000), pituitary (Korbonits et al. 2001), kidney (Mori et al. 2000), placenta (Gualillo et al. 2001), hypothalamus (Kojima et al. 1999), testes, ovaries, pancreas and other tissues (Rindi et al. 2004). Although the majority of circulating ghrelin is produced in the stomach, other sources may increase ghrelin secretion in a compensatory manner. After gastrectomy, for example, plasma ghrelin level is surprisingly reduced only by 65% (Ariyasu et al. 2001).

Based on rodent experiments (Date et al. 2000; Tolle et al. 2001) and clinical studies (Arvat et al. 2000; Peino et al. 2000), it is evident that ghrelin is indeed a potent GH-releasing agent. However, soon after its identification, ghrelin was found to have widespread activity beyond stimulating GH secretion (natural GHS), including stimulation of lactotroph and corticotroph secretion probably coupled with modulatory influence on gonadal axis (Broglio et al. 2003; Otto et al. 2005), orexigenic effect coupled with control of energy expenditure (Kojima et al. 1999; Tschop et al. 2000; Wren et al. 2001), influence on sleep and behavior (Tolle et al. 2002), influence on gastro-entero-pancreatic functions and secretions (Broglio et al. 2003), influence on the endocrine pancreatic function as well as on glucose metabolism (Date et al. 2002), cardiovascular functions and modulation of cell proliferation (Broglio et al. 2003). Ghrelin further interacts with other hormonal systems such as HPA and HPT axes.

Differential effects of ghrelin might be mediated by separate ghrelin receptor (GHS-R) subtypes as suggested by Ghigo et al. (2001). This group further showed that the orexigenic effects of ghrelin are independent of its ability to stimulate GH secretion, suggesting distinct GHS-R-subtypes. In the brain, receptors for ghrelin were detected in multiple hypothalamic nuclei as well as in the hippocampus, substantia nigra, ventral tegmental area, and dorsal and medial raphe nuclei (Tannenbaum et al.1998; Kojima et al. 1999; Shuto et al. 2001). I.c.v. administered ghrelin increases food intake and body weight gain as well as inducing GH release (Nakazato et al. 2001; Shintani et al. 2001).

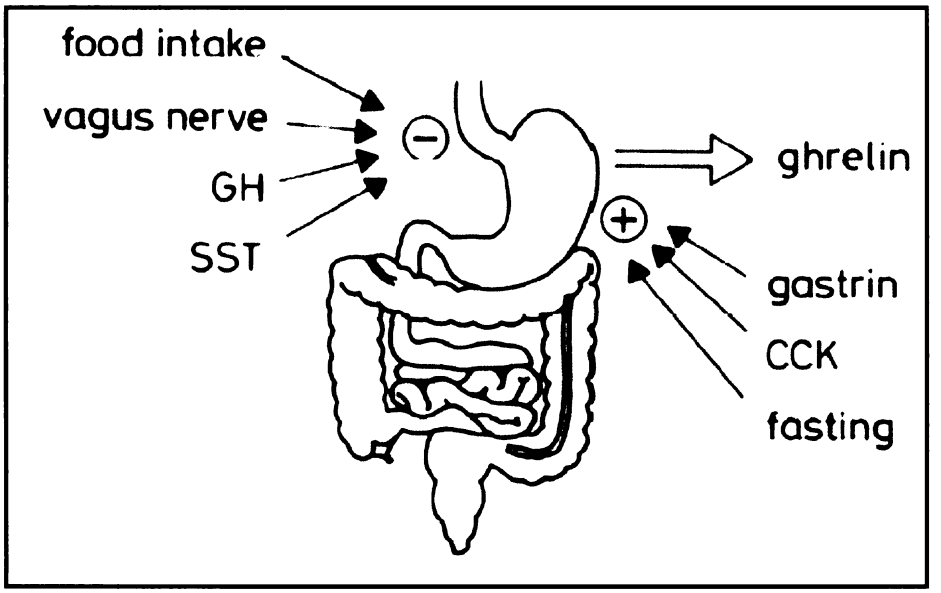


Figure 7. Regulation of gastric-derived ghrelin by different signals. SST = somatostatin, CCK = cholecystokinin, GH = growth hormone.
Source: Casanueva F, Diaguez C. Ghrelin: a new hormone implicated in the regulation of growth hormone secretion and body energy homeostasis. Growth, Genetics and Hormones Journal 2004 20(1).

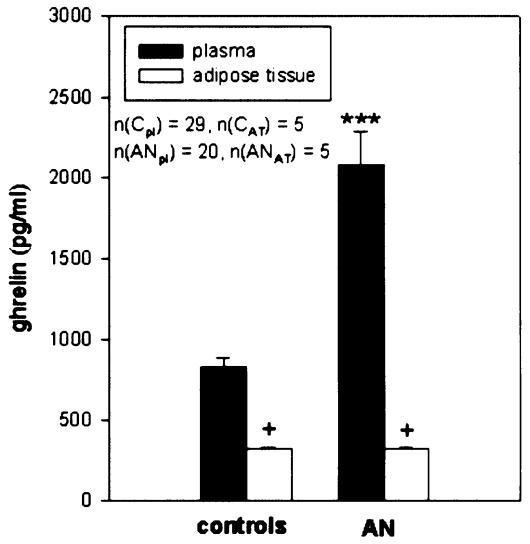


Fig. 8. Ghrelin levels (pg/ml) in plasma and in extracellular space of abdominal AT in healthy controls (C, n = 29) and in patients with anorexia nervosa (AN, n = 20). Plasma samples were taken at 0800 h and microdialysate samples (n(C) = 5, n(AN) = 5) were collected between 0800 h and 0930 h. Values are means ± SEM. pl = plasma, AT= adipose tissue, $^{***}p < 0.005$ vs. controls, $^{+}p < 0.05$ vs. plasma levels.

In humans, circulating ghrelin levels are decreased in chronic (obesity) (Tschop et al. 2001a) and acute (caloric) (Ariyasu et al. 2001; Cummings et al. 2001; Tschop et al. 2001b) states of positive energy balance, whereas plasma levels of ghrelin are increased by fasting (Tschop et al. 2000; Cummings et al. 2001) and in cachectic patients with AN (Ariyasu et al. 2001; Shiiya et al. 2002; **Fig. 8; Nedvidkova, Krykorkova et al. 2003**). Furthermore, ghrelin levels increase in response to weight loss resulting from low caloric diets (Wisse et al. 2001; Cummings et al. 2002), mixed lifestyle modifications (Hansen et al. 2002), cancer anorexia (Shimizu et al. 2003) and chronic failure of the heart (Nagaya et al. 2001), liver (Tacke et al. 2003), or kidneys (Yoshimoto et al. 2002). The first publications comparing ghrelin circulating levels between females and males did not show any difference when gender was taken into the account (Ariyasu et al. 2001). However, recently was found that the 24-h ghrelin concentrations were 3-fold higher in women in the follicular phase of their menstrual cycle when compared to men of the same age (Barkan et al. 2003).

3.3.3. Body weight-related ghrelin regulation and model of ghrelin as an adiposity signal

Peripheral signals communicate the status of body fat stores to the brain, and fluctuations in them trigger compensatory changes in food intake and energy expenditure that resist alterations in body weight (Cummings and Shannon 2003). An adiposity signal that participates in energy homeostasis should manifest the following qualities:

A, it should circulate in proportion to body energy stores, and consequently, should be modulated reciprocally by increases or decreases in these stores

B, it should gain access to the brain and interact there with receptors and signal transduction systems in neurons that participate in body weight regulation

C, exogenous administration of the compound should alter food intake and/or energy expenditure, and chronic infusions should change body weight (or at least fat mass)
D, blockade of the signal should render the opposite effects

Until recently, leptin and insulin were the only hormones shown to satisfy these criteria (Cummings and Shannon 2003). Somewhat surprisingly for a gut hormone, ghrelin fulfills all of the established criteria for peripheral adiposity signals, and may thus be an unique orexigenic counterpart to leptin and insulin in long-term body weight regulation. Circulating ghrelin levels correlate inversely with the size of energy stores over a very wide weight range, extending from emaciated victims of AN to super-obese humans and rodents with genetically absent leptin signaling (Ariyasu et al. 2001; Otto et al. 2001; Tschop et al. 2001a; Cummings et al. 2002; Shiiya et al. 2002; **Nedvidkova, Krykorkova et al. 2002, 2003**). Central targets of ghrelin action are located in regions of the hypothalamus and brainstem that are not well protected by the blood brain barrier and are known to regulate energy homeostasis (Cummings and Shannon 2003; Faulconbridge et al. 2003). The best documented of these targets are neurons in the arcuate nucleus of the hypothalamus that coexpress NPY and AgRP, both prototypic anabolic neuropeptides that promote positive energy balance (Kamegai et al. 2001; **Fig. 9**). Almost all arcuate NPY/AgRP neurons express the ghrelin receptor (Willesen et al. 1999), and ghrelin clearly activates these cells (Dickson and Luckman 1997; Nakazato et al. 2001; Cowley et al. 2003). Furthermore, pharmacological blockade of either NPY or AgRP signaling attenuates the orexigenic action of ghrelin (Nakazato et al. 2001; Shintani et al. 2001). Peripheral or central ghrelin administration stimulates short-term food intake as potently as does any known agent (Asakawa et al. 2001; Wren et al. 2001), and chronic or repeated infusions increase body weight (Tschop et al. 2000; Nakazato et al. 2001; Wren et al. 2001). In addition to stimulating food intake, ghrelin can decrease energy expenditure (Asakawa et al. 2001), fat catabolism (Tschop et al. 2000) and temperature (Lawrence et al. 2002). Thus, ghrelin affects all aspects of the energy homeostasis system in a conserted manner to promote weight gain.

Additionally, an alternative and equally feasible pathway for ghrelin signaling from the stomach is via an ascending neural network through the vagus nerve and brain stem nuclei that ultimately reaches the hypothalamus (Asakawa et al. 2001). Vagotomy or atropine administration abolished the gastric acid secretion induced by i.c.v. administration of ghrelin (Date et al. 2001). In addition, i.c.v. administration of ghrelin stimulated firing of gastric vagal efferent fibers (Date et al. 2001). These two results together suggest that ghrelin stimulates gastric acid secretion via the vagus nerve.

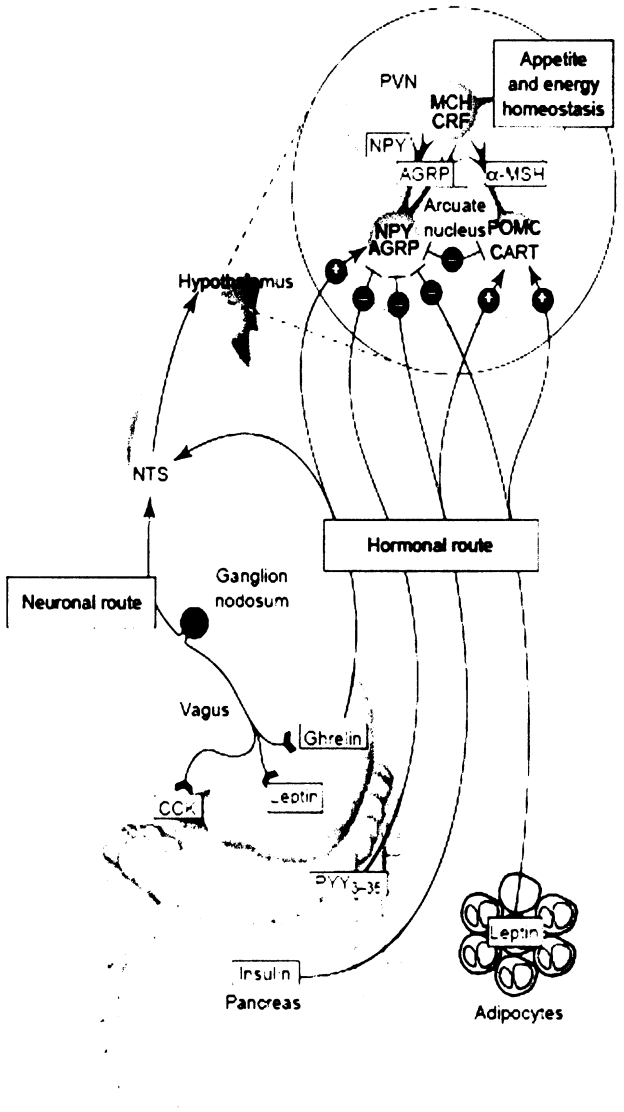


Figure 9. The possible dual hormonal and neuronal route for ghrelin as an orexigenic (apetite-stimulating) signal from the gastrointestinal (GI) tract to the hypothalamus. Ghrelin stimulates food intake, balancing against several inhibitory signals (e.g., insulin, leptin and PYY) from the periphery to the arcuate nucleus of the hypothalamus. Ghrelin as an hormone can, in principle, increase food intake by either stimulating neuropeptide Y (NPY) and agouti-related protein (AgRP)-containing neurons in the arcuate nucleus or acting indirectly through the dorsal vagal complex in the nucleus tractus solitarius (NTS) in the brain stem. Note the inhibitory pathway from NPY/AgRP-containing neurons to proopiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART)-containing neurons, through which ghrelin receptor activity can indirectly inhibit the latter neurons. Ghrelin also acts by stimulating mainly gastric afferent vagal fibers, conceivably in a paracrine manner when released from the endocrine cells in the gut. CCK, cholecystokinin; CRF, corticotropin-releasing factor; MCH, melanin-concentrating hormone; PVN, paraventricular nucleus.
Source: Holst B, Schwartz TW. Constitutive ghrelin receptor activity as a signaling set-point in appetite regulatin. Trends in Pharmacological Sciences 2004 25(3).

3.3.4. Prandial ghrelin regulation and possible roles for ghrelin in meal initiation

The hypothesis that circulating ghrelin is a physiological meal initiator predicts that its levels should rise before, and fall after, every meal, and peak concentrations should be sufficiently high to stimulate appetite. Consistent with this model, ghrelin levels in healthy human are indeed rapidly suppressed by nutrient ingestion (Tschop et al. 2001b; Shiiya et al. 2002; **Nedvidkova, Krykorkova et al. 2003**), and 24-hour plasma profiles reveal marked preprandial increases and postprandial decreases associated with every meal (Cummings et al. 2001). Ghrelin levels increase before meals to values that have been shown to stimulate appetite and food intake when generated by peripheral ghrelin administration in humans and rodents (Wren et al. 2001), suggesting that these levels are sufficient to play a physiological role in normal pre-meal hunger.

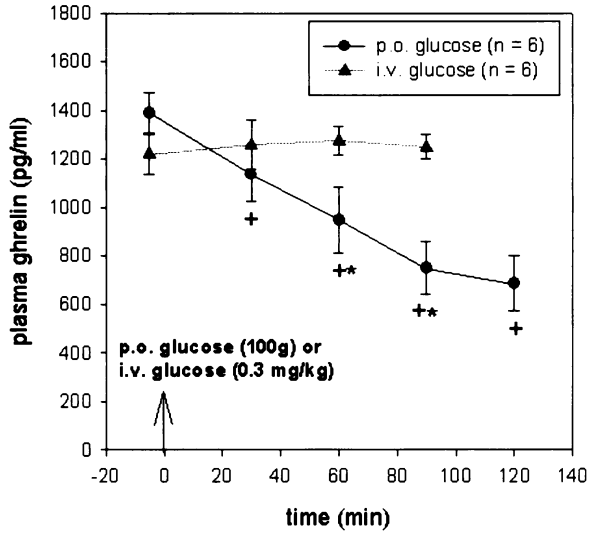


Fig. 11. The effect of either intravenous (i.v., 0.3 mg/kg) or oral (p.o., 100g in 250 ml of tea) glucose administration on plasma ghrelin levels (pg/ml) in healthy control women (C, n = 6). Values are means ± SEM.
$^{*}p < 0.05$ vs. i.v. glucose, $^{+}p < 0.05$ vs.basal levels.

Our group found that both a standard mixed meal and dieting fiber of the same volume are equally effective in suppressing plasma ghrelin in healthy women and that this postprandial ghrelin response is abolished in patients with AN (**Fig. 10 A,B; Nedvidkova, Krykorkova et al. 2003; Nedvidkova, Dostalova et al. 2004a**). These findings suggest that the acute response of ghrelin to feeding is independent of meal nutritional value, but is rather related to the presence of food in the stomach. We supported the hypothesis by observation that only p.o., but not i.v., glucose administration leads to reduction of plasma ghrelin in healthy women **(Fig. 11; Dostalova, Nedvidkova et al. 2003)**. Although ghrelin decrease after food consumption found in our study occured independently on the caloric value of the meal, the maximum response of ghrelin was more rapid to fiber than to caloric meal. Shiiya et al. (2002) observed reduction in plasma ghrelin levels after both p.o. and i.v. glucose administration, but not after water load. Such a discrepancy in data could be in part explained by higher dose of i.v. glucose used in the study of Shiiya et al. (2002). It remains questionable, whether water load could be compared with food consumption accordingly. Another point that is making, is the effect of caloric load of the meal on plasma ghrelin.

Although not confirmed in our study (Nedvidkova, Krykorkova et al. 2003), Callahan et al. (2004) concluded that the depth of postprandial ghrelin suppression is proportional to ingested caloric load. However, the experimental protocol used in this study differed from that used by our group (meal consumption in 09:00 - 09:15 h; macronutrient distribution: 35% of carbohydrate, 45% of fat, 20% of protein).

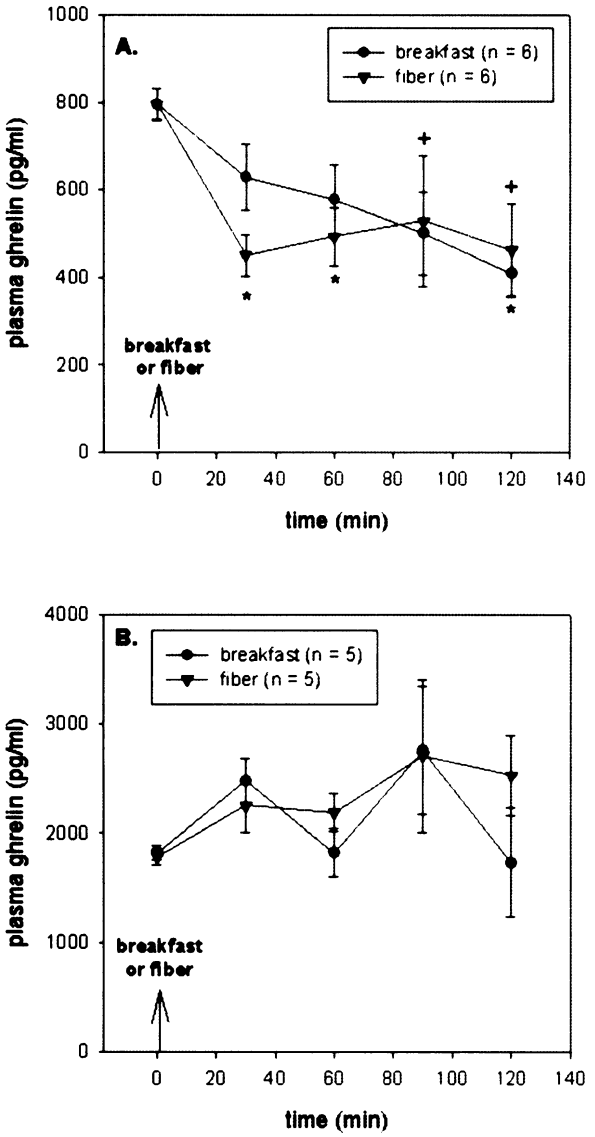


Fig.10. The effect of either caloric (standardized breakfast; 585 kcal, 32.6 g of fat, 17.6 g of protein, 50.0 g of carbohydrate) or non-caloric (fiber; 0.11 kcal, 4 g of Psyllium in 250 ml of water) meal of the same volume on plasma ghrelin levels (pg/ml) in healthy controls (C, n = 6; A) and in patients with anorexia nervosa (AN, n = 5; B). Values are means ± SEM. $^{+}p < 0.05$ vs. basal levels in breakfast, $^{*}p < 0.05$ vs. basal levels in fiber.

The postprandial ghrelin response was found to be mediated by meal macronutrient composition, with carbohydrates being most effective at suppressing ghrelin concentrations (Erdmann et al. 2003; Greenman et al. 2004). In agreement with these findings, our results **(Fig. 10A; Fig. 11)** has shown that in healthy women, p.o. glucose administration of less caloric load (393.4 kcal) leads to the equal decrease in plasma ghrelin as standard mixed meal (585 kcal; 50.0 g of carbohydrate) of the same volume (in 120. min: breakfast ≈ 51 %, glucose ≈ 49%). Fat, fruit, and vegetable ingestion significantly increased ghrelin levels (Erdmann et al. 2004). There is also evidence that incorporating a high-carbohydrate, low-fat diet for weight loss may mitigate the increase in ghrelin concentrations observed during energy restriction (Weigle et al. 2003). Although the ingestion of a physiological dose of essential amino acids has been shown to increase ghrelin concentrations (Groschl et al. 2003; Knerr et al. 2003), there is a conflicting evidence as to whether a protein-rich meal has the same effect (Erdmann et al. 2003; Greenman et al. 2004).

The impaired ghrelin response to food intake in patients with AN may be explained by the chronic adaptation of these patients to food restriction with a single meal not to be sufficient to change this status **(Nedvidkova, Krykorkova et al. 2003)**. Somewhat surprisingly, obese subjects, similarly to patients with AN, do not exhibit the decline in plasma ghrelin levels after a standard mixed meal (English et al. 2002).

3.3.5. Ghrelin in anorexia nervosa

Extremely underweight women with untreated AN manifest ghrelin levels that are among the highest reported in any humans (Ariyasu et al. 2001; Otto et al. 2001; Shiiya et al. 2002; Tanaka et al. 2003a; **Fig. 8; Nedvidkova, Krykorkova et al. 2003**). Plasma ghrelin levels in these patients decrease with nutritional therapy to a degree commensurate with the increase in BMI, and levels can be fully normalized with adequate renutrition (Otto et al. 2001; Tolle et al. 2003). The findings suggest a compensatory response of circulating ghrelin to energy deficit resulting from other causes that drive this psychiatric disorder. Interestingly, constitutionally thin women without body image disturbance or psychological disorders, with a low BMI, do not display elevated plasma ghrelin concentrations (Nagaya et al. 2001). This confirms that circulating ghrelin levels depend on body fat mass. It remains to be demonstrated whether they are also directly influenced by feeding behavior and/or energy intake habits.

Patients with AN who binge and purge have higher ghrelin values than nonpurgative anorectics (Tanaka et al. 2003b). Thus, elevated ghrelin levels in AN patients who vomit frequently may result from an additional stimulatory effect, beyond weight loss, rendered by forced emptying of nutrients from the foregut – i.e., short-term ghrelin regulation. Alternatively, there may be a direct, perhaps autonomic, effect of vomiting itself on ghrelin.

3.3.6. Ghrelin relationship to leptin

Regulation of ghrelin secretion, as well as its biological effects appear to be opposite to those of leptin (Kalra et al. 1999; Schwartz et al. 2000; Tschop et al. 2000). During food deprivation when leptin levels rapidly decline (Saladin et al. 1995) and NPY/AgRP production is elevated, but POMC neurons are suppressed (Saladin et al. 1995; Schwartz et al. 1997; Sahu 1998), circulating ghrelin levels increase (Tschop et al. 2000; Arvat et al. 2001; Tschop et al. 2001b) suggesting that leptin and ghrelin coregulate hypothalamic peptinergic systems in opposite ways. Furthermore, plasma leptin levels in patients with AN show significant negative relationship to plasma ghrelin levels, but this relationship is not observed

in healthy women, suggesting the possible important role of the interactions between these two hormones in the states of extreme energy deficit, such as AN, rather than under the states of energy balance **(Dostalova et al. unpublished results)**. Interestingly, low concentrations of ghrelin were detected in the subcutaneous adipose tissue of both patients with AN and healthy women (**Nedvidkova, Krykorkova et al. 2002**). These observations support the hypothesis that ghrelin as a „hunger signal" is the counterpart of leptin aiming to prevent further energy deficit. However, considering the extreme complexity of hypothalamic interactions of different peptinergic circuits and peripheral hormone receptors, it is necessary to determine the hierarchy and direction of signaling flow within these systems to understand ghrelin's central effects on metabolic regulation. For that, a multidisciplinatory approach is mandatory. From a teleological point of view, ghrelin and leptin might really be complementary players of one regulatory system that has developed to inform the central nervous system about the current status of acute and chronic energy balance (Tschop et al. 2000; Asakawa et al. 2001; Shintani et al. 2001). In addition, a specific role for ghrelin might be to ensure the provision of calories that GH requires for growth and repair.

Table 5: Possible clinical application of ghrelin

GH deficiency
Diagnosis of pituitary function
Child and adult GH deficiency
Eating disorder
AN
Bulimia nervosa
Prader-Willi syndrome
Gastrointestinal disease
Cardiovascular disease
Heart failure
Dilated cardiomyopathy
Osteoporosis
Aging
Catabolic state or chronic wasting syndrome
Cachexia (cancer, cardiac cachexia)
AIDS
Postoperative patients

Source:Kojima M, Kangawa K. Ghrelin:structure and function. Physiol Rev 2005 85:495-522.

3.4. RESISTIN

3.4.1. Discovery of resistin

Thiazolidinediones (TZDs) have been clinically identified as drugs that lower blood glucose and insulin levels, leading to improved insulin sensitivity in type2 diabetics (Day 1999; Reginato and Lazar 1999). Although TZDs have been shown to regulate a number of factors contributing to insulin resistance and regulation of metabolism, it is plausible that hirherto unidentified TZD-regulated genes also contribute to their antidiabetic and insulin-sensitizing effects. To test this possibility a substantive screen was performed on 3T3-L1 adipocytes (Rangwala and Lazar 2000). This screen led to the identification of a novel mRNA that is downregulated by rosiglitazone (Steppan et al. 2001). The novel gene encoded for a 114 amino acid polypeptide with an amino terminal signal sequence, suggesting that this is a secreted molecule. Named resistin, the protein was found to contain a pattern of 11 cysteine residues in a unique motiv (X11-c-x8-c-x-x3-c-x10-c-x-c-c-c-x9-cc-x6) (Steppan et al. 2001). Resistin has been found to be secreted as a dimer (Rajala et al. 2002). When the cys-26 residue of resistin is mutated to an alanine, it is secreted as a monomer, suggesting that this residue is critical for dimerization. Similar cysteine rich motifs are common among secreted growth factors (Banerjee and Lazar 2001).

3.4.2. Murine resistin

In vitro studies showed that resistin is induced during adipocyte differentiation and, as suggested by its signal sequence, is secreted into the media (Steppan et al. 2001). In mice, resistin is highly and specifically expressed in white adipose tissue (WAT) (Steppan et al. 2001). Mouse studies also showed that resistin is detectable in the serum, demonstrating that it can circulate in blood. Treatment of mice with TZDs lowered resistin protein levels, similar to the effects observed *in vitro* (Steppan et al. 2001).

In both environmental (high-fat-diet-induced) and genetic (leptin-deficient, ob/ob; leptin receptor-deficient, db/db) models of obesity in mice resistin serum levels were elevated (Steppan et al. 2001). Additionally, both WAT mRNA and serum protein levels of resistin dropped during fasting and increased during refeeding. Regulation of resistin by nutritional status is interesting because it parallels that of leptin (Ahima and Flier 2000). In mice with diet-induced obesity, immunoneutralization of resistin resulted in a 20% drop in blood glucose, and improved insulin sensitivity as measured by insulin tolerance testing (Steppan et al. 2001). Resistin also inhibited the differentiation of 3T3-L1 preadipocytes and was strongly induced in streptozotocin-diabetic mice following treatment with insulin (Kim et al. 2001). In

Sprague-Dawley rats a gene expression profile screening approach identified resistin as one of the most strongly upregulated genes following a week of 60% high-fat diet (Li et al. 2002).

