Endocr (2008) 33:77–83
DOI 10.1007/s12020-008-9056-z

ORIGINAL PAPER

Influence of short- and long-term treadmill exercises on levels of ghrelin, obestatin and NPY in plasma and brain extraction of obese rats

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Received: 22 December 2007 / Accepted: 19 March 2008 / Published online: 4 April 2008

Abstract This study aims to clarify the effects of exercise on levels of appetite regulatory hormones in plasma and hypothalamus of obese rats. Diet-induced obese rats undergo short- (40 min) and long-term (40 min, 5 days/week for 8 weeks) exercises. The rats ran at a speed of 20 m/min on a 5° slope treadmill. Rats undergoing short-term exercise were divided into C, E0, E1, E3, E12, and E24. Rats undergoing long-term exercise (LE) were compared to long-term control (LC). Concentrations of ghrelin, obestatin, and neuropeptide Y (NPY) were measured using radio immuno-assay. Expression of ghrelin receptor (GHSR-1a), putative obestatin receptor (GPR-39), and NPY in the hypothalamus was measured by quantitative RT-PCR. After short-term exercise, the plasma concentrations of ghrelin and obestatin were not changed, but NPY decreased. Ghrelin and obestatin in the hypothalamus decreased, and recovered 12 until 24 h. NPY increased and recovered after 24 h. Expression of GHSR-1a and NPY was not changed and GPR-39 was not observed. In LE, these changes are different in plasma and hypothalamus. It would be concluded appetite and body weight of obese rats are decreased by exercise through reduced level of ghrelin in the hypothalamus. Obestatin seems to have no effect in exercise-induced change in appetite.

Keywords Obesity · Exercise · Appetite · Ghrelin · Obestatin · NPY

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Introduction

Ghrelin is an endogenous ligand for the growth hormone secretagogue receptor [1]. Considerable and unequivocal evidence shows that ghrelin plays critical roles in the short- and long-term regulation of appetite and body weight [2]. Levels of circulating ghrelin increase markedly before each meal and decrease rapidly after food intake [3–7], and exogenous ghrelin administration stimulates appetite and food intake in both rodents and humans [3, 8, 9]. In addition, ghrelin stimulates gastrointestinal motility [10, 11], gastric acid secretion [12] and pancreatic exocrine secretion [13]. It can also decrease locomotor activity [14], energy expenditure [3, 10], fat catabolism, and lipolysis [3, 15, 16], adipocyte apoptosis [17], and can directly promote adipogenesis [3, 8, 15, 17]. Thus, ghrelin affects the multiple steps involved in appetite and food intake, energy expenditure and fuel utilization, and promotes weight gain and fat accumulation. It is hypothesized that elevated levels of ghrelin may contribute to the pathogenesis of obesity [18].

Recently, Zhang et al. [19] reported that the GHRL (ghrelin/obestatin preprohormone) gene also encoded another 23-amino acid secreted peptide, termed "obestatin." The biological activity of obestatin requires the amidation of its carboxyl terminus, and it can then bind to and activate the orphan receptor GPR39 [19]. It is interesting to note that obestatin, though derived from the same peptide precursor as ghrelin, suppresses food intake, inhibits jejunal contraction, reduces body-weight gain and antagonizes the action of ghrelin when both peptides are co-administered [19]. It is therefore suggested that the delicate balance of ghrelin and obestatin is important in the regulation of energy homeostasis and body-weight control. Subsequent investigations, however, reach a different conclusion. Unlike for ghrelin, fasting rodents for 48 h and

then re-feeding has no effect on obestatin levels [19]. Receptor GPR39 is expressed in several tissues in human and rodents including stomach, small intestine, pituitary and hypothalamus [19, 20]. It is also found that GPR39 expresses in rodent amygdala, hippocampus, auditory cortex, and other brain regions but not in hypothalamus [21]. Two separate research groups have failed to activate the GPR39 receptor with obestatin [22, 23]. In addition, ghrelin induces wakefulness in rodents [24] and subsequent research has shown that centrally administered obestatin increases time in non-rapid-eye-movement sleep; hence, obestatin has a sleep-promoting effect [25]. Although the original publications of two groups have partially confirmed the effects of obestatin on food intake and gastric emptying [26, 27], other groups have failed to replicate these findings [28, 29]. Its significance in the short-term regulation of food intake therefore remains unclear and contradictory. It also warrants more research in this area.

