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Effects of acute ethionine injection on plasma ghrelin and obestatin levels in trained male rats

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Abstract

Ghrelin and obestatin are orexigenic and anorexigenic peptides, respectively, that are secreted from the stomach mucosa into the circulation. These peptides have opposing actions on food intake, weight gain, and adiposity. It is thought that ghrelin is sensitive to a negative energy environment and also plays a considerable role in short- and long-term energy balance and glucose homeostasis. It has been suggested that the levels of ghrelin and obestatin are upregulated by fasting, hypoglycemic status, and a physical-exercise–induced energy deficit. Ethionine (ETH), the ethyl analogue of methionine, has been shown to increase food intake, decrease adenosine triphosphate (ATP) and glycogen levels, and inhibit protein synthesis in the liver. The purpose of this study was to examine the effect of a single dose of ETH (0.7 mg/g of body weight) injection on resting plasma total ghrelin and obestatin concentrations in male trained rats. Thirty-two adult Wistar male rats weighing 180 to 200 g were randomly assigned to control (n = 16) and training (n =16) groups. The training group was exercised for 10 weeks (25 m/min, 0% grade, 60 minutes, and 5 d/wk). Seventy-two hours after the last exercise session, rats were injected with either saline (NaCl) or ETH and then killed. Ethionine compared with a NaCl injection resulted in significant ($P < .013$) reductions in resting hepatic ATP and glycogen levels, and in a significant ($P < .001$) increase in concentrations of plasma total ghrelin but not obestatin. The results indicate that ETH-induced liver ATP and glycogen deficiency could exert a powerful regulatory influence on plasma total ghrelin, but this is not the case for obestatin. Findings demonstrate the short-term energy-regulating capacity of ghrelin.

1. Introduction

Ghrelin and obestatin are respective orexigenic and anorexigenic peptides. They are derived from the same precursors, a preproghrelin gene (117–amino acid) and proghrelin (99–amino acid) peptide synthesized in endocrine (A-like) cells of the gastric mucosa [1]. Ghrelin, a stomach-derived 28–amino acid and ligand for the growth hormone secretagogue receptor–1a, was initially isolated from the rat and then human stomach [2,3]. It is also expressed by extragastric tissues [4,5]. Ghrelin is recognized as a regulator of food intake, weight gain, growth hormone release, and energy/glucose homeostasis [2,6]. In addition to ghrelin, another peptide, obestatin, is an amidated, 23–amino acid peptide that is encoded by the ghrelin gene [5,7,8]. It has been shown that intraperitoneal injection of obestatin suppresses food intake in a time- and dose-dependent manner [7]. Distribution and biological activity of obestatin as well as its role in energy balance, glucose homeostasis, energy expenditure, and body weight gain have been studied in rodents [9-11]. Plasma ghrelin and obestatin levels are altered under several conditions including anorexia nervosa [12], obesity [13], diabetes [14], fasting and refeeding [15],

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high- and low-carbohydrate diets [16-18], weight reduction [19,20], and physical exercise [21-29].

Ethionine (ETH), the ethyl analogue of methionine, when injected to female and male rats is known to perturb liver metabolism [30-32]. It is well documented that administration of ETH in rats results in significant and dramatic reductions in plasma glucose and in levels of liver adenosine triphosphate (ATP) and glycogen, as well as inhibition of liver gluconeogenesis and reduction of protein synthesis. It also increases plasma glucagon, food intake, hepatic oxidative stress, and plasma lipids [33-38]. Thus, ETH administration has served as a useful model to study liver metabolism and to induce liver ATP and glycogen depletion.

Exercise training is known to affect liver ATP and glycogen levels and alter circulating ghrelin levels. To our knowledge, despite the growing information about the effects of ghrelin on liver metabolism and plasma metabolites, there is no information about the effect of ETH-induced liver ATP and glycogen deficiency on plasma and liver total ghrelin and obestatin concentrations, which are thought to increase during fasting and other negative energy balance circumstances. Therefore, the present study was conducted to examine the effect of ETH-induced reductions in liver ATP and glycogen on plasma total ghrelin and obestatin in trained and untrained rats at rest. Based upon previous findings from our laboratories [26-28], we hypothesized that ETH-induced reductions in hepatic glycogen and ATP levels would be accompanied by increases in plasma levels of total ghrelin but no change in obestatin. A secondary hypothesis was that trained animals would reveal an attenuated response to the ETH treatment in terms of less reduction in liver glycogen and ATP and less increase in ghrelin.

