# Univerzita Karlova v Praze

## 1. lékařská fakulta

**Autoreferát disertační práce**

**PERORÁLNÍ PODÁNÍ ACIPIMOXU BĚHEM FYZICKÉ ZÁTĚŽE ZPŮSOBUJE NEGATIVNÍ ZPĚTNOVAZEBNÝ MECHANISMUS RŮSTOVÉHO HORMONU NA SEKRECI GHRELINU U PACIENTEK S MENTÁLNÍ BULIMIÍ A ZDRAVÝCH ŽEN: ÚLOHA LIPOLÝZY**

**ACIPIMOX DURING SHORT-TERM EXERCISE EXERTS A NEGATIVE FEEDBACK OF GROWTH HORMONE ON GHRELIN SECRETION IN PATIENTS WITH BULIMIA NERVOSA AND IN HEALTHY WOMEN: THE ROLE OF LIPOLYSIS**

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Disertační práce bude nejméně pět pracovních dnů před konáním obhajoby zveřejněna k nahlížení veřejnosti v tištěné podobě na Oddělení pro vědeckou činnost a zahraniční styky Děkanátu 1. lékařské fakulty.

## ABSTRAKT

**Název: Perorální podání acipimoxu během fyzické zátěže způsobuje negativní zpětnovazebný mechanismus růstového hormonu na sekreci ghrelinu u pacientek s mentální bulimií a zdravých žen: Úloha lipolýzy**

**Úvod**: Poruchy příjmu potravy, ke kterým patří mentální bulimie (MB) a mentální anorexie, jsou charakterizovány abnormálním jídelním chováním. Hlavními rysy u MB jsou opakované záchvaty přejídání a nepřiměřené kompenzační způsoby ve snaze zabránit váhovému přírůstku. Orexigenní peptid ghrelin produkovaný žaludkem působí jako sekretagog růstového hormonu (STH). Potenciální zpětnovazebný mechanismus STH a ghrelinu mezi žaludkem a hypofýzou byl nedávno zaznamenán. Acipimox (Aci), analog kyseliny nikotinové, inhibuje lipolýzu v tukové tkáni (TT) a tím snižuje plazmatické hladiny glycerolu a volných mastných kyselin. Fyzická zátěž a Aci jsou stimulátory sekrece STH. Předpokládáme, že negativní zpětnovazebný mechanismus ze zvýšených hladin STH během zátěže může způsobovat snížené hladiny ghrelinu. Domníváme se, že u pacientek s MB odlišná funkce sympatické nervové aktivity za bazálních podmínek a po fyzické zátěži může přispívat ke zvýšené lipolýze, metabolickému rozvratu a abnormálnímu metabolismu TT. Porušená signalizace mezi trávicí soustavou, centrálním nervovým systémem a TT se může podílet na patogeneze MB. **Cíle**: Cílem studie bylo vyhodnotit plazmatické hladiny STH a ghrelinu za bazálních podmínek a během fyzické zátěže bez nebo po perorálním podání Aci u pacientek s MB a zdravých žen. Současně jsme stanovili plazmatické hladiny volných mastných kyselin a glycerolu a *in situ* a *in vivo* jsme sledovali extracelulární koncentrace glycerolu v podkožní (sc) abdominální TT za bazálních podmínek a během fyzické zátěže bez nebo po perorálním podání Aci. **Metodika**: Zkoumali jsme odpovědi plazmatického STH, ghrelinu, volných mastných kyselin, glycerolu a dialyzovaného glycerolu u pacientek s MB a zdravých žen (ZŽ) během fyzické zátěže po perorálním podání antilipolytického Aci nebo placeba. Sedm ZŽ a sedm pacientek s MB bylo zahrnuto do randomizované, placebem kontrolované, jednoduše zaslepené studie. Perorální užití Aci nebo placeba bylo 60 minut před fyzickou zátěží (45 minut, 2W/kg aktivní tělesné hmoty, [ATH]). STH, ghrelin, volné mastné kyseliny, glycerol v plazmě a glycerol v extracelulární tekutině a plazmě byly stanoveny komerčními kity. Glycerol byl měřen *in vivo* v podkožní tukové tkáni mikrodialyzační technikou. **Výsledky**: Fyzická zátěž indukovala zvýšení STH a volných mastných kyselin u obou skupin a snížení ghrelinu jen u pacientek s MB. Perorální podání Aci během fyzické zátěže vedlo ke zvýšení STH a poklesu ghrelinu a volných mastných kyselin u obou skupin. U pacientek s MB fyzická zátěž indukovala signifikantně vyšší stimulaci produkce extracelulárního glycerolu v sc TT, zatímco perorální podání Aci během cvičení vedlo k většímu poklesu dialyzovaného glycerolu u pacientek s MB oproti kontrolám. Plazmatické hladiny glycerolu byly fyzickou zátěží bez podání Aci zvýšeny podobně u obou skupin. Plazmatické hladiny glycerolu byly po podání Aci a během fyzické zátěže suprimovány více u pacientek s MB. **Závěry**: V předkládané randomizované, placebem kontrolované, jednoduše zaslepené mikrodialyzační studii jsme prokázali, že Aci indukovaná suprese ghrelinu během zátěže u obou skupin vzbuzuje inhibiční zpětnovazebný mechanismus STH na sekreci ghrelinu. Pozátěžové zvýšení extracelulárního glycerolu v sc abdominální TT je mnohem více suprimováno akutním podáním Aci u pacientek s MB než u kontrol, což ukazuje na hypersenzitivitu sympatické nervové aktivity v sc abdominální TT u pacientek s MB. Současně jsem nalezli facilitovaný obrat plazmatického glycerolu během fyzické zátěže po podání Aci u pacientek s MB. Aci účinkuje na nezávislém mechanismu volných mastných kyselin. Nižší bazální lipolýza v tukové tkáni u pacientek s MB může být způsobena protektivním mechanismem, který zabraňuje vyčerpání energetických zásob organismu.