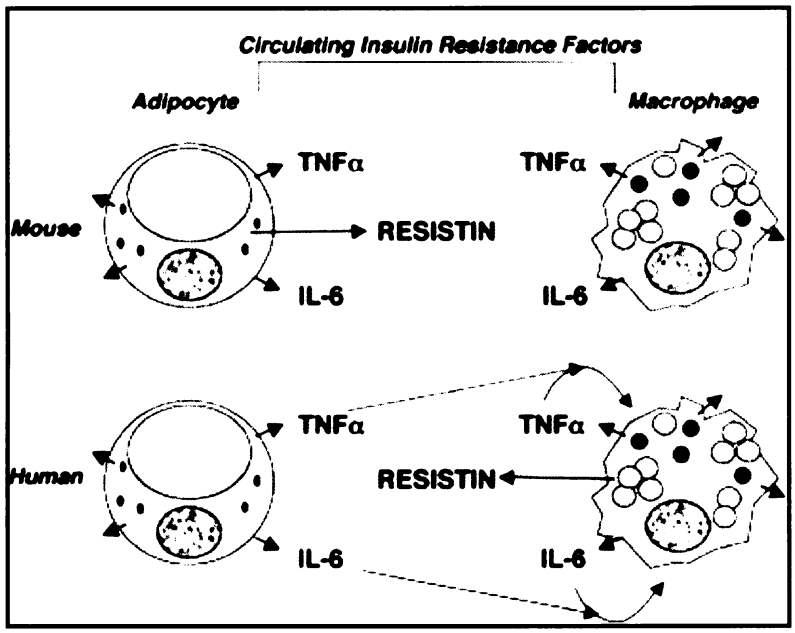


Figure 12. Model to explain hyperresistinemia in mice and humans with obesity despite the species differences in the source of plasma resistin. Circulating inflammatory cytokines TNF-α and IL-6 are depicted because of their role in resistin induction in human macrophages and their implied role in insulin resistance. Other cytokines and inflammatory markers may also contribute to insulin resistance and/or resistin induction.
Source: Lehrke et al.: An inflammatory cascade leading to hyperresistinemia in humans. PloS Med 2004 1: e45.

3.4.2.1. Regulation of resistin gene expression

The discovery of resistin as a new adipose-specific secreted molecule implicated in diabetes and obesity generated excitement in the field. A number of groups have investigated regulation of resistin in animal models of diabetes and obesity and by a variety of agents known to modulate insulin sensitivity. These results are summarized in **Table 6.**

3.4.3. Human resistin

Based upon the studies of resistin regulation and function in rodents a great deal of interest was generated in determining how the rodent studies apply to the mechanism of type2 diabetes in humans. Translating the findings of murine resistin biology to human pathophysiology has been complicated, however, by the fact that only two human homoloques have been found for the three mouse genes (Steppan and Lazar 2002). Overall, human resistin is only 53% identical with its murine counterpart, but identity is highest in the C-terminal signature sequence region (Steppan and Lazar 2002). Furthermore, unlike murine resistin, human resistin appears to be expressed at low levels in adipocytes, but is readily detectable in mononuclear blood cells (Nagaev and Smith 2001; Savage et al. 2001).

Studies in human brought rather contradictory results. Monocytes isolated from peripheral blood and differentiated *in vitro* to macrophages show a fourfold induction of resistin mRNA; treatment of these cells with rosiglitazone downregulates resistin expression by up to 80% (Patel et al. 2003). Nagaev and Smith (2001) detected only a very low level of resistin in fat or muscle samples of 42 individuals from control, insulin-resistant, or type2 diabetic patient groups and did not find any correlation with insulin resistance. Savage et al. (2001) were unable to detect resistin in lean patients, but found increased levels of resistin mRNA in subcutaneous fat of morbidly obese individuals. However, similarly to the results of Nagaev and Smith (2001), resistin mRNA levels were detected in only 4 of 14 subjects in freshly isolated adipocytes and were not correlated with BMI (Savage et al. 2001). In this study was also found that 24-h treatment of freshly isolated mononuclear cells with PPARγ agonists failed to have any effect on resistin levels, in contrast to the effects in mice and those reported by Patel et al. (2003). Furthermore, Savage et al. (2001) found that a patient with clinically severe insulin resistance due to a mutation in PPARγ has no detectable resistin in subcutaneous fat. Janke et al. (2002) found that resistin is highly expressed in pre-adipocytes isolated from plastic surgery patients, and that resistin expression decreased during adipogenesis, contrary to the results in mice. Janke et al. (2002) failed to find any correlation between adipose tissue resistin expression and body weight or insulin sensitivity. In healthy normal-weight women, the relationship between plasma resistin and BMI, body fat, plasma leptin levels and insulin sensitivity was not confirmed (**Dostalova, Kunesova et al. subm. to press**). Several groups have investigated whether human resistin, as mouse resistin, is increased in visceral adipose depots. Both Savage et al. (2001) and McTernan et al. (2002) failed to see a difference between resistin expression in subcutaneous and omental abdominal adipose tissue, although McTernan et al. (2002) detected 4.2-fold higher resistin expression and protein than in peripheral fat depots in thigh and breast.

3.4.4. Resistin and anorexia nervosa

It seems possible that according to the „thrifty gene" hypothesis, humans have evolved under selective pressure favoring the ability to store energy and survive periodic starvation (Spiegelman and Flier 2001). Resistin´s regulatory pattern in fasting and refeeding paradigms minnor that of leptin, which has been shown to be critical in the physiological response to long-term fasting (Ahima et al. 1996). Our group found significantly decreased plasma resistin levels and markedly increased resistin levels in the extracellular space of abdominal adipose tissue of patients with a restrictive type of AN in comparison to healthy women (**Fig. 13; Nedvidkova, Dostalova et al. 2004b; Dostalova, Kunesova et al. subm. to press**). However, Housova et al. (2005) have found that patients with either a restrictive or a purging type of AN show no significant difference in plasma resistin levels compared to healthy women. Interestingly, Skilton et al. (2005) found that pathophysiologically relevant concentrations of resistin, within the range of concentrations exhibited by patients with AN, increase endothelial cell adhesion molecule expression, possibly contributing to increased atherosclerosis risk in these patients.

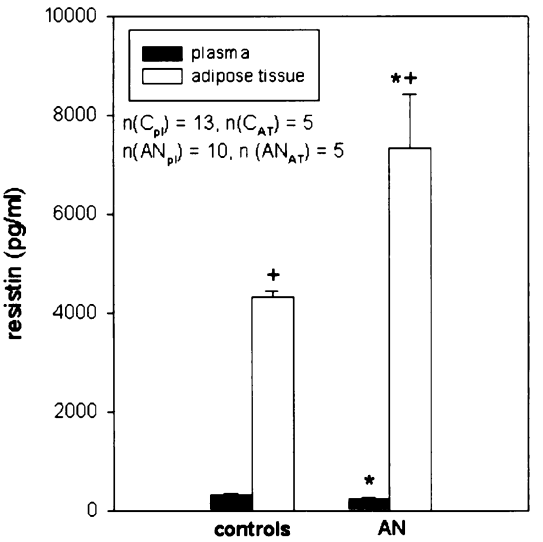


Fig. 13. Resistin levels (pg/ml) in plasma and in extracellular space of abdominal AT in healthy control women (C, n = 13) and in patients with anorexia nervosa (AN, n = 10). Plasma samples were taken at 0800 h and microdialysate samples (n(C) = 5, n(AN) = 5) were collected between 0800 h and 0930 h. PL = plasma, AT = adipose tissue. Values are means ± SEM. $^{*}p < 0.05$ vs. controls, $^{+}p < 0.05$ vs. plasma levels.

Table 6. Regulation of murine resistin gene expression

	Model system	References
Suppressors		
Thiazolidinediones	3T3-L1s, mice, rats	Steppan et al. 2001
Obesity	mice	Way et al. 2001
Insulin	3T3-L1s	Haugen et al. 2001
Tumor necrosis factor-α	3T3-L1s	Rajala et al. 2002
Isoproterenol	3T3-L1s	Fasshauer et al. 2001
Epinephrine	3T3-L1s	Shojima et al. 2002
Somatotropin	3T3-L1s	Shojima et al. 2002
Fasting	mice	Steppan et al. 2001
Lipopolysaccharide	mice	Rajala et al. 2002
Inducers		
Thiazolidinediones	mice	Way et al. 2001
Glucose	3T3-L1s, rats	Rajala et al. 2002
Insulin	mice	Kim et al. 2001
Dexamethasone	3T3-L1s, mice	Haugen et al. 2001
Hyperprolactinemia	mice	Ling et al. 2001
Testosterone	mice	Ling et al. 2001
Growth hormone	SDR rats	Delhanty et al. 2002
Refeeding	Mice	Steppan et al. 2001
Lipopolysaccharide	3T3-L1s, rats	Lu et al. 2002
No effect		
IL-6	3T3-L1s	Rajala et al. 2002
Lipopolysaccharide	3T3-L1s, mice	Rajala et al. 2002
Angiotensin-2	3T3-L1s	Fasshauer et al. 2001
Growth hormone	3T3-L1s	Fasshauer et al. 2001
β-agonists	3T3-L1s, mice	Moore et al. 2001

Source: RR Banerjje, MA Lazar. Resistin: molecular history and prognosis. J Mol Med 2003 81:218-226.

4. RESULTS AND DISCUSSION

The present Thesis was mainly focused on the alterations of leptin, resistin and ghrelin levels in plasma and in extracellular fluid from abdominal adipose tissue of patients with AN. The next point of interest was the effect of acute energy disbalance (food intake; a single bout of exercise) on plasma levels of leptin and ghrelin. The results obtained in patients with AN were compared with those found in healthy age-matched women. The samples of extracellular fluid from subcutaneous adipose tissue of patients with AN and of healthy women were taken by *in vivo* microdialysis after previous modification of this method *in vitro*.

The main results of the Thesis could be summarized in the following points:

I. Microdialysis is a suitable technique for *in vivo* sampling of leptin and resistin from human subcutaneous adipose tissue. For the sampling of leptin (16 kDa) microdialysis membrane with 100 kDa cut-off and adding 40 g/l of dextran-70 in a perfusion fluid is needed, whereas for resistin (12.5 kDa) usually available microdialysis system (microdialysis membrane with 20 kDa cut-off; perfusion fluid with no colloid added) can be used. Thus, the molecular weight of the studied substance is an important, but not the only, limiting factor in the set up of microdialysis experiment **(Article A; Article D)**.

II. The assesment of relative recovery of the substance for the specific type of microdialysis catheter and for the selected perfusion rate is necessary step before starting *in vivo* microdialysis sampling. The perfusion rate of the microdialysis experiment should be chosen with respect for a limitation of the amount of a single sample for analytical assesment, for the existence of indirect relationship between perfusion rate and the percent of relative recovery of the substance and last, but not least, for the proband undergoing the microdialysis experiment (the total duration of the experiment) **(Article A)**.

III. Patients with AN have markedly decreased total plasma leptin levels, whereas soluble plasma leptin receptor (sOb-R) is significantly increased in these patients. Thus, during chronic starvation, low circulating leptin levels together with up-

regulation of the sOb-R in patients with AN may translate into reduced leptin availability to peripheral and brain tissue **(Article B)**.

IV. *In vivo* free leptin levels in the extracellular space of abdominal adipose tissue of patients with AN are not significantly different in comparison to healthy women. Thus, markedly reduced plasma levels of total leptin in patients with AN do not directly reflect leptin levels in the dialysate from abdominal adipose tissue of these patients **(Article B)**.

V. Patients with AN have markedly elevated fasting plasma ghrelin levels. Plasma ghrelin is significantly negatively related to body fat content in both patients with AN and in healthy women. Thus, the regulation of ghrelin during chronic starvation is opposite to the regulation of leptin in this state **(Article C)**.

VI. In healthy women, the consumption of either a standardized mixed meal or non-caloric fiber of the same volume leads to a significant decrease of plasma ghrelin levels with the maximum ghrelin response occuring more rapidly (in 30 min) to fiber than to caloric meal **(Article C)**.

VII. Short-term response of plasma ghrelin to feeding, which in healthy normal-weight women is independent of food caloric value, is impaired in patients with AN **(Article C)**.

VIII. Patients with AN have significantly decreased plasma resistin levels and significantly increased resistin levels in the extracellular space of abdominal adipose tissue. Plasma resistin levels are not directly related to body mass index, body fat content, plasma leptin levels and insulin sensitivity in either patients with AN or in healthy women **(Article D)**.

IX. A single bout of moderate-intensity cycle ergometer exercise (2 $W.kg^{-1}$ of lean body mass; 45 min) leads to a significant reduction of plasma leptin levels in patients with AN when measured immediately after and 90 min after the end of the exercise bout. The reduction of plasma leptin levels observed during exercise in healthy women is not a direct result of exercise, but rather the naturally occuring

early morning decrease of plasma leptin that is lacking in patients with AN. Neither norepinephrine nor insulin are the salient factors responsible for the sensitive and rapid leptin response on exercise in patients with AN **(Article E)**.

The list of enclosed articles:

A. **Dostalova I**, Pacak K, Nedvidkova J 2003 Application of in vivo microdialysis to measure leptin concentrations in adipose tissue. *International Journal of Biological Macromolecules* 32(3-5):205-208.

B. **Dostalova I**, Kopsky V, Duskova J, Papezova H, Pacak K, Nedvidkova J 2005 Leptin concentrations in the abdominal subcutaneous adipose tissue of patients with anorexia nervosa assessed by in vivo microdialysis. *Regulatory Peptides* 128(1):63-68.

C. Nedvidkova J, **Krykorkova I**, Bartak V, Papezova H, Gold PW, Alesci S, Pacak K 2003 Loss of meal-induced decrease in plasma ghrelin levels in patients with anorexia nervosa. *Journal of Clinical Endocrinology and Metabolism*. 88(4):1678-1682.

D. **Dostalova I**, Kunesova M, Duskova J, Papezova H, Nedvidkova J Adipose tissue resistin levels in patients with anorexia nervosa. Subm. *Nutrition.*

E. **Dostalova I**, Bartak V, Papezova H, Nedvidkova J The effect of acute exercise on plasma leptin levels in patients with anorexia nervosa. Subm. *European Journal of Applied Physiology*.

ARTICLE A

Dostalova I, Pacak K, Nedvidkova J

Application of in vivo microdialysis to measure leptin concentrations in adipose tissue.

International Journal of Biological Macromolecules. 2003 32(3-5):205-208.

ELSEVIER

International Journal of Biological Macromolecules 32 (2003) 205–208

INTERNATIONAL JOURNAL OF
Biological
Macromolecules
STRUCTURE, FUNCTION AND INTERACTIONS

www.elsevier.com/locate/ijbiomac

Application of in vivo microdialysis to measure leptin concentrations in adipose tissue

I. Dostálová[a,b], K. Pacák[c], J. Nedvídková[a,*]

[a] *Institute of Endocrinology, Narodni trida 8, Praque 116 94, Czech Republic*
[b] *Faculty of Science, Charles University, Prague 116 94, Czech Republic*
[c] *Pediatric and Reproductive Endocrinology Branch, NICHD, NIH, Bethesda, USA*

Received 21 October 2002; received in revised form 10 April 2003; accepted 24 April 2003

Abstract

Microdialysis is a relatively new in vivo sampling technique, which allows repeated collecting of interstitial fluid and infusion of effector molecules into the tissue without influencing whole body function. The possibility of using microdialysis catheter with a large-pore size dialysis membrane (100 kDa) to measure concentrations of the adipocyte-derived peptide hormone leptin in interstitial fluid of adipose tissue was explored. Krebs–Henseleit buffer with 40 g/l dextran-70 was used to prevent perfusion fluid loss across the dialysis membrane. The relative recovery of leptin in vitro was determined using CMA/65 microdialysis catheter (100 kDa cut-off, membrane length 30 mm; CMA, Stockholm, Sweden) and four perfusion rates were tested (0.5, 1.0, 2.0, 5.0 μl/min). Furthermore, the microdialysis catheter CMA/65 was inserted into subcutaneous abdominal adipose tissue of 11 healthy human subjects and leptin concentrations in the interstitial fluid of adipose tissue in vivo were measured. The present findings are the first documentation on the use of microdialysis to study local leptin concentrations in the interstitial fluid of adipose tissue.

Keywords: Leptin; Microdialysis; Adipose tissue

1. Introduction

Knowledge about the local leptin concentrations in adipose tissue, the main source of its production, in humans is of great importance due to the role of leptin in energy homeostasis regulation and local disruptions in its production in adipose tissue during conditions influencing fat depots in organism such as long-term fasting or obesity. A simple and direct approach to obtain this information in vivo can be the use of microdialysis, which is based on the similarity of the dialysis membrane with blood capillary. Rosdahl et al. [4] have shown that large-pore dialysis membrane (100 kDa) can be used to study interstitial concentrations of metabolites in skeletal muscle only if 40 g/l of dextran-70 is included in the perfusate to prevent perfusion fluid loss. Furthermore, it is important to establish to what extent the measured concentration of a compound in the dialysate reflects its true level in the extracellular space. Under most circumstances, microdialysis only allows measurement of a fraction of the concentration of a substance in the extracellular space [1]. Microdialysis probes can be characterized by the assessment of their recovery in vitro, as described by Lonnroth et al. [11]. We tested leptin recovery in vitro at different flow rates using large-pore dialysis membrane to explore its possibilities for studying local leptin concentrations in interstitial fluid of human adipose tissue in vivo.

2. Methods

2.1. In vitro experiments

These experiments were performed to determine the relative leptin recovery in vitro. Microdialysis catheter CMA/65 with 100 kDa cut-off and membrane length 30 mm (CMA, Stockholm, Sweden) connected to CMA/107 microdialysis pump was used to set up the experiment. The microdialysis syringe was filled with Krebs–Henseleit buffer (KHB), which consists of 140 mmol/l NaCl, 3.9 mmol/l

* Corresponding author. Tel.: +420-2-24905-272; fax: +420-2-24905-325.
E-mail address: jnedvidkova@endo.cz (J. Nedvídková).

0141-8130/$ – see front matter © 2003 Elsevier B.V. All rights reserved.
doi:10.1016/S0141-8130(03)00055-2

KCl, 2.5 mmol/l $CaCl_2 \cdot 2H_2O$, 1.2 mmol/l $MgSO_4 \cdot 7H_2O$, 0.8 mmol/l $Na_2HPO_4 \cdot 2H_2O$, 0.8 mmol/l KH_2PO_4 and 40 g/l dextran-70 (NaOH was used to obtain pH 7.4). Dextran-70 at a concentration of 40 g/l was used to prevent perfusion fluid loss. Catheters were immersed in test tubes containing standard leptin concentration (10 ng/ml), which was determined from the average leptin concentrations in human plasma. The temperature was maintained by digital block heater at 37–38 °C. Four perfusion rates have been tested (0.5, 1.0, 2.0, 5.0 μl/min). Fifteen independent samples were taken at each rate, collected at 45-min intervals. Smaller perfusion rates were not used because of the large volume of sample, which we usually need to measure leptin by an analytical method. The relative recovery in vitro (%) was calculated from the ratio of leptin concentration in dialysate to leptin concentration in perfusate multiplied by 100.

2.2. *In vivo experiments*

Eleven healthy women (mean BMI 20.9 ± 1.47 kg/m^2; weight 60.4 ± 5.41 kg, height 169.9 ± 4.76 cm, percent body fat 19.5 ± 3.58%, age 23.3 ± 2.57 years) were recruited for this study. All subjects included in the study were non-smokers, had no allergies, and had been free of medications for at least 3 weeks before the experiment. Volunteers had no history of weight loss because of eating disorder, obesity, hypertension, gastrointestinal disease or a psychiatric disorder in the past and had normal values in physical examination and in electrocardiography. Blood tests confirmed a normal blood count, liver and renal functions. Volunteers were in the first 2 weeks of the follicular phase of their menstrual cycle. All subjects provided written informed consent prior to participating in the study, which was approved by the Human Ethic Review Committee, Institute of Endocrinology, Prague.

All subjects were admitted to the Institute of Endocrinology at 7.00 a.m. They underwent a short medical survey (blood pressure and heartbeat, EKG, respiration hearing). The percent body fat was determined by bioimpedancy (TANITA, Japan) and by anthropometric measurement. Microdialysis catheter was introduced into abdominal adipose tissue at 7.15 a.m. The experiments were performed with the subjects in supine position in a room kept at 24–25 °C.

2.3. *Microdialysis*

Microdialysis catheter CMA/65 with 100 kDa cut-off and membrane length 30 mm (CMA, Stockholm, Sweden) with polyethylene sulfone membrane connected to CMA/107 microdialysis pump was inserted into abdominal subcutaneous adipose tissue after local lidocain administration. Microdialysis syringe was filled with sterile KHB with dextran to prevent perfusion fluid loss [2]. The perfusion rate was 2 μl/min. Microvials with interstitial fluid samples were collected at two 45-min intervals (8.00–9.30 a.m.) at least 45 min after catheter introduction and bed rest to reach steady-state conditions. Samples were kept on ice during the experiment, immediately chilled and stored at −80 °C. Leptin concentrations were determined by high sensitive RIA (Linco Research, MO, USA) with the limit of sensitivity 0.05 ng/ml. The resulting leptin concentration in the microdialysate is the mean of two measurements at the two intervals, taking into account leptin recovery.

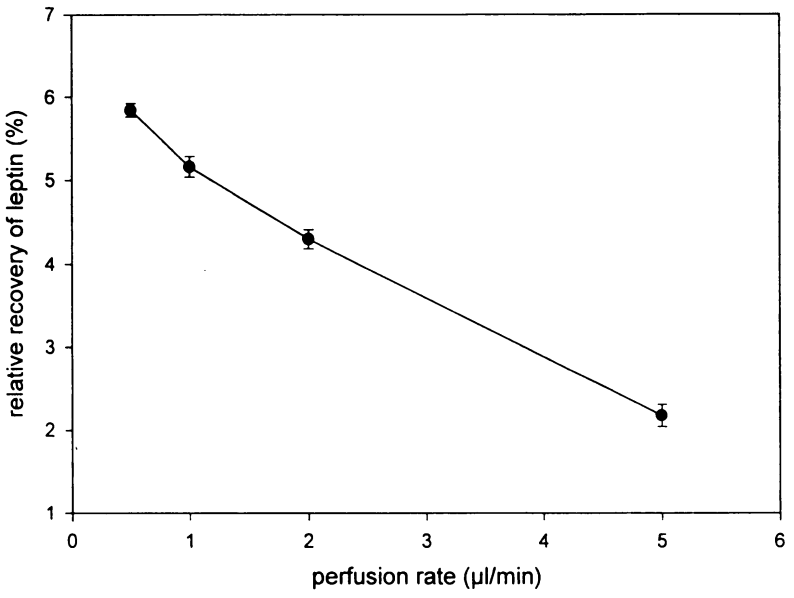


Fig. 1. The relative leptin recovery in vitro (%) was indirectly proportional to perfusion rate (μl/min). Four perfusion rates have being tested (0.5, 1.0, 2.0, 5.0 μl/min). Results are shown as mean ± S.D., $n = 15$ at each rate.

3. Results

3.1. In vitro recovery

The relative leptin recovery was 5.85 ± 0.08% at perfusion rate of 0.5 μl/min, 5.17 ± 0.12% at perfusion rate of 1.0 μl/min, 4.30 ± 0.11% at perfusion rate of 2.0 μl/min and 2.18 ± 0.13% at perfusion rate of 5.0 μl/min (n = 15 for each rate). The relative leptin recovery in vitro was indirectly proportional to the perfusion rate (Fig. 1).

3.2. Leptin concentrations in the interstitial fluid of adipose tissue in healthy women

Concentrations of leptin in the interstitial fluid of adipose tissue in healthy women (n = 11) were 2.59 ± 1.99 ng/ml using perfusion rate of 2 μl/min, as discussed above. The microdialysate volumes of samples measured at 45-min intervals were 84.58 ± 7.22 μl, which confirms the view that 40 g/l dextran-70 added is adequate to prevent perfusate loss. Leptin concentrations in the interstitial fluid of adipose tissue were analyzed by high sensitive RIA (Linco Research, MO, USA), sensitivity limit of an assay was 0.05 ng/ml.

4. Discussion

The present study was designed to explore the possibility of using microdialysis catheters with a large-pore dialysis membrane (100 kDa) to investigate the concentrations of large peptide adipocyte derived hormone leptin in interstitial fluid of adipose tissue. The 100 kDa membrane should allow the study of larger molecules than conventional membranes with a pore size ≤ 20 kDa, which, with two exceptions [3,4], has been used in all previous microdialysis investigations. Furthermore, the largest molecule used previously to diffuse across the 100 kDa pore membrane, was insulin (5808 Da) [4].

On the other hand, the 100 kDa membrane has the disadvantage that it allows a greater loss of perfusate into the interstitial space. The problem with the perfusate explains, why 100 kDa pore membrane was not used in previous microdialysis research.

In our study, similarly to Rosdahl et al. [4,5] Krebs–Henseleit buffer with dextran-70 (40 g/l) as a perfusate was used to prevent perfusion fluid loss. Rosdahl et al. [4] have shown that the loss of perfusate from the microdialysis catheter could be prevented if a colloid is added to the perfusate and that this membrane is suitable for recordings of molecules in the interstitial space and that large molecules, such as insulin, diffuse across the dialysis membrane. Leptin (16 kDa) is almost three times larger than insulin (5.8 kDa). Despite this fact we assumed the accuracy of the previous observations that a pore size of microdialysis membrane three times greater than is the molecular weight of the molecule is sufficient to permit diffusion across the dialysis membrane [6]. Our results confirm that a 100 kDa pore membrane is applicable for supervision of leptin in the interstitial fluid of adipose tissue.

It is important to establish to what extent the measured concentration of a particular compound in the dialysate reflects its true level in the extracellular space. Under most circumstances microdialysis only allows measurement of a fraction of the concentration of a substance in the extracellular space [1]. Microdialysis probes can be characterized by the assessment of their recovery in vitro, as described by Lonnroth et al. [11]. The relative recovery of leptin measured in vitro is defined as a ratio between leptin concentration in the dialysate and the concentration in the fluid surrounding the probe (expressed as percentage) [1]. We have shown that the relative leptin recovery reach the order of percent values and that the relative recovery is indirectly proportional to the perfusion rate. Despite our attempt to simulate the tissue conditions in measuring the relative leptin recovery, it was not possible to reach the same conditions as in vivo. On the other hand, the relative recovery was measured at different rates and a negative relationship between the perfusion rate and leptin concentration in microdialysate was observed. This observation, together with at least 15 independent measurements with minimal deviations at each perfusion rate, confirm the suitability of the procedure.

Our results achieved in human subcutaneous adipose tissue in vivo using a protocol with 2 μl/min perfusion flow show that perfusate loss does not occur when 40 g/l dextran-70 is included in the perfusate. The selection of the perfusion rate of 2 μl/min for in vivo experiments has two main reasons. First, the minimal volume sample to assay leptin is 100 μl. If a smaller perfusion rates were used, it would take too much time to collect the required volume of one leptin sample for the assay and we would lose the acute reflection, e.g. after local drug application. Secondly, the relative recovery measured at this rate is sufficient to calculate leptin concentrations. At higher rates, the relative recovery of leptin is too low to give optimal results. Despite our results, which show reduced leptin concentrations in the interstitial fluid of adipose tissue compared to leptin concentrations in plasma in healthy women, diurnal decrease of leptin concentrations in plasma is in agreement with a decrease in leptin concentrations in interstitial fluid of adipose tissue in the morning (not shown). Furthermore, leptin expression in other tissues, including human pituitary [7], stomach [8], placenta [9] and mammary epithelial cells [10] could contribute to circulating leptin levels.

5. Conclusions

Microdialysis has been usually used for monitoring small biologically active molecules in peripheral tissues. The present study has for the first time shown that the in vivo microdialysis is suitable for monitoring large peptide adipocyte-derived hormones, such as leptin, in inters-

titial fluid of human adipose tissue provided that a 100 kDa pore microdialysis membrane was used with addition of dextran-70 in the perfusate. Our results could be applied for measuring local changes in the production of adipocyte-derived hormones which are important in energy homeostasis regulation and could be important in clinical research engaged in eating disorders or obesity.

Acknowledgements

This study was supported by Grant No. 303/00/1555 of the Grant Agency of Czech republic. We thank Diana Riegerová for excellent technical assistance.

References

[1] Lafontan M, Arner P. Trends Pharmacol Sci. Elsevier, 17; 1996. p. 309–13.
[2] Rosdahl H, Ungerstedt U, Henriksson J. Acta Physiol Scand 1997;159:261–2.
[3] Sjostrand M, Holmang A, Lonnroth P. Am J Physiol 1999;276: E151–4.
[4] Rosdahl H, Hamrin K, Ungerstedt U, Henriksson J. Int J Biol Macromol 2000;28:69–73.
[5] Rosdahl H, Ungerstedt U, Jorfeldt L, Henriksson J. J Physiol 1993;471:637–57.
[6] Torto N, Bang J, Richardson S, Nilsson GS, Gorton L, Laurell T, et al. J Chromatogr A 1998;806(2):265–78.
[7] Jin L, Burguera BG, Couce ME, Scheithauer BW, Lamson J, Eberhardt NL, et al. J Clin Endocrinol Metab 1999;84:2903–11.
[8] Mix H, Manns MP, Wagner S, Widjaja A, Brabant G. Gastroenterology 1999;117(2):509.
[9] Masuzaki H, Ogawa Y, Sagawa N, Hosada K, Matsumoto T, Mise H, et al. Nat Med 1997;3:1029–33.
[10] Smith-Kirwin SM, O'Connor DM, De Johnson J, DeLancey ED, Hassink SG, Funanage VL. J Clin Endocrinol Metab 1998;83: 1810–3.
[11] Lonnroth P, Jansson PA, Fredholm BB, Smith U. Am J Physiol 1989;256:E250–5.