Ample evidence indicates that exercise causes weight loss in obese individuals. As the balance of ghrelin and obestatin is involved in the pathogenesis caused by obesity, the effect of exercise on levels of ghrelin and obestatin in the circulation and the metabolic regulatory area of brain, the hypothalamus, requires investigation. In this experiment, we measured the levels of ghrelin, obestatin, and neuropeptide Y (NPY, a well-established peptide regulating appetite) in the plasma and hypothalamus of obese rats after short- and long-term periods of exercise, aiming to clarify the mechanism of exercise in weight control.

Results

Influences of short-term exercise

The influences of short-term exercise on meal size of obese rats

Almost every group had a great decline in meal size after acute exercise, except for E0. E1 ate little for 1 h after exercise. The meal size of groups E3, E12, and E24 was recovered with the greater time following exercise. The effect of exercise on meal size is given in Table 1.

Table 1 The meal size of obese rats after short-term exercise

Groups	Decrease (g)	Decreasing rate (%)
E1 ($n = 8$)	2	100
E3 ($n = 8$)	2.5	83
E12 ($n = 8$)	14.2	70
E24 ($n = 8$)	16	64

E1, 1 h post-exercise; E3, 3 hour post exercise; E12, 12 h post-exercise; E24, 24 h post-exercise

The influence of short-term exercise on serum lipid levels of obese rats

The level of GLU decreased greatly in group E1 ($P < 0.01$). There was no significant difference seen in other groups. The level of TG decreased markedly in E12 but was not changed greatly in other groups. CHOL, HDL-C, and LDL-C levels changed by different degrees. These changes were listed in Table 2.

In Fig. 1, it can be seen that the concentrations of plasma ghrelin (a) and obestatin (b) have not changed markedly, but NPY (c) decreased greatly in each group after acute exercise.

In Fig. 2, it can be seen that the concentrations of ghrelin and obestatin (a) in the hypothalamus decreased greatly at 0, 1, and 3 h post-exercise, then increased sharply for the E12 group and were restored for the E24 group. The changes of ghrelin and obestatin were proportionally similar. NPY (b) levels increased greatly after acute exercise but were restored for the E24 group.

In Fig. 3, it can be seen that GHSR-1a mRNA and NPY mRNA in the hypothalamus did not change after acute exercise. GPR-39 mRNA could not be detected on the hypothalamus in any group.

Influences of long-term exercise

In Table 3, it can be seen that after 8 weeks of exercise, the weight of both LC and LE groups increased, although the increase in the LE group was not as great.

The meal size of the LE group decreased on every test day compared to the LC group. These results are given in Table 4.

In Fig. 4, it can be seen that 8 weeks of exercise had almost no effect on serum index of obese rats.

After 8 weeks of exercise, the concentrations of plasma ghrelin and obestatin had not changed in the LE group compared to LC group. However, NPY decreased sharply after long-term exercise. Results were different in the hypothalamus; however, the concentrations of ghrelin and obestatin decreased sharply while NPY increased greatly. These results can be seen in Figs. 5 and 6.

In Fig. 7, it can be seen that GHSR-1a mRNA in the hypothalamus did not change compared to LC group. However, NPY mRNA increased sharply after long-term exercise.

Discussion

Obesity represents one of the most urgent global health threats as well as being one of the leading causes of death throughout industrialized nations. It has been reported that

Table 2 The serum index of obese rats after short-term exercise

Group	Index				
	GLU (mmol/l)	TG (mmol/l)	CHOL (mmol/l)	HDL-C (mmol/l)	LDL-C (mmol/l)
C ($n = 6$)	7.51 ± 0.38	0.79 ± 0.17	2.07 ± 0.27	0.59 ± 0.09	0.24 ± 0.07
E0 ($n = 8$)	7.48 ± 0.58	0.64 ± 0.15	1.77 ± 0.18*	0.48 ± 0.03*	0.29 ± 0.10
E1 ($n = 8$)	5.97 ± 0.64**	0.61 ± 0.14	1.62 ± 0.16**	0.42 ± 0.04**	0.23 ± 0.04
E3 ($n = 8$)	6.98 ± 0.82	0.61 ± 0.27	1.75 ± 0.25	0.48 ± 0.08*	0.27 ± 0.06
E12 ($n = 8$)	6.84 ± 0.85	0.55 ± 0.20*	1.82 ± 0.42	0.48 ± 0.11	0.32 ± 0.13
E24 ($n = 8$)	6.24 ± 0.65	0.67 ± 0.34	1.75 ± 0.42	0.52 ± 0.16	0.34 ± 0.18**