**Klíčová slova**: Mentální bulimie • Acipimox • Růstový hormon • Ghrelin • Volné mastné kyseliny • Glycerol • Fyzická zátěž • Mikrodialýza • Tuková tkáň

## ABSTRACT

**Title: Acipimox during Short-Term Exercise Exerts A Negative Feedback of Growth Hormone on Ghrelin Secretion in Patients with Bulimia Nervosa and in Healthy Women: The Role of Lipolysis**

**Objective**: Eating disorders, such as bulimia nervosa (BN) and anorexia nervosa (AN), are characterized by abnormal eating behavior. The main features of BN are binge-eating and inappropriate compensatory methods to prevent weight gain. The appetite-modulating peptide ghrelin is secreted by the stomach and shows a strong release of growth hormone (GH). A potential GH-ghrelin feedback loop between stomach and the pituitary has been recently reported. Acipimox (Aci), an analogue of nicotinic acid, inhibits lipolysis in adipose tissue (AT) and reduces plasma glycerol and free fatty acids (FFA) levels. Exercise and Aci are stimulators of GH secretion. We suppose that a negative feedback from increased GH levels during exercise may play a role in reducing plasma ghrelin levels. We surmised that altered baseline activity and exercise-induced activation of the sympathetic nervous system (SNS) results in excessive stimulation of lipolysis associated with negative energy balance and may lead to abnormal AT metabolism in patients with BN. Disruption of the gut-brain-AT axis might be involved in the pathogenesis of BN. **The Aims**: The aim of this study was to evaluate plasma GH and plasma ghrelin levels under resting conditions and in response to exercise alone or together with Aci administration in patients with BN and healthy women. Simultaneously, we measured plasma FFA and plasma glycerol levels in circulation and subcutaneous (sc) abdominal AT glycerol levels using a microdialysis technique *in situ* and *in vivo* under basal conditions and after exercise alone or together with systemic administration of Aci. **Study Design and Methods**: We investigated responses of plasma GH, ghrelin, FFA, glycerol and AT glycerol concentrations to exercise in BN patients and healthy women (C) given the anti-lipolytic drug Aci or placebo. Seven BN and seven C women were recruited for this randomized, placebo-controlled, single-blind study. Aci or placebo was given 60 minutes before the exercise (45 min, 2 W/kg of lean body mass [LBM]). GH, ghrelin, FFA, glycerol plasma concentrations and microdialysate glycerol concentrations were measured using commercial kits. Glycerol was measured *in vivo* in sc abdominal AT using microdialysis. **Results**: The exercise induced an increase in plasma GH and FFA in both groups and a decrease in plasma ghrelin only in BN patients. Exercise with Aci administration resulted in plasma GH increase, and in plasma ghrelin and FFA decrease in both groups. The exercise induced a higher increase of extracellular glycerol concentrations in sc abdominal AT of BN patients, while exercise with Aci administration induced a higher decrease of extracellular glycerol in BN patients compared to the C group. The exercise induced similar increases in plasma glycerol levels in both groups. The exercise with Aci administration resulted in plasma glycerol decrease more in BN patients. **Conlusions**: In conclusion, we confirm the results of a randomized, placebo-controlled, single-blind, microdialysis study, *i.e.* that the Aci-induced suppression in plasma ghrelin levels during short-term exercise in both groups suggests an inhibitory feedback of GH on ghrelin secretion in both groups. The post-exercise rise (45 minute) in AT glycerol is much more attenuated by acute Aci treatment in BN patients and that hypersensitivity of SNS in sc abdominal AT may exist in patients with BN. Simultaneously, we found facilitated turnover of plasma glycerol after short-term exercise together with Aci administration in BN. Aci effects a FFA-independent mechanism. Lower basal lipolysis in AT in BN patients may be due to the protective mechanism that prevents the exhaustion of energy reserves. **Key Words**: Bulimia nervosa • Acipimox • Growth hormone • Ghrelin • Free fatty acids • Glycerol • Exercise • Microdialysis • Adipose tissue

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| **Aci** | Acipimox (5-Methylpyrazine-2-carboxylic acid 4-oxide) |
| **AM** | Ante meridiem |
| **AN** | Anorexia nervosa |
| **ATGL** | Adipose triglyceride lipase (Desnutrin) |
| **AT** | Adipose tissue |
| **BMI** | Body mass index |
| **BN** | Bulimia nervosa |
| **C** | Controls |
| **cAMP** | 3´, 5´- cyclic adenosine monophosphate |
| **DSM-IV** | Diagnostic and Statistical Manual of Mental Disorders |
| **ECG** | Electrocardiogram |
| **EDTA** | Ethylenediamine tetra-acetate |
| **FFA** | Free fatty acids |
| **$G_i$-type** | G-inhibitory type G protein-coupled receptors |
| **GH** | Growth hormone |
| **$G_s$-type** | G-stimulatory type G protein-coupled receptors |
| **HM74A** | Nicotinic acid receptor in humans |
| **HSL** | Hormone-sensitive lipase |
| **i.v.** | Intravenous |
| **LBM** | Lean body mass |
| ***n*** | Number of subjects |
| **NS** | Not significant |
| **PM** | Post meridiem |
| ***P*** | Probability |
| **p.o.** | Per os |
| **S.E.M.** | Standard error of the mean |
| **SNS** | Sympathetic nervous system |
| **sc** | Subcutaneous |
| **% BF** | Percentage of body fat |

# 1. INTRODUCTION

Bulimia nervosa (BN) and anorexia nervosa (AN) are eating disorders characterized by severe disturbances in eating behavior. AN is characterized by self induced starvation and refusal to gain and maintain a minimal normal body weight (weight criterion for the diagnosis is under 85% of normal body weight) while for BN repeated episodes of binge eating followed by inappropriate compensatory behavior, such as self-induced vomiting, laxative and diuretics misuse, fasting or excessive exercise are typical (Diagnostic and Statistical Manual of Mental Disorders [DSM-IV], American Psychiatric Association, 1994). These disorders affect 2-3% of young women and adolescents (Hsu 1996). AN is highly morbid pathologic condition with the highest mortality rate among psychiatric disorders (Vitiello and Lederhendler 2000). In addition to that the multiple negative social and psychological impacts of eating disorders are considerable.

The cause of pathogenesis of BN and AN, however, remains unknown. The phenomenon of binge eating *i.e.* consumption of large amounts of food in a short time period accompanied by a sensation of losing control over eating in BN, and/or intense fear of losing control over eating and becoming overweight in BN suggest a deficit in the normal mechanisms that turn off eating. There are two sub-types of BN. Purging bulimia is the more common of the two and involves self-induced vomiting, which may include use of emetics, such as syrup of ipecacuanha, and self-induced purging, which may include use of laxatives, diuretics and enemas to rapidly remove food from the body before it can be ingested. Non-purging bulimia, which occurs in only approximately 6%-8% of cases, which involves excessive exercise or fasting after a binge to offset the caloric intake after eating. Purging sub-type bulimics may also exercise or fast but as a secondary form of weight control.