2. Materials and methods

2.1. Animals

All experiments involving the animals were conducted according to the policy of Iranian Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes; and the protocol was approved by the Ethics Committee of the School of Medicine Sciences, Tarbiat Modares University (TMU), Tehran, Iran. Thirty-two Wistar male rats (10 weeks old) initially weighing 180 to 200 g were used for this study. Animals were obtained from Pasteur's Institute (Tehran, Iran) and maintained in the Central Animal House, School of Medical Sciences of TMU. The animals were housed in a 46-L cage with 5 animals per cage, and light was controlled using a 12-hour:12-hour light-dark cycle. Temperature was 22°C ± 1.4°C, and humidity was 55.6% ± 4.0%. Animals were fed a pellet rodent diet ad libitum and had free access to water. Animals were randomly assigned into control (n = 16) and training (n = 16) groups. The control group remained sedentary, whereas the training group underwent a moderate running exercise program.

2.2. Exercise training protocol

Treadmill training began with familiarization of rats with the apparatus for 4 days by placing the rats on a motorized-driven treadmill (Iranian Model, 14 lanes, designed by Dr Abbass Ghanbari-Niaki, Physical Education and Sports Sciences Department of TMU, Tehran, Iran). The training group was exercised 5 d/wk for 10 weeks as described previously [26,39]. Exercise intensity was progressively increased from 10 min/d at 15 m/min, 0% slope, up to 60 min/d at 25 m/min, 0% slope, for the last 5 weeks of the program. This condition corresponded to a moderate intensity of about of 65% of maximal oxygen consumption [40].

2.3. Tissue collection

On the day of the experiment, food was removed from the cage at least 4 hours before the beginning of the experiment. Rats weighing 300 to 320 g were injected (intraperitoneally) with either DL-ETH (0.7 mg/g of body weight) or an equivalent volume of saline (0.9% NaCl). The dose of ETH was chosen because it has been used successfully when injected intraperitoneally in several previous studies [31,35]. DL-Ethionine was obtained from Sigma-Aldrich (Winston Park, Oakville, Ontario, Canada). DL-Ethionine was first solved in physiologic saline (35 mg/mL) of low pH (5.5-5.6), and the pH was adjusted thereafter to 7.0 to 7.4 with NaOH (1 N) solution [35]. The solutions were injected 2 times with a 2-hour interval between injections. The second injection was followed by an additional 2-hour period. This was done to allow the ETH to have its full effect before collection of blood and liver samples. Animals were anesthetized with a mixture of ketamine (80 mg/kg) and Rompun (Alfasan, Woerden, Holland; 10 mg/kg; xylazine) (0.02-0.05 mL, intravenously). After complete anesthesia, the abdominal cavity was opened, a small piece of liver (400-500 mg) from the median lobe was immediately freeze-clamped using liquid nitrogen precooled aluminum tongs, and the sample was excised and immersed in liquid nitrogen (<15 seconds) for determination of liver ghrelin, ATP, and glycogen concentrations. All frozen liver pieces were stored at −80° C until analysis was performed. Blood was collected directly from the heart in test tubes containing EDTA, separated by centrifugation, frozen, and stored −80° C until biochemical analysis was performed.

2.4. Plasma glucose, total ghrelin and obestatin levels, ratios, liver ATP and glycogen concentrations

Plasma glucose was determined by an enzymatic, colorimetric method (glucose oxidase–amino antipyrine; Pars Azmoun, Tehran, Iran); and the intraassay coefficient of variation and sensitivity of the method were 1.3% and 1 mg/dL, respectively. Plasma total ghrelin and obestatin levels were determined by rat enzyme immunoassay (EIA) methods (Phoenix Pharmaceuticals, Burlingame, CA, and Peninsula Laboratories, San Carlos, CA, respectively). The

intraassay coefficient of variation and sensitivity of the EIA for ghrelin were 8.9% and 0.008 ng/mL. For obestatin, the EIA intraassay coefficient of variation and sensitivity were 5.4% and 0.02 ng/mL. Liver ATP concentration was determined using a Biaffin (Kassel, Germany) ATP Sensitive Bioluminescence Kit. Glycogen was determined using a commercial kit (Glycogen Colorimetric Kit; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Because obestatin is thought to oppose the orexigenic effects of ghrelin [10] and because previous investigators have shown greater obestatin-ghrelin ratios in anorexic patients [41] and lower ghrelin-obestatin ratios in obese women [42], we thought it important to examine ghrelin-obestatin ratios in trained/untrained, ETH-treated/-untreated animals.