C, control group; E0, post-exercise; E1, 1 h post-exercise; E3, 3 h post-exercise; E12, 12 h post-exercise; E24, 24 h post-exercise
*$P < 0.05$; **$P < 0.01$

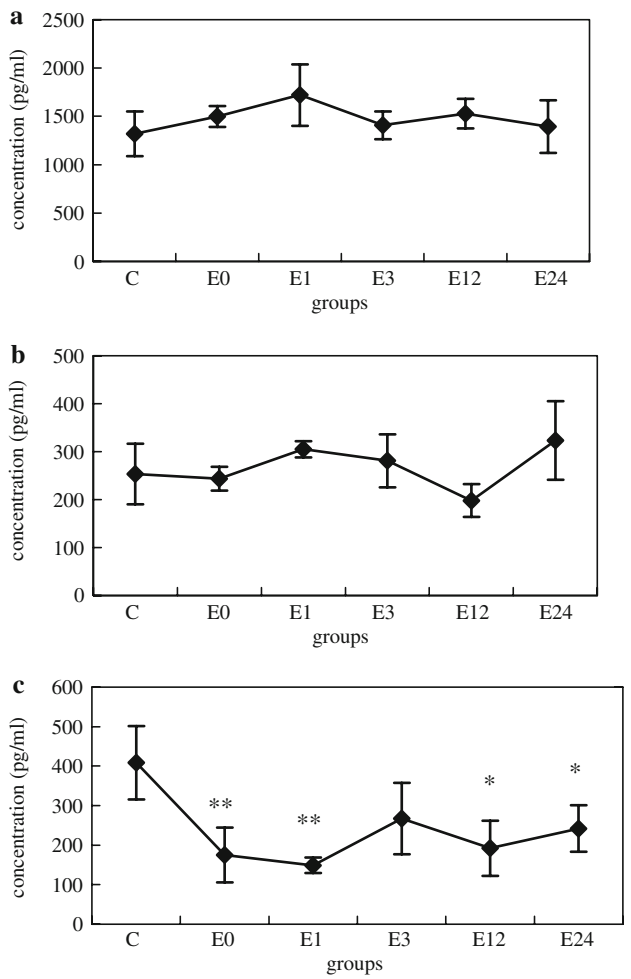

Fig. 1 The concentrations of plasma ghrelin (**a**), obestatin (**b**), and NPY (**c**) after short-term exercise. The concentrations of plasma ghrelin and obestatin have not changed markedly, but NPY decreased greatly in each group after acute exercise. C, control group; E0, post-exercise; E1, 1 h post-exercise; E3, 3 h post-exercise; E12, 12 h post-exercise; E24, 24 h post-exercise. *$P < 0.05$, **$P < 0.01$