Ghrelin is produced primarily in the stomach and plasma ghrelin levels rise during fasting and decrease after feeding (Cummings 2006). It was shown that the efferent vagus nerve contributes to the fasting-induced increase in ghrelin secretion and that higher ghrelin stimulates the afferent vagus nerve and promotes food intake. These findings demonstrate that the vagal circuit between the brain and stomach has an important role in regulating plasma ghrelin levels (Nonogaki 2008). Ghrelin is also a potent secretagogue for growth homone (GH) and i.v. ghrelin administration stimulates GH release in a dose-dependent fashion in humans (Takaya et al. 2000) and there may be a positive association mediated by ghrelin, alternatively, a negative feedback action such that inhibition of plasma ghrelin levels occurs when plasma GH levels are high (Vestergaard et al. 2007). Indeed, a dysfunction of the ghrelin feedback systems might lead to the pathophysiology of eating disorders, such as BN and AN (Nonogaki 2008).

Exercise and anti-lipolytic drug Acipimox (Aci) are enhancers of GH (Dall et al. 2002, Kok et al. 2004, Stokes et al. 2010). In spite of numerous studies the control of exercise-induced GH release remains uncertain yet, but the final signal pathway is surmised to involve either GH-releasing hormone secretion or inhibition of somatostatin release (Giustina and Veldhuis 1998). It was shown that Aci potentiates GH response to GH-releasing hormone by lowering plasma free fatty acids (FFA) in humans (Lee et al. 1995) and that plasma FFA have an independent suppressive effect on plasma ghrelin levels (Gormsen et al. 2006). Exercise stimulates GH release, however, there are conflicting reports regarding the acute effects of short-term exercise on plasma ghrelin levels, the most recent member of the family of GH regulators (Stokes et al. 2010). Changes in plasma ghrelin levels after a single bout of exercise did not differ from those observed at rest (Schmidt et al. 2004, Burns et al. 2007). However, other authors have documented plasma ghrelin levels decrease after exercise (Ballard et al. 2009, Stokes et al. 2010) or increased after exercise (Borer et al. 2005, Jürimäe et al. 2007, Erdmann et al. 2007).

Catecholamines of the sympathetic nervous system (SNS) play an important role in the regulation of adipose tissue (AT) lipolysis, which is a key step in the metabolic processes leading to the decrease of fat mass. Catecholamines influence lipolysis *via* beta-adrenergic G-protein-coupled receptors of adipocytes; norepinephrine stimulates lipolysis through the activation of beta adrenergic $G_s$-type G protein-coupled receptors and epinephrine exhibits a higher affinity and inhibits lipolysis through activation of the alpha$_2$-adrenergic $G_i$-type G protein-coupled receptors as has been described *in vitro* (Wellman 2000). In humans, subcutaneous (sc) fat cells alpha$_2$-adrenoceptors numerically predominate over beta-adrenoceptors and therefore lower catecholamine concentrations can cause inhibition of lipolysis (Mauriege et al. 1987, Lawrence and Coppack 2000). In our previous studies (Nedvídková et al. 2004, Barták et al. 2004), we found increased norepinephrine concentrations and increased production of glycerol as an index of lipolysis rate in sc abdominal AT in patients with AN, measured *in vivo* by microdialysis.

Acipimox (Aci) (5-Methylpyrazine carboxylic acid 4-oxide; Olbetam) is a nicotinic acid-derived anti-lipolytic drug devoid of major side effects, and has been used in a number of human trials (Ball et al. 1986, Fulcher et al. 1992), but the cellular mechanism by which Aci exerts its main effect (*i.e.* suppression of lipolysis from AT) is not fully known. It is supposed that the action of Aci is mediated through suppression of intracellular cyclic adenosine monophosphate (cAMP) and the reduction of the association of hormone-sensitive lipase (HSL) with its triacylglycerol substrate in the lipid droplet of adipocytes. Importantly, on the basis of above mentioned mechanism in action of catecholamines, anti-lipolytic influences include alpha$_2$-adrenergic $G_i$- type G protein-coupled receptors activation and activation of nicotinic acid receptors HM74A which are associated with $G_i$-type G protein-coupled receptors, too (Karpe and Frayn 2004). Moreover, anti-lipolytic effect of Aci is the result of the suppression of adipocyte lipases, such as HSL and adipose triglyceride lipase (ATGL, *i.e.* desnutrin), although a direct link between Aci and either HSL or ATGL has not been demonstrated yet (Soudijn et al. 2007). Interestingly, *in vitro* ATGL gene expression was not regulated by cAMP suggesting that activation and/or inhibition of ATGL could mediate alternative non cAMP-dependent signal pathway (Villena et al. 2004). Indeed, Aci may bypass the membrane receptor control to reach the target regulatory machinery *via* alternative non cAMP-dependent signal transduction pathways.

Microdialysis has been used to determine the extracellular concentrations and local changes of tissue metabolism (glycerol, catecholamines, hormones, etc.) *in vivo* (Nedvídková et al. 2003, 2004, Dostálová et al. 2003, 2009). A microdialysis catheter is an artificial blood vessel system which can be placed in the extracellular space of various tissues such as AT *in situ*. (Arner 1999). Conceptually, microdialysis is simple. A tubular dialysis membrane is introduced into the tissue, and a liquid is perfused that allows bi-directional exchange with the interstitial fluid outside of the tube. Endogenous compounds in the interstitial fluid that enter the microdialysate can be assayed, so that concentrations in the microdialysate reflect concentrations in the interstitial fluid (Pacak et al. 1995 a, b). The composition of the perfusate should resemble that of human extracellular fluid.

Therefore, the present randomized, placebo-controlled, single-blind, microdialysis study was designed to examine either the effects of anti-lipolytic drug Aci administration or placebo during short-term exercise on plasma ghrelin, GH, FFA and glycerol levels in BN patients. The purpose of this study was to find out whether GH secretion is regulated by peripheral ghrelin and vice versa under exercise alone or together with Aci administration. At the same time, we measured AT glycerol using microdialysis. Healthy women were used as the control group.