2.5. Statistics

All the data are reported as mean ± SE. Statistical analyses were performed using a 2-way analysis of variance. Least significant difference post hoc test was used in the event of a significant ($P < .05$) F ratio. All statistical analyses were performed with SPSS (Version 13; SPSS, Chicago, IL).

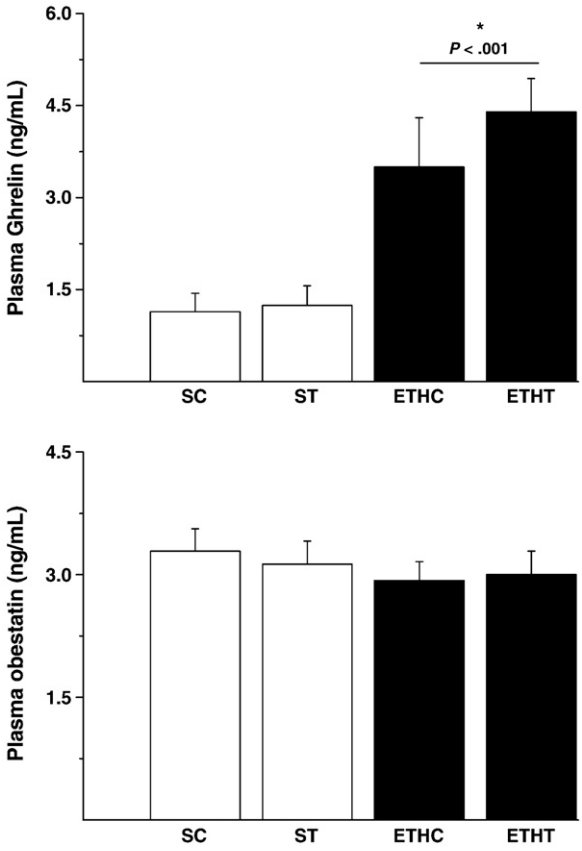

Fig. 1. Plasma total ghrelin and obestatin concentrations in saline- and ETH-treated rats. SC indicates saline-control; ST, saline-trained; ETHC, ETH-control; and ETHT, ETH-trained. *Ethionine vs saline. Data are expressed as mean ± SE for 8 animals per group. Plasma total ghrelin was significantly higher in ETH-treated rats.