ARTICLE B

Dostalova I, Kopsky V, Duskova J, Papezova H, Pacak K, Nedvidkova J

Leptin concentrations in the abdominal subcutaneous adipose tissue of patients with anorexia nervosa assessed by in vivo microdialysis.

Regulatory Peptides. 2005 128(1):63-68.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Regulatory Peptides 128 (2005) 63-68
REGULATORY PEPTIDES
www.elsevier.com/locate/regpep

Leptin concentrations in the abdominal subcutaneous adipose tissue of patients with anorexia nervosa assessed by in vivo microdialysis

Ivana Dostálová[a,b], Vojtěch Kopský[a], Jaroslava Dušková[c], Hana Papežová[d], Karel Pacák[e], Jara Nedvídková[a,*]

[a]*Institute of Endocrinology, Laboratory of Clinical and Experimental Neuroendocrinology, Narodni 8, Z116 94, Prague 1, Czech Republic*
[b]*Charles University, Faculty of Sciences, Viničná 7, 128 00 Prague, Czech Republic*
[c]*Charles University, 1st Faculty of Medicine, Institute of Pathology, Studničkova 2, 128 00 Prague 2, Czech Republic*
[d]*Charles University, 1st Faculty of Medicine, Psychiatric Clinic, Ke Karlovu 11, 120 00 Prague 2, Czech Republic*
[e]*Pediatric and Reproductive Endocrinology Branch, NICHD, NIH, 10 Center Drive, MSC 1583, Bethesda, MD 20892-1583, USA*

Received 25 February 2004; received in revised form 6 December 2004; accepted 11 December 2004
Available online 27 January 2005

Abstract

Objective: The adipocyte-derived hormone leptin is involved in energy metabolism and body weight regulation. Plasma leptin concentrations are significantly reduced in patients with anorexia nervosa (AN) and with severe malnutrition. Whether reduced plasma leptin is reflected by its decreased production by the adipose tissue is unknown.
Methods: In the present study we measured leptin concentrations locally in the abdominal subcutaneous adipose tissue of 9 female AN patients and 11 healthy controls by in vivo microdialysis.
Results: Adipose tissue free leptin levels were not different in patients with AN compared to controls (2.59±1.99 vs 2.36±0.25 ng/ml, *P>0.05*). Plasma leptin soluble receptor (sOb-R) levels were significantly higher in patients with AN than in healthy subjects (58.05±38.69 vs 12.79±5.08 U/ml, *P<0.01*). The area of adipocyte in AN was considerably smaller than in the controls (183±104.01 μm^2 compared to 2145.8±1003.41).
Conclusions: We conclude that decreased plasma leptin levels in patients with AN are not directly related to dialysate leptin levels in the abdominal subcutaneous adipose tissue.

Keywords: Microdialysis; Anorexia nervosa; Leptin; Adipose tissue

1. Introduction

Leptin is a hormone with multiple functions, including predominantly long-term regulation of body weight, energy balance and body temperature by acting as a hunger suppressant signal to the central nervous system [1,2]. In eating disorders, leptin is involved in the development of its symptoms rather than the pathogenesis itself [3]. Initially thought to be expressed and secreted exclusively by adipocytes, leptin has also been found in other human tissues, including the pituitary, stomach, placenta and mammary gland [4–8]. Nevertheless, adipose tissue remains its main source responsible for 95% of leptin production [9]. Leptin levels can be influenced by such conditions as satiety, amount of adipose tissue or diurnal cycles. These influences are mediated mostly by sympathetic regulators, insulin, glucocorticoids, and glucose entry into adipocytes [10]. These influences result in great inter- and intrapersonal variations of leptin levels.

Plasma leptin exists in two forms: free and protein-bound, which reflects leptin binding to its soluble receptor (sOb-R), the major leptin binding activity in plasma [11–13]. Expression of sOb-R and plasma leptin correlates significantly and inversely with age, IGF-I levels, pubertal stage and body composition [14,15,16]. In various eating

* Corresponding author. Tel.: +420 224905272; fax: +420 224905325.
E-mail address: jnedvidkova@endo.cz (J. Nedvídková).

0167-0115/$ - see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.regpep.2004.12.029

disorders, plasma sOb-R levels were found to vary in the manner opposite to leptin levels [17]. The relationship between bound and free leptin may become clinically relevant in physiological and pathophysiological states that cause rapid changes in total plasma leptin, such as fasting [13]. Leptin as index of adiposity is reduced by caloric restriction and weight loss [18]. It was found that while physical exercise does not induce changes in circulating leptin, fasting reliably affects serum leptin levels [19].

Anorexia nervosa (AN) is a psychiatric disorder characterized by intensive fear of gaining weight, despite the patient's weight being normal or even below normal. This abnormal behavior leads to voluntary reduction of food intake, which results in severe weight and fat loss in affected patients, thus endangering their lives [20]. Hyperactivity often plays a role in developing and maintaining AN and represents an obstacle to weight gain in refeeding [21]. Plasma leptin concentrations are significantly decreased in subjects with AN [22,23]. It has been suggested that this decrease may reflect fat loss during starvation [22,24]. Interestingly, weight gain results in significantly increased plasma leptin [22,23] and decreased plasma sOb-R in AN patients [15]. Leptin levels can also be influenced by lowered insulin levels in AN patients [25], increased activity of sympathetic nervous system (SNS) in abdominal adipose tissue [26] and other factors. Holtkamp et al. [27] found that therapeutically induced weight gain in AN patients accompanied by high serum leptin levels has prognosis of repeated weight loss.

However, all these studies were based on measurement of plasma total and free leptin concentrations but not on measurement of leptin concentrations of local specific adipose tissue. Microdialysis is a powerful and safe technique that allows detection of in vivo local changes in interstitial fluid concentrations of various molecules, including hormones [28–31]. In previous study [26], we documented increased SNS activity, especially increased norepinephrine (NE) levels in adipose tissue of AN patients, but not in the whole body where the SNS activity was rather decreased. It is known that catecholamines inhibit leptin production [32]. Thus, we hypothesized that the increased NE concentration in the extracellular space of abdominal adipose tissue of AN patients may serve to protect the organism from increased lipolysis by lowering leptin production there. Therefore, we decided to determine leptin levels in interstitial space of adipose tissue in AN patients where the sympathetic activity is alternated.

2. Subjects and methods

2.1. Study subjects

Nine women with AN (age: 24.4±3.59 years; BMI: 15.4±1.59 kg/m^2) and eleven healthy women (age: 23.3±2.57 years; BMI: 20.9±1.47 kg/m^2) were enrolled in this study. All subjects were non-smokers, had no allergies, and had been free of medications for at least three weeks prior to the study. Healthy women had no history of obesity, hypertension, gastrointestinal diseases, eating disorders or other psychiatric disorders, and had normal physical examination and electrocardiogram. Blood tests confirmed normal blood count, liver and renal functions. Patients with AN were diagnosed according to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994) [33] after detailed medical and psychiatric evaluation. All patients with AN restrictive type were examined after two weeks of hospitalization on the Department of Psychiatry, still in acute state. Patients with AN were clinically stabile and in relatively good health, except for their eating disorder. All healthy women were in the first two weeks of the follicular phase of their menstrual cycle whereas all AN patients had amenorrhea. All subjects provided written informed consent prior to participating in the study, which was approved by the Human Ethic Review Committee, Institute of Endocrinology, Prague.

2.2. Experimental procedures

Upon enrolment, subjects were prohibited to drink coffee and alcohol and were asked to fast and drink only water the night prior to the study. All subjects were admitted to the Institute of Endocrinology at 7:00 a.m. After a short medical examination (blood pressure, heart and respiratory rates measurement, EKG), percentage of body fat (% BF) was estimated by anthropometry measurement and bioimpedancy (TANITA, Japan). All subjects were then placed in supine position on a comfortable bed in a room kept at 23–25 °C and a venous catheter was placed in the antecubital vein. A microdialysis catheter CMA/65 (CMA, Stockholm, Sweden) with a membrane length 30 mm and with a molecular cut-off of 100 kDa was inserted subcutaneously under sterile conditions, 8–10 cm lateral to the umbilical scar after local anesthesia with 0.1% lidocain. Immediately after insertion, the catheter was perfused with physiologic solution using a portable pump (CMA 107). Sterile Krebs–Henseleit buffer (KHB, consisting of 140 mmol/l NaCl, 3.9 mmol/l KCL, 2.5 mmol/l $CaCl_2 \cdot 2H_2O$, 1.2 mmol/l $MgSO_4 \cdot 7H_2O$, 0.8 mmol/l $Na_2HPO_4 \cdot 2H_2O$, 0.8 mmol/l KH_2PO_4) was used as perfusion fluid. KHB pH was adjusted to 7.4 with NaOH and dextran (40g/l) was added to prevent perfusion fluid loss. A constant perfusion rate of 2 μl/min was maintained throughout the study. Blood and microdialysate collection started at least 45 min after catheter insertion and bed rest to reach steady state conditions. Microdialysate samples were collected at 45 min intervals between 8:00 and 9:30 a.m. Blood samples for leptin and sOb-R assay were collected into chilled polypropylene tubes containing Na_2EDTA and antilysin. Plasma was immediately separated from whole blood by centrifugation at 3000 rpm for 20 min at 4 °C and stored at −20 °C until being thawn and assayed.

2.3. Subcutaneous blood flow estimation

Subcutaneous adipose tissue blood flow in the abdominal region was estimated by the ethanol washout method. According to this method, differences between ethanol concentrations in the perfusate (inflow) and dialysate (outflow) indirectly reflect local changes in blood flow [34,35]. The application of this procedure to the adipose tissue has been previously validated by comparison with the ^{133}Xe washout [34]. Briefly, in our study ethanol (50 mmol/l) was added to the perfusion fluid and its escape from the perfusate into the adipose tissue interstitial fluid was assessed by measuring changes in dialysate/perfusate ratio.

Ethanol was measured using a standard enzymatic assay (Sigma Diagnostics, USA). For simplicity, the microdialysate ethanol concentration/perfusate ethanol concentration ratio is referred to as "ethanol ratio".

2.4. Calculation of relative leptin recovery

Before starting microdialysis perfusion, the relative leptin recovery (RLR) was calculated in vitro at a temperature of 37–38 °C and maintained by a digital block heater to simulate body temperature. The CMA/65 catheter was immersed into a solution containing a concentration of leptin equivalent to the average plasma level, and a microdialysis syringe was filled with KHB supplemented with dextran-70 (40g/l) as described above. Different perfusion rates (0.5, 1.0, 2.0, 5.0 μl/min) were tested to investigate possible relative recovery vs perfusion rate dependency. At each rate, RLR was calculated from 15 samples collected in perfusion rate-dependent intervals according to the formula: RLR (%)=(leptin concentration in dialysate/leptin concentration in standard solution)×100. A perfusion rate of 2 μl/min was selected for in vivo microdialysis, based on the calculated in vitro RLR corrected for experiment duration. The procedure is described in detail in our previous report [31].

2.5. Biopsy

Thick needle aspiration samples were fixed in buffered formalin and embedded in paraffin. Four micrometer sections were stained H and E and the adipocyte areas were measured with the LUCIA (Laboratory Universal Computer Image Analysis-Laboratory Imaging Prague) image analysis program.

2.6. Analytical procedures

Leptin was measured by RIA (Linco Research, St.Charles, Missouri, USA). Samples were initially incubated with 0.05 M of a phosphate-buffered saline solution (0.025 M of EDTA, 0.08% of sodium azide, 0.05% of Triton X-100 and 1% of RIA grade BSA, pH 7.4) and with anti-leptin anti-serum for 24 h at room temperature. Next, ^{125}I-leptin was added and samples were incubated for additional 24 h at room temperature. Antibody-bound leptin was then precipitated by 1 ml of cold (4 °C) precipitating reagent (goat anti-rabbit IgG serum, 3% PEG and 0.05% Triton X-100 in 0.05M Phosphosaline, 0.025M EDTA, 0.08% sodium azide) and tubes were incubated for 20 min at 4 °C and centrifuged for 30 min at 3000 rpm. After centrifugation, supernatants were decanted and radioactivity of pellets was quantified in a γ-counter. The detection limit of the assay was 0.05 ng/ml, with intra-assay coefficient of variations (CVs) of 5.25% and 5.97%, and interassay CVs of 8.9% and 8.67% for low (0.44 ng/ml) and high (4.24 ng/ml) leptin levels, respectively. All assays were run twice in duplicate.

Plasma sOb-R was measured by ELISA (BioVendor Laboratory Medicine). Plasma samples, recombinant human sOb-R standards and quality controls were diluted 1:2 with dilution buffer and incubated in microtitration wells coated with monoclonal anti-human sOb-R antibody for 1 h at room temperature on an orbital microplate shaker at 300 rpm. The wells were then washed 3× with a washing buffer. After washing, a monoclonal anti-human sOb-R antibody, labeled with horseradish peroxidase, was added and incubated with the immobilized antibody–leptin receptor complex for 1 h at room temperature and 300 rpm shaking. Next, the wells were washed 4× and the substrate solution was added. After an additional 5–10 min incubation at room temperature, the reaction was stopped by adding 0.2 M of H_2SO_4. Absorbance was measured at 450 nm with a spectrophotometer (Dynex, USA). The detection limit of the assay was 0.4 Unit recombinant human sOb-R/ml of sample, with intra- and interassay CVs of 4.7% and 3.9%, respectively.

2.7. Data analysis

All results are presented as mean±S.D. Microdialysate leptin concentrations, calculated as average of the two values measured at 45 min intervals between 8:00 and 9:30 a.m., were corrected for in vitro RLR. For evaluation of relations between leptin concentrations and anorexia, ANCOVA model was used with adjustment to constant BMI and/or percent of body fat. To achieve Gaussian data distribution and to stabilize the variance, leptin plasma and adipose tissue levels were transformed by logarithmic and rank transformation, respectively. Plasma and adipose tissue microdialysate leptin levels relationship was analysed using Spearman correlation.

3. Results

Baseline characteristics of the study subjects, including anthropometric measurements, are summarized in Table 1. At a perfusion rate of 2 μl/min, the in vitro RLR was 4.29±0.38%. Local adipose tissue blood flow was similar in

Table 1
Characteristics of the study subjects

	Controls (n=11)	AN (n=9)
Age	23.3±2.57	24.4±3.59
Body weight (kg)	60.4±5.41	43.6±7.04[a]
Body height (cm)	169.9±4.76	168.0±6.30
BMI (kg/m^2)	20.9±1.47	15.4±1.59[a]
Body fat (%)	19.5±3.58	4.1±0.85[a]
Total fat skinfold (mm)	120.5±25.17	42.1±18.78[a]
Abdominal fat skinfold (mm)	16.4±6.18	5.8±3.59[a]

Values are mean±S.D., AN=anorexia nervosa, BMI=body mass index.
[a] Values significantly different from control subjects, p<0.05.

AN subjects and the controls (ethanol ratio: 41.2±6.67% vs 40.9±5.26%). Plasma leptin concentrations were significantly decreased in AN patients when compared to the controls (1.9±0.81 vs 8.0±3.38 ng/ml *P<0.0001*) whereas dialysed leptin levels from the abdominal subcutaneous adipose tissue were not significantly different in the two groups (2.6±1.99 vs 2.4±0.25 ng/ml) (Fig. 1). Plasma and adipose tissue microdialysate leptin levels did not correlate. Plasma sOB-R levels were significantly increased in AN patients compared to healthy controls (58.1±38.69 vs 12.8±5.08 U/ml, P<0.01) (Fig. 2). The images of biopsies are shown (Fig. 3). The area of adipocyte in the controls was 2145.8±1003.41 μm^2 and 183±104.01 μm^2 in AN.

4. Discussion

AN is a severe psychiatric disorder associated with high morbidity and mortality. Only recently, the neuroendocrine

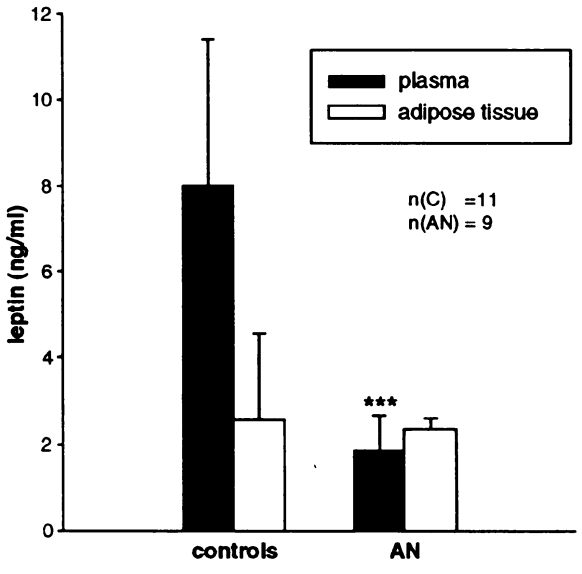


Fig. 1. Plasma and subcutaneous adipose tissue free leptin concentrations (mean±S.D.) in patients with anorexia nervosa (AN) and healthy controls (C). Plasma samples were collected at 8:00 a.m. whereas microdialysate samples were collected every 45 min between 8:00 and 9:30 a.m. ***P<0.01 compared with controls.

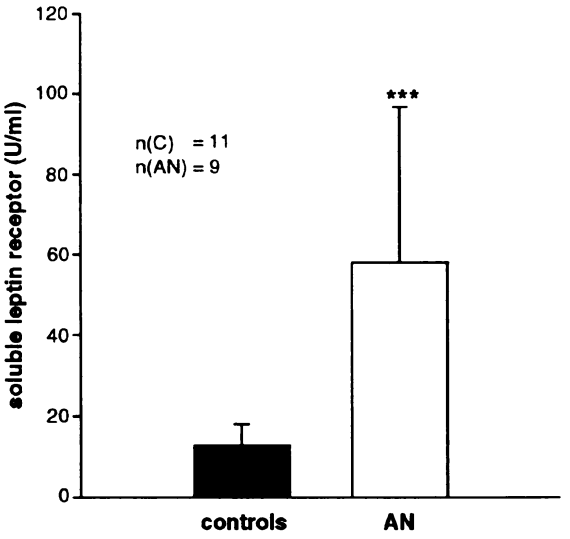


Fig. 2. Plasma leptin receptor (sOb-R) levels (mean±S.D.) in patients with anorexia nervosa (AN) and healthy controls (C). Plasma samples were collected at 8:00 a.m. ***P<0.01 compared with controls.

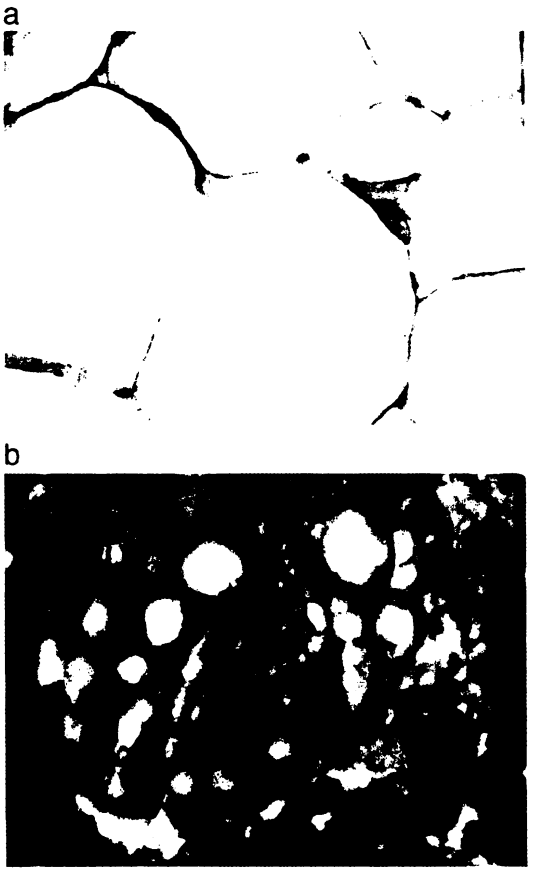


Fig. 3. Biopsies of subcutaneous adipose tissue from subject with normal weight (a) and from an anorexia nervosa patient (b).

regulation of energy metabolism and feeding behavior in eating disorders, such as AN, has become object of intense investigation [3]. Our group, for example, has previously described abnormal plasma ghrelin response to food intake in AN patients [36] and increased norepinephrine levels in extracellular space of abdominal subcutaneous adipose tissue [26].

In the present study, we found significantly decreased plasma leptin concentration, and increased plasma soluble Ob-R (sOb-R) in AN patients, in agreement with the observations already reported [17,20,37]. Obviously, during chronic starvation, low circulating leptin levels together with up-regulation of the sOb-R in AN patients may translate into reduced leptin availability to peripheral and brain tissues [15]. It is also needed to point out that plasma sOb-R levels are independently regulated by many different physiological and pathophysiological conditions [14] and sOb-R may modulate actions of leptin in tissues in which direct action of leptin has been demonstrated [38].

Contrary to our expectations, there was no difference in local subcutaneous adipose tissue dialysate leptin concentrations between AN patients and healthy controls, which could mean that malnutrition and severe weight loss in AN either do not interfere with changes in leptin levels in the subcutaneous adipose tissue or their influence is somehow compensated. We expected decrease of interstitial leptin levels mainly due to increased norepinephrine concentrations that we found in abdominal subcutaneous adipose tissue [26].

The higher than expected local leptin concentrations in AN patients could be explained by the increased number of smaller adipocytes in the vicinity of the microdialysing membrane. Our minibiopsies of abdominal adipose tissue in AN patients and in the controls showed that the number of adipocytes per volume is higher in AN patients than the number of adipocytes in the controls (Fig. 1a,b). Therefore, if SNS influences leptin production per cell, rather than per volume, high local concetrations, similar to controls, still can be achieved in AN. On the other hand, less leptin is secreted into circulation due to reduced volume of abdominal adipose tissue.

The influence of sOb-R on adipose tissue interstitial leptin concentrations is unequivocal at the present state of knowledge. It does not penetrate the microdialysis membrane due to its size so its local concentrations in vivo cannot be determined. Although it is tempting to speculate about them being proportional to serum concentrations, it does not necessarily have to be the case. Up to date, little is known about its site of synthesis.

We are aware that there are many influences to leptin concentrations [10,32] and many of those factors are alternated in AN [3,26] as well as the serum leptin levels. Our microdialysis measurements had shown that the local leptin concentration is not affected. This supports the view that, in the case of AN, the diminished size of adipose tissue is the major contributor to low serum leptin levels.

Acknowledgement

The study was supported by a grant agency of Czech Republic, GA CR, No. 303/03/0376. We would like to thank Mrs. Diana Riegerova, Jana Novotna and Romana Bajtlova for their technical assistance and to Dr. M. Kunešová MD, PhD for performing biopsies.

References

[1] Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science 1995;269:546–9.
[2] Friedman JM, Halaas JL. Leptin and the regulation of the body weight in mammals. Nature 1998;395:763–70.
[3] Monteleone P, DiLieto A, Castaldo E, Maj M. Leptin functioning in eating disorders. CNS Spectr 2004;9:523–9.
[4] Jin L, Burguera BG, Couce ME, Scheithauer BW, Lamson J, Eberhardt NL, et al. Leptin and leptin receptor expression in the normal and neoplastic human pituitary: evidence of a regulatory role of leptin on pituitary cell proliferation. J Clin Endocrinol Metab 1999;84:2903–11.
[5] Mix H, Widjaja A, Jandl O, Cornberg M, Kaul A, Goke M, et al. Expression of leptin and leptin receptor isoform in the human stomach. Gut 2000;47:481–6.
[6] Masuzaki H, Ogawa Y, Sagawa N, Hosada K, Matsumoto T, Mise H, et al. Non-adipose tissue production of leptin: leptin as a novel placenta derived hormone in humans. Nat Med 1997;3:1029–33.
[7] Senaris R, Garcia-Caballero T, Casabiell X, Callero R, Castro R, Considine RV, et al. Synthesis of leptin in human placenta. Endocrinology 1997;138:4501–4.
[8] Smith-Kirwin SM, O'Connor DM, De Johnson J, DeLancey ED, Hassink SG, Funanage VL. Leptin expression in human mammary epithelial cells breast milk. J Clin Endocrinol Metab 1998;83:1810–3.
[9] Frayn KN, Karpe F, Fielding BA, Macdonald IA, Coppack SW. Integrative physiology of human adipose tissue. Int J Obes Relat Metab Disord 2003;27:875–88.
[10] Coleman RA, Herrmann TS. Nutritional regulation of leptin in humans. Diabetologia 1999;42:639–46.
[11] Lammert A, Kiess W, Bottner A, Glasow A, Kratzsch J. Soluble leptin receptor represents the main leptin binding activity in human blood. Biochem Biophys Res Commun 2001;283:982–8.
[12] Landt M, Horowitz JF, Coppack SW, Klein S. Effect of short-term fasting on free and bound leptin concentrations in lean and obese women. J Clin Endocrinol Metab 2001;86:3768–71.
[13] Sinha MK, Opentanova I, Ohannesian JP, Kolaczynski JW, Heiman ML, Hale J, et al. Evidence of free and bound leptin in human circulation. Studies in lean and obese subjects and during short-term fasting. J Clin Invest 1996;15(98):1277–82.
[14] Chan JL, Bluher S, Yiannakouris N, Suchard MA, Kratzsch J, Mantzoros CS. Regulation of circulating soluble leptin receptor levels by gender, adiposity, sex steroids, and leptin: observational and interventional studies in humans. Diabetes 2002;51:2105–12.
[15] Kratzsch J, Lammert A, Bottner A, Seidel B, Mueller G, Thiery J. Circulating soluble leptin receptor and free leptin index during childhood, puberty, and adolescence. J Clin Endocrinol Metab 2002;87:4587–94.
[16] Monteleone P, Martiadis V, Colurcio B, Maj M. Leptin secretion is related to chronicity and severity of the illness in bulimia nervosa. Psychosom Med 2002;64:874–9.
[17] Monteleone P, Fabrazzo M, Tortorella A, Fuschino A, Maj M. Opposite modifications in circulating leptin and soluble leptin receptor across the eating disorder spectrum. Mol Psychiatry 2002;7(6):641–6.

[18] Mori TA, Burke V, Puddey IB, Shaw JE, Beilin LJ. Effect of fish diets and weight loss on serum leptin concentration in overweight, treated-hypertensive subjects. J Hypertens 2004;22:1983–90.
[19] Piccione G, Bertolucci C, Foa A, Caola G. Influence of fasting and exercise on the daily rhythm of serum leptin in the horse. Chronobiol Int 2004;21:405–17.
[20] Ferron F, Considine RV, Peino R, Lado IG, Diaguez C, Casanueva FF. Serum leptin concentrations in patients with anorexia nervosa, bulimia nervosa and non-specific eating disorders correlate with the body mass index but are independent of the respective disease. Clin Endocrinol 1997;46:289–93.
[21] Holtkamp K, Hebebrand J, Herpertz-Dahlmann B. The contribution of anxiety and food restriction on physical activity levels in acute anorexia nervosa. Int J Eat Disord 2004;36:163–71.
[22] Casanueva FF, Diaguez C, Popovic V, Peino R, Considine RV, Caro JF. Serum immunoreactive leptin concentrations in patients with anorexia nervosa before and after partial weight recovery. Biochem Mol Med 1997;60:116–20.
[23] Nedvidkova J, Papezova H, Haluzik M, Schreiber V. Interaction between serum leptin levels and hypothalamo–hypophyseal–thyroid axis in patients with anorexia nervosa. Endocr Res 2000;26:219–30.
[24] Hebebrand J, Hinney A, Roth H, Ballauff A, Remschmidt H. Will leptin provide an insight into weight regulation of patients with anorexia nervosa? Eur Eat Disord Rev 1997;5:221–5.
[25] Misra M, Miller KK, Almazan C, Ramaswamy K, Aggarwal A, Herzog DB, et al. Hormonal and body composition predictors of soluble leptin receptor, leptin, and free leptin index in adolescent girls with anorexia nervosa and controls and relation to insulin sensitivity. J Clin Endocrinol Metab 2004;89:3486–95.
[26] Nedvidkova J, Dostalova I, Bartak V, Papezov H, Pacak K. Increased subcutaneous abdominal tissue norepinephrine levels in patients with anorexia nervosa: an in vivo microdialysis study. Physiol Res 2004;53:409–13.
[27] Holtkamp K, Hebebrand J, Mika C, Heer M, Heussen N, Herpertz-Dahlmann B. High serum leptin levels subsequent to weight gain predict renewed weight loss in patients with anorexia nervosa. Psychoneuroendocrinology 2004;29:791–7.
[28] Jansson PA, Fowelin JP, von Schenck HP, Smith UP, Lonnroth PN. Measurement by microdialysis of the insulin concentration in subcutaneous interstitial fluid. Importance of the endothelial barrier for insulin. Diabetes 1993;42:1469–73.
[29] Rosdahl H, Ungerstedt U, Jorfeldt L, Henriksson J. Interstitial glucose and lactate balance in human skeletal muscle and adipose tissue studied by microdialysis. J Physiol 1993;471:637–57.
[30] Sjorstrand M, Holmang A, Strindberg L, Lonnroth P. Estimation of muscle interstitial insulin, glucose, and lactate in type 2 diabetic subjects. Am J Physiol, Endocrinol Metab 2000;2798:E1097–1103.
[31] Dostalova I, Pacak K, Nedvidkova J. Application of in vivo microdialysis to measure leptin concentrations in adipose tissue. Int J Biol Macromol 2003;32:205–8.
[32] Rayner DV, Trayhurn P. Regulation of leptin production: sympathetic nervous system interactions. J Mol Med 2001;79:8–20.
[33] Diagnostic and Statistical manual of Mental Disorders, 4th Edition, American Psychiatric Association, 1994.
[34] Enoksson S, Nordenstrom J, Bolinder J, Arner P. Influence of local blood flow on glycerol levels in human adipose tissue. Int J Obes Relat Metab Disord 1995;19:350–4.
[35] Lafontan M, Arner P. Application of in situ microdialysis to measure metabolic and vascular responses in adipose tissue. Trends Pharmacol Sci 1996;17:309–13.
[36] Nedvidkova J, Krykorkova I, Bartak V, Papezova H, Alesci S, Gold WP, et al. Loss of meal-induced fall in plasma ghrelin in patients with anorexia nervosa. J Clin Endocrinol Metab 2003;88:1678–82.
[37] Eikelis N, Lambert G, Wiesner G, Kaye D, Schlaich M, Morris M, et al. Extra-adipocyte leptin release in human obesity and its relation to sympathoadrenal function. Am J Physiol, Endocrinol Metab 2004; 286:E744–52.
[38] Yang G, Ge H, Boucher A, Yu X, Li C. Modulation of direct leptin signaling by soluble leptin receptor. Mol Endocrinol 2004 (June);18(6):1354–62.