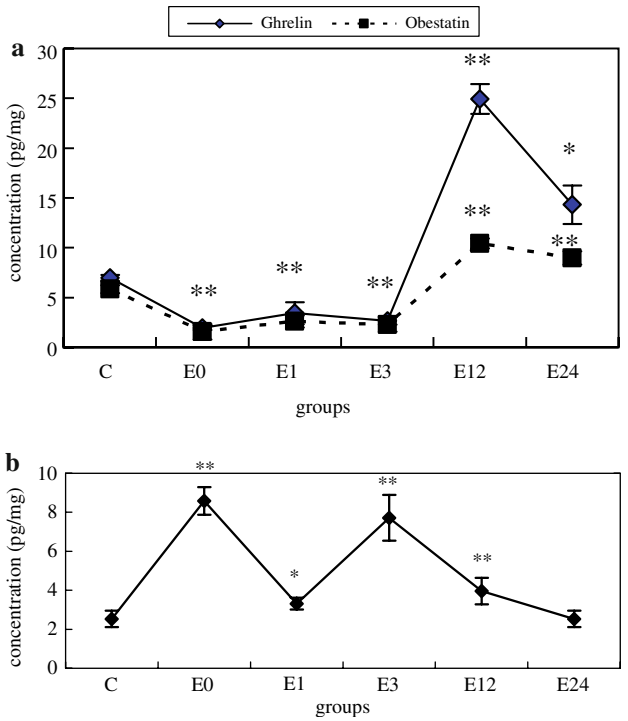

Fig. 2 The concentrations of brain ghrelin, obestatin (**a**) and NPY (**b**) after short-term exercise. The concentrations of ghrelin and obestatin in the hypothalamus decreased greatly at 0, 1, and 3 h post-exercise, then increased sharply for the E12 group and were restored for the E24 group. The changes of ghrelin and obestatin were proportionally similar. NPY levels increased greatly after acute exercise but were restored for the E24 group. The concentrations of hypothalamic ghrelin, obestatin (**a**) and NPY (**b**) after short-term exercise. C, control group; E0, post-exercise; E1, 1 h post-exercise; E3, 3 h post-exercise; E12, 12 h post-exercise; E24, 24 h post-exercise. *$P < 0.05$, **$P < 0.01$

exercise is the best method to reduce weight for the obese and over-weight. Exercise expends energy and influences appetite, although the mechanisms for the improvement are not clear. Several hormones have been identified to play an important role in regulating appetite and body weight.

Ghrelin has been shown to stimulate food intake after acute systemic (intraperitoneal) or intracerebroventricular administration. However, the concentration of ghrelin administrated intraperitoneally was 1,000-fold that injected intracerebroventricularly [30].

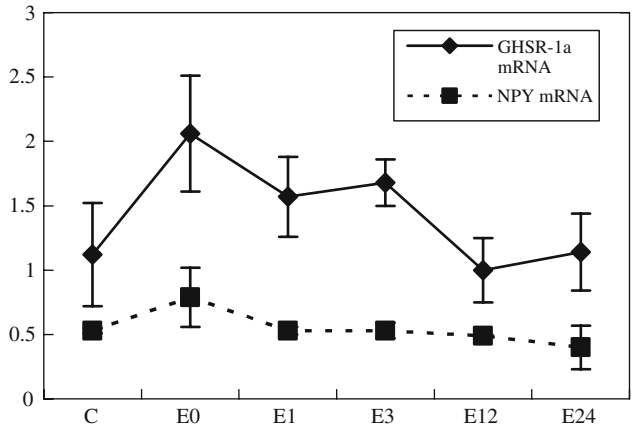

Fig. 3 Influences of GHSR-1a and NPY mRNA on hypothalamus after short-term exercise. GHSR-1a mRNA and NPY mRNA in the hypothalamus did not change after acute exercise. GPR-39 mRNA could not be detected on the hypothalamus in any group. C, control group; E0, post-exercise; E1, 1 h post-exercise; E3, 3 h post-exercise; E12, 12 h post-exercise; E24, 24 h post exercise

Table 3 Influences of body weight of obese rats after long-term exercise

Group	LC ($n = 10$)	LE ($n = 10$)
Weight pre-exercise (g)	440.5 ± 35.4	439.6 ± 28.1
Weight post-exercise (g)	589.7 ± 52.1	478.7 ± 37.4**
Weight increasing (g)	149.2	39.1

LC, long-term control group; LE, long-term exercise group
*$P < 0.05$; **$P < 0.01$

Table 4 Influence of meal size of obese rats after long-term exercise

Weeks	LC (g)	LE (g)	Reduction (g)	Reducing rate (%)
1	23.03	16.95	6.08	26.40
2	31.04	20.46	10.58	34.10
3	28.44	19.64	8.80	30.95
4	25.27	19.60	5.67	22.45
5	23.42	18.42	5.00	21.34
6	23.82	20.40	3.41	14.33
7	24.06	19.64	4.43	18.39
8	23.84	20.01	3.84	16.07