# 2. THE WORKING HYPOTHESES AND THE AIMS

## *2.1. The Working Hypotheses*

Ghrelin is secreted by the stomach and stimulates the pituitary gland secretion of GH *via* the vagal circuit between the central nervous system and stomach. The purpose of this study was to test the hypothesis that a ghrelin-GH loop exists in which either an elevation or suppression in plasma ghrelin levels during exercise alone or currently with anti-lipolytic Aci administration is exerted by a positive or negative feedback action, respectively, between the stomach-ghrelin-pituitary-GH axis in patients with BN and in healthy women (Kraemer and Castracane 2007).

The sympathoadrenal system is considered to be an important system that regulates AT metabolism. Thus, function alterations of this system may contribute to dysregulation of adipocyte metabolism. In the present study, we used *in vivo* microdialysis technique to measure interstitial AT glycerol levels to assess local lipolysis in patients with BN. On the basis of our previous studies, we hypothesize, that higher sensitivity of SNS to anti-lipolytic drug Aci during exercise in sc abdominal AT may exist in BN patients, and that Aci influences the same signal transduction pathway as norepinephrine (Wang-Fisher et al. 2002), the major representative of SNS.

## *2.2. The Aims*

1. To find whether GH may play an inhibitory role on ghrelin secretion during exercise alone or during exercise after systemic administration of Aci.

2. To find the gut-brain-AT function in BN patients compared to the controls with the attention to exercise alone or together with Aci administration responses of GH, ghrelin, FFA and glycerol in circulation.

3. To determine the abdominal AT function and its relationship to gut-brain hormones and lipid metabolites on tissue level using *in vivo* microdialysis technique in patients with BN and compared to healthy women.

4. To determine glycerol turnover, as the index of local abdominal AT and systemic lipolysis by the measurements of extracellular and plasma levels of glycerol under basal conditions and during exercise alone or together with Aci administration in bulimic patients and compared to healthy women.

5. To find if Aci acts through a FFA-dependent mechanism.

# 3. STUDY DESIGN AND METHODS

The study was performed in accordance with the Declaration of Helsinki and was approved by the Ethics Committee of the Institute of Endocrinology in Prague. Each participant signed an informed consent form before entering the study.

## *3.2. Bulimic Patients and Healthy Women*

Seven women with BN (mean ± S.E.M.; age: 24.33 ± 1.38 years; body mass index (BMI): 20.63 ± 0.80 kg/m$^2$; percentage of body fat [% BF]: 24.83 ± 1.92) and seven healthy women (age: 25.83 ± 1.69 years; BMI: 19.98 ± 0.44 kg/m$^2$; % BF: 24.5 ± 0.47) were recruited for this study. All subjects included in the study were nonsmokers, had no allergies and had been free of medications for at least two weeks prior to the study. Healthy volunteers had no history of cardiovascular disease, eating disorders or other psychiatric diseases. All healthy women were in the first two weeks of the follicular phase of their menstrual cycle. Patients with BN were diagnosed according to the 4$^{th}$ edition of the DSM-IV, American Psychiatric Association, 1994. All BN patients were clinically stable and in relatively good health, except for their eating disorder and amenorrhoea. They were investigated after 1 week of hospitalization at the Department of Psychiatry of the Charles University, Prague.

## *3.3. Experimental Protocol; Blood Sampling*

Laboratory screening conducted before initiation of the study confirmed normal values for blood count, fasting blood glucose, and liver and renal function tests. All subjects consumed a standardized dinner at 6:00 PM and were then asked to fast overnight. Reported duration of sleep in the night preceding blood sampling was comparable in all studied subjects (approximately 8 hours).The subjects were admitted to the Institute of Endocrinology at 7:00 AM. After a short medical examination (blood pressure, heart, and respiratory rate measurement, electrocardiogram, [ECG]), % BF was estimated by anthropometric measurements and bioimpedance (TANITA, Tokyo, Japan). At 7:30 AM, after overnight fasting, a venous catheter was inserted into antecubital vein. Before starting the test, all individuals remained in supine position for 45 minutes. Then their blood was withdrawn for the estimation of basal values of the plasma ghrelin, GH, FFA and glycerol concentrations. Blood samples were collected into chilled tubes containing $Na_2$EDTA and antilysin. Plasma was immediately separated by centrifugation at 4 $^{o}$C and stored at -80 $^{o}$C until being assayed. In the first week, 60 min after the blood withdrawal, all subjects received placebo and, after another 60 min, underwent a 45-min low- to moderate-intensity exercise bout on an electromagnetically braked bicycle ergometer (Cateye EC 1600, Japan) at a power output of 2 W/kg of lean body mass (LBM), which was intended to be below the aerobic-anaerobic threshold. Another blood withdrawal followed immediately after the exercise and then after another 90 min during which all subjects assumed a resting supine position on a comfortable

bed. In the following week the same women were randomized to receive Aci capsules (two 250 mg capsules of Aci; total 500 mg – 5-Methylpyrazine-2-carboxylic acid 4-oxide, molecular weight: 154.1, Olbetam capsules, Farmitalia Carlo Erba, Milan, Italy) 1 hour before a single exercise bout (exercise plus Aci). Blood withdrawal followed again immediately after the exercise and then after another 90 min under the same conditions.

## *3.4. Experimental Protocol; Microdialysate Sampling*

The *in vivo* microdialysis technique was used to examine the exercise stimulated lipolysis by measurement of dialysate glycerol alone or with Aci randomly received (500 mg p.o., Olbetam capsules, Farmitalia Carlo Erba, Milan, Italy) for two consecutive weeks. A CMA-60 microdialysis probe (CMA Microdialysis, Stockholm, Sweden) with membrane length 3 cm and molecular weight cut-off 20 kDa was inserted sc under sterile conditions (8-10 cm left of the umbilicus at least 60 min before microdialysate sampling). Sterile Ringer solution was used as perfusate, a constant perfusion rate of 2 µl/min was maintained throughout the study using a CMA 107 microdialysis pump (CMA Microdialysis, Stockholm, Sweden). Each probe contained an inner cannula, to deliver perfusate into the AT, and an outer cannula, to remove dialysate after that had equilibrated with the surrounding interstitial fluid. Microdialysate samples were collected every 15-30 min over a 6-h period, 120 min before exercise (basal values), 45 min during the exercise and 90 min after the exercise. Microvials were placed on ice immediatelly after the collection, and stored at -80 °C until analysis.