ARTICLE C

Nedvidkova J, **Krykorkova I**, Bartak V, Papezova H, Gold PW, Alesci S, Pacak K

Loss of meal-induced decrease in plasma ghrelin levels in patients with anorexia nervosa.

Journal of Clinical Endocrinology and Metabolism. 2003 88(4):1678-1682.

0013-7227/03/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 88(4):1678–1682

doi: 10.1210/jc.2002-021669

Loss of Meal-Induced Decrease in Plasma Ghrelin Levels in Patients with Anorexia Nervosa

JARA NEDVÍDKOVÁ, IVANA KRYKORKOVÁ, VLADIMÍR BARTÁK, HANA PAPEZOVÁ, PHILIP W GOLD, SALVATORE ALESCI, KAREL PACAK

Institute of Endocrinology, Charles University, Prague, Czech Republic (J.N., I.K., V.B.), Faculty of Natural Sciences, Charles University, Prague, Czech Republic (I.K., V.B.), Department of Psychiatry, Charles University, Prague, Czech Republic (H.P.), Pediatric and Reproductive Endocrinology Branch, NICHD, NIH, Bethesda, USA (S.A., K.P.), Clinical Neuroendocrinology Branch, NIMH, NIH, Bethesda, USA (S.A., P.W.G.), Sezione di Endocrinologia, Dipartimento Clinico Sperimentale di Medicina e Farmacologia, University of Messina, Messina, Italy (S.A.).

ABSTRACT Studies have shown that ghrelin plays a major role in energy homeostasis and modulation of feeding behavior. However, little is known about the influence of food consumption on plasma ghrelin levels in humans. Therefore, we investigated responses of plasma ghrelin to food intake, meal volume and meal nutritional value in healthy volunteers and women with anorexia nervosa (AN). After overnight fasting, all subjects received either a standardized breakfast or fiber. Plasma ghrelin levels were measured before and after the meal. Fasting plasma ghrelin was significantly higher in AN patients than in controls (1,800.6 ± 47.0 *vs* 795.9 ± 24.3 pg/ml, $P < 0.001$) (606.8 ± 15.8 *vs* 268.2 ± 8.2 pmol/l, $P < 0.001$), and correlated negatively with percentage of body fat in both groups. Ghrelin levels markedly fell after consumption of either a standardized meal or fiber in controls, but not in anorexic women. Thus, we concluded that the acute plasma ghrelin response to food intake, which in healthy individuals is independent of meal caloric value, is impaired in women with AN. This abnormality may be part of a chronic adaptation to prolonged food restriction, which attempts to restore a normal feeding conduct by maintaining the drive to eat.

Introduction

Ghrelin is a novel peptide hormone identified as the endogenous ligand of the growth hormone secretagogue receptor. Predominantly synthesized by the neuroendocrine-like oxyntic cells of the gastric mucosa, ghrelin is also found in the intestine, pancreas, and the arcuate nucleus of the hypothalamus (1-3).

In addition to strongly stimulating growth hormone (GH) release both *in vitro* and *in vivo* (4-6), ghrelin seems to be involved in the control of energy metabolism and feeding behavior in both rodents and humans. In rodents, systemic and intracerebroventricular injections of ghrelin increase food intake, fat deposition and weight gain, while ghrelin expression increases after prolonged fasting (7,8). In healthy humans, plasma ghrelin concentrations increase before meals, reach a nadir within one hour after meals, and are higher in lean than in obese subjects (9-11). Similarly to rodents, when administered intravenously to humans, ghrelin stimulates appetite and increases food intake (12,13). At least in rodents, the orexigenic properties of ghrelin, which are independent of GH-release stimulation, appear to be partially mediated by activation of arcuate nucleus neurons expressing neuropeptide Y and agouti-related protein, two orexigenic peptides (14-16).

Anorexia nervosa (AN) is a highly morbid pathologic condition with the highest mortality rate among other psychiatric disorders (17). Patients with AN experience hunger, but are prevented from eating by intense fear of losing control over eating and becoming overweight. This abnormal feeding behavior leads to marked weight loss and several metabolic disturbances, which in most cases are life-threatening (17-19). Compared to healthy subjects, patients with AN have higher fasting plasma ghrelin levels, which return back to normal after weight gain, suggesting a role for this hormone in the pathogenesis of their psychiatric disorder (10,20,21). However, responses of plasma ghrelin to eating in AN subjects have not been explored. Therefore, we studied the response of plasma ghrelin to food intake, meal volume and nutritional value in patients with AN and healthy controls.

Study Design and Methods

Subjects. Five women with AN (age: 24.3 ±

Received 10/25/02. Accepted 01/15/03.

2.69 years; BMI: 15.21 ± 1.54 kg/m^2; mean ± SEM) and six healthy women (age: 22.95 ± 4.75 years; BMI 21.63 ± 1.24 kg/m^2), were recruited for this study. All participants provided written informed consent before being enrolled in the study, which was approved by the Human Ethic Review Committee, Institute of Endocrinology, Charles University, Prague. All subjects included in the study were non-smokers, had no allergies, and had been free of medications for at least two weeks prior to the study. Healthy volunteers had no history of obesity, hypertension, gastrointestinal disease, eating disorders or other psychiatric disorders. They had normal physical examination, electrocardiogram, blood count, liver and renal functions. Patients with AN were diagnosed according to the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994). All AN patients were clinically stable and in relatively good health, except for their eating disorder. All subjects were asked to fast and drink only water on the night prior to the study.

Protocol. All participants were hospitalized in the Department of Psychiatry at Charles University, Prague, for the entire duration of the study. Subjects were weighed and measured without shoes. Waist and hip circumferences were determined at the level of the umbilicus and greater trochanters, respectively. Percentage of body fat (% BF) was estimated by bioimpedance (22). To test the influence of both food volume and food nutritional value on plasma ghrelin levels, all subjects received a standard solid meal (585 kcal (2,439 kj), 32.6 g of fat, 17.6 g of protein, 50.0 g of carbohydrate) modified from Paris-Bockel et al. (23) or non-caloric fiber (0.11 kcal (0.46 kj), 4 g of Psyllium) at 8:00 AM on two consecutive days. Blood samples were drawn before the meal and at specific intervals (30, 60, 90 and 120 min) after the meal. Samples were collected into chilled polypropylene tubes containing Na_2EDTA and antilysin. Plasma was immediately separated by centrifugation at 4 °C and stored at –80 °C until being assayed.

Ghrelin assay. Plasma ghrelin was measured by a commercial RIA kit that uses ^{125}I–labeled bioactive ghrelin as a tracer, and a rabbit polyclonal antibody raised against human full-length octanoylated ghrelin (Phoenix Pharmaceuticals, Inc., Belmont, CA). Sensitivity, inter-assay CV and intra-assay CV were 1 pg/ml, 6.2% and 9%, respectively.

Statistical analyses. All data are presented as mean ± SEM. Statistical differences between groups were analyzed by unpaired t-test or Mann-Whitney Rank sum test. Correlations between parameters were examined using Spearman's rank correlation coefficient. Group by time interaction for ghrelin response to meal was analyzed by one-way repeated measure ANOVA followed by Dunnett's test for multiple comparisons with baseline. A P value < 0.05 denoted statistical significance.

Results

Baseline characteristics of the study subjects are summarized in Table 1. Fasting plasma ghrelin was more than two-fold higher in patients with AN than in controls (1,800.6 ± 47.0 *vs* 795.9 ± 24.3 pg/ml, P < 0.001) (606.8 ± 15.8 *vs* 268.2 ± 8.2 pmol/l, P < 0.001) (Table 1) and correlated negatively with % BF in both controls (R^2 = - 0.49; P < 0.05) and AN (R^2 = - 0.54; P < 0.05).

Following the standardized breakfast, plasma ghrelin concentrations continuously and markedly decreased in control subjects from 795.9 ± 35.8 before meal to 408.5 ± 52.1 pg/ml (268.2 ± 12.0 and 137.7 ± 17.6 pmol/l, respectively) two hours after the meal (P < 0.05). Similarly, plasma ghrelin fell from 795.8 ± 36.4 at baseline to 450.2 ± 36.4 (268.1 ± 12.3 and 151.7 ± 12.3 pmol/l, respectively) 30 min after consumption of Psyllium fiber (P < 0.05), remaining constantly low thereafter (Figure 1A). In contrast, ingestion of either standard breakfast or Psyllium fiber was accompanied by either non-significant increases or no changes in plasma ghrelin over two hours in patients with AN (*breakfast:* baseline: 1,820 ± 68.6, 30 min: 2477 ± 199, 60 min: 1818 ± 220, 90 min: 2,754 ± 588, 120 min: 1,730 ± 495; *fiber:* baseline: 1780 ± 71, 30 min: 2,250 ± 242, 60 min: 2,184 ± 173, 90 min: 2707 ± 706, 120 min: 2524 ± 369 pg/ml (613.3 ± 23.1, 834.7 ± 67.0, 612.7 ± 74.1, 928.0 ± 198.1, 583.0 ± 166.8; and 599.9 ± 23.9, 758.9 ± 81.6, 736.0 ± 58.3, 912.3 ± 237.9, 850.6 ± 124.4 pmol/l) (Figure 1B). The percentage changes in ghrelin responses after consumption of either the standardized breakfast or Psyllium fiber were 36.0 and 26.0 at 30 min; - 0.2 and 23.5 at 60 min; 51.3 and 52.0 at 90 min; and – 5.0 and 41.8 in two hours, respectively.

Discussion

Eating disturbances, such as AN and bulimia nervosa, are severe clinical conditions associated with increased morbidity and mortality. Patients affected by

these disorders often deny their illness, an element that, in addition to the complexity of the disease, makes most of the current treatments unsuccessful. New hopes have arisen through by the recent progress in understanding the neuroendocrine regulation of energy metabolism and feeding behavior.

The newly characterized hormone ghrelin appears to play a major role in the control of energy homeostasis and adipogenesis (7,8). In support of this hypothesis and in agreement with previous reports (10,20,24), we found in our study that fasting plasma ghrelin levels correlate negatively with both BMI and % BF. In humans, ghrelin also functions as an appetite stimulant. In fact, its plasma levels rise before meals, inducing hunger, and fall after food intake (9, 11). In our study, consumptions of the standard 585 kcal (2,439 kj) breakfast or fiber were equally effective in suppressing plasma ghrelin in healthy women. These findings suggest that the acute response of plasma ghrelin to feeding is independent of meal nutritional value, but rather related to the presence of food in the stomach. However, it has been shown that in normal subjects, plasma ghrelin also decreases after intravenous administration of glucose, suggesting that ghrelin secretion may not be influenced by stomach expansion (10). Further studies are required to characterize the cellular and molecular mechanisms underlying ghrelin responses to food intake.

The importance of ghrelin in the control of feeding behavior in humans is further supported by the occurrence of altered levels of this orexigenic hormone in patients with different eating disorders. In fact, fasting plasma ghrelin was found to be decreased in obesity (10,11) and increased in AN and bulimia nervosa (10,20,21). Our study confirms that women with AN have significantly increased fasting plasma ghrelin when compared to healthy controls. This abnormality may result from a chronic adaptation compensating the abnormal feeding conduct, in order to preserve energy homeostasis. Baseline plasma ghrelin levels measured in the present study were higher than those reported by Otto et al. (20). Such a difference might be explained by different experimental procedures, including sample preparation and timing of the assay (in our study we analyzed samples no later than 5 days after sampling) and the small number of patients in our study.

We also showed, for the first time, that in patients with AN plasma ghrelin levels remain high after food intake, and do not fall even two hours after meal consumption. This impaired response may indicate that in subjects with AN, because of the chronic food restriction and the consequent adaptation, a single meal is not sufficient to suppress the drive to eat, in order to regain a normal weight and replenish the energy stores. However, weight gain in anorexic patients is associated with decreased plasma ghrelin (20). It is therefore conceivable that, in these patients, correction of the abnormal feeding behavior over a prolonged period of time may restore the normal acute response of plasma ghrelin to single meals.

In summary, we have shown that the short-term response of plasma ghrelin to feeding, which in healthy subjects is independent of food caloric value, is impaired in women with AN. Further research is needed to clarify the basic mechanism underlying this and other abnormalities associated with chronic food deprivation.

Acknowledgments

We are grateful to Zdenka Fajtova and Diana Riegerova for their technical assistance. We also would like to thank Shiromi Perera and Christina Kukura for their editorial assistance. This study was supported by the grant agency GA CR, No. 303/00/1555.

References

1. Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Atamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K 2001 Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in human. J Clin Endocrinol Metab 86: 4753-4758
2. Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S 2002 Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. Diabetes 51:124-129
3. Kojima M, Hosoda H, Kangawa K 2001 Purification and distribution of ghrelin: the natural endogenous ligand for the growth hormone secretagogue receptor. Horm Res 56: 93-97
4. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans.

Endocrinology 141:4255-61
5. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H 1999 Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 402:656-660
6. Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Okawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K 2000 Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab 85:4908-4911
7. Tschöp M, Smiley DL, Heiman ML 2000 Ghrelin induces adipocyty in rodents. Nature 407:908-913
8. Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR 2000 The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141:4325-4328
9. Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS 2002 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714-1749
10. Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukura S 2002 Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab 87:240-244
11. Tschöp M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML 2001 Circulating ghrelin levels are decreased in human obesity. Diabetes 50:707-709
12. Arvat E, Di Vito L, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E 2000 Preliminary evidence that ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. J Endocrinol Invest 23:493-495.
13. Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR 2001 Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 86:5992
14. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I 2000 Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. Endocrinology 141:4797-4800
15. Pinkney J, Williams G 2002 Ghrelin gets hungry. Lancet 359:1360-1361.
16. Williams G, Bing C, Cai XJ, Harrold JA, King PJ, Liu XH 2001 The hypothalamus and the control of energy homeostasis: different circuits, different purposes. Physiol Behav 74:683-701
17. Vitiello B, Lederhendler I 2000 Research on eating disorders: current status and future prospects. Biol Psychiatry 47:777-786
18. de Rosa, Corsello SM, de Rosa E, Della Casa S, Ruffilli MP, Grasso P, Pasargiklian E 1983 Endocrine study of anorexia nervosa. Exp Clin Endocrinol 82: 160-172
19. de Tournemire R, Alvin P 2002 Anorexia nervosa and severe malnutrition: nutritional practice guidelines in pediatrics. Arch Pediatr 9:429-433
20. Otto B, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, Heiman ML, Lehnert P, Fichter M, Tschöp M 2001 Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. Eur J Endocrinol 145:669-673
21. Cuntz U, Fruhauf E, Wawarta R, Tschöp M, Folwaczny C, Riepl R, Lehnert P, Fichter M, Otto B 2002 A role for the novel weight-regulating hormone ghrelin in anorexia nervosa. Am Clin Lab 21:22-23
22. Bolanowski M, Nilsson BE 2001 Assessment of human body composition using dual-energy x-ray absorptiometry and bioelectrical impedance analysis. Med Sci Monit 7:1029-1033
23. Paris-Bockel D, Comte F, Jeanmougin P, Keller B, Schlienger JL 1983 Breakfast tolerance test: application to the detection of gestational diabetes. Sem Hop 59:2167-2172
24. English PJ, Wilding JP 2002 Ghrelin: sweet regulation? Clin Sci 103:329-330

Correspondence:
Karel Pacak, M.D., Ph.D., D.Sc.
Chief, Unit on Clinical Neuroendocrinology
Pediatric and Reproductive Endocrine Branch
Bldg 10, Room 9D 42, 10 Center Drive, MSC 1583
NICHD, NIH, Bethesda, Maryland, MD 20892-1583
Tel: 301-402-4594, Fax: 301-402-4712
E-mail: karel@mail.nih.gov

Table 1. Baseline characteristics of the study subjects (mean ± SEM). C = controls; AN = anorexia nervosa; BMI = body mass index; %BF = percentage of body fat; NS = not significant.

	C (n=6)	AN (n=5)	P value
Age (years)	22.95 ± 4.75	24.3 ± 2.69	NS
BMI (kg/m^2)	21.63 ± 1.24	15.21 ± 1.54	<0.05
%BF	24.6 ± 1.59	7.2 ± 0.88	<0.001
Fasting plasma ghrelin (pg/ml)*	795.9 ± 24.3	1800.6 ± 47.0	<0.001

*To convert values for plasma ghrelin to pmol/l multiply by 0.337.

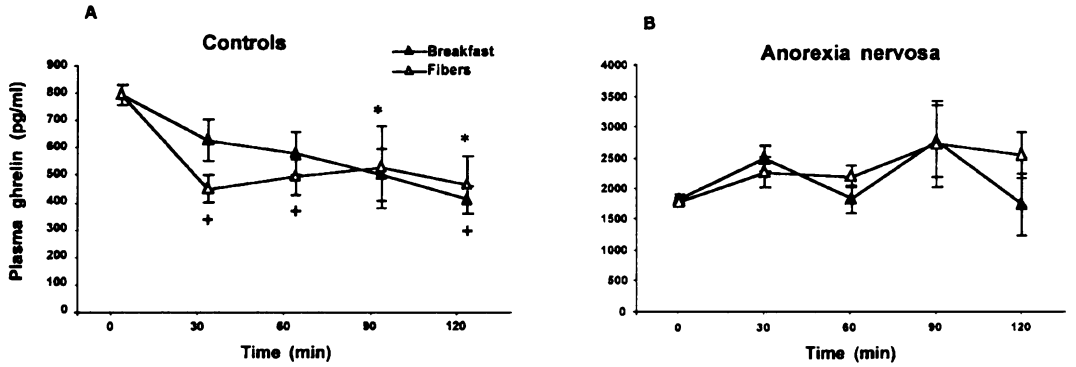


Figure 1. A. Plasma ghrelin (mean ± SEM) response to food intake in healthy women. Following a standardized breakfast, ghrelin plasma levels decreased markedly and continuously over two hours. Similarly, ghrelin levels fell thirty minutes after consumption of fibers, remaining low thereafter. $^*P < 0.05$ and $^+P < 0.05$ *vs* baseline after standardized breakfast and Psyllium fibers, respectively. **B.** Plasma ghrelin (mean ± SEM) response to food intake in women with anorexia nervosa (AN). Ingestion of a single standardized meal or fiber did not induce any significant change in plasma ghrelin over two hours. To convert values for plasma ghrelin to pmol/l multiply by 0.337.

ARTICLE D

Dostalova I, Kunesova M, Duskova J, Papezova H, Nedvidkova J

Adipose tissue resistin levels in patients with anorexia nervosa.

Subm. *Nutrition.*

Adipose tissue resistin levels in patients with anorexia nervosa

IVANA DOSTALOVA (1,2), MARIE KUNESOVA (1), JAROSLAVA DUSKOVA (3), HANA PAPEZOVA (4), JARA NEDVIDKOVA (1)

(1) Institute of Endocrinology, Laboratory of Clinical and Experimental Neuroendocrinology, Narodni 8, 116 94 Prague 1, Czech Republic (2) Charles University, Faculty of Sciences, Vinicna 7, 128 00 Prague 2, Czech Republic (3) Charles University, Institute of Pathology, 1st Faculty of Medicine, Studnickova 2, 128 00 Prague 2 (4) Charles University, Psychiatric Clinic, 1st Faculty of Medicine, Ke Karlovu 11, 120 00 Prague 2, Czech Republic.

ABSTRACT

Background. Resistin is a specific fat-derived hormone that affects fuel homeostasis and insulin action in rodents. However, its role in human physiology and pathophysiological conditions, such as malnutrition, remains uncertain. **Material and methods.** To enhance understanding of the role of resistin in the pathophysiology of anorexia nervosa (AN), we measured plasma resistin levels in ten women with a restrictive type of AN and in thirteen healthy age-matched women (C). Furthermore, we measured resistin levels in the subcutaneous adipose tissue of 5 women from AN group and 5 women from C group, respectively, using *in vivo* microdialysis technique (CMA/107 pump, CMA/60 catheters, CMA Microdialysis AB, Solna, Sweden). **Results.** Body mass index (BMI), percent body fat (%BF), fasting plasma leptin and insulin and HOMA-IR index were severely decreased in patients with AN compared to C group. Plasma resistin levels were significantly reduced in patients with AN ($P < 0.05$), whereas subcutaneous adipose tissue resistin levels were significantly increased in patients with AN compared to C group ($P < 0.05$). In both groups, plasma resistin levels showed no significant relationship to resistin in the dialyzate, %BF, BMI, HOMA-IR and fasting plasma leptin levels. **Conclusions.** We demonstrated that AN is associated with decreased plasma resistin levels and increased resistin levels in extracellular space of the abdominal adipose tissue. Plasma resistin levels in either patients with AN or in healthy normal-weight women were not directly related to BMI, %BF, plasma leptin levels and insulin sensitivity.

Key words: **resistin, anorexia nervosa, microdialysis**

INTRODUCTION

Resistin has been proposed as a fuel homeostasis and an insulin action regulator in rodents [1], and may also be involved in hematopoesis, immune function [2] and inflammatory processes [3,4].

Whether or not human resistin is implicated in insulin resistance is still uncertain. Much speculations remain not only due to the differences observed between human and mouse tissue expression of the gene [5]. Studies of resistin gene expression in human adipose tissue have been inconsistent [6,7] and the role of adipose tissue resistin gene expression in human insulin resistance was not confirmed [8]. Resistin expression tended to be higher in obese than in lean subjects [7,9], and was higher in abdominal than in other adipose depots [9,10]. Plasma resistin levels were found not to be different between non-diabetic obese and lean subjects and correlated with insulin resistance, but not with body mass index (BMI) [11]. On the other hand, numerous other studies have not reported any relationship between circulating resistin levels and insulin resistance [12,13,14].

Initially, resistin was supposed to be under tight nutritional control, being decreased by fasting and re-increased by refeeding, suggesting that this factor may be an adipose sensor for the nutritional status in rodents [1,15]. Although Valsamikis et al. [16] have highlighted that serum resistin levels are reduced with modest weight loss in obese individuals, other studies in human seem not to support the original hypothesis [17,18]. Furthermore, some of authors showed that resistin expression in human white adipose tissue was negatively regulated by cholesterol [19] and by estrogen [20] and was not related to adiposity, blood pressure, fasting plasma glucose level [13], acute fasting and leptin administration [12].

Anorexia nervosa (AN) is a psychiatric disorder characterized by intensive fear of gaining weight leading to deliberate food intake reduction and life threatening weight and fat loss in affected patients [21]. As expected, severe malnutrition in AN is associated with altered glucose and lipid metabolism, and multiple endocrine perturbations [22]. Some of these abnormalities may be linked to altered adipocytokine production [2]. In the study of Housova et al. [18] plasma resistin levels in patients with AN have not been found significantly different from either controls or patients with bulimia nervosa

and have shown no significant relationship to BMI or fat content. However, *in vivo* resistin levels in human white adipose tissue have not been explored. Our group has previously shown that despite significantly decreased plasma leptin levels in patients with AN, leptin levels in adipose tissue of patients with AN are unchanged [23], which demonstrated the importance of regional *in vivo* measurements for better understanding of the systemic hormone function.

Microdialysis is a powerful and safe technique, which allows to detect *in vivo* local changes in interstitial fluid concentrations of various molecules, including hormones [24]. The spectrum of molecules and tissues studied by microdialysis is getting wider. For example, in our previous study [25], we documented increased sympathetic nervous system (SNS) activity, especially increased norepinephrine (NE) levels, in adipose tissue of patients with AN. More recently, our group has adopted the microdialysis technique to study *in vivo* local adipocytokines production in adipose tissue [26].

Based on our previous results, we hypothesized that resistin concentrations in extracellular space of abdominal adipose tissue of patients with AN may be altered and may contribute to regional changes in adipose tissue metabolism in AN. Therefore, we decided to determine resistin levels in interstitial space of adipose tissue in patients with AN by *in vivo* microdialysis.

SUBJECTS AND METHODS

Study subjects

Ten women with a restrictive type of AN (age: 24.4 ± 1.59 years; BMI: 15.6 ± 0.55 kg/m^2, percent body fat (%BF): 6.9 ± 1.05) and thirteen healthy control women (age 23.3 ± 1.27 years; BMI: 20.7 ± 0.35 kg/m^2, %BF: 21.5 ± 1.64) were enrolled in this study. All subjects were non-smokers, had no allergies, and had been free of medications for at least three weeks prior to the study. Healthy women had no history of obesity, hypertension, gastrointestinal diseases, eating disorders or other psychiatric disorders, and had normal physical examination and electrocardiogram (ECG). Blood tests confirmed normal blood count, liver and renal functions. Patients with AN were diagnosed according to the 4th edition of the Diagnostic and Statistical Manual of Mental Disorder [27] after detailed medical and psychiatric evaluation. All patients with a restrictive type of AN were examinated after two weeks of hospitalization in the Department of Psychiatry, still in acute state. Patients with AN were clinically stable and in relative good health, except for their eating disorder. All healthy women were in the first two weeks of the follicular phase of their menstrual cycle, whereas all patients with AN had amenorrhea. All subjects provided written informed consent prior to participation in the study, which was approved by the Human Ethic Review Committee, Institute of Endocrinology, Prague, Czech Republic, and was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

Experimental procedures

Upon enrollment, subjects were prohibited to drink coffee and alcohol, and were asked to fast and drink only water the night prior to the study. All subjects were admitted to the Institute of Endocrinology at 07:00 h. After a short medical examination (blood pressure, heart and respiratory rates measurement, ECG), %BF was estimated by anthropometry measurement and bioimpedance (TANITA, Japan). All subjects were then placed in supine position on a comfortable bed in a room kept at 23-25 ^{0}C, and a venous catheter was placed in the antecubital vein. A microdialysis catheter CMA/60 (CMA Microdialysis AB, Solna, Sweden) with a membrane length 30 mm and with a molecular cut-off of 20 kDa was inserted subcutaneously under sterile conditions, 8-10 cm lateral to the umbilical scar after local anesthesia with 0.1% lidocain. Immediately after insertion, the catheter was perfused with physiologic solution using a portable pump (CMA 107). Sterile Krebs-Henseleit buffer [26] was used as perfusion fluid. A constant perfusion rate of 2 μl/min was maintained throughout the study. Blood and microdialysate collection started at least 45 minutes after catheter insertion and bed rest to reach steady state conditions. Microdialysate samples were collected at 45 minute intervals between 08:00 and 09:30 h. Blood samples for resistin and leptin assay were collected into chilled polypropylene tubes containing Na_2EDTA and antilysin. Plasma was immediately separated from whole blood by centrifugation at 3,000 rpm for 20 min at 4°C, and stored at –20 °C until being thawn and assayed.

Subcutaneous blood flow estimation

Subcutaneous adipose tissue blood flow in the abdominal region was estimated by the ethanol washout method [28,29]. Briefly, in our study ethanol (50 mmol/l) was added to the perfusion fluid and its escape from the perfusate into the adipose tissue interstitial fluid was assessed by measuring changes in dialysate/perfusate ethanol ratio. Ethanol was measured using a standard enzymatic assay (Sigma Diagnostics Inc., St.Louis, Missouri, USA).