LC, long-term control group; LE, long-term exercise group

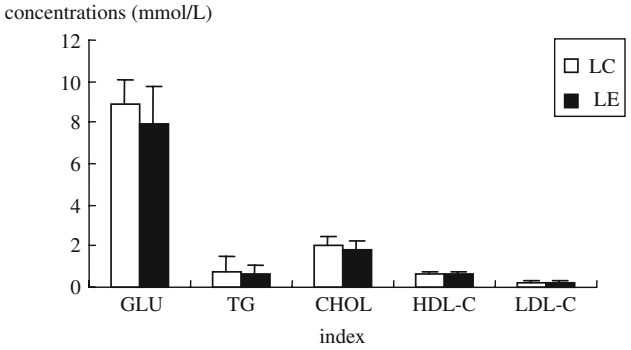

Fig. 4 The serum index of obese rats after long-term exercise. Eight-weeks of exercise had almost no effect on serum index of obese rats. LC, long-term control group; LE, long-term exercise group

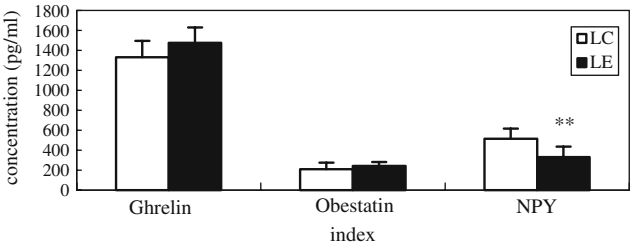

Fig. 5 The concentrations of plasma ghrelin, obestatin, and NPY after long-term exercise. After 8-weeks of exercise, the concentrations of plasma ghrelin and obestatin had not changed in the LE group compared with LC group. However, NPY decreased sharply after long-term exercise. Results were different in the hypothalamus; however, the concentrations of ghrelin and obestatin decreased sharply while NPY increased greatly. LC, long-term control group; LE, long-term exercise group. $P < 0.01$

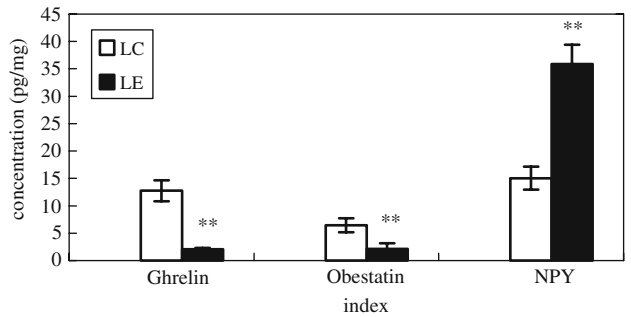

Fig. 6 The concentrations of hypothalamic ghrelin, obestatin, and NPY after long-term exercise. LC, long-term control group; LE, long-term exercise group. **$P < 0.01$

Regarding the effect of exercise on plasma ghrelin concentrations, different researchers have come to variable conclusions. Some researchers reported that acute exercise had no effect on plasma ghrelin concentrations [31–35], while other studies concluded that plasma ghrelin concentrations increased after physical exercise [36–40].

There are no extended studies on levels of ghrelin in the hypothalamus during or after exercise, although this area of the brain is central to the control of appetite and body weight. Several studies have however reported that physical exercise under normal conditions induces hyperphagia by increasing energy intake in humans and stimulating NPY activity in the rat hypothalamus [41, 42]. It is therefore highly possible that ghrelin, obestatin, and NPY levels are affected by exercise and these changes lead to meal size and weight changes.

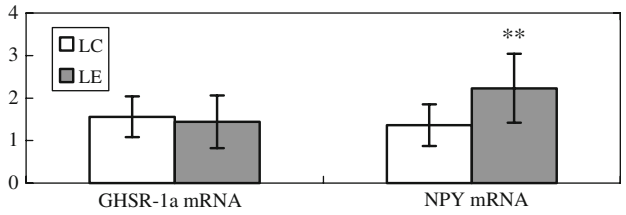

Fig. 7 GHSR-1a and NPY mRNA on hypothalamic after long-term exercise. GHSR-1a mRNA in the hypothalamus did not change compared with LC group. However, NPY mRNA increased sharply after long-term exercise. LC, long-term control group; LE, long-term exercise group. $^{**}P < 0.01$