## *3.5. Hormonal and Biochemical Assays*

Plasma GH concentrations were measured by a commercial RIA kit (Immunotech, Prague, Czech Republic). Intra- and inter-assay variability was 1.5% and 14%, respectively, sensitivity was 0.1 µIU/ml.Total plasma ghrelin was determined by commercially available RIA kits (Linco Research, Inc., St. Charles, Missouri, U.S.A.). The intra- and inter-assay variability for total ghrelin was 6.4 % and 16.3 %, respectively, and the sensitivity was 93.0 pg/ml. Glycerol in plasma and in the dialysate was analyzed with a radiometric kit (Randox Laboratories, GY 105, Montpellier, France). Plasma FFA were estimated colorimetrically with a commercial kit (Randox Laboratories, FA 115, Montpellier, France). All assays were run twice in duplicate.

## *3.6. Statistical Analysis*

Values are expressed as the means ± S.E.M. All statistical comparisons were performed using a statistical program: General Linear Repeated Measures with Status as the between Factor and Aci and Time as the within Factors. Correlations between parameters

were examined using Spearman´s rank correlation coefficient. The difference between medians (Mann-Whitney and Wilcoxon Rank-Sum tests) was applied to compare baseline values with those during exercise. A $P$ value < 0.05 denoted statistical significance.

# 4. RESULTS

## *4.1. Tables*

Baseline characteristics of the study subjects, including anthropometric, hormonal and biochemical measurements, are summarized in Table 1. The exercise-induced changes in plasma of the study subjects after Aci treatment or with placebo are shown in Table 2, Table 3 and Table 4, respectively.

### *4.1.1. Baseline and Exercise-Induced Plasma GH Concentrations Alone or Together with Aci Administration*

Mean baseline fasting plasma GH concentrations were significantly increased in BN patients compared to the controls (11.2 ± 0.9 *vs.* 7.1 ± 0.5 mIU/l in the controls, $P < 0.05$) (Table 1). In both groups plasma GH concentration increased in response to the 45-min exercise (13.1 ± 4.3 *vs.* 11.3 ± 2.2 mIU/l in the controls, $P < 0.001$). Next week the administration of Aci 60 min before 45-min exercise increased plasma GH in both groups even further (73.7 ± 23.1 *vs.* 40.9 ± 8.7 mIU/l in the controls, $P < 0.0001$). 90 min after the exercise alone, plasma GH levels significantly decreased, more in the controls than in BN patients (2.0 ± 0.5 *vs.* 0.7 ± 0.2 mIU/l in the controls, $P < 0.0001$). In contrast, 90 min after the exercise plus Aci plasma GH levels were significantly elevated in both groups (28.9 ± 7.5 *vs.* 21.4 ± 8.1 mIU/l in the controls, $P < 0.0001$) (Table 2).

### *4.1.2. Baseline and Exercise-Induced Plasma Ghrelin Concentrations Alone or Together with Aci Administration*

Mean baseline fasting plasma ghrelin concentrations were similar in BN patients and in the controls (1099 ± 218 *vs.* 1112 ± 273 pg/ml in the controls) (Table 1). In BN patients plasma ghrelin levels significantly decreased after the 45-min exercise compared to the controls (812 ± 104.4 *vs.* 1189.7 ± 254.8 pg/ml in the controls, $P < 0.05$). In both groups plasma ghrelin levels decreased after the exercise plus Aci (690.5 ± 92.7 *vs.* 932 ± 115.2 pg/ml in the controls, $P < 0.01$). In BN patients plasma ghrelin levels remained significantly decreased 90 min after the exercise compared to the controls (952.3 ± 77.7 *vs.* 1322.2 ± 240.5 pg/ml in the controls, $P < 0.05$). In both groups plasma ghrelin levels were still significantly decreased 90 min after the exercise plus Aci (768.7 ± 73.1 *vs.* 836.7 ± 137.7 pg/ml in the controls, $P < 0.01$) (Table 2).

### 4.1.3. Baseline and Exercise-Induced Plasma FFA Concentrations Alone or Together with Aci Administration

Mean baseline fasting plasma FFA concentrations were similar in BN patients and the controls (0.79 ± 0.3 *vs.* 0.86 ± 0.3 mmol/l in the controls) (Table 1). In both groups plasma FFA concentrations significantly increased after the exercise (1.60 ± 0.28 *vs.* 1.54 ± 0.13 mmol/l in the controls, $P < 0.0001$) and decreased to basal levels after the exercise plus Aci compared with the exercise alone (0.79 ± 0.14 *vs.* 0.82 ± 0.05 mmol/l in the controls, $P < 0.05$). In both groups plasma FFA concentrations were nearly basal values 90 min after the exercise alone (0.83 ± 0.1 *vs.* 0.79 ± 0.05 mmol/l in the controls) and significantly decreased below basal values 90 min after the exercise plus Aci (0.26 ± 0.02 *vs.* 0.25 ± 0.02 mmol/l in the controls, $P < 0.0001$) (Table 3).

### 4.1.4. Baseline and Exercise-Induced Plasma Glycerol Concentrations Alone or Together with Aci Administration

Mean baseline fasting plasma glycerol levels were significantly lower in BN patients compared to the controls (82.2 ± 26.0 *vs.* 117 ± 33.0 μmol/l in the controls, $P < 0.05$) (Table 1). In both groups plasma glycerol levels significantly increased after the exercise (256.0 ± 56.0 *vs.* 318.0 ± 34.0 μmol/l in the controls, $P < 0.0001$), and dropped down to baseline values 90 min after the exercise (74.0 ± 4.2 *vs.* 85.0 ± 7.3 μmol/l in the controls). Plasma glycerol levels significantly decreased after the exercise plus Aci compared with the exercise alone, more in BN patients (114.0 ± 13 *vs.* 157.8 ± 18.4 μmol/l in the controls, $P < 0.001$). In both groups plasma glycerol concentrations were significantly suppressed below baseline values 90 min after the exercise plus Aci (57.0 ± 6.2 *vs.* 44.0 ± 4.7 μmol/l in the controls, $P < 0.01$) (Table 3).