Calculation of relative resistin recovery and HOMA index

Before starting microdialysis perfusion, the relative resistin recovery (RRR) was calculated *in vitro*. The procedure is described in detail in our previous report [26].

Insulin resistance (IR) was estimated using the homeostasis model assessment (HOMA) using the formula : IR = fasting insulin x fasting glucose / 22.5 [30].

Analytical procedures

Plasma and microdialysate resistin was measured by a commercial human resistin RIA kit (Phoenix Pharmaceuticals, Inc., Belmont, CA, USA). The detection limit of the assay was 100 pg/ml and the intra- and interassay variability was 4.5% and 7.4%, respectively. Plasma leptin was assayed by commercially available RIA kit (Linco Research, St.Charles, Missouri, USA). Sensitivity was 0.05 ng/ml and the intra- and interassay variability was 4.6 % and 8.7 %, respectively. Plasma insulin was measured by a commercial RIA (Immunotech, Prague, Czech Republic). Sensitivity was 0.5 μIU/ml and the intra- and interassay variability was 3.4% and 4.3%, respectively. Plasma glucose levels were measured in on Cobas Integra 400 plus (Roche Diagnostics, GmbH, Mannheim, Germany). All assays were run twice in duplicate.

Data analysis

All results are presented as mean ± S.E.M. Microdialysate resistin concentrations were corrected for *in vitro* RRR. For evaluations of relations between resistin concentrations and anorexia, ANCOVA model was used with adjustment to constant BMI and/or percent body fat. To achieve Gaussian data distribution and to stabilize the variance, resistin plasma and adipose tissue levels were transformed by logarithmic and rank transformation, respectively. Plasma resistin and other paramethers relationship was analyzed using Spearman correlation. A *P* value of 0.05 or less denoted statistical significance.

RESULTS

Characteristics of the study subjects

Baseline characteristics of the study subjects, including anthropometric measurements, are summarized in Table 1. Patients with AN were extremely malnutrished as evidenced by severely decreased BMI, %BF and total and abdominal skinfold relative to control (C) group (Table 1).

In vitro relative resistin recovery and abdominal adipose tissue blood flow

At a perfusion rate of 2 μl/min, the in vitro RRR was 3.9 ± 0.18 % (Table 2). Local adipose tissue blood flow was similar in AN subjects and C group (ethanol ratio: 42.3 ± 1.25% vs. 39.8 ± 0.98%).

Plasma and in vivo abdominal adipose tissue concentrations of resistin, plasma glucose, insulin, leptin and HOMA index

Fasting plasma resistin levels were significantly lower in patients with AN than in C group (243.8 ± 21.90 vs. 312.6 ± 15.73 pg/ml, $P < 0.05$) whereas fasting dialyzed resistin levels from the abdominal subcutaneous adipose tissue were significantly increased in patients with AN compared to controls (7320.0 ± 1090.78 vs. 4319.2 ± 128.10 pg/ml, $P < 0.05$) (Fig.1A,B). Detailed data on plasma and adipose tissue resistin levels of subjects undergoing microdialysis performance are given in Table 3A for controls and in Table 3B for patients with AN, respectively. Fasting plasma insulin (2.1 ± 0.33 vs. 4.5 ± 0.24 mIU/l, $P < 0.05$) and leptin (1.7 ± 0.30 vs. 7.8 ± 0.89 ng/ml, $P < 0.05$) were significantly decreased in patients with AN compared to controls, whereas fasting plasma glucose was not significantly different between groups (4.1 ± 0.08 vs. 4.6 ± 0.10 mmol/l). Insulin resistance estimated by HOMA was significantly decreased in patients with AN than in controls (0.39 ± 0.06 vs. 0.89 ± 0.05, $P < 0.05$) (table 1).

Relationship of plasma resistin with other parameters

Plasma resistin levels were not significantly related to any other parameters studied, including dialyzed resistin levels in the abdominal adipose tissue, BMI, %BF, HOMA-IR and fasting plasma leptin levels in either patients with AN or C group.

DISCUSSION

The most important finding of this study is that while plasma resistin concentrations were significantly decreased in severely malnourished patients with a restrictive type of AN, resistin concentrations found in the extracellular space of subcutaneous abdominal adipose tissue of these patients were much higher compared to healthy age-matched women. Furthermore, plasma resistin did not correlate with any other parameters studied, including BMI, %BF, fasting plasma insulin and leptin and HOMA-IR either in patients with AN or in healthy women.

AN is a severe psychiatric disorder associated with high morbidity and mortality. Only recently, the neuroendocrine regulation of energy metabolism and feeding behavior in eating disorders, such as AN, has become object of intense investigation [31]. Our group, for example, has previously described abnormal plasma ghrelin response to food intake in patients with AN [32] and increased NE levels in extracellular space of abdominal subcutaneous adipose tissue [25].

In the only other study published so far plasma resistin concentrations in patients with a restrictive type of AN tended to be lower than in control women, but in contrast to our results the difference did not reach statistical significance [18]. Results found in healthy normal-weight women demonstrated that a modest decrease in energy intake sustained over several weeks has no significant effect on plasma resistin levels [17] and that plasma resistin levels are not associated with markers of insulin resistance and are not regulated by fasting, leptin administration [12], adiposity and fasting plasma glucose levels [13]. On contrary, Valsamakis et al. [16] have highlighted that serum resistin levels are

reduced with modest weight loss, but the results were found in obese individuals.

Studies examined the relationship between BMI and plasma resistin levels brought rather contradictory results. While some studies have found such results as correlation [33], others failed to determine such an effect [11,12,18]. Here we show that despite altered resistin levels in both plasma and extracellular space of abdominal adipose tissue of patients with AN, plasma resistin levels demonstrated no signifficant relationship to either BMI or %BF. We therefore suggest, that neither reduction of fat mass nor several weight loss associated with AN is an important factor affecting plasma resistin levels in these patients. Possible explanation of low resistin concentrations in plasma of patients with AN could be diminished production in bone marrow [5], eventually altered stability or clearance of resistin. Although plasma resistin levels in both groups have shown no relationship to resistin levels found in extracellular space of subcutaneous abdominal adipose tissue, we may not exclude the existence of relationship between plasma and adipose tissue resistin because of the small number of microdialysated probands in our study.

Although the initial report by Steppan et al [1] suggested that resistin might constitute the link between obesity and insulin resistance/diabetes, resistin's role in human physiology is currently unclear and probably different from that in mice. Also the producing cell type, which in human are the macrophages and the site of the highest production which is bone marrow [5], differ from mice. Nevertheless, the evidence of the production of resistin by human adipocytes was also demonstrated [7,9].

Our microdialysis experiments revealed increased levels of resistin in the subcutaneous adipose tissue of patients with AN. These results are free of bias by differences in blood flow, as was verified by ethanol washout method. Reasons for the resistin increase in adipose tissue of patients with AN might be multiple due to its pleiotropic character. Interestingly, minibiopsies of abdominal adipose tissue in patients with AN and in control women performed previously by our group showed that the number of adipocytes per volume is higher in patients with AN than the number of adipocytes in control women [23]. Since, beside the macrophages, resistin is secreted by adipocytes [9], the high adipose tissue resistin concentrations in patients with AN could be in part explained by the increased number of small adipocytes in the vicinity of the microdialysing membrane. However, it remains of interest, whether resistin shows a higher expression in subcutaneous adipocytes of patients with AN.

The finding of increased resistin production in abdominal adipose tissue of patients with AN may have interesting etiopathogenetic consequences. The changes of resistin levels in patients with AN may reflect defective mononuclear-macrophage functions [2] and/or a number of mononuclear cells. Early cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6), are responsible for secondary induction or enhancement of resistin expression in macrophages [34]. Kahl et al. [35] found a significant increase in TNF-α and IL-6 mRNA expression in whole blood of patients with AN at admission.

In conclusion, we demonstrated that AN is associated with decreased plasma resistin levels and increased resistin levels in the extracellular space of subcutaneous abdominal adipose tissue. Plasma resistin levels in either patients with AN or in healthy normal-weight women were not directly related to BMI, %BF, plasma leptin levels and insulin sensitivity. Further investigation is needed to define the role of resistin in eating disturbances, such as AN.

Acknowledgements: We are grateful to Diana Riegerova for her technical assistance. This study was supported by the grant agency GA CR, No. 303/03/0376.

References

1. 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.
2. Brichard SM, Delporte ML, Lambert M. Adipocytokines in anorexia nervosa: a review focusing on leptin and adiponectin. Horm Metab Res 2003;35:337-342.
3. Steppan CM, Lazar MA. Resistin and obesity-associated insulin resistance. Trends Endocrinol Metab 2002;13:18-23.
4. Bokarewa M, Nagaev I, Dahlberg L, Smith U, Tarkowski A. Resistin, an adipokine with potent proinflammatory properties. J Immunol 2005;174:5789-5795.
5. Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C et al. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. Biochem Biophys Res Commun 2003;300: 472-476.
6. Nagaev I, Smith U. Insulin resistance and type2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. Biochem Biophys Res Commun 2001;285:561-564.
7. Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV et al. Resistin/Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. Diabetes 2001;50:2199-2202.
8. Janke J, Engeli S, Gorzelniak K, Luft FC, Sharma AM. Resistin gene expression in

human adipocytes is not related to insulin resistance. Obes Res 2002;10:1-5.
9. McTernan PG, McTernan CL, Chetty R, Jenner K, Fischer FM, Lauer MN et al. Increased resistin gene and protein expression in human abdominal adipose tissue. J Clin Endocrinol Metab 2002;87:2407-2410.
10. Fain JN, Cheema PS, Bahouth SW, Lloyds HM. Resistin release by human adipose tissue explants in primary culture. Biochem Biophys Res Commun 2003;300:674-678.
11. Silha JV, Krsek M, Skrha JV, Sucharda P, Nyomba BL, Murphy LJ. Plasma resistin, adiponectin and leptin levels in lean and obese subjects: correlations with insulin resistance. Eur J Endocrinol 2003;149:331-335.
12. Lee JH, Chan JL, Yiannakouris N, Kontogianni M, Estrada E, Seip R et al. Circulating resistin levels are not associated with obesity or insulin resistance in humans and are not regulated by fasting or leptin administration:cross-sectional and interventional studies in normal, insulin-resistant, and diabetic subjects. J Clin Endocrinol Metab 2003;88:4848-4856.
13. Chen CC, Li TC, Li CI, Liu CS, Wang HJ, Lin CC. Serum resistin level among healthy subjects: relatioship to anthropometric and metabolic parameters. Metabolism 2005;54:471-475.
14. Hasegawa G, Ohta M, Ichida Y, Obayashi H, Shigeta M, Yamasaki M et al. Increased serum resistin levels in patients with type 2 diabetes are not linked with markers of insulin resistance and adiposity. Acta Diabetol 2005;42:104-109.
15. Kim KH, Lee K, Moon YS, Sul HS. A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. J Biol Chem 2001;276:11252-11256.
16. Valsamikis G, McTernan PG, Chetty R, Al Daghri N, Field A, Hanif W et al. Modest weight loss and reduction in waist circumference after medical treatment are associated with favorable changes in serum adipocytokines. Metabolism 2004;53:430-434.
17. Wolfe BE, Jimerson DC, Orlova C, Mantzoros CS. Effect of dieting on plasma leptin, soluble leptin receptor, adiponectin and resistin levels in healthy volunteers. Clin Endocrinol (Oxf) 2004;61:332-338.
18. Housova J, Anderlova K, Krizova J, Haluzikova D, Kremen J, Kumstyrova T et al. Serum adiponectin and resistin concentrations in patients with rescrictive and binge/purge form of anorexia nervosa and bulimia nervosa. J Clin Endocrinol Metab 2005;90:1366-1370.
19. Jove M, Planavila A, Cabrero A, Novell F, Ros E, Zambon D et al. Reductions in plasma cholesterol levels after fenofibrate treatment are negatively correlated with resistin expression in human adipose tissue. Metabolism 2003;52:351-355.
20. Huang SW, Seow KM, Ho LT, Chien Y, Chung DY, Chang CL et al. Resistin mRNA levels are downregulated by estrogen in vivo and in vitro. FEBS Lett 2005;579:449-454.
21. Ferron F, Considine RV, Peino R, Lado IG, Diaguez C, Casanueva FF. Serum leptin concentrations in patients with anorexia nervosa, bulimia nervosa and non-specific eating disorders correlate with the body mass index but are independent of the respective disease. Clin Endocrinol 1997;46:289-293.
22. Stoving RK, Hangaard J, Hansen-Nord M, Hagen C. A review of endocrine changes in anorexia nervosa. J Psychiatr Res 1999;33:139-152.
23. Dostalova I, Kopsky V, Duskova J, Papezova H, Pacak K, Nedvidkova J. Leptin concentrations in the abdominal subcutaneous adipose tissue of patients with anorexia nervosa assessed by in vivo microdialysis. Regul Pept 2005;128:63-68.
24. Hansen TK, Gravholt CH, Orskov H, Rasmussen MH, Christiansen JS, Jorgensen JO. Dose dependency of the pharmacokinetics and acute lipolytic actions of growth hormone. J Clin Endocrinol Metab 2002;87: 4691-4698.
25. Nedvidkova J, Dostalova I, Bartak V, Papezova H, Pacak K. Increased subcutaneous abdominal tissue norepinephrine levels in patients with anorexia nervosa: an in vivo microdialysis study. Physiol Res 2004;53: 409-413.
26. Dostalova I, Pacak K, Nedvidkova J. Application of in vivo microdialysis to measure leptin concentrations in adipose tissue. Int J Biol Macromol 2003;32:205-208.
27. Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). 4th ed. Washington, DC 1994: American Psychiatric Association.
28. Enoksson S, Nordenstrom J, Bolinder J, Arner P. Influence of local blood flow on glycerol levels in human adipose tissue. Int J Obes Relat Metab Disord 1995;19:350-354.
29. Lafontan M, Arner P. Application of in situ microdialysis to measure matabolic and vascular response in adipose tissue. Trends Pharmacol Sci 1996;17:309-313.

30. Matthews DR, Hosker JP, Rudenski AS, Nazlor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and β-cellfunction from fasting plasma glucose and insulin concentrations in man. Diabetologia 1985;28:412-419.
31. Monteleone P, DiLieto A, Castaldo E, Maj M. Leptin functioning in eating disorders. CNS Spectr 2004;9:523-529.
32. Nedvidkova J, Krykorkova I, Bartak V, Papezova H, Alesci S, Gold WP et al. Loss of meal-induced fall in plasma ghrelin in patients with anorexia nervosa. J Clin Endocrinol Metab 2003;88:1678-1682.
33. Degawa-Yamauchi M, Bovenkerk JE, Juliar BE, Waston W, Kerr K, Jones R et al. Serum resistin (FIZZ3) protein is increased in obese humans. J Clin Endocrinol Metab 2003; 88:5452-5455.
34. Lehrke M, Reilly MP, Millington SC, Iqbal N, Rader DJ, Lazar MA. An inflammatory cascade leading to hyperresistinemia in humans. Plos Med 2004;1:e45.
35. Kahl KG, Kruse N, Rieckmann P, Schmidt MH. Cytokine mRNA expression patterns in the disease course of female adolescents with anorexia nervosa. *Psychoneuroendocrinology* 2004;29:13-20.

Address all correspondence and requests for reprints to:
Jara Nedvidkova, PhD
Institute of Endocrinology
Narodni 8
11694 Prague
Czech Republic
Tel.: + 420-224905272 Fax: + 420-224905325
Email: **jnedvidkova@endo.cz**

Table 1 Clinical, anthropometric, and major laboratory characteristics of the study subjects

	C (n = 13)	AN (n = 10)
Age (years)	23.3 ± 1.27	24.4 ± 1.59
BMI (kg/m^2)	20.7 ± 0.35	15.6 ± 0.55*
Body fat (%)	21.5 ± 1.64	6.9 ± 1.05*
Total fat skinfold (mm)	120.5 ± 12.17	42.1 ± 4.78*
Abdominal fat skinfold (mm)	12.5 ± 2.13	4.8 ± 1.59*
Resistin$_{PL}$ (pg/ml)	312.6 ± 15.73	243.8 ± 21.9*
Resistin$_{AT}$ (pg/ml)	4319.2 ± 128.10	7320.0 ± 1090.78*
Leptin (ng/ml)	7.8 ± 0.89	1.7 ± 0.30*
Fasting insulin (mIU/l)	4.5 ± 0.24	2.1 ± 0.33*
Fasting glucose (mmol/l)	4.6 ± 0.10	4.1 ± 0.08
HOMA-IR	0.89 ± 0.05	0.39 ± 0.06*

Values are expressed as means ± S.E.M., C = controls, AN = anorexia nervosa, BMI = body mass index, PL = plasma, AT = adipose tissue, * Values significantly diffent from C group, $p < 0.05$

Table 2 Relative resistin recovery (RRR, %) *in vitro* at four different perfusion rates (0.5, 1.0, 2.0, 5.0 µl/min). The type of catheter CMA/60 with a molecular cut-off of 20 000 Da and type of portable pump CMA/107 (CMA Microdialysis AB, Solna, Sweden) were used for the performance. Fifteen samples were taken at each perfusion rate. The perfusion rate of 2µl/min was chosen for *in vivo* trials.

Number of samples	Perfusion rate (µl/min)	RRR (%)
15	0.5	6.4 ± 0.05
15	1.0	5.3 ± 0.12
15	2.0	3.9 ± 0.18
15	5.0	2.4 ± 0.15

Values are expressed as means ± S.E.M.

Table 3A,B Detailed data on plasma and adipose tissue resistin levels (pg/ml) of the 5 subjects from control (C) group (C1-C5, **A**) and of 5 subjects from anorexia nervosa (AN) group (AN1-AN5, **B**) undergoing microdialysis study.

A.

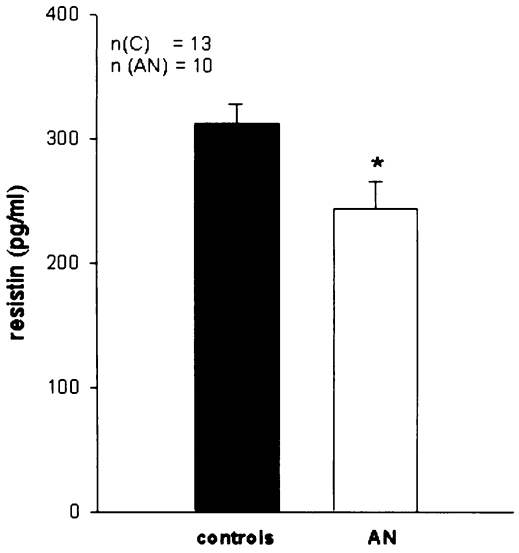
Control subjects (n = 5)	Plasma resistin (pg/ml)	Adipose tissue resistin (pg/ml)
C1	432.2	4703.9
C2	393.0	4408.0
C3	323.1	4324.3
C4	390.1	3909.6
C5	317.0	4252.3
Mean ± SEM	371.1 ± 22.14	4319.6 ± 128.09

B.

AN (n = 5)	Plasma resistin (pg/ml)	Adipose tissue resistin (pg/ml)
AN1	202.8	5920.1
AN2	240.9	10049.9
AN3	253.9	9890.7
AN4	282.0	5659.3
AN5	250.9	5080.0
Mean ± SEM	246.1 ± 12.79*	7320.0 ± 1090.78*

Values are expressed as means ± S.E.M., C = controls, AN = anorexia nervosa.
* Values significantly diffent from C group, $p < 0.05$

1A



1B

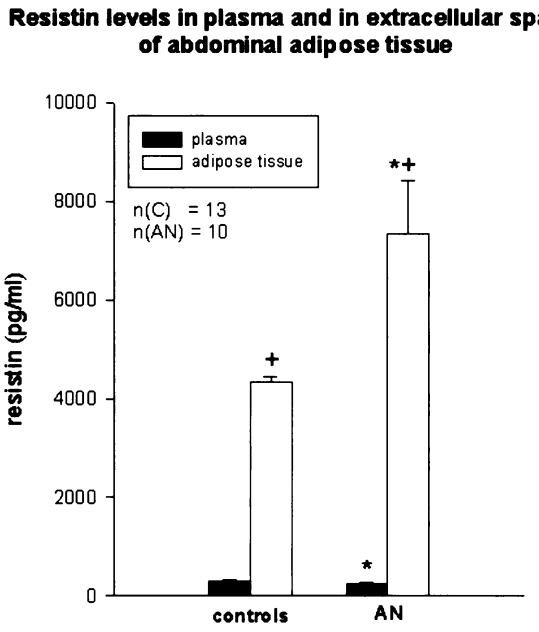


Figure 1 Plasma resistin concentrations (pg/ml) in patients with anorexia nervosa (AN, n = 10) and in healthy age-matched women (C, n = 13) **(A)**. Resistin concentrations (pg/ml) in plasma and in extracellular space of abdominal adipose tissue in patients with anorexia nervosa (AN, n = 10) and in healthy age-matched women (C, n = 13). Resistin concentrations in extracellular space of abdominal adipose tissue were measured in 5 women from AN group and in 5 women from C group, respectively **(B)**. Values are expressed as means ± SEM. *$p < 0.05$ compared to C group, $^{+}p < 0.05$ vs. plasma levels.

ARTICLE E

Dostalova I, Bartak V, Papezova H, Nedvidkova J

The effect of acute exercise on plasma leptin levels in patients with anorexia nervosa.

Subm. *European Journal of Applied Physiology.*

The effect of acute exercise on plasma leptin levels in patients with anorexia nervosa

IVANA DOSTALOVA (1,2), VLADIMIR BARTAK (1), HANA PAPEZOVA (3), JARA NEDVIDKOVA (1)

(1) Institute of Endocrinology, Laboratory of Clinical and Experimental Neuroendocrinology, Narodni 8, 116 94 Prague 1, Czech Republic (2) Charles University, Faculty of Sciences, Vinicna 7, 128 00 Prague 2, Czech Republic (3) Charles University, Psychiatric Clinic, 1st Faculty of Medicine, Ke Karlovu 11, 120 00 Prague 2, Czech Republic.

ABSTRACT

Objective: The adipocyte-derived hormone leptin is involved in energy metabolism and body weight regulation. Plasma leptin concentrations are markedly reduced in malnutrished patients with anorexia nervosa (AN). However, the effect of an acute exercise on circulating leptin levels in patients with AN has not been explored.

Methods: We investigated the effect of 45 min cycle ergometer exercise (2 $W.kg^{-1}$ of lean body mass (LBM)) on plasma leptin, norepinephrine (NE), glycerol and insulin levels in 10 patients with a restrictive type of AN (body mass index (BMI): 15.7 ± 0.47 $kg.m^{-2}$, percent body fat (%BF): 7.1 ± 0.88) and in 15 healthy age-matched women (C) (BMI: 21.2 ± 0.42 $kg.m^{-2}$, %BF: 24.3 ± 0.79). Over 3 h and 30 min, baseline, immediately postexercise and 90 min postexercise blood samples were collected from an intravenous catheter. A control trial conducted the next day consisted of the same blood sampling protocol without exercise.

Results: Basal and exercise-induced plasma leptin levels were markedly decreased in the AN group compared to the C group ($p<0.05$). Plasma leptin levels immediately after and 90 min after an exercise bout were significantly reduced compared to basal leptin levels in both AN and C group ($p<0.05$). Compared to the control trial, leptin levels were significantly reduced immediately and 90 min postexercise in the AN group but not in the C group ($p<0.05$). Basal and exercise-induced increases in plasma glycerol as well as NE levels did not significantly differ between the groups. Basal and exercise-induced plasma insulin levels were significantly reduced in the AN group compared to the C group ($p<0.05$).

Conclusions: We demonstrated that a single bout of moderate-intensity exercise significantly reduces plasma leptin levels in patients with AN, but not in healthy women, when sampled immediately after and 90 min after an exercise bout. Neither NE nor insulin are responsible for the different response of leptin to exercise in AN.

Key words: leptin, anorexia nervosa, exercise.

INTRODUCTION

Leptin is a hormone with multiple functions, including predominantly long-term regulation of body weight, energy balance and body temperature (Campfield et al. 1995; Friedman et al. 1998). It is produced predominantly by adipocytes and causes satiety by regulating hypothalamic neurotransmission and energy expenditure (Kiely et al. 2005). However, large variations in plasma leptin levels have been noted for a given level of body fat (Boden et al. 1996; Chin-Chance et al. 2000; Chan et al. 2003) suggesting that other factors may be involved in the regulation of leptin levels such as catecholamines, insulin, free fatty acids, food intake, gender, sex, etc. Recent data have provided evidence on the regulatory role of leptin between energy intake and energy expenditure in humans (Doucet et al. 2000; Hilton and Loucks 2000; McMurray et al. 2005). Exercise with concomitant alterations in energy balance may influence leptin metabolism. However, in humans, the effects of acute exercise differ depending upon the volume and type of exercise. Either a reduction (Elias et al. 2000; Jurimae et al. 2005; Keller et al. 2005) or no change (Perusse et al. 1997; Racette et al. 1997; Zafeiridis et al. 2003; Ozcelik et al. 2004; Sartorio et al. 2004) have been reported following a single bout of exercise.

Exercise alteres concentrations of certain hormones that may alter leptin concentrations, including insulin, cortisol, catecholamines, growth hormone etc. (Fisher et al. 2001; Kraemer et al. 2002; Bartak et al. 2004). Among these lipolytic NE and antilipolytic insulin play an important counteracting role in the regulation of leptin production. As the sympathetic nervous system (SNS) was established to have a tonic inhibitory action on leptin synthesis (Rayner et al. 2001), adrenergic regulation may contribute to the rapid decline in circulating leptin that occurs when the SNS is activated, such as during fasting or exercise (Rayner et al. 2001; Hulver et al. 2003; Ricci et al. 2005). On contrary, it has been demonstrated that leptin expression occurs after elevation of insulin in response to feeding (Saladin et al. 1995), and a decline in leptin levels follows reduction in insulin during fasting (Boden et al.1996). This decline of leptin during fasting should be reversed by insulin administration (Govoni et al. 2005).

AN is a psychiatric disorder characterized by intensive fear of gaining weight leading to deliberate food intake reduction and life threatening weight and fat loss in affected patients (Ferron et al. 1997). As expected, severe malnutrition in AN is associated with altered glucose and lipid metabolism, and multiple endocrine perturbations. Some of these

abnormalities may be linked to altered adipocytokine production (Brichard et al. 2003). Several studies have previously described markedly reduced basal plasma leptin levels in malnutrished and underweight anorectic patients (Casanueva et al. 1997; Nedvidkova et al. 2000; Dostalova et al. 2005).

However, responses of plasma leptin to a single bout of exercise in patients suffering from AN have not been explored. We hypothesized that in underweight patients with AN, who have markedly low plasma leptin (Dostalova et al. 2005) and insulin levels (Dostalova et al. unpublished results) and increased exercise-induced adipose tissue NE and glycerol levels (Bartak et al. 2004), the production of leptin during a single bout of exercise could be altered. Therefore we decided to investigate the effect of a single bout of exercise on plasma leptin, NE, insulin and glycerol levels in patients with AN. To account for a diurnal variation of leptin and for the effect of 12 h fasting of the subjects, a control trial consisted of the same blood sampling protocol without exercise was conducted the next day after exercise.

SUBJECTS AND METHODS

Study subjects

Ten women with a restrictive type of AN (age: 22.1 ± 1.0 years; BMI: 15.7 ± 0.47 $kg.m^{-2}$, percent body fat (%BF): 7.1 ± 0.88) and fifteen healthy age-matched women (age: 21.3 ± 0.9 years; BMI: 21.2 ± 0.42 $kg.m^{-2}$, %BF: 24.3 ± 0.79) were enrolled in this study. All subjects included in the study were non-smokers, had no allergies, and had been free of medications for at least three weeks prior to the study. Professional athletes were not included in the study. Healthy women had no history of obesity or malnutrition, hypertension, gastrointestinal diseases, eating disorders or other psychiatric disorders, and had normal physical examination and electrocardiogram (ECG). Blood tests confirmed normal blood count, liver and renal functions. Patients with AN were diagnosed according to the 4th edition of the Diagnostic and Statistical Manual of Mental Disorders (American Psychiatric Association, 1994) after detailed medical and psychiatric evaluation. All patients with a restrictive type of AN were examinated after two weeks of hospitalization on the Department of Psychiatry and were clinically stable and in relatively good health, except for their eating disorder. All healthy women were studied during days 7 to 10 of their menstrual cycle, whereas all AN patients had amenorrhea. All subjects were asked to fast and drink only water on the night before the study and not to use stimulant drugs, such as caffeine, nicotine or alcohol, for 2 days before the experiment. All participants provided written informed consent prior to participating in the study, which was approved by the Human Ethic Review Committee, Institute of Endocrinology, Prague, Czech Republic, and was performed in accordance with the guidelines proposed in the Declaration of Helsinki.