In this study, it was found that the rat meal size decreased sharply after short-term exercise and was restored at 12–24 h post-exercise. Serum GLU and TG decreased only 1 h post-exercise. Plasma ghrelin concentrations were not changed at 24 h after short-term exercise. This is consistent with previous published data [32]. The level of ghrelin in the hypothalamus decreased during the first 12 h after short-term exercise, then increased briefly before returning to pre-exercise level 24 h after exercise. The reason for the discrepancy between plasma and hypothalamic ghrelin concentrations is not clear. It is possible that negative energy balance caused by high intensity, short-term exercise may cause the increased hypothalamic ghrelin concentration and hyperphagia 12 h post-exercise. In our study, it was also found that plasma NPY decreased and hypothalamic NPY increased after short-term exercise in obese rats. NPY stimulates food intake and is secreted mostly by the hypothalamus. Plasma NPY was irrelevant to feeding of obese rats. Increased hypothalamic NPY stimulates feeding and reduces energy expenditure after exercise, which is probably a protective feedback mechanism. The fact that there are no changes to ghrelin receptor GHSR-1a and NPY mRNA in the hypothalamus indicate that acute exercise cannot increase their synthesis. Acute exercise does not decrease serum GLU and TG immediately after exercise. This is because obese rats can motivate surplus energy of body after exercise, so hypothalamic ghrelin concentration decreased sharply. After 1 and 3 h, levels of GLU and TG decreased. This leads to hypothalamic ghrelin and NPY concentrations increasing.

After 8-week long-term exercise, the LE group's body weight decreased sharply compared with LC. This is attributed to the decreased meal size and to energy expenditure. It may also be attributed to the decreased hypothalamic ghrelin concentration according to the molecular biology mechanism; however, the plasma ghrelin concentration did not change compared with LC. It can be concluded that it is the change of hypothalamic ghrelin concentration that leads to the rats' body weight decrease after 8 weeks exercise, but not plasma ghrelin concentration. Ghrelin receptor GHSR-1a having no change indicated that long-term exercise could not induce the increasing of ghrelin synthesis. At the same time, NPY and NPY mRNA increased, indicating that NPY synthesis and release augment after long-term exercise. The levels of brain NPY remained continuously high after the exercise, indicating such high-intensity exercise was in a state of exhaustion. It may be contributed to the stimulation of brain NPY neurons by reducing energy expenditure. Hypothalamic ghrelin are decreased after 8 weeks exercise, but NPY is increased. The decrease in ghrlein in hypothalamus was induced by exercise and such decrease led to reduction in food intake. The energy deficit caused by exercise and food intake resulted in the increase in NPY on hypothalamus. The increase in NPY may be a protective feedback to avoid exhaustion.

The functions of obestatin and its receptor have not yet been stated clearly in the literature. In this study, GPR-39 mRNA expression was not identified in the hypothalamus before or after exercise. The change in obestatin levels was consistent with that for ghrelin in short-term and long-term exercise models. As there was no GPR-39 found in our study, it is not clear whether there is any effect of obestatin in the hypothalamus on food-intake or energy balance. As changing pattern is similar between ghrelin and obestatin, it is hard to propose an opposite effects between two peptides.

From theses results, it would be concluded that appetite and body weight of obese rats are decreased by exercise through a reduced level of ghrelin in the hypothalamus. Obestatin seems to have no effect in this exercise-induced change in appetite. GPR39 was not observed in the hypothalamus. How did exercise decrease ghrelin in hypothalamus is still not clear and awaits future research.

Materials and methods

Animals and obesity model

Diet-induced obese male Sprague–Dawley rats weighing between 404 and 464 g were housed under standard light cycle (12-h light phase, 12-h dark phase) and at controlled ambient conditions (temperature 22°C; humidity 70%). These animals had ad libitum access to a high-fat diet (Beijing Keao Xieli Co., changed every morning) and tap water. All experiments were conducted in accordance with the internationally accepted principles for the care and use of laboratory animals and approved by the Beijing Ethical Committee for Animal Research.

Experimental design I: influences of short-term exercise on obese rats

Forty-six obese rats were randomly divided into six groups, namely C (control, six rats), E0, E1, E3, E12, and E24

(number of hours after exercise, eight rats for each group). All groups were engaged in short-term exercise, with the exception of the control group. Rats were trained to run on a motor-driven treadmill at different time on the daytime and they were killed on the light cycle. The treadmill slope was 5°, the speed was 20 m/min and duration was 40 min after 3 days' adaptation at 20 min/day. On the days of adaptation, the rats were forced by administration of sound (15–30 db) and mild electric shock presented to the rats' tails when the rats stopping running. The rats accustomed to running on the treadmill after 3 days' adaptation. After that, the sound and electric shock have no effect on them and not required. On the exercising day, the sound and electric shock were not needed and not applied. The control group remained in their cage during the periods when their counterparts were exercising. E0 were tested at 0 min post-exercise, E1 were tested 1 h post-exercise, E3 were tested 3 h post-exercise, E12 were tested 12 h post-exercise and E24 were tested 24 h post-exercise.