### 4.1.5. Baseline and Exercise-Induced AT Glycerol Concentrations Alone or Together with Aci Administration

Mean baseline AT glycerol levels were significantly decreased in BN patients compared to the controls (36.39 ± 4.15 *vs.* 41.21 ± 4.43 μmol/l in the controls, $P < 0.05$) (Table 1). In both groups, but much more in BN patients AT glycerol concentrations increased significantly after 45 minute exercise with placebo (148.6 ± 23.2 *vs*. 82.2 ± 11.82 μmol/l in the controls, $P < 0.0001$). In both groups, AT glycerol levels decreased significantly to the basal values after 45 minute exercise with Aci administration (38.3 ± 5.39 *vs.* 41.6 ± 4.21 μmol/l in the controls; $P < 0.0001$ for BN patients, $P < 0.01$ for the controls, respectively). In the controls, AT glycerol concentrations were nearly to the basal values 90 minutes after the exercise with placebo, while AT glycerol concentrations in BN patients decreased significantly under the basal values (29.0 ± 2.9 *vs*. 40.5 ± 3.81 μmol/l in the controls, $P < 0.05$). In both groups, AT glycerol levels decreased significantly under the basal levels 90 minutes after the exercise with Aci administration in both groups (20.4 ± 3.01 *vs*. 33.9 ± 3.16 μmol/l in the controls, $P < 0.01$) (Table 4).

## *4.2. Correlations Between Parameters*

### *4.2.1. The relationship of biochemical parameters during basal conditions and after the exercise with Aci administration (45 min) in patients with BN and in healthy control women*

As expected, fasting plasma glycerol concentrations positively correlated with plasma FFA concentrations in BN patients (r = 0.69, *P* = 0.0001) and in the controls (r = 0.87, *P* = 0.0001). Plasma glycerol concentrations positively correlated with plasma FFA concentrations after the exercise plus Aci in BN patients (r = 0.91, *P* = 0.004) and the controls (r = 0.93, *P* = 0.002).

**Table 1.** Anthropometric and major laboratory characteristics of the study subjects (means ± S.E.M.). C = controls; BN = bulimia nervosa; GH = growth hormone; FFA = free fatty acids; BMI = body mass index; % BF = percentage of body fat; NS = not significant; $^{\$}$ = $P < 0.05$ BN *vs.* control subjects (C); *n* = the number of subjects.

| | **C (*n* = 7)** | **BN (*n* = 7)** | ***P* value** |
|---|---|---|---|
| **Age (years)** | 25.83±1.69 | 24.33±1.38 | NS |
| **BMI (kg/m$^2$ )** | 19.98±0.44 | 20.63±0.80 | NS |
| **% BF** | 24.50±0.47 | 24.83±1.92 | NS |
| **GH (mIU/l)** | 7.1±0.5 | 11.2±0.9$^{\$}$ | <0.05 |
| **Ghrelin (pg/ml)** | 1112±273 | 1099±218 | NS |
| **FFA (mmol/l)** | 0.86±0.3 | 0.79±0.3 | NS |
| **Plasma Glycerol (µmol/l)** | 117.0 ± 33 | 82.2 ± 26$^{\$}$ | < 0.05 |
| **Dialysate Glycerol (µmol/l)** | 41.21 ± 4.43 | 36.39 ± 4.15$^{\$}$ | < 0.05 |

**Table 2.** Effect of exercise (45 min, 2 W/kg of lean body mass [LBM]) alone or together with Acipimox (Aci) administration on plasma growth hormone (GH) and ghrelin in the controls (C) ($n$ = 7) and bulimia nervosa (BN) patients ($n$ = 7).

| | 0 min | | 45 min | 45 min | 90 min | 90 min |
|---|---|---|---|---|---|---|
| | Basal | | Exercise | Exercise | Post-exercise | Post-exercise |
| | | | + placebo | + Aci | + placebo | + Aci |
| **GH** (mIU/l) | | | | | | |
| C group | 7.1±0,5 | | 11.3±2.2*** | 40.9±8.7**** | 0.7±0.2**** | 21.4±8.1**** |
| BN group | 11.2±0.9$^{\$}$ | | 13.1±4.3*** | 73.7±23.1****$^{\$}$ | 2.01±0.5****$^{\$}$ | 28.9±7.5****$^{\$}$ |
| **Ghrelin** (pg/ml) | | | | | | |
| C group | 1112±273 | | 1189.7±254.8 | 932.0±115.2** | 1322.2±240.5 | 836.7±137.7** |
| BN group | 1099±218 | | 812.0±104.4*$^{\$}$ | 690.5±92.7**$^{\$}$ | 952.3±77.7*$^{\$}$ | 768.7±73.1** |

* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, **** = $P < 0.0001$ *vs.* resting (baseline) values

$^{\$}$ = $P < 0.05$ BN *vs.* control subjects (C)

$^{+}$ = $P < 0.05$ exercise together with Aci administration *vs.* exercise alone, 45 minute

$^{++}$ = $P < 0.01$ exercise together with Aci administration *vs.* exercise alone, 45 minute

$^{+++}$ = $P < 0.001$ exercise together with Aci administration *vs*. exercise alone, 45 minute

$^{\#}$ = $P < 0.05$ post-exercise together with Aci administration *vs.* exercise alone, 90 minute

**Table 3.** Effect of exercise (45 min, 2 W/kg of lean body mass [LBM]) alone (placebo) or together with Acipimox (Aci) administration on plasma glycerol and free fatty acids (FFA) levels in the controls (C) ($n$ = 7) and bulimia nervosa (BN) patients ($n$ = 7). Values are means ± S.E.M.; $n$ = the number of subjects.

| | 0 min | 45 min | 45 min | 90 min | 90 min |
|---|---|---|---|---|---|
| | Baseline | Exercise | Exercise | Post-exercise | Post-exercise |
| | | + placebo | + Aci | + placebo | + Aci |
| **Plasma Glycerol** (μmol/l) | | | | | |
| C group | 117.0 ± 33 | 318.0 ± 34**** | 157.8 ± 18.4$^{++}$ | 85.0 ± 7.3 | 44.0 ± 4.7**$^{\#}$ |
| BN group | 82.2 ± 26$^{\$}$ | 256.0 ± 56****$^{\$}$ | 114.0 ± 13.0$^{\$+++}$ | 74.0 ± 4.2 | 57.0 ± 6.2**$^{\$\#}$ |
| **FFA** (mmol/l) | | | | | |
| C group | 0.86±0.3 | 1.54±0.13**** | 0.82±0.05$^{+}$ | 0.79±0.05 | 0.25±0.02****$^{\#}$ |
| BN group | 0.79±0.3 | 1.6±0.28**** | 0.79±0.14$^{+}$ | 0.83±0.1 | 0.26±0.02****$^{\#}$ |