Experimental procedures

Upon enrollment to the study, subjects were asked to fast (from 20:00 h) and drink only water the night prior to the study. All subjects were admitted to the Institute of Endocrinology at 07:00 h in two following days. After a short medical examination (blood pressure, heart and respiratory rates measurement, ECG), %BF was estimated by anthropometry measurement and bioimpedancy (TANITA, Japan). All subjects were then placed in supine position on a comfortable bed in a room kept at 23-25 ^{0}C. An intravenous catheter was placed in the antecubital vein, and a normal saline lock was attached. Blood collection started at least 45 min after catheter insertion and bed rest to reach steady state condition. First blood samples were collected at 08:00 h after 12 h of fasting (resting levels), than immediately after the exercise bout (at 10:00 h) and finally 90 min after the end of the exercise bout (at 11:30 h). Similarly, the next day blood samples were taken at 08:00 h, 10:00 h and 11:30 h, respectively, without the exercise stimulation. All blood samples were taken when subjects were in a supine position. For each blood draw, the first 3 ml of blood (with saline from the catheter lock) was withdrawn into a discard tube preceding a 20 ml draw. The catheter was after each draw flushed with physiological saline (3 ml) to maintain patency. On the first day, subjects underwent 45 min physical exercise on an electromagnetically braked bicycle ergometer (Cateye, Japan) at a power output of 2 $W.kg^{-1}$ of LBM. After finishing an exercise bout all subjects assumed a resting supine position on a comfortable bed for further 90 min. The metabolic rate during exercise was measured as oxygen consumption using a modified paramagnetic oxygen analyzer Spirolyt (Junkalor, Germany). ECG, heart rate and blood pressure were monitored using an Eagle 3000 cardiomonitor (Marquette, Milwaukee, WI). Blood samples for leptin assay were collected into chilled polypropylene tubes containing Na_2EDTA and antilysin. Plasma was immediately separated from whole blood by centrifugation at 3,000 rpm for 20 min at 4°C, and stored at –20 °C until being thawn and assayed.

Analytical procedures

Plasma leptin concentrations were determined by a commercial RIA (Linco Research, St.Charles, Missouri, USA). The detection limit of the assay was 0.05 $ng.ml^{-1}$, with intraassay coefficient of variations (CVs) of 5.25% and 5.97%, and interassay CVs of 8.9% and 8.67% for low (0.44 $ng.ml^{-1}$) and high (4.24 $ng.ml^{-1}$) leptin levels, respectively.

Plasma NE was assayed by high-performance liquid chromatography, using electrochemical detection after purification on alumina (Holmes et al.

1994). Plasma glycerol was measured by radiometric kit (Randox Laboratories, GY105, Montpellier, France). Plasma insulin was measured by a commercial RIA (Immunotech, Czech Republic, respectively). Sensitivity was 3.7 pmol.l^{-1} and the intra- and interassay variability was 3.4% and 4.3%, respectively. Plasma glucose levels were measured in on Cobas Integra 400 plus (Roche Diagnostics, GmbH, Mannheim). All assays were run twice in duplicate.

Data analysis

All data are presented as mean ± SEM. Data were analyzed by two-way ANOVA with factors AN and time, and using Bonferroni intervals. Tukey's interaction between factors was incorporated into the statistical model. Statistical differences between groups were analyzed by the Mann-Whitney rank-sum test. Statistical differences between the phases of the experiment were analyzed by Wilcoxon's paired test. A P-value equal to or less than 0.05 denoted statistical significance. All statistics were performed using Statgraphics Plus 3.3 (Manugistics Rockville, MA).

RESULTS

The baseline characteristics and the circulatory response of the study subjects on exercise stimulation are shown in Table 1 and Table 2, respectively. Patients with AN were extremely malnutrished as evidenced by severely decreased BMI and body fat content relative to the C group (Table 1). Baseline plasma insulin levels were significantly reduced in AN compared to the C group, whereas fasting plasma glucose did not significantly differ between the groups studied (Table 1). The oxygen consumption under basal conditions and throughout the exercise, respectively, was comparatible in the groups studied (Table 2). The resting heart rate, systolic and diastolic blood pressure were markedly lower in patients with AN than these variables in the C group (Table 2). Exercise stimulation led to significant excess in heart beat and systolic blood pressure, but not in diastolic blood pressure in both groups when compared to resting values (Table 2).

Basal and exercise-stimulated plasma leptin concentrations

Basal plasma leptin levels were markedly decreased in the AN group relative to the C group (Table 1). Plasma leptin levels measured immediately after an exercise bout and after 90 min of recovery significantly decreased in both groups compared to basal levels (Fig. 1a).

Comparision of exercise-stimulated and control trial leptin levels

When compared the exercise-stimulated to control trial plasma leptin levels, the decreases found in the C group throughout exercise (either immediately or 90 min after the end of the exercise bout), were nearly the same during control trial (Fig. 1a,b). On contrary, immediately postexercise and 90 min postexercise plasma leptin levels were significantly reduced in patients with AN compared to control trial leptin levels (Fig. 1a,b). For the C group, leptin levels declined to the same extent during the control and exercise trials. However, in the AN group, leptin concentrations only declined during the exercise trial (Fig. 1a,b).

Basal and exercise-induced plasma NE, glycerol and insulin concentrations

Basal and exercise-induced plasma NE (Fig. 2) and glycerol (Fig. 3) levels, were not significantly different between the groups. Plasma NE as well as glycerol levels significantly increased during exercise and fall to the levels conformable to those observed under basal conditions in 90 min after the end of an exercise bout (Fig. 2, 3). Basal and exercise-induced plasma insulin levels were significantly reduced in patients with AN compared to the C group (Fig. 4). In both group, insulin levels were not significantly changed immediately postexercise and 90 min postexercise compared to basal levels (Fig. 4).

Relationship of plasma leptin with other parameters

Plasma leptin levels correlated positively with BMI ($R = 0.68$, $p = 0.03$) and negatively with plasma glycerol levels ($R = - 0.82$, $p = 0.04$) in AN, but not in the C group. We did not confirm significant correlation between baseline, exercise-stimulated and post exercise leptin levels, respectively, and %BF, plasma NE, insulin and glucose levels in any group studied.

DISCUSSION

The most important finding of this study is that a single bout of moderate-intensity exercise significantly reduces plasma leptin levels in underweight patients with a restrictive type of AN, but not in healthy normal-weight women in comparison with leptin levels found in these subjects during control trial. Furthermore, basal and exercise-induced plasma NE as well as glycerol levels were not significantly different in patients with AN compared to C group. Basal and exercise-induced plasma insulin levels were significantly reduced in patients with AN compared to C group. In both groups, NE as well as glycerol levels were significantly increased and plasma insulin levels were not changed immediately postexercise compared to basal levels.

Studies that have investigated effects of short-term exercise on leptin have shown either reductions (Kraemer et al. 1999; Elias et al. 2000; Jurimae et al. 2005) or no change (Perusse et al. 1997) in leptin concentrations. In the study of Jurimae et al. (2005) relatively short term intense exercise significantly decreased plasma leptin levels and in the study of

Elias et al. (2000) acute exhaustive exercise in male volunteers led to decline in plasma leptin levels. However, a control trial was not conducted in these studies to determine whether diurnal changes accounted for observed reductions. In the study of Kraemer et al. (1999) a 30 min of exercise at 80% of VO_2max was associated with reduced leptin concentrations in postmenopausal females, but the reductions were due to the circadian rhythms of leptin as determined from control trial samples from the same subjects. Thus, reported reductions in leptin levels during a single bout of exercise could be attributed to circadian rhythms. The absence of any reduction in leptin reported in short-term exercise study may be due to the limited energy expenditure of these exercise bouts (Hilton and Loucks 2000; Kraemer et al. 2002) or the protocol of these studies excluded prolonged postexercise blood sampling. Previous studies that utilized aerobic running exercise in lean healthy men have reported delayed leptin reduction in 24 and/or 48 h after exercise, but not immediately after the exercise bout (Van Aggel-Leijssen et al. 1999; Essig et al. 2000).

A positive attribute of our study was that the effects of exercise were compared with those obtained during a control trial with the same subjects to control for diurnal chnges and effects of overnight fasting. Accounting for these factors is important for the validity of the data (Zafeiridis et al. 2003). It is well established that circulating leptin levels in humans follow a diurnal pattern, with zenith and nadir at about midnight and shortly after the morning breakfast, respectively (Schoeller et al. 1997; Van Aggel-Leijssen et al. 1999). The reduction in plasma leptin levels that we observed during exercise in the C group appears to be a continuation of the natural latenight/early morning decline in plasma leptin levels (Schoeller et al. 1997), since both exercise and control trial leptin concentrations declined in nearly the same manner. Another possibility could be that the observed decrease of plasma leptin levels in the C group is due to prolonged overnight fasting in both exercise and control trials with exercise of low energy expenditure having no effect on leptin levels (Hilton and Loucks 2000). Moreover, it is also possible that if our subjects had exercised for a longer period and/or we had measured plasma leptin levels for longer time, an exercise-induced reduction in leptin might have taken place in healthy women. Patients with AN could have altered timing of the leptin regulation in acute energy disbalance.

We did not find any reduction in plasma leptin levels during control trial in patients with AN. Balligand et al. (1998) had previously demonstrated that AN is associated with the complete lack of diurnal leptin pulsation and short-term refeeding is not sufficient to restore this abnormality. We suppose that patients with AN are adapted on a state of chronic starvation with markedly low leptin and insulin levels and prolonged fasting is not sufficient to further decrease plasma leptin levels in these patients. However, this observation does not explain the rapid decrease of plasma leptin levels after a single bout of exercise that we observed in patients with AN.

Leptin responds to the difference between intake and expenditure in healthy women (Hilton and Loucks 2000). In other words, the threshold for energy deficit (when acute exercise energy expenditure exceeds energy intake) must be reached in order for leptin levels to be suppressed by exercise. Subjects in our study underwent the exercise trial after 12 h of overnight fasting at a power output of 2 $W.kg^{-1}$ of LBM. Compared to other studies, the intensity of exercise in our study is low, but the intensity and duration of exercise had been limited by the physical condition of extremely malnutrished patients with AN. As the previous diet, meal schedule and physical activity of patients with AN had been controlled during the two week of hospitalization, the results of our study could not be influenced by before-exercise-trial fasting and/or extreme physical activity of patients with AN. However, we could not varify the unitary daily caloric intake/energy expenditure in patients with AN. The daily caloric value had been chosen individually with respect for the pre-hospitalization caloric intake and psychical attitude to food intake of the patient. But, we confirmed that all patients with AN included in the study consumed their diet as an oral meals six time a day within the usual 12 h span and adhered sedentary behavior for two weeks prior exercise trial.

To our best knowledge, there are no published data on the influnce of a single bout of exercise on plasma leptin levels in patients with AN. Previously published studies have shown that leptin decrease under the effect of exercise is independent of weight reduction (Ishii et al. 1998; Pasman et al. 1998). These studies included the training program with diet therapy or only diet therapy lasting for 6 weeks (Ishii et al. 1998) or a very low energy diet and training program for 4 months (Pasman et al. 1998). However, patients with AN are in a state of chronic, several-years-persisting, malnutrition associated with chronic metabolic and endocrine adaptation to this starvation status. Rapid exercise-induced leptin decline in patients with AN could be a part of chronic adaptation to energy deficit in these patients with many factors influencing this abnormally sensitive and prompt response to energy expenditure. This response of leptin to exercise parallels the prompt leptin increase after consumption of a standardized mixed meal (585 kcal, 32.6 g of fat, 17.6 g of protein, 50.0 g of carbohydrate) in patients with AN, not seen in healthy women (Dostalova et al. unpublished results).

Exercise has been shown to alter concentrations of certain hormones and neurotransmitters that may influence leptin concentrations (Kraemer et al. 2002;

Zafeiridis et al. 2003; Bartak et al. 2004; Stafford et al. 2005). Among these catecholamines have been shown to inhibit leptin production as well as plasma leptin levels (Pinkney et al. 1998; Couillard et al. 2002), whereas insulin has been shown to stimulate leptin (Govoni et al. 2005). Although we had initially hypothesized that the activity of the SNS and insulin could be important in the regulation of leptin during acute exercise, the finding of different exercise-induced leptin response in AN, yet there was no difference in NE and insulin responses to exercise between groups, indicates that something other than NE (SNS activity) and insulin was responsible for the different leptin responses. However, we could not exclude the possibility that chronically low insulin levels that we found in patients with AN affect the glucose uptake in the adipocyte and thus, production of leptin in adipose cells (Flier et al. 1997). Kolaczynski et al. (1996) concluded that insulin does not stimulate leptin production acutely, but they demonstrated a long-term effect of insulin on leptin production both *in vivo* and *in vitro*.

Although we failed to find differences in NE and glycerol responses to exercise between AN and C group, our group has previously found that AN is associated with *in vivo* increased basal as well as exercise-stimulated SNS activity, especially NE levels, and unchanged basal, but significantly increased exercise-stimulated lipolysis in the subcutaneous abdominal adipose tissue (Bartak et al. 2004). Regulation of lipolysis by exercise is potentially relevant to regulation of circulating leptin levels, because lipolysis and leptin production during exercise have been found to be inversely controlled (Duclos et al. 1999). Thus, we could not exclude the influence of excited activity of SNS and of increased lipolysis in the abdominal adipose tissue of patients with AN during an acute exercise (Bartak et al. 2004) on exercise leptin production in these patients.

In summary, we demonstrated that patients with AN, who have significantly decreased fasting plasma leptin levels, show a significant fall in leptin levels immediately after and 90 min after a single bout of exercise. On the other hand, the decreases in plasma leptin observed during both exercise and control trials in healthy women could be explained by prolonged overnight fasting and/or circadian variation of leptin lacking in AN. Neither NE nor insulin are the salient factors responsible for the different response of leptin to exercise in AN. Further investigation is needed to classify the particular role of altered regulation mechanisms on plasma leptin in the state of acute energy disbalance in patients with AN.

Acknowledgements

We thank Diana Riegerova for her technical assistance and Vojtech Kopsky, PhD for the proof reading of the article. This study was supported by the grant agency GA CR, No. 303/03/037

REFERENCES

Balligand JL, Brichard SM, Brichard V, Desager JP, Lambert M (1998) Hypoleptinemia in patients with anorexia nervosa: loss of circadian rhythm and unresponsiveness to short-term refeeding. Eur J Endocrinol 138:415-420

Bartak V, Vybiral S, Papezova H, Dostalova I, Pacak K, Nedvidkova J (2004) Basal and exercise-induced sympathetic nervous activity and lipolysis in adipose tissue of patients with anorexia nervosa. Eur J Clin Invest 34:371-377

Boden G, Chen X, Mozzoli M, Ryan I (1996) Effect of fasting on serum leptin in normal human subjects. J Clin Endocrinol Metab 81:3419-3423

Brichard SM, Delporte ML, Lambert M (2003) Adipocytokines in anorexia nervosa: a review focusing on leptin and adiponectin. Horm Metab Res 35:337-342

Campfield LA, Smith FJ, Gisez Y, Devos R, Burn P (1995) Recombinant mouse OB protein:evidence for a peripheral signal linking adiposity and central neural networks. Science 269:546-549

Casanueva FF, Diaguez C, Popovic V, Peino R, Considine RV, Caro JF (1997) Serum immunoreactive leptin concentrations in patients with anorexia nervosa before and after partial weight recovery. Biochemistry and Molecular Medicine 60:116-120

Chan JL, Heist K, DePaoli AM, Veldhuis JD, Mantzoros CS (2003) The role of falling leptin levels in the neuroendocrine and metabolic adaptation to short-term starvation in healthy men. J Clin Invest 111:1409-1421

Chin-Chance C, Polonsky KS, Schoeller DA (2000) Twenty-four-hour leptin levels respond to cumulative short-term energy imbalance and predict subsequent intake. J Clin Endocrinol Metab 85:2685-2691

Couillard C, Mauriege P, Prud'homme D, Nadeau A, Tremblay A, Bouchard C, Despres JP (2002) Plasma leptin response to an epinephrine infusion in lean and obese women. Obes Res 10:6-13

Doucet E, St Pierre S, Almeras N, Mauriege P, Richard D, Tremblay A (2000) Changes in energy expenditure and substrate oxidation resulting from weight loss in obese men and women: is there an important contribution of leptin? J Clin Endocrinol Metab 85:1550-1556

Duclos M, Corcuff JB, Fuffie A, Roger P, Manier G (1999) Rapid leptin decrease in immediate post-exercise recovery. Clin Endocrinol (Oxf) 50:337-342

Elias AN, Pandian MR, Wang L, Suarez E, James N, Wilson AF (2000) Leptin and IGF-I levels in unconditioned male volunteers after short-term exercise. Psychoneuroendocrinology 25:453-461

Essig DA, Alderson NL, Ferguson MA, Bartoli WP, Durstine JL (2000) Delayed effects of exercise

on the plasma leptin concentrations. Metabolism 49:395-399

Ferron F, Considine RV, Peino R, Lado IG, Diaguez C, Casanueva FF (1997) Serum leptin concentrations in patients with anorexia nervosa, bulimia nervosa and non-specific eating disorders correlate with the body mass index but are independent of the respective disease. Clinical Endocrinology 46:289-293

Fisher JS, Van Pelt RE, Zinder O, Landt M, Kohrt WM (2001) Acute exercise effect on postabsorptive serum leptin. J Appl Physiol 91(2):680-686

Flier JS (1997) Leptin expression and action: new experimental paradigms. Proc Natl Acad Sci USA 94:4242-4245

Friedman JM, Halaas JL (1998) Leptin and the regulation of the body weight in mammals. Nature 395:763-770

Govoni N, Galeati G, Castellani G, Tamanini C (2005) Leptin concentrations in plasma and follicular fluid from prepubertal gilts as influenced by fasting, refeeding and insulin. Horm Metab Res 2005 37(3):152-158

Hickey MS, Calsbeek DJ (2001) Plasma leptin and exercise: recent findings. Sports Med 31:583-589

Hilton LK, Loucks AB (2000) Low energy availability, not exercise stress, suppresses the diurnal rhythm of leptin in healthy young women. Am J Physiol Endocrinol Metab 2000 278(1):E43-49

Holmes C, Eisenhofer G, Goldstein DS (1994) Improved assay for plasma dihydroxyphenylacetic acid and other catechols using high-performance liquid chromatography with electrochemical detection. J Chromatogr B Biomed Appl 653:131-138

Hulver MW, Houmard JA (2003) Plasma leptin and exercise. Sports Med 33:473-482

Ishii T, Yamakita T, Yamagami K, Yamamoto T, Miyamoto M, Kawasaki K, Hosoi M, Yoshioka K, Sato T, Tanaka S, Fujii S (2001) Effect of exercise training on serum leptin levels in type 2 diabetic patients. Metabolism 2001 50(10):1136-1140

Jurimae J, Jurimae T (2005) Leptin repsonses to short term exercise in college level male rowers. Br J Sports Med 39:6-9

Keller P, Keller C, Steensberg A, Robinson LE, Pedersen BK (2005) Leptin gene expression and systemic levels in healthy men:effect of exercise, carbohydrate, interleukin-6, and epinephrine. J Appl Physiol 98:1805-1812

Kiely JM, Noh JH, Graewin SJ, Pitt HA, Swartz-Basile DA (2005) Altered intestinal motility in leptin-deficient obese mice. J Surg Res 124:98-103

Kolaczynski JW, Nyce MR, Considine RV, Boden G, Nolan JJ, Henry R, Mudaliar SR, Olefsky J, Caro JF (1996) Acute and chronic effects of insulin on leptin production in humans: Studies in vivo and in vitro. Diabetes 45(5):699-701

Kraemer RR, Johnson LG, Haltom R, Kraemer GR, Hebert EP, Gimpel T, Castracane VD (1999) Serum leptin concentrations in response to acute exercise in postmenopausal women with and without hormone replacement therapy. Proc Soc Exp Biol Med 221:171-177

Kraemer RR, Chu H, Castracane VD (2002) Leptin and exercise. Exp Biol Med (Maywood) 227:701-708

McMurray RG, Hackney AC (2005) Interactions of metabolic hormones, adipose tissue and exercise. Sports Med 35:393-412

Nedvidkova J, Papezova H, Haluzik M, Schreiber V (2000) Interaction between serum leptin levels and the hypothalamo-hypophyseal-thyroid axis in patients with anorexia nervosa. Endocr Res 26:219-230

Ozcelik O, Celik H, Ayar A, Serhatlioglu S, Kelestimur H (2004) Investigation of the influence of training status on the relationship between the acute exrcise and serum leptin levels in obese females. Neuro Endocrinol Lett 25:381-385

Pasman WJ, Westerterp-Plantenga MS, Saris WH (1998) The effect of exercise training on leptin levels in obese males. Am J Physiol 274(2 Pt 1):E280-286

Perusse L, Collier G, Gagnon J, Leon AS, Rao DC, Skinner JS, Wilmore JH, Nadeau A, Zimmet PZ, Bouchard C (1997) Acute and chronic effects of exercise on leptin levels in humans. J Appl Physiol 83:5-10

Pinkney JH, Coppack SW, Mohamed-Ali V (1998) Effect of isoprenaline on plasma leptin and lipolysis in humans. Clin Endocrinol 48:407-411

Racette SB, Coppack SW, landt M, Klein S (1997) Leptin production during moderate-intensity aerobic exercise. J Clin Endocrinol Metab 82:2275-2277

Rayner DV (2001) The sympathetic nervous system in white adipose tissue regulation Proc Nutr Soc 60:357-364

Ricci MR, Lee MJ, Russell CD, Wang Y, Sullivan S, Schneider SH, Brolin RE, Fried SK (2005) Isoproterenol decreases leptin release from rat and human adipose tissue through posttranscriptional mechanisms. Am J Physiol Endocrinol Metab 288:E798-804

Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, Auwerx J (1995) Transient increase in obese gene expression after food intake or insulin administration. Nature 377:527-529

Sartorio A, Agosti F, De Col A, Marinone PG, Maffiuletti NA (2004) Leptin levels do not change following acute exercise in healthy adults

and in obese children. J Endocrinol Invest 27:117-120

Schoeller DA, Cella LK, Sinha MK, Caro JF (1997) Entrainment of the diurnal rhythm of plasma leptin to meal timing. J Clin Invest 100:1882-1887.

Stafford DE (2005) Altered hypothalamic-pituitary-ovarian axis function in young female athletes: implications and recommendations for management. Treat Endocrinol 4(3):147-154

Van Aggel-Leijssen DPC, van Baak MA, Tenenbaum R, Campfield LA, Saris WHM (1999) Regulation of average 24h human plasma leptin level:the influence of exercise and physiological changes in energy balance. Int J Obes 23:151-158

Zafeiridis A, Smilios I, Considine RV, Tokmakidis SP (2003) Serum leptin responses after acute resistance exercise protocols. J Appl Physiol 94:591-597

Address all correspondence and requests for reprints to:
Jara Nedvidkova, PhD
Institute of Endocrinology
Narodni 8
11694 Prague
Czech Republic
Tel.: + 420-224905272 Fax: + 420-224905325
Email: **jnedvidkova@endo.cz**

TABLE 1 Characteristics of the study subjects

	C (n = 15)	AN (n = 10)
Age (years)	21.3 ± 0.9	22.1 ± 1.0
Height (cm)	171.9 ± 1.86	170.8 ± 1.11
Weight (kg)	62.2 ± 1.54	45.8 ± 1.89*
BMI ($kg.m^{-2}$)	21.2 ± 0.42	15.7 ± 0.47*
Body fat (%)	24.3 ± 0.79	7.1 ± 0.88*
Leptin ($ng.ml^{-1}$)	7.4 ± 0.81	1.5 ± 0.32*
NE ($pg.ml^{-1}$)	151.6 ± 18.26	177.1 ± 22.37
Glycerol ($\mu mol.l^{-1}$)	135.8 ± 17.41	139.9 ± 19.08
Insulin ($pmol.l^{-1}$)	28.3 ± 4.53	14.2 ± 3.67*
Glucose ($mmol.l^{-1}$)	4.7 ± 0.08	4.1 ± 0.11

Values are means ± S.E.M., C = control group, AN = anorexia nervosa, BMI = body mass index, n = number of probands. *$p < 0.05$ vs. C subjects.

TABLE 2 Circulatory response of the study subjects

		C (n = 15)	AN (n =10)
Heart beat ($b.min^{-1}$)	rest	83 ± 2.5	71 ± 2.9*
	exercise	$107 \pm 3.1^{+}$	$102 \pm 1.9^{+}$
Systolic blood pressure (kPa)	rest	15 ± 0.4	12 ± 0.4*
	exercise	$17 \pm 0.3^{+}$	$14 \pm 0.4^{*+}$
Diastolic blood pressure (kPa)	rest	10 ± 0.4	8 ± 0.4*
	exercise	10 ± 0.3	8 ± 0.4*
Oxygen consumption ($W.kg^{-1}$)	rest	0.8 ± 0.2	0.8 ± 0.1
	exercise	$2.26 \pm 0.2^{+}$	$2.39 \pm 0.1^{+}$

Values are means ± S.E.M., C = control group, AN = anorexia nervosa, n = number of probands. *$p < 0.05$ vs. C subjects , $^{+}$ $p < 0.05$ vs. resting values.

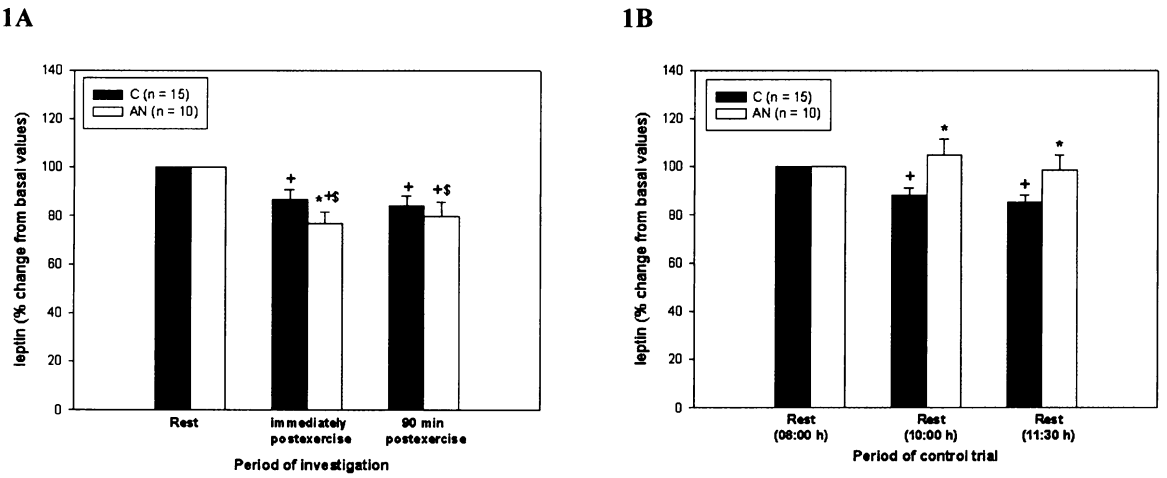


Figure 1. Changes in plasma leptin levels (%) under the effect of a single bout of exercise (2 $W.kg^{-1}$ of lean body mass; 45 min; **A**) and during the control trial **(B)** in patients with anorexia nervosa (AN, n = 10) and in healthy women (C, n = 15). Plasma leptin levels were measured at rest (08:00 h), immediately after the end of the exercise bout (10:00 h) and after 90 min of recovery (11:30 h). Values are expressed as means ± SEM. $^{+}p < 0.05$ vs. basal levels, $^{*}p < 0.05$ vs. C group, $^{\$}p < 0.05$ vs. control trial levels.

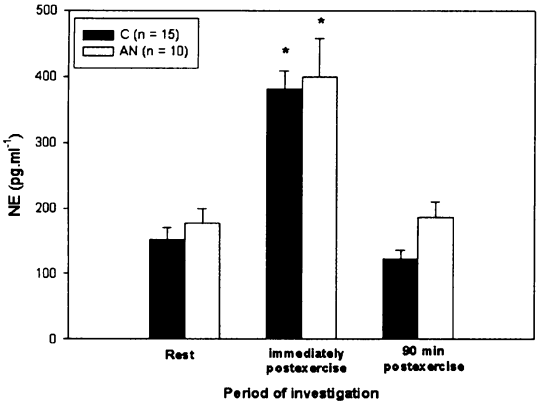


Figure 2. The effect of a single bout of exercise (2 $W.kg^{-1}$ of lean body mass; 45 min) on plasma norepinephrine (NE, $pg.ml^{-1}$) levels in patients with anorexia nervosa (AN, n = 10) and in healthy women (C, n = 15). Plasma NE levels were measured at rest (08:00 h), immediately after the end of the exercise bout (10:00 h) and after 90 min of recovery (11:30 h). Values are expressed as means ± SEM. $^{*}p < 0.05$ vs. basal levels.