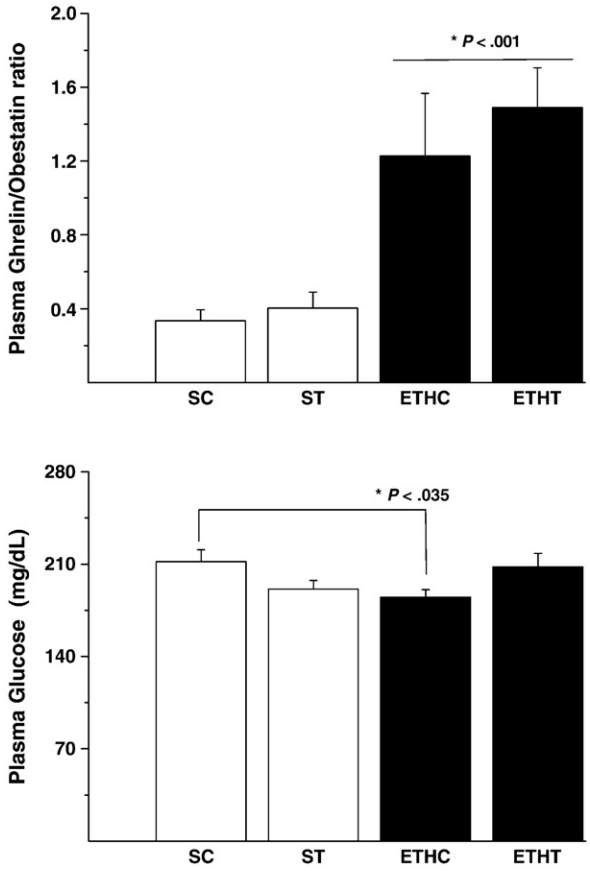

Fig. 2. Plasma total ghrelin and obestatin ratio and plasma glucose concentrations in saline- and ETH-treated rats. *Ethionine vs saline. Data are expressed as mean ± SE for 8 animals per group. Plasma total ghrelin-obestatin ratio was significantly higher in ETH-treated rats.

3. Results

After the administration of ETH in the control and trained animals, plasma total ghrelin concentrations were significantly higher compared with saline-injected rats; however, obestatin levels were not different among groups (Fig. 1A, B). In addition, the plasma ghrelin-obestatin ratio was significantly greater ($P < .001$) in ETH-treated rats when compared with saline groups, whereas a reduction in plasma glucose was significant in the ETH-treated trained rats but not the ETH-treated control (nonexercised) rats (Fig. 2). Liver ATP levels were significantly decreased by approximately 25% after ETH administration in ETH-treated compared with saline-injected rats (Fig. 3A). The administration of ETH also resulted in a significant decrease by approximately 27% in liver glycogen concentrations in control as well as trained animals (Fig. 3B).

4. Discussion

Ethionine treatment elicited the expected reductions in liver glycogen and ATP content, which is consistent with

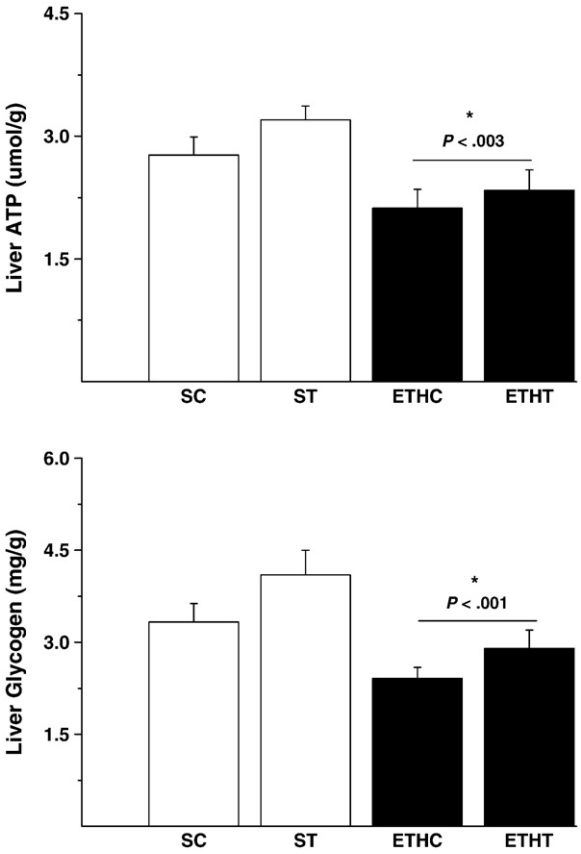

Fig. 3. Liver ATP and glycogen concentrations in saline- and ETH-treated rats. *Ethionine vs saline. Data are expressed as mean ± SE for 8 animals per group. The levels of ATP and glycogen were significantly lower in ETH-treated rats.

previous research [31,32,34-36]. Thus, the main findings of the study were that ETH-induced liver ATP and glycogen deficiency (1) exerts a strong regulatory influence on plasma total ghrelin and (2) does not affect obestatin levels, and that (3) exercise training does not alter these results. The first 2 findings were consistent with our hypotheses; the third was not. These data demonstrate the short-term energy-regulating capacity of ghrelin. However, obestatin findings reveal that it is not as sensitive to changes in liver glycogen and ATP content as ghrelin. There was no effect of exercise training on plasma ghrelin and obestatin, suggesting that exercise training was not a sufficient stimulus to alter orexigenic/anorexigenic peptide levels under ETH treatment conditions.