** = $P < 0.01$, **** = $P < 0.0001$ *vs.* resting (baseline) values
$ = $P < 0.05$ BN *vs.* control subjects (C)
++ = $P < 0.01$ exercise together with Aci administration *vs.* exercise alone, 45 minute
+++ = $P < 0.001$ exercise together with Aci administration *vs.* exercise alone, 45 minute
# = $P < 0.05$ post-exercise recovering phase together with Aci administration *vs.* post-exercise recovering phase alone, 90 minute

**Table 4.** Dialysate glycerol concentration in subcutaneous (sc) abdominal adipose tissue (AT) during basal conditions and during exercise (45 min, 2W/ kg of lean body mass [LBM] alone or together with Acipimox (Aci) administration in the controls (C) (*n* = 7) and bulimia nervosa patients (BN) (*n* = 7). Values are means ± S.E.M.; *n* = the number of subjects.

| | 0 min | 45 min | 45 min | 90 min | 90 min |
|---|---|---|---|---|---|
| | Baseline | Exercise | Exercise | Post-exercise | Post-exercise |
| | | + placebo | + Aci | + placebo | + Aci |
| **Dialysate Glycerol** (μmol/l) | | | | | |
| C group | 41.21 ± 4.43 | 82.2 ± 11.82**** | 41.6 ± 4.21++ | 40.5 ± 3.81 | 33.9 ± 3.16**# |
| BN group | 36.39 ± 4.15$ | 148.6 ± 23.2****$$ | 38.3 ± 5.39++++ | 29.0 ± 2.9$ | 20.4 ± 3.01**$# |

** = $P < 0.01$, **** = $P < 0.0001$ *vs.* resting (baseline) values
$ = $P < 0.05$ BN *vs.* control subjects (C)
$$ = $P < 0.01$ BN *vs.* control subjects (C)
++ = $P < 0.01$ exercise together with Aci administration *vs.* exercise alone, 45 minute
++++ = $P < 0.0001$ exercise together with Aci administration *vs.* exercise alone, 45 minute
# = $P < 0.05$ post-exercise recovering phase together with Aci administration *vs.* post-exercise recovering phase alone, 90 minute

# 5. DISCUSSION

The most important finding of the present study is that basal fasting GH plasma levels were increased but not fasting basal ghrelin levels in BN patients. GH plasma levels similarly increased after acute exercise with placebo in both groups but short-term exercise induced suppression of plasma ghrelin levels only in BN patients. Anti-lipolytic drug Aci administered 60 minute before starting short-term exercise induced important increase of plasma GH levels in both groups and significant suppression of plasma ghrelin in BN patients and healthy women (Table 2).

Studies have reported a decrease (Toshinai et al. 2007, Malkova et al. 2008, Smitka et al. 2008, Stokes et al. 2010, Nedvídková et al. 2011) or an increase (Erdmann et al. 2007, Jürimäe et al. 2007) in plasma ghrelin levels following exercise, whereas others have not observed any changes (Kraemer et al. 2003, Burns et al. 2007, Ueda et al. 2009). These results may depend on several factors, such as duration or intensity of exercise, body composition, nutritional status, as well as the timing of food intake. As ghrelin is a secretagogue for GH, there could be potentially an inhibitory feedback loop such that suppression of ghrelin occurs when plasma GH levels are high (Vestergaard et al. 2007). It has been shown that resistance exercise increases GH levels to a higher degree than aerobic exercise (Consitt et al. 2007), which may explain why resistance exercise compared with aerobic exercise appears to have a greater inhibitory effect on plasma ghrelin levels. In our study, it is possible that minor increase in plasma GH levels induced by moderate intensity exercise did not invoke GH feedback inhibition of ghrelin secretion in healthy women. Conversely, in BN patients having increased basal fasting GH levels and unchanged plasma ghrelin levels, further increase of plasma GH levels induced by exercise alone invoked GH feedback inhibition of plasma ghrelin levels in BN patients. Therefore, it can be suggested that in bulimic women, but not in healthy women, plasma ghrelin concentrations best reflect nutritional status rather than specific patterns of disordered eating behavior (Troisi et al. 2005). The decrease of plasma ghrelin levels after exercise alone would mean a change in sensitivity ghrelin to GH and/or impaired metabolic status in BN patients.

In contrast to exercise alone, anti-lipolytic drug Aci administered 60 minute before starting short-term exercise induced not only important increase in plasma GH levels but also the decrease in plasma ghrelin levels in both groups. Thus, it is possible that only high absolute plasma GH levels after exercise together with Aci administration results in a significant decrease of plasma ghrelin levels also in healthy women. Another possibility is that plasma ghrelin decline reflects changes in plasma FFA and glycerol levels during exercise in combination with Aci administration in both groups. Ghrelin is a meal initiating signal and in line with this view the suppression of lipolysis *via* inhibition of the HSL in AT would suppress ghrelin secretion since FFA mobilization is increased in response to both fasting and exercise, and blocked by food intake (Vestergaard et al. 2005). Interestingly, Gormsen et al. (2006) reported that FFA reduce ghrelin levels independently of GH levels in humans. However, we found decreased plasma ghrelin levels immediately after the exercise in BN patients, while plasma FFA and glycerol concentrations increased in the same range as in healthy women in whom plasma ghrelin did not change. Furthermore, the decrease in plasma ghrelin levels after exercise together with Aci administration in both groups, when FFA levels returned to basal values, or in post-exercise state when FFA concentrations were infraphysiologically suppressed and GH levels were still higher than basal levels in both groups. These observations lead us to suggestion that elevated GH levels induced by the exercise together with Aci administration do not appear to be directly mediated *via* FFA and to influence ghrelin secretion in both groups. Thus, we conclude that FFA probably are not ghrelin enhancers. This is evidence for the view to suggest a FFA-independent mechanism of Aci.