Experimental design II: influences of long-term exercise on obese rats

Twenty obese rats were randomly divided into two groups: long-term control group (LC = 10 rats) and 8-week long-term exercise group (LE = 10 rats). The rats ran on a motor-driven treadmill at a speed of 20 m/min, 50 slopes for 40 min at 8:00–8:40 am per day except every Saturday and Sunday for 8 weeks. The control group remained in their cage during experimental days. They were killed after 24 h rest at 8:00 am on the last exercise day.

Preparation of plasma and tissue

All rats were placed under deep anesthesia with 2% (0.25 mg/100 g, i.p.) Pentobarnital Na. Blood was drawn from the abdominal aortia. A proportion of blood taken was injected into tubes for standard serum collected for GLU, TG, CHOL, HDL and LDL measurement. A proportion was collected in cooled tubes containing 4 M ethylene glycol-bis (2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid and 3 M GSH (Glutathione), mixed thoroughly and then centrifuged at 4,000 rpm/min for 20 min at 4°C. The plasma layer was carefully transferred to a fresh tube kept at −20°C for radio immuno-assay (RIA). Rats' hypothalamus were stripped and divided into two parts from the middle line. The left was preserved −70°C for quantitative RT-PCR and the right was boiled at 100°C isotonic Na chloride for 5 min. The boiled tissue was weighed and homogenated using 0.5 ml HCl (1 mol/l), standing 100 min at room temperature, then neutralized with 0.5 ml NaOH (1 mol/l). Finally, it was centrifuged at 4,000 rpm/min for 20 min at 4°C and the upper layer was carefully transferred to a fresh tube kept at −20°C for RIA.

Measurement of food intake and serum levels

The meal size was measured by weight, by subtracting the food remaining from that served. Every treatment group was given access to food at the same time with the control group on light cycle. E12 and E24 groups exercised ahead of time with other groups. Serum GLU, TG, CHOL, HDL, and LDL were measured by using auto-biochemistry instruments (HITACHI-7020).

Radioimmunoassay

The concentrations of ghrelin, obestatin, and NPY in plasma and hypothalamus were assayed using a commercial RIA (Phoenix Biotech Co., LTD). The total plasma immunoreactive peptide concentrations were measured with samples that had never been previously thawed, using the modification of a commercial RIA that employs a polyclonal antibody raised against full-length rat ghrelin and obestatin and ^{125}I-labeled peptides as a tracer (Phoenix Pharmaceuticals, Belmont, CA).

RNA extraction and RT-PCR

Total RNA was extracted by Trizol Reagent (Invitrogen, Inc.). Rat hypothalamus expression of the mRNA encoding GHSR 1a, GPR-39, and NPY was assessed by quantitative RT-PCR. Actin was used as a control for PCR and we normalized all data to the actin control. The oligonucleotide specific primers for rat are as follows. GHSR1a(Sense:5′CAGCGTCTTCTTCTTTCTAC3′;Antisense:5′TGGACATGAGCACCATCTTC3′);GPR-39(Sense:5′CCAAGCGTCCGCACTGTAAC3′; Antisense:5′CCATGTCACCACGATCAGTC3′),NPY(Sense:5′GTGGACTGACCCTCGCTCTA3′;Antisense:5′TGCATTGGGTGGGACAGG3′) and *β*-actin (Sense:5′CCCATCTATGAGGGTTACGC3′;Antisense:5′CATCGTACTCCTGCTTGCTG3′).

Statistical method

All values are expressed as means ± SEM unless otherwise indicated. The data were analyzed at time points 0, 1, 3, 12, and 24 h after exercise and 8 -week exercise. The data were compared by independent samples *t*-test using SSPS11.5. Values of $P < 0.05$ were considered significant.

Acknowledgments This work was supported by grants from the State Sport General Administration of China, The Science and Technology Foundation (Project 05