Dietary restriction has previously been used to study ghrelin and obestatin dynamics [10,15-17,43]. Although diet modification will elicit declines in levels of liver glycogen and ATP, it will also expose the stomach mucosa (a major source of ghrelin and obestatin) to different substrate concentrations. Moreover, fasting changes the mobilization of carbohydrates and fats to skeletal muscle and other tissues to a greater degree than would ETH administration (via the liver). The importance of the present study is that ETH treatment mainly affects liver metabolism and thus isolates the effects of liver metabolism on the orexigenic peptides to a greater degree than investigated in previous studies. Findings demonstrate that liver glycogen content alone appears to substantially alter ghrelin but not obestatin levels.

The mechanisms by which a single dose of ETH decreases liver glycogen and ATP levels have been studied extensively. It is thought that the action of ETH on liver metabolism, particularly on ATP and glycogen depletion, is mediated via inhibition of enzymatic reactions (ATP plus methionine and *S*-adenosyltransferase). The reactions are required for subsequent conversion of methionine to *S*-adenosylmethionine as a methyl donor in the numerous methylation processes, and *S*-adenosylhomocysteine is hydrolyzed to homocysteine by releasing the adenosyl moiety. The released adenosine is reused to produce ATP, whereas homocysteine undergoes remethylation to methionine. However, during the conversion of ETH to *S*-adenosylethionine, it competes with methionine for *S*-adenosyltransferases. This, in turn, increases the levels of *S*-adenosylethionine and decreases the level of *S*-adenosylmethionine in the liver and other tissues, which is accompanied by severe and rapid decline of the liver ATP in female more than male rats [30,44-46]. Drug- and nutrient-induced liver ATP and glycogen deficiency has been shown to increase food intake in male and female rats [47-52]. Thus, data from the present study suggest that liver glycogen deficiency may induce hunger by increasing circulating levels of ghrelin, an orexigenic hormone.

It is also well known that liver and muscle glycogen are primary sources for the resynthesis of ATP during fasting and exercise [53-56]. Mamedova et al [56] reported that glycogen depletion resulted in a considerable decrease (40%) of ATP in myotubes, indicating that the cells were suffering from metabolic stress immediately after glycogen depletion. The effect of fasting (but not specifically liver glycogen/ATP depletion) on plasma total ghrelin and obestatin concentrations has been studied previously. For example, Guo et al [15] reported increased plasma total ghrelin and obestatin levels in rats fasted for 48 hours when compared with these orexigenic peptide levels in ad libitum–fed rats (4.21 ± 0.18 vs 2.84 ± 0.11 ng/mL). It has also been shown that low-carbohydrate diets have an impact on the levels of liver and muscle glycogen and plasma total ghrelin concentrations in human and animal subjects [16,17,57,58]. Collectively, these data and data from the present investigation suggest that carbohydrate levels in the liver provide an important signal for ghrelin release.

Concerning the training effect, it is known that exercise training will increase liver glycogen and ATP levels [26]. Indeed, mean liver glycogen and ATP content was higher in the trained saline-treated rats when compared with control saline-treated group. The same pattern was also observed in trained and control ETH-treated rats. We hypothesized that this training-induced increase in liver glycogen and ATP would attenuate ETH-induced reduction in liver glycogen and thus attenuate the increase in plasma ghrelin levels. However, this did not occur. Thus, there may be a critical

level of decline in liver glycogen and/or ATP content that affects plasma ghrelin levels for which a training-induced attenuation of that decline may not be able to compensate.

In summary, this is the first study demonstrating that liver glycogen and ATP deficiency was accompanied by increases in total plasma ghrelin, which is considered an important initiator of food intake. Findings also revealed that reductions in liver glycogen and ATP do not affect plasma obestatin, nor does exercise training affect these results. The present study partially addresses ghrelin as an important element in a signaling pathway involved in liver glycogen and ATP deficiency–induced food intake and reveals that hepatic glycogen depletion may affect feeding behavior. Data also indicate that ghrelin is more sensitive to liver energy deficit than obestatin. This suggests a pronounced role for ghrelin in short-term energy balance. Further investigation of the effects of greater liver glycogen and ATP deficiency as well as more strenuous exercise on concentrations of these peptides is warranted.

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