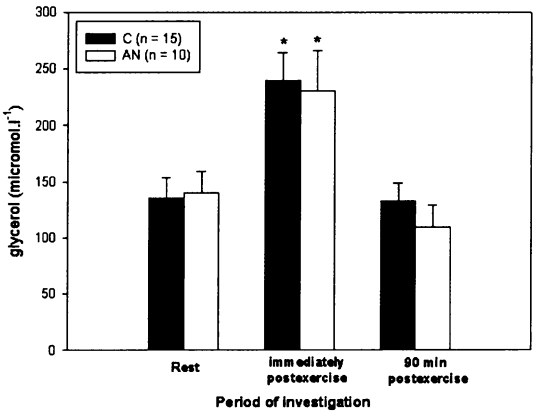


Figure 3. The effect of a single bout of exercise (2 $W.kg^{-1}$ of lean body mass; 45 min) on plasma glycerol ($micromol.l^{-1}$) levels in patients with anorexia nervosa (AN, n = 10) and in healthy women (C, n = 15). Plasma glycerol levels were measured at rest (08:00 h), immediately after the end of the exercise bout (10:00 h) and after 90 min of recovery (11:30 h). Values are expressed as means ± SEM. $^{*}p < 0.05$ vs. basal levels.

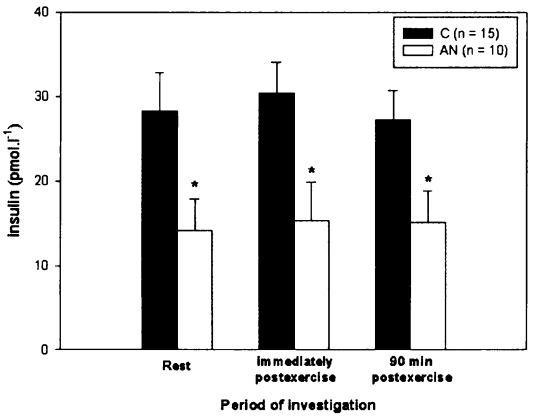


Figure 4. The effect of a single bout of exercise (2 $W.kg^{-1}$ of lean body mass; 45 min) on plasma insulin ($pmol.l^{-1}$) levels in patients with anorexia nervosa (AN, n = 10) and in healthy women (C, n = 15). Plasma insulin levels were measured at rest (08:00 h), immediately after the end of the exercise bout (10:00 h) and after 90 min of recovery (11:30 h). Values are expressed as means ± SEM. $^{*}p < 0.05$ vs. C group.

5. CONCLUSIONS

Severe malnutrition and food restriction associated with AN lead to loss of fat mass and delayed gastric emptying. The present Thesis was mainly focused on the roles of adipocytokines leptin and resistin and gastrointestinal hormone ghrelin in the adaptive response to starvation (long-term regulation of energy balance) and in the acute regulation of energy intake/expenditure (caloric- or non-caloric meal; single bout of exercise). The concentrations of leptin, resistin and ghrelin were measured in plasma of patients with a restrictive type of AN and compared with those obtained in healthy normal-weight women. Furthermore, *in vivo* concentrations of leptin and resistin in the subcutaneous abdominal adipose tissue, the site of production of these proteins, were measured in both AN and control women and compared with those found in plasma of these subjects. Before starting human studies, microdialysis had been tested and modified *in vitro* to be applicable for *in vivo* sampling of leptin and resistin from human subcutaneous adipose tissue.

The primary results of this Thesis have shown that *in vivo* microdialysis technique could be used, beside to well-known sampling of small molecules, such as glycerol, to sampling of adipocytokines leptin and resistin. Furthermore, it was pointed out that there are meaningful differences between systemic (plasma) and local (adipose tissue) levels of leptin and resistin. Plasma leptin and resistin levels were found to be significantly decreased in patients with AN, whereas leptin and resistin levels in the subcutaneous adipose tissue of these patients were found to be unchanged and increased, respectively. Moreover, the regulation of leptin as well as ghrelin in the states of acute energy disbalance, such as meal consumption and a single bout of exercise, has been found to be altered in patients with AN.

The results of the present Thesis confirm the roles of leptin, resistin and ghrelin in the pathophysiology of AN. The alterations of systemic (plasma) as well as of local (adipose tissue) levels of these proteins could play a part in the adaptive response to starvation and in the disrupted regulation of acute energy intake/expenditure of patients with AN that leads to inability of these patients to gain and mainly maintain normal weight. However, many questions about the function of these factors in human physiology as well as in the pathophysiological conditions, such as malnutrition, still remain for future experiments.

I evision that the results of this Thesis may advance the understanding of hormone-induced regulations of adipose tissue metabolism and delayed gastric emptying in patients with AN and so take a step forward to introduce more specific and effective pharmacological interventions in AN.

6. REFERENCES

Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, Flier JS. Role of leptin in the neuroendocrine response to fasting. *Nature.* 1996 382(6588):250-252.

Ahima RS, Dushay J, Flier SN, Prabakaran D, Flier JS. Leptin accelerates the onset of puberty in normal female mice. *J Clin Invest.* 1997 99(3):391-395.

Ahima RS, Flier JS. Leptin. *Annu Rev Physiol.* 2000 62:413-437.

Ahima RS, Hileman SM. Postnatal regulation of hypothalamic neuropeptide expression by leptin: implications for energy balance and body weight regulation. *Regul Pept.* 2000 92(1-3):1-7.

Ahima RS. Leptin and the neuroendocrinology of fasting. *Front Horm Res.* 2000 26:42-56.

Aparicio T, Kermorgant S, Darmoul D, Guilmeau S, Hormi K, Mahieu-Caputo D, Lehy T. Leptin and Ob-Rb receptor isoform in the human digestive tract during fetal development. *J Clin Endocrinol Metab.* 2005 90(11):6177-6184.

Ariyasu H, Takaya K, Tagami T, Ogawa Y, Hosoda K, Akamizu T, Suda M, Koh T, Natsui K, Toyooka S, Shirakami G, Usui T, Shimatsu A, Doi K, Hosoda H, Kojima M, Kangawa K, Nakao K. Stomach is a major source of circulating ghrelin, and feeding state determines plasma ghrelin-like immunoreactivity levels in humans. *J Clin Endocrinol Metab.* 2001 86(10):4753-4758.

Arvat E, Di Vito L, Broglio F, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E. Preliminary evidence that Ghrelin, the natural GH secretagogue (GHS)-receptor ligand, strongly stimulates GH secretion in humans. *J Endocrinol Invest.* 2000 23(8):493-495.

Arvat E, Maccario M, Di Vito L, Broglio F, Benso A, Gottero C, Papotti M, Muccioli G, Dieguez C, Casanueva FF, Deghenghi R, Camanni F, Ghigo E. Endocrine activities of ghrelin, a natural growth hormone secretagogue (GHS), in humans: comparison and interactions with hexarelin, a nonnatural peptidyl GHS, and GH-releasing hormone. *J Clin Endocrinol Metab.* 2001 86(3):1169-1174.

Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M. Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. *Gastroenterology.* 2001 120(2):337-345.

Ashworth CJ, Hoggard N, Thomas L, Mercer JG, Wallace JM, Lea RG. Placental leptin. *Rev Reprod.* 2000 5(1):18-24.

Audi L, Mantzoros CS, Vidal-Puig A, Vargas D, Gussinye M, Carrascosa A. Leptin in relation to resumption of menses in women with anorexia nervosa. *Mol Psychiatry.* 1998 3(6):544-547.

Balligand JL, Brichard SM, Brichard V, Desager JP, Lambert M. Hypoleptinemia in patients with anorexia nervosa: loss of circadian rhythm and unresponsiveness to short-term refeeding. *Eur J Endocrinol.* 1998 138(4):415-420.

Banerjee RR, Lazar MA. Dimerization of resistin and resistin-like molecules is determined by a single cysteine. *J Biol Chem.* 2001 276(28):25970-25973.

Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM. Leptin enters the brain by a saturable system independent of insulin. *Peptides.* 1996 17(2):305-311.

Banks WA, Niehoff ML, Martin D, Farrell CL. Leptin transport across the blood-brain barrier of the Koletsky rat is not mediated by a product of the leptin receptor gene. *Brain Res.* 2002 950(1-2):130-136.

Banks WA, Coon AB, Robinson SM, Moinuddin A, Shultz JM, Nakaoke R, Morley JE. Triglycerides induce leptin resistance at the blood-brain barrier. *Diabetes.* 2004 53(5):1253-1260.

Barkan AL, Dimaraki EV, Jessup SK, Symons KV, Ermolenko M, Jaffe CA. Ghrelin secretion in humans is sexually dimorphic, suppressed by somatostatin, and not affected by the ambient growth hormone levels. *J Clin Endocrinol Metab.* 2003 88(5):2180-2184.

Bartak V, Dostalova I, Nedvidkova J, Vybiral S, Pacak K. *In vivo* adrenergic regulation of lipolysis and plasma leptin levels in patients with anorexia nervosa during exercise. 8th Symposium on Catecholamines and Other Neurotransmitters in Stress, June-July 28-3 2003, Smolenice Castle, Slovakia, printed in Endocrine Regulations, 37(2) **2003**:87.

Bartak V, Vybiral S, Papezova H, Dostalova I, Pacak K, Nedvidkova J. Basal and exercise-induced sympathetic nervous activity and lipolysis in adipose tissue of patients with anorexia nervosa. *Eur J Clin Invest.* 2004 34:371-377

Bates SH, Myers MG Jr. The role of leptin receptor signaling in feeding and neuroendocrine function. *Trends Endocrinol Metab.* 2003 14(10):447-452.

Boden G, Chen X, Mozzoli M, Ryan I. Effect of fasting on serum leptin in normal human subjects. *J Clin Endocrinol Metab.* 1996 81(9):3419-3423.

Bowers CY. Unnatural growth hormone-releasing peptide begets natural ghrelin. *J Clin Endocrinol Metab.* 2001 86(4):1464-1469.

Brichard SM, Delporte ML, Lambert M. Adipocytokines in anorexia nervosa: a review focusing on leptin and adiponectin. *Horm Metab Res.* 2003 35(6):337-342.

Broglio F, Gottero C, Arvat E, Ghigo E. Endocrine and non-endocrine actions of ghrelin. *Horm Res.* 2003 59(3):109-117.

Callahan HS, Cummings DE, Pepe MS, Breen PA, Matthys CC, Weigle DS. Postprandial suppression of plasma ghrelin level is proportional to ingested caloric load but does not predict intermeal interval in humans. *J Clin Endocrinol Metab.* 2004 89(3):1319-1324.

Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. *Science.* 1995 269(5223):546-549.

Casanueva FF, Dieguez C, Popovic V, Peino R, Considine RV, Caro JF. Serum immunoreactive leptin concentrations in patients with anorexia nervosa before and after partial weight recovery. *Biochem Mol Med.* 1997 60(2):116-120.

Chan JL, Heist K, DePaoli AM, Veldhuis JD, Mantzoros CS. The role of falling leptin levels in the neuroendocrine and metabolic adaptation to short-term starvation in healthy men. *J Clin Invest.* 2003 111(9):1409-1421.

Chehab FF, Lim ME, Lu R. Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nat Genet.* 1996 12(3):318-320.

Chin-Chance C, Polonsky KS, Schoeller DA. Twenty-four-hour leptin levels respond to cumulative short-term energy imbalance and predict subsequent intake. *J Clin Endocrinol Metab.* 2000 85(8):2685-2691.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL, Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med.* 1996 334(5):292-295.

Coppack SW. Pro-inflammatory cytokines and adipose tissue. *Proc Nutr Soc.* 2001 60(3):349-356.

Couillard C, Mauriege P, Imbeault P, Prud'homme D, Nadeau A, Tremblay A, Bouchard C, Despres JP. Hyperleptinemia is more closely associated with adipose cell hypertrophy than with adipose tissue hyperplasia. *Int J Obes Relat Metab Disord.* 2000 24(6):782-788.

Couillard C, Mauriege P, Prud'homme D, Nadeau A, Tremblay A, Bouchard C, Despres JP. Plasma leptin response to an epinephrine infusion in lean and obese women. *Obes Res.* 2002 10(1):6-13.

Cowley MA, Smart JL, Rubinstein M, Cerdan MG, Diano S, Horvath TL, Cone RD, Low MJ. Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus. *Nature.* 2001 411(6836):480-484.

Cowley MA, Cone RD, Enriori P, Louiselle I, Williams SM, Evans AE. Electrophysiological actions of peripheral hormones on melanocortin neurons. *Ann N Y Acad Sci.* 2003 994:175-186.

Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes.* 2001 50(8):1714-1719.

Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, Purnell JQ. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. *N Engl J Med.* 2002 346(21):1623-1630.

Cummings DE, Shannon MH. Roles for ghrelin in the regulation of appetite and body weight. *Arch Surg.* 2003 138(4):389-396.

Cunningham MJ, Clifton DK, Steiner RA. Leptin's actions on the reproductive axis: perspectives and mechanisms. *Biol Reprod.* 1999 60(2):216-222.

Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. *Endocrinology.* 2000 141(11):4255-4261.

Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochem Biophys Res Commun.* 2001 280(3):904-907.

Date Y, Nakazato M, Hashiguchi S, Dezaki K, Mondal MS, Hosoda H, Kojima M, Kangawa K, Arima T, Matsuo H, Yada T, Matsukura S. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes.* 2002 51(1):124-129.

Day C. Thiazolidinediones: a new class of antidiabetic drugs. *Diabet Med.* 1999 16(3):179-192.

De Vos P, Saladin R, Auwerx J, Staels B. Induction of ob gene expression by corticosteroids is accompanied by body weight loss and reduced food intake. *J Biol Chem.* 1995 270(27):15958-15961.

Dickson SL, Luckman SM. Induction of c-fos messenger ribonucleic acid in neuropeptide Y and growth hormone (GH)-releasing factor neurons in the rat arcuate nucleus following systemic injection of the GH secretagogue, GH-releasing peptide-6. *Endocrinology.* 1997 138(2):771-777.

Dostalova I, Nedvidkova J, Bartak V, Papezova H, Alesci S, Gold PW, Pacak K. Loss of meal-induced fall in plasma ghrelin in patients with anorexia nervosa. IV. Interdisciplinary Eating Disorders Conference, March 20-22 2003, Prague, printed in *Česká a Slovenská Psychiatrie* **2003** 99(1):38-39.

Dostalova I, Pacak K, Nedvidkova J. Application of in vivo microdialysis to measure leptin concentrations in adipose tissue. *Int J Biol Macromol* **2003** 32(3-5):205-208.

Dostalova I, Kopsky V, Duskova J, Papezova H, Pacak K, Nedvidkova J. Leptin concentrations in the abdominal subcutaneous adipose tissue of patients with anorexia nervosa assessed by in vivo microdialysis. *Regul Pept* **2005** 128(1):63-68.

Dostalova I, Bartak V, Papezova H, Nedvidkova J. The effect of acute exercise on plasma leptin levels in patients with anorexia nervosa. Subm. *Eur J Appl Physiol.*

Dostalova I, Kunesova M, Duskova J, Papezova H, Nedvidkova J. Adipose tissue resistin levels in patients with anorexia nervosa. Subm. *Nutrition.*

Doucet E, Imbeault P, St-Pierre S, Almeras N, Mauriege P, Richard D, Tremblay A. Appetite after weight loss by energy restriction and a low-fat diet-exercise follow-up. *Int J Obes Relat Metab Disord.* 2000 24(7):906-914.

Drewes C, Nauck MA, Horn R, Holst J, Schmiegel W, Brabant G. A liquid mixed meal or exogenous glucagon-like peptide 1 (GLP-1) do not alter plasma leptin concentrations in healthy volunteers. *Acta Diabetol.* 1997 34(3):230-234.

Ducy P, Amling M, Takeda S, Priemel M, Schilling AF, Beil FT, Shen J, Vinson C, Rueger JM, Karsenty G. Leptin inhibits bone formation through a hypothalamic relay: a central control of bone mass. *Cell.* 2000 100(2):197-207.

Elias AN, Pandian MR, Wang L, Suarez E, James N, Wilson AF. Leptin and IGF-I levels in unconditioned male volunteers after short-term exercise. *Psychoneuroendocrinology.* 2000 25(5):453-461

Elimam A, Marcus C. Meal timing, fasting and glucocorticoids interplay in serum leptin concentrations and diurnal profile. *Eur J Endocrinol.* 2002 147(2):181-188.

Elmquist JK, Maratos-Flier E, Saper CB, Flier JS. Unraveling the central nervous system pathways underlying responses to leptin. *Nat Neurosci.* 1998 1(6):445-450.

English PJ, Ghatei MA, Malik IA, Bloom SR, Wilding JP. Food fails to suppress ghrelin levels in obese humans. *J Clin Endocrinol Metab.* 2002 87(6):2984.

Erdmann J, Lippl F, Schusdziarra V. Differential effect of protein and fat on plasma ghrelin levels in man. *Regul Pept.* 2003 116(1-3):101-107.

Erdmann J, Topsch R, Lippl F, Gussmann P, Schusdziarra V. Postprandial response of plasma ghrelin levels to various test meals in relation to food intake, plasma insulin, and glucose. *J Clin Endocrinol Metab.* 2004 89(6):3048-3054.

Essig DA, Alderson NL, Ferguson MA, Bartoli WP, Durstine JL. Delayed effects of exercise on the plasma leptin concentrations. *Metabolism* 2000 49:395-399.

Farooqi S, Rau H, Whitehead J, O'Rahilly S. ob gene mutations and human obesity. *Proc Nutr Soc.* 1998 57(3):471-475.

Faulconbridge LF, Cummings DE, Kaplan JM, Grill HJ. Hyperphagic effects of brainstem ghrelin administration. *Diabetes.* 2003 52(9):2260-2265.

Feighner SD, Tan CP, McKee KK, Palyha OC, Hreniuk DL, Pong SS, Austin CP, Figueroa D, MacNeil D, Cascieri MA, Nargund R, Bakshi R, Abramovitz M, Stocco R, Kargman S, O'Neill G, Van Der Ploeg LH, Evans J, Patchett AA, Smith RG, Howard AD. Receptor for motilin identified in the human gastrointestinal system. *Science.* 1999 284(5423):2184-2188

Flier JS, Elmquist JK. A good night's sleep: future antidote to the obesity epidemic? *Ann Intern Med.* 2004 141(11):885-886.

Fries JH. Food faddism--the dilemma of diet. *Ann Allergy.* 1977 39(4):288-289.

Fruhbeck G. Pivotal role of nitric oxide in the control of blood pressure after leptin administration *Diabetes.* 1999 48(4):903-908.

Gainsford T, Willson TA, Metcalf D, Handman E, McFarlane C, Ng A, Nicola NA, Alexander WS, Hilton DJ. Leptin can induce proliferation, differentiation, and functional activation of hemopoietic cells. *Proc Natl Acad Sci U S A.* 1996 93(25):14564-14568.

Ghigo E, Arvat E, Giordano R, Broglio F, Gianotti L, Maccario M, Bisi G, Graziani A, Papotti M, Muccioli G, Deghenghi R, Camanni F. Biologic activities of growth hormone secretagogues in humans. *Endocrine.* 2001 14(1):87-93.

Goldstone AP, Howard JK, Lord GM, Ghatei MA, Gardiner JV, Wang ZL, Wang RM, Girgis SI, Bailey CJ, Bloom SR. Leptin prevents the fall in plasma osteocalcin during starvation in male mice. *Biochem Biophys Res Commun.* 2002 295(2):475-481.

Greenman Y, Golani N, Gilad S, Yaron M, Limor R, Stern N. Ghrelin secretion is modulated in a nutrient- and gender-specific manner. *Clin Endocrinol (Oxf).* 2004 60(3):382-388.

Groschl M, Knerr I, Topf HG, Schmid P, Rascher W, Rauh M. Endocrine responses to the oral ingestion of a physiological dose of essential amino acids in humans. *J Endocrinol.* 2003 179(2):237-244.

Gualillo O, Caminos J, Blanco M, Garcia-Caballero T, Kojima M, Kangawa K, Dieguez C, Casanueva F. Ghrelin, a novel placental-derived hormone. *Endocrinology.* 2001 142(2):788-794.

Halaas JL, Gajiwala KS, Maffei M, Cohen SL, Chait BT, Rabinowitz D, Lallone RL, Burley SK, Friedman JM. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science.* 1995 269(5223):543-546.

Hamilton BS, Paglia D, Kwan AY, Deitel M. Increased obese mRNA expression in omental fat cells from massively obese humans. *Nat Med.* 1995 1(9):953-956.

Hansen TK, Dall R, Hosoda H, Kojima M, Kangawa K, Christiansen JS, Jorgensen JO. Weight loss increases circulating levels of ghrelin in human obesity. *Clin Endocrinol (Oxf).* 2002 56(2):203-206.

Havel PJ, Townsend R, Chaump L, Teff K. High-fat meals reduce 24-h circulating leptin concentrations in women. *Diabetes.* 1999 48(2):334-341.

Hebebrand J, Blum WF, Barth N, Coners H, Englaro P, Juul A, Ziegler A, Warnke A, Rascher W, Remschmidt H. Leptin levels in patients with anorexia nervosa are reduced in the acute stage and elevated upon short-term weight restoration. *Mol Psychiatry.* 1997 2(4):330-334.

Heini AF, Lara-Castro C, Schneider H, Kirk KA, Considine RV, Weinsier RL. Effect of hydrolyzed guar fiber on fasting and postprandial satiety and satiety hormones: a double-blind, placebo-controlled trial during controlled weight loss. *Int J Obes Relat Metab Disord.* 1998 22(9):906-909.

Hickey MS, Calsbeek DJ. Plasma leptin and exercise: recent findings. *Sports Med.* 2001 31(8):583-589.

Hileman SM, Pierroz DD, Masuzaki H, Bjorbaek C, El-Haschimi K, Banks WA, Flier JS. Characterizaton of short isoforms of the leptin receptor in rat cerebral microvessels and of brain uptake of leptin in mouse models of obesity. *Endocrinology.* 2002 143(3):775-783.

Holtkamp K, Mika C, Grzella I, Heer M, Pak H, Hebebrand J, Herpertz-Dahlmann B. Reproductive function during weight gain in anorexia nervosa. Leptin represents a metabolic gate to gonadotropin secretion. *J Neural Transm.* 2003 110(4):427-435.

Hosoda H, Kojima M, Matsuo H, Kangawa K. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. *Biochem Biophys Res Commun.* 2000 279(3):909-913.

Hosoda H, Kojima M, Mizushima T, Shimizu S, Kangawa K. Structural divergence of human ghrelin. Identification of multiple ghrelin-derived molecules produced by post-translational processing. *J Biol Chem.* 2003 278(1):64-70.

Hosoya M, Moriya T, Kawamata Y, Ohkubo S, Fujii R, Matsui H, Shintani Y, Fukusumi S, Habata Y, Hinuma S, Onda H, Nishimura O, Fujino M. Identification and functional characterization of a novel subtype of neuromedin U receptor. *J Biol Chem.* 2000 275(38):29528-29532.

Housova J, Anderlova K, Krizova J, Haluzikova D, Kremen J, Kumstyrova T, Papezova H, Haluzik M. Serum adiponectin and resistin concentrations in patients with restrictive and binge/purge form of anorexia nervosa and bulimia nervosa. *J Clin Endocrinol Metab.* 2005 90(3):1366-1370.

Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou M, Liu KK, McKee KK, Pong SS, Chaung LY, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJ, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta

SK, Schaeffer JM, Smith RG, Van der Ploeg LH. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science.* 1996 273(5277):974-977.

Hsu LK Epidemiology of the eating disorders. *Psychiatr Clin North Am.* 1996 19(4):681-700.

Hube F, Lietz U, Igel M, Jensen PB, Tornqvist H, Joost HG, Hauner H. Difference in leptin mRNA levels between omental and subcutaneous abdominal adipose tissue from obese humans. *Horm Metab Res.* 1996 28(12):690-693.

Ishikawa M, Kitayama J, Nagawa H. Enhanced expression of leptin and leptin receptor (OB-R) in human breast cancer. *Clin Cancer Res.* 2004 10(13):4325-4331.

Jakimiuk AJ, Skalba P, Huterski D, Tarkowski R, Haczynski J, Magoffin DA. Leptin messenger ribonucleic acid (mRNA) content in the human placenta at term: relationship to levels of leptin in cord blood and placental weight. *Gynecol Endocrinol.* 2003 17(4):311-316.

Janik JE, Curti BD, Considine RV, Rager HC, Powers GC, Alvord WG, Smith JW 2nd, Gause BL, Kopp WC. Interleukin 1 alpha increases serum leptin concentrations in humans. *J Clin Endocrinol Metab.* 1997 82(9):3084-3086.

Janke J, Engeli S, Gorzelniak K, Luft FC, Sharma AM. Resistin gene expression in human adipocytes is not related to insulin resistance. *Obes Res.* 2002 10(1):1-5.

Jurimae J, Jurimae T. Leptin repsonses to short term exercise in college level male rowers. *Br J Sports Med.* 2005 39(1):6-9.

Kalra SP, Dube MG, Pu S, Xu B, Horvath TL, Kalra PS. Interacting appetite-regulating pathways in the hypothalamic regulation of body weight. *Endocr Rev.* 1999 20(1):68-100.

Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I. Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. *Diabetes.* 2001 50(11):2438-2443.

Kanu A, Fain JN, Bahouth SW, Cowan GS Jr. Regulation of leptin release by insulin, glucocorticoids, G(i)-coupled receptor agonists, and pertussis toxin in adipocytes and adipose tissue explants from obese humans in primary culture. *Metabolism.* 2003 52(1):60-66.

Kastin AJ, Pan W. Dynamic regulation of leptin entry into brain by the blood-brain barrier. *Regul Pept.* 2000 92(1-3):37-43.

Keim NL, Stern JS, Havel PJ. Relation between circulating leptin concentrations and appetite during a prolonged, moderate energy deficit in women. *Am J Clin Nutr.* 1998 68(4):794-801.

Keller P, Keller Ch, Steensberg A, Robinson LE, Pedersen BK. Leptin gene expression and systemic levels in healthy men:effect of exercise, carbohydrate, interleukin-6, and epinephrine. *J Appl Physiol.* 2005 98:1805-1812.

Kim KH, Lee K, Moon YS, Sul HS. A cysteine-rich adipose tissue-specific secretory factor inhibits adipocyte differentiation. *J Biol Chem.* 2001 276(14):11252-11256.

Kmiec Z, Pokrywka L, Kotlarz G, Kubasik J, Szutowicz A, Mysliwski A. Effects of fasting and refeeding on serum leptin, adiponectin and free fatty acid concentrations in young and old male rats. *Gerontology.* 2005 51(6):357-362.

Knerr I, Groschl M, Rascher W, Rauh M." Endocrine effects of food intake: insulin, ghrelin, and leptin responses to a single bolus of essential amino acids in humans. *Ann Nutr Metab.* 2003 47(6):312-318.

Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature.* 1999 402(6762):656-660.

Kojima M, Haruno R, Nakazato M, Date Y, Murakami N, Hanada R, Matsuo H, Kangawa K. Purification and identification of neuromedin U as an endogenous ligand for an orphan receptor GPR66 (FM3). *Biochem Biophys Res Commun.* 2000 276(2):435-438.

Kolaczynski JW, Nyce MR, Considine RV, Boden G, Nolan JJ, Henry R, Mudaliar SR, Olefsky J, Caro JF. Acute and chronic effects of insulin on leptin production in humans: Studies in vivo and in vitro. *Diabetes.* 1996 45(5):699-701.

Korbonits M, Trainer PJ, Little JA, Edwards R, Kopelman PG, Besser GM, Svec F, Grossman AB. Leptin levels do not change acutely with food administration in normal or obese subjects, but are negatively correlated with pituitary-adrenal activity. *Clin Endocrinol (Oxf).* 1997 46(6):751-757.

Korbonits M, Bustin SA, Kojima M, Jordan S, Adams EF, Lowe DG, Kangawa K, Grossman AB. The expression of the growth hormone secretagogue receptor ligand ghrelin in normal and abnormal human pituitary and other neuroendocrine tumors. *J Clin Endocrinol Metab.* 2001 86(2):881-887.

Kratzsch J, Lammert A, Bottner A, Seidel B, Mueller G, Thiery J, Hebebrand J, Kiess W. Circulating soluble leptin receptor and free leptin index during childhood, puberty, and adolescence. *J Clin Endocrinol Metab.* 2002 87(10):4587-4594.

Kristensen P, Judge ME, Thim L, Ribel U, Christjansen KN, Wulff BS, Clausen JT, Jensen PB, Madsen OD, Vrang N, Larsen PJ, Hastrup S. Hypothalamic CART is a new anorectic peptide regulated by leptin. *Nature.* 1998 393(6680):72-76.

Krizova J, Papezova H, Haluzikova D, Parizkova J, Jiskra J, Kotrlikova E, Haas T, Haluzik M. Soluble leptin receptor levels in patients with anorexia nervosa. *Endocr Res.* 2002 28(3):199-205.

LaPaglia N, Steiner J, Kirsteins L, Emanuele M, Emanuele N. Leptin alters the response of the growth hormone releasing factor- growth hormone--insulin-like growth factor-I axis to fasting. *J Endocrinol.* 1998 159(1):79-83.