The physiological significance of exercise-induced suppression in ghrelin levels is not still clear. Indeed, lower concentration of ghrelin during exercise may provide benefit by suppressing appetite and directing substrate use towards the lipolysis (Vestergaard et al. 2007).

This is the first randomized microdialysis study to evaluate the effect of antilipolysis on AT and plasma glycerol during short-term exercise in healthy women and patients with BN. The exercise induced a higher increase of glycerol concentrations in sc abdominal AT of BN patients, while exercise with Aci administration induced a higher decrease of extracellular

glycerol in BN patients compared to the C group. The exercise induced similar increases in plasma glycerol levels in both groups. The exercise with Aci administration resulted in plasma glycerol decrease more in BN patients.

Furthermore, we observed lower both baseline AT and plasma glycerol levels in patients with BN when compared to healthy women. (Table 1) These findings are in concordance with previous and recent reports suggesting the higher activity of SNS in AT and disrupted adrenergic regulation of lipolysis occuring both receptor and postreceptor levels in sc abdominal AT in AN (Nedvídková et al. 2003, 2004).

Interestingly, we found a discrepancy between plasma glycerol and local (dialysate) glycerol levels (Table 3, Table 4) in BN patients, and we determined a significantly higher dialysate glycerol level during exercise in BN compared to the controls. Currently, it is well known that local (tissue) lipolysis does not reflect plasma glycerol levels during exercise in BN patients (Barták et al. 2004). This discrepancy could be possibly explained by the fact that plasma glycerol concentration reflects the net amount of this parameter released from different sources, whereas dialysate glycerol concentration determinates the quantity released in AT. Aci acutely received during the exercise led to much more abolished lipolysis in sc abdominal AT in BN than in the controls, which leads us to suggesting that altered lipolysis in BN may result from local modification of adrenergic activities. Thus, Aci and catecholamines act *via* their inhibition on cAMP production in AT, rather than *via* alternative cAMP-independent pathways (Wang-Fisher et al. 2002, Soudijn et al. 2007), and up-regulation of receptor subtypes and/or their sensitivity or affinity are much more effective in abolishing lipolysis in BN.

Furthermore, we found even higher plasma glycerol levels after the exercise combined with Aci administration in the controls (Table 3). These observations lead us to suggesting that glycerol is not easily remetabolized and the decrease of plasma glycerol after the exercise associated with Aci administration is exerted by altered activity of SNS in BN, and/or by facilitated turnover of plasma glycerol which would reflect metabolic status in this eating disorder. However, Gianotti et al. (2000) studied effect of Aci on basal plasma lipolysis in AN and that overall lipolysis was inhibited by Aci in both groups but persisted higher in AN than in healthy women.

Endocrine disturbances and a dysfunction within the ghrelin-GH secretion may also take part in the etiopathogenesis of either bulimia or AN. The mechanism of altered ghrelin-GH secretion in BN is a complex and not entirely understood. Further studies are necessary to confirm these findings and to clarify the role of lipolysis and effects of the GH-ghrelin release during short-term exercise in BN.

# 6. CONCLUSIONS

In conclusion, this study shows that exercise-induced elevation in GH levels and Aci-induced GH release are not mediated by ghrelin. However, this report adds to the hypothesis that the decrease in ghrelin levels observed after Aci administration during exercise may be consistent with a negative feedback of GH on ghrelin secretion in both BN patients and healthy women (Smitka et al. 2008, Nedvídková et al. 2011). Simultaneously, the present data support the hypothesis that exercise-induced elevation in GH levels suppresses ghrelin levels only in BN patients, but did not support the hypothesis that exercise-induced minor increase in GH levels feedback inhibits ghrelin secretion in healthy women. These findings lead us to

suggestion that BN patients are abnormally sensitive to negative energy balance and because ghrelin is one of the peptides that functions to regulate energy homeostasis, therefore, ghrelin may be a potential discriminator between patients with endocrine disturbances and chronic perturbations in energy imbalance, such as BN and AN patients, but not in healthy women. Our results support the hypothesis that exercise and Aci-induced GH and ghrelin release is not mediated by FFA and FFA did not inhibit lipolysis in a feedback fashion (Coiro et al. 2007).

Altogether, our results support the hypothesis that higher sensitivity of SNS to anti-lipolytic drug Aci in sc abdominal AT exists in BN patients, and that Aci influences the same signal transduction pathway as norepinephrine, the major representative of SNS, *i.e.* that Aci acts *via* its inhibition on cAMP production, rather than *via* alternative cAMP-independent pathways (Wang-Fisher et al. 2002, Villena et al. 2004, Soudijn et al. 2007). Likewise, it can be concluded based on this randomized, placebo-controlled, single-blind, microdialysis study that pharmacological antilipolysis in sc abdominal AT during short-term exercise is much higher in patients with BN. Simultaneously, we found facilitated turnover of plasma glycerol after short-term exercise together with Aci administration which would reflect abnormal metabolic status in BN. Lower basal lipolysis in AT in BN patients may be due to the protective mechanism before the exhaustion of energy reserves.

Thus, changes in FFA levels did not respond to changes in GH and ghrelin levels. The mechanistic pathways through which Aci exerts its effect on GH secretion remains elusive. These observations led us to suggesting that Aci affects a FFA-independent mechanism. Using the *in situ* and *in vivo* microdialysis technique we documented that post-exercise rise in sc abdominal AT glycerol was much more attenuated by acute Aci treatment in BN patients and that acute Aci received during short-term exercise overrode lipolytic effects of GH in sc abdominal AT greater in BN patients.

The present microdialysis study has a high impact on understanding of mechanisms that may contribute to altered functions of the AT in patients with BN. The results of our study should contribute further to the development of a new generation of drugs, such as ghrelin synthetic analogues that could alter synaptic cleft concentrations of norepinephrine and epinephrine and therefore lipid mobilization and energy expenditure. Thus, modification of the ghrelin pathway with receptor antagonists and agonists might pave the way for new drug treatments for BN and AN patients because current long-term pharmacological therapy of these patients is frequently unsuccessful.

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Abstracts:

Nedvídková J, **Smitka K**, Kvasničková H, Papežová H, Pacak K: Exercise alone or together with acipimox induces increase in plasma growth hormone (GH) and decrease in plasma ghrelin in healthy women and women with bulimia nervosa. The Endocrine Society´s 89th Annual Meeting, Toronto, Canada, June 2-5, 2007.

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