Lawrence CB, Snape AC, Baudoin FM, Luckman SM. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. *Endocrinology.* 2002 143(1):155-162.

Lee GH, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JI, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature.* 1996 379(6566):632-635.

Li J, Yu X, Pan W, Unger RH. Gene expression profile of rat adipose tissue at the onset of high-fat-diet obesity. *Am J Physiol Endocrinol Metab.* 2002 282(6):E1334-1341.

Licinio J, Mantzoros C, Negrao AB, Cizza G, Wong ML, Bongiorno PB, Chrousos GP, Karp B, Allen C, Flier JS, Gold PW. Human leptin levels are pulsatile and inversely related to pituitary-adrenal function. *Nat Med.* 1997 3(5):575-579.

Lin L, Martin R, Schaffhauser AO, York DA. Acute changes in the response to peripheral leptin with alteration in the diet composition. *Am J Physiol Regul Integr Comp Physiol.* 2001 280(2):R504-509.

Lu S, Guan JL, Wang QP, Uehara K, Yamada S, Goto N, Date Y, Nakazato M, Kojima M, Kangawa K, Shioda S. Immunocytochemical observation of ghrelin-containing neurons in the rat arcuate nucleus. *Neurosci Lett.* 2002 321(3):157-160.

Machinal F, Dieudonne MN, Leneveu MC, Pecquery R, Giudicelli Y. In vivo and in vitro ob gene expression and leptin secretion in rat adipocytes: evidence for a regional specific regulation by sex steroid hormones. *Endocrinology.* 1999 140(4):1567-1574.

Machinal-Quelin F, Dieudonne MN, Pecquery R, Leneveu MC, Giudicelli Y. Direct in vitro effects of androgens and estrogens on ob gene expression and leptin secretion in human adipose tissue. *Endocrine.* 2002 18(2):179-184.

Mantzoros C, Flier JS, Lesem MD, Brewerton TD, Jimerson DC. Cerebrospinal fluid leptin in anorexia nervosa: correlation with nutritional status and potential role in resistance to weight gain. *J Clin Endocrinol Metab.* 1997 82(6):1845-1851.

Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M, Kangawa K. Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun.* 2000 276(3):905-908.

McKee KK, Tan CP, Palyha OC, Liu J, Feighner SD, Hreniuk DL, Smith RG, Howard AD, Van der Ploeg LH. Cloning and characterization of two human G protein-coupled receptor genes (GPR38 and GPR39) related to the growth hormone secretagogue and neurotensin receptors. *Genomics.* 1997 46(3):426-434.

McTernan PG, McTernan CL, Chetty R, Jenner K, Fisher FM, Lauer MN, Crocker J, Barnett AH, Kumar S. Increased resistin gene and protein expression in human abdominal adipose tissue. *J Clin Endocrinol Metab.* 2002 87(5):2407-2410.

Misra M, Miller KK, Almazan C, Ramaswamy K, Aggarwal A, Herzog DB, Neubauer G, Breu J, Klibanski A. Hormonal and body composition predictors of soluble leptin receptor, leptin, and free leptin index in adolescent girls with anorexia nervosa and controls and relation to insulin sensitivity. *J Clin Endocrinol Metab.* 2004 89(7):3486-3495.

Mix H, Widjaja A, Jandl O, Cornberg M, Kaul A, Goke M, Beil W, Kuske M, Brabant G, Manns MP, Wagner S. Expression of leptin and leptin receptor isoforms in the human stomach. *Gut.* 2000 47(4):481-486.

Montague CT, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S. Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature.* 1997 387(6636):903-908.

Monteleone P, Fabrazzo M, Tortorella A, Fuschino A, Maj M. Opposite modifications in circulating leptin and soluble leptin receptor across the eating disorder spectrum. *Mol Psychiatry.* 2002 7(6):641-646.

Mori K, Yoshimoto A, Takaya K, Hosoda K, Ariyasu H, Yahata K, Mukoyama M, Sugawara A, Hosoda H, Kojima M, Kangawa K, Nakao K. Kidney produces a novel acylated peptide, ghrelin. *FEBS Lett.* 2000 486(3):213-216.

Moschos S, Chan JL, Mantzoros CS. Leptin and reproduction: a review. *Fertil Steril.* 2002 77(3):433-444.

Myers MG Jr. Leptin receptor signaling and the regulation of mammalian physiology. *Recent Prog Horm Res.* 2004 59:287-304.

Nagaev I, Smith U. Insulin resistance and type 2 diabetes are not related to resistin expression in human fat cells or skeletal muscle. *Biochem Biophys Res Commun.* 2001 285(2):561-4.

Nagaya N, Uematsu M, Kojima M, Date Y, Nakazato M, Okumura H, Hosoda H, Shimizu W, Yamagishi M, Oya H, Koh H, Yutani C, Kangawa K. Elevated circulating level of ghrelin in cachexia associated with chronic heart failure: relationships between ghrelin and anabolic/catabolic factors. *Circulation.* 2001 104(17):2034-2038.

Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature.* 2001 409(6817):194-198.

Nedvidkova J, Papezova H, Haluzik M, Schreiber V. Interaction between serum leptin levels and hypothalamo-hypophyseal-thyroid axis in patients with anorexia nervosa. *Endocr Res.* 2000 26(2):219-230.

Nedvidkova J, Krykorkova I, Papezova H, Sedlak J, Pacak K. Ghrelin levels in adipose tissue and plasma in patients with anorexia nervosa. 10th Meeting of the European Neuroendocrine Association ENEA 2002, September 12-14 **2002**, Munich.

Nedvidkova J, Krykorkova I, Bartak V, Papezova H, Gold PW, Alesci S, Pacak K. Loss of meal-induced decrease in plasma ghrelin levels in patients with anorexia nervosa. *J Clin Endocrinol Metab.* **2003** 88(4):1678-1682.

Nedvidkova J, Dostalova I, Papezova H, Hainer V. Plasma ghrelin levels in anorexia nervosa: effect of meal. 13th European Congress on Obesity ECO 2004, May 26-29 2004, Prague, printed in *Int J Obes Relat Metab Disord* **2004a** 28(S1).

Nedvidkova J, Dostalova I, Papezova H, Marek J, Pacak K. Increased subcutaneous adipose tissue resistin levels in anorexia nervosa assessed by *in vivo* microdialysis. The Endocrine Society's 86th Annual Meeting ENDO 2004, June 16-19 **2004b**, New Orleans.

Olive JL, Miller GD. Differential effects of maximal- and moderate-intensity runs on plasma leptin in healthy trained subjects. *Nutrition.* 2001 17(5):365-369.

Otto B, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, Heiman ML, Lehnert P, Fichter M, Tschop M. Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. *Eur J Endocrinol.* 2001 145(5):669-673.

Otto B, Spranger J, Benoit SC, Clegg DJ, Tschop MH. The many faces of ghrelin: new perspectives for nutrition research? *Br J Nutr.* 2005 93(6):765-771.

Ozata M, Uckaya G, Beyhan Z, Ozdemir IC. Plasma leptin levels in male patients with idiopathic central diabetes insipidus. *J Endocrinol Invest.* 1999 22(6):451-454.

Ozcelik O, Celik H, Ayar A, Serhatlioglu S, Kelestimur H. Invetsigation of the influence of training status on the relationship between the acute exrcise and serum leptin levels in obese females. Neuro *Endocrinol Lett.* 2004 25(5):381-385

Patel L, Buckels AC, Kinghorn IJ, Murdock PR, Holbrook JD, Plumpton C, Macphee CH, Smith SA. Resistin is expressed in human macrophages and directly regulated by PPAR gamma activators. *Biochem Biophys Res Commun.* 2003 300(2):472-476.

Peino R, Baldelli R, Rodriguez-Garcia J, Rodriguez-Segade S, Kojima M, Kangawa K, Arvat E, Ghigo E, Dieguez C, Casanueva FF. Ghrelin-induced growth hormone secretion in humans. *Eur J Endocrinol.* 2000 143(6):R11-14.

Pelleymounter MA, Cullen MJ, Baker MB, Hecht R, Winters D, Boone T, Collins F. Effects of the obese gene product on body weight regulation in ob/ob mice. *Science.* 1995 269(5223):540-543.

Perusse L, Collier G, Gagnon J, Leon AS, Rao DC, Skinner JS, Wilmore JH, Nadeau A, Zimmet PZ, Bouchard C. Acute and chronic effects of exercise on leptin levels in humans. *J Appl Physiol.* 1997 83(1):5-10.

Pinkney JH, Coppack SW, Mohamed-Ali V. Effect of isoprenaline on plasma leptin and lipolysis in humans. *Clin Endocrinol (Oxf).* 1998 48(4):407-411.

Popovic V, Damjanovic S, Dieguez C, Casanueva FF. Leptin and the pituitary. *Pituitary.* 2001 4(1-2):7-14.

Racette SB, Coppack SW, Landt M, Klein S. Leptin production during moderate-intensity aerobic exercise. *J Clin Endocrinol Metab.* 1997 82(7):2275-2277.

Rajala MW, Lin Y, Ranalletta M, Yang XM, Qian H, Gingerich R, Barzilai N, Scherer PE. Cell type-specific expression and coregulation of murine resistin and resistin-like molecule-alpha in adipose tissue. *Mol Endocrinol.* 2002 16(8):1920-1930.

Rangwala SM, Lazar MA. Transcriptional control of adipogenesis. *Annu Rev Nutr.* 2000 20:535-559.

Reginato MJ, Lazar MA. Mechanisms by which thiazolidinediones enhance insulin action. *Trends Endocrinol Metab.* 1999 10(1):9-13.

Rentsch J, Chiesi M. Regulation of ob gene mRNA levels in cultured adipocytes. *FEBS Lett.* 1996 379(1):55-59.

Rindi G, Torsello A, Locatelli V, Solcia E. Ghrelin expression and actions: a novel peptide for an old cell type of the diffuse endocrine system. *Exp Biol Med (Maywood).* 2004 229(10):1007-1016.

Romon M, Lebel P, Velly C, Marecaux N, Fruchart JC, Dallongeville J. Leptin response to carbohydrate or fat meal and association with subsequent satiety and energy intake. *Am J Physiol.* 1999 277(5 Pt 1):E855-861.

Rosenbaum M, Nicolson M, Hirsch J, Heymsfield SB, Gallagher D, Chu F, Leibel RL. Effects of gender, body composition, and menopause on plasma concentrations of leptin. *J Clin Endocrinol Metab.* 1996 81(9):3424-3427.

Rosenbaum M, Murphy EM, Heymsfield SB, Matthews DE, Leibel RL. Low dose leptin administration reverses effects of sustained weight-reduction on energy expenditure and circulating concentrations of thyroid hormones. *J Clin Endocrinol Metab.* 2002 87(5):2391-2394.

Saad MF, Damani S, Gingerich RL, Riad-Gabriel MG, Khan A, Boyadjian R, Jinagouda SD, el-Tawil K, Rude RK, Kamdar V. Sexual dimorphism in plasma leptin concentration. *J Clin Endocrinol Metab.* 1997 82(2):579-584.

Sahu A. Evidence suggesting that galanin (GAL), melanin-concentrating hormone (MCH), neurotensin (NT), proopiomelanocortin (POMC) and neuropeptide Y (NPY) are targets of leptin signaling in the hypothalamus. *Endocrinology.* 1998 139(2):795-798.

Saladin R, De Vos P, Guerre-Millo M, Leturque A, Girard J, Staels B, Auwerx J. Transient increase in obese gene expression after food intake or insulin administration. *Nature.* 1995 377(6549):527-529.

Sarraf P, Frederich RC, Turner EM, Ma G, Jaskowiak NT, Rivet DJ 3rd, Flier JS, Lowell BB, Fraker DL, Alexander HR. Multiple cytokines and acute inflammation raise mouse leptin levels: potential role in inflammatory anorexia. *J Exp Med.* 1997 185(1):171-175.

Savage DB, Sewter CP, Klenk ES, Segal DG, Vidal-Puig A, Considine RV, O'Rahilly S. Resistin / Fizz3 expression in relation to obesity and peroxisome proliferator-activated receptor-gamma action in humans. *Diabetes.* 2001 50(10):2199-2202.

Sharp CW, Freeman CP. The medical complications of anorexia nervosa. *Br J Psychiatry.* 1993 162:452-462.

Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukura S. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *J Clin Endocrinol Metab.* 2002 87(1):240-244.

Shimizu Y, Nagaya N, Isobe T, Imazu M, Okumura H, Hosoda H, Kojima M, Kangawa K, Kohno N. Increased plasma ghrelin level in lung cancer cachexia. *Clin Cancer Res.* 2003 9(2):774-778.

Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K. Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. *Diabetes.* 2001 50(2):227-232.

Shuto Y, Shibasaki T, Wada K, Parhar I, Kamegai J, Sugihara H, Oikawa S, Wakabayashi I. Generation of polyclonal antiserum against the growth hormone secretagogue receptor (GHS-R): evidence that the GHS-R exists in the hypothalamus, pituitary and stomach of rats. *Life Sci.* 2001 68(9):991-996.

Schoeller DA, Cella LK, Sinha MK, Caro JF. Entrainment of the diurnal rhythm of plasma leptin to meal timing. *J Clin Invest.* 1997 100(7):1882-1887.

Schwartz MW, Seeley RJ, Woods SC, Weigle DS, Campfield LA, Burn P, Baskin DG Leptin increases hypothalamic pro-opiomelanocortin mRNA expression in the rostral arcuate nucleus. *Diabetes.* 1997 46(12):2119-2123.

Schwartz MW, Woods SC, Porte D Jr, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature.* 2000 404(6778):661-671.

Sierra-Honigmann MR, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR. Biological action of leptin as an angiogenic factor. *Science.* 1998 281(5383):1683-1686.

Simon C, Gronfier C, Schlienger JL, Brandenberger G. Circadian and ultradian variations of leptin in normal man under continuous enteral nutrition: relationship to sleep and body temperature. *J Clin Endocrinol Metab.* 1998 83(6):1893-1899.

Sinha MK, Sturis J, Ohannesian J, Magosin S, Stephens T, Heiman ML, Polonsky KS, Caro JF. Ultradian oscillations of leptin secretion in humans. *Biochem Biophys Res Commun.* 1996 228(3):733-738.

Skilton MR, Nakhla S, Sieveking DP, Caterson ID, Celermajer DS. Pathophysiological levels of the obesity related peptides resistin and ghrelin increase adhesion molecule expression on human vascular endothelial cells. *Clin Exp Pharmacol Physiol.* 2005 32(10):839-844.

Slieker LJ, Sloop KW, Surface PL, Kriauciunas A, LaQuier F, Manetta J, Bue-Valleskey J, Stephens TW. Regulation of expression of ob mRNA and protein by glucocorticoids and cAMP. *J Biol Chem.* 1996 271(10):5301-5304.

Solberg R, Aas V, Thoresen GH, Kase ET, Drevon CA, Rustan AC, Reseland JE. Leptin expression in human primary skeletal muscle cells is reduced during differentiation. *J Cell Biochem.* 2005 96(1):89-96.

Spiegelman BM, Flier JS. Obesity and the regulation of energy balance. *Cell.* 2001 104(4):531-543.

Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. *Nature.* 2001 409(6818):307-312.

Steppan CM, Lazar MA. Resistin and obesity-associated insulin resistance. *Trends Endocrinol Metab.* 2002 13(1):18-23.

Stoving RK, Vinten J, Handberg A, Ebbesen EN, Hangaard J, Hansen-Nord M, Kristiansen J, Hagen C. Diurnal variation of the serum leptin concentration in patients with anorexia nervosa. *Clin Endocrinol (Oxf).* 1998 48(6):761-768.

Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K, Terashima Y. A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. *Biochem Biophys Res Commun.* 2002 295(2):255-260.

Tacke F, Brabant G, Kruck E, Horn R, Schoffski P, Hecker H, Manns MP, Trautwein C. Ghrelin in chronic liver disease. *J Hepatol.* 2003 38(4):447-454.

Tanaka M, Naruo T, Yasuhara D, Tatebe Y, Nagai N, Shiiya T, Nakazato M, Matsukura S, Nozoe S. Fasting plasma ghrelin levels in subtypes of anorexia nervosa. *Psychoneuroendocrinology.* 2003a 28(7):829-835.

Tanaka M, Naruo T, Nagai N, Kuroki N, Shiiya T, Nakazato M, Matsukura S, Nozoe S. Habitual binge/purge behavior influences circulating ghrelin levels in eating disorders. *J Psychiatr Res.* 2003b 37(1):17-22.

Tannenbaum GS, Lapointe M, Beaudet A, Howard AD. Expression of growth hormone secretagogue-receptors by growth hormone-releasing hormone neurons in the mediobasal hypothalamus. *Endocrinology.* 1998 139(10):4420-4423.

Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI. Identification and expression cloning of a leptin receptor, OB-R. *Cell.* 1995 83(7):1263-1271.

Tolle V, Zizzari P, Tomasetto C, Rio MC, Epelbaum J, Bluet-Pajot MT. In vivo and in vitro effects of ghrelin/motilin-related peptide on growth hormone secretion in the rat. *Neuroendocrinology.* 2001 73(1):54-61.

Tolle V, Bassant MH, Zizzari P, Poindessous-Jazat F, Tomasetto C, Epelbaum J, Bluet-Pajot MT. Ultradian rhythmicity of ghrelin secretion in relation with GH, feeding behavior, and sleep-wake patterns in rats. *Endocrinology.* 2002 143(4):1353-1361.

Tolle V, Kadem M, Bluet-Pajot MT, Frere D, Foulon C, Bossu C, Dardennes R, Mounier C, Zizzari P, Lang F, Epelbaum J, Estour B. Balance in ghrelin and leptin plasma levels in anorexia nervosa patients and constitutionally thin women. *J Clin Endocrinol Metab.* 2003 88(1):109-116.

Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. *Nature.* 2000 407(6806):908-913.

Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML. Circulating ghrelin levels are decreased in human obesity. *Diabetes.* 2001a 50(4):707-709.

Tschop M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, Folwaczny C. Post-prandial decrease of circulating human ghrelin levels. *J Endocrinol Invest.* 2001b 24(6):RC19-21.

Van Aggel-Leijssen DPC, van Baak MA, Tenenbaum R, Campfield LA, Saris WHM. Regulation of average 24h human plasma leptin level:the influence of exercise and physiological changes in energy balance. *Int J Obes.* 1999 23:151-158.

Van Harmelen V, Reynisdottir S, Eriksson P, Thorne A, Hoffstedt J, Lonnqvist F, Arner P. Leptin secretion from subcutaneous and visceral adipose tissue in women. *Diabetes.* 1998 47(6):913-917.

Warren MP, Vande Wiele RL. Clinical and metabolic features of anorexia nervosa. *Am J Obstet Gynecol.* 1973 117(3):435-449.

Weigle DS, Duell PB, Connor WE, Steiner RA, Soules MR, Kuijper JL. Effect of fasting, refeeding, and dietary fat restriction on plasma leptin levels. *J Clin Endocrinol Metab.* 1997 82(2):561-565.

Weigle DS, Cummings DE, Newby PD, Breen PA, Frayo RS, Matthys CC, Callahan HS, Purnell JQ. Roles of leptin and ghrelin in the loss of body weight caused by a low fat, high carbohydrate diet. *J Clin Endocrinol Metab.* 2003 88(4):1577-1586.

Willesen MG, Kristensen P, Romer J. Co-localization of growth hormone secretagogue receptor and NPY mRNA in the arcuate nucleus of the rat. *Neuroendocrinology.* 1999 70(5):306-316.

Wisse BE, Frayo RS, Schwartz MW, Cummings DE. Reversal of cancer anorexia by blockade of central melanocortin receptors in rats. *Endocrinology.* 2001 142(8):3292-3301.

Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DG, Ghatei MA, Bloom SR. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology.* 2000 141(11):4325-4328.

Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR. Ghrelin causes hyperphagia and obesity in rats. *Diabetes.* 2001 50(11):2540-2547.

Yoshimoto A, Mori K, Sugawara A, Mukoyama M, Yahata K, Suganami T, Takaya K, Hosoda H, Kojima M, Kangawa K, Nakao K. Plasma ghrelin and desacyl ghrelin concentrations in renal failure. *J Am Soc Nephrol.* 2002 13(11):2748-2752.

Zafeiridis A, Smilios I, Considine RV, Tokmakidis SP. Serum leptin responses after acute resistance exercise protocols. *J Appl Physiol.* 2003 94:591-597.

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(6505):425-432.

7. THE LIST OF ALL PUBLICATIONS OF THE AUTHOR

Original articles in impacted journals

Dostalova I, Kopsky V, Duskova J, Papezova H, Pacak K, Nedvidkova J. Leptin concentrations in the abdominal subcutaneous adipose tissue of patients with anorexia nervosa assessed by in vivo microdialysis. *Regul Pept.* 2005 128(1):63-68.

Nedvidkova J, **Dostalova I**, Bartak V, Papezova H, Pacak K. Increased subcutaneous abdominal tissue norepinephrine levels in patients with anorexia nervosa: an in vivo microdialysis study. *Physiol Res.* 2004 53(4):409-413.

Nedvidkova J, Haluzik M, Bartak V, **Dostalova I**, Vlcek P, Racek P, Taus M, Behanova M, Svacina S, Alesci S, Pacak K. Changes of noradrenergic activity and lipolysis in the subcutaneous abdominal adipose tissue of hypo- and hyperthyroid patients: an in vivo microdialysis study. *Ann N Y Acad Sci.* 2004 1018:541-549.

Bartak V, Vybiral S, Papezova H, **Dostalova I**, Pacak K, Nedvidkova J. Basal and exercise-induced sympathetic nervous activity and lipolysis in adipose tissue of patients with anorexia nervosa. *Eur J Clin Invest.* 2004 34(5):371-377.

Haluzik M, Nedvidkova J, Bartak V, **Dostalova I**, Vlcek P, Racek P, Taus M, Svacina S, Alesci S, Pacak K. Effects of hypo- and hyperthyroidism on noradrenergic activity and glycerol concentrations in human subcutaneous abdominal adipose tissue assessed with microdialysis. *J Clin Endocrinol Metab.* 2003 88(12):5605-5608.

Dostalova I, Pacak K, Nedvidkova J. Application of in vivo microdialysis to measure leptin concentrations in adipose tissue. *Int J Biol Macromol.* 2003 32(3-5):205-208.

Nedvidkova J, **Krykorkova I**, Bartak V, Papezova H, Gold PW, Alesci S, Pacak K. Loss of meal-induced decrease in plasma ghrelin levels in patients with anorexia nervosa. *J Clin Endocrinol Metab.* 2003 88(4):1678-1682.

Reviews

Krykorkova I, Nedvidkova J. Recently discovered hormones with a role in energy homeostasis. *Cas Lek Cesk.* 2003 142(2):80-83.

Dostalova I, Nedvidkova J. Leptin and ghrelin: alterations in anorexia nervosa. Subm. *Physiol Res.*

Articles submitted to press

Dostalova I, Kunesova M, Duskova J, Papezova H, Nedvidkova J. Adipose tissue resistin levels in patients with anorexia nervosa. Subm. *Nutrition.*

Dostalova I, Bartak V, Papezova H, Nedvidkova J. The effect of acute exercise on plasma leptin levels in patients with anorexia nervosa. Subm. *Eur J Appl Physiol.*

Smitka K, **Dostalova I**, Papezova H, Kvasnickova H, Nedvidkova J. The altered response of ghrelin and growth hormone on exercise in bulimia nervosa patients. Subm. *J Clin Endocrinol Metab.*

Abstracts

21 abstracts

APPENDIX - microdialysis

Figure I. The *in vivo* microdialysis trial in healthy control woman. The microdialysis catheter (on photo CMA/60 type of catheter with 30 mm membrane length and 20 kDa cut-off) is inserted subcutaneously under sterile conditions after local anesthesia with 0.1% lidocain. Catheter is perfused with sterile Ringer solution using a portable pump (CMA/107). A constant perfusion rate of 2 µl/min is maintained during the trial.

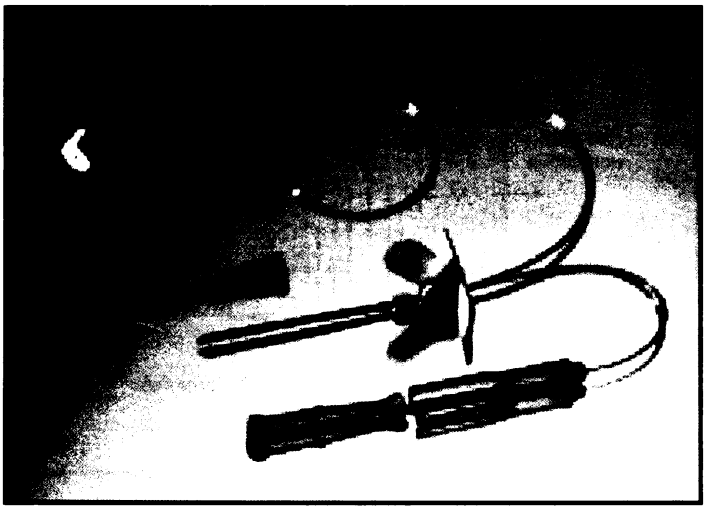
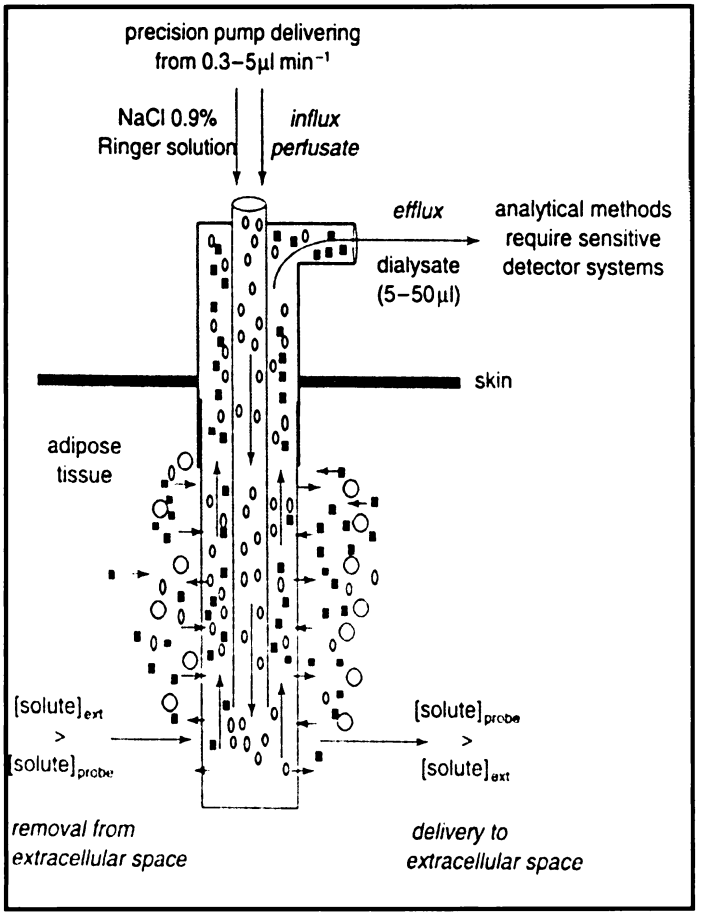


Figure II. The detail of CMA/60 microdialysis catheter.

APPENDIX - microdialysis



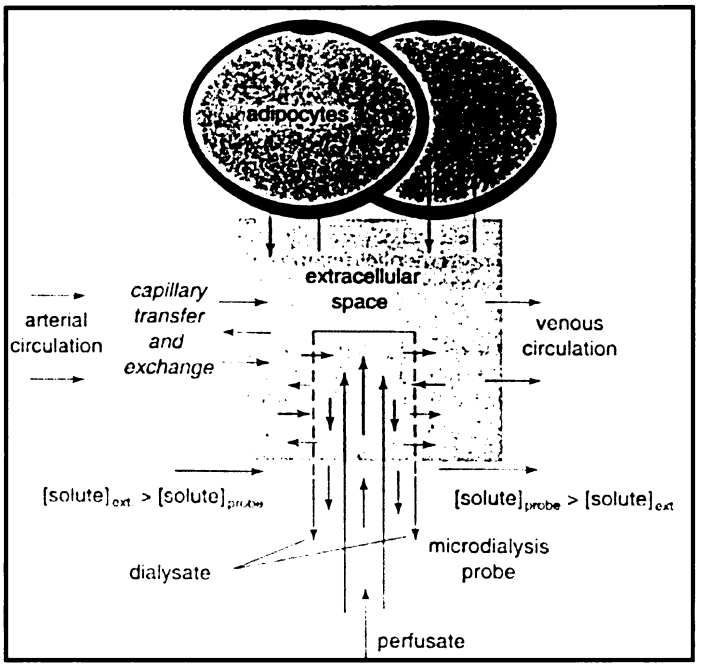


Figure III A, B. The principle of function of the microdialysis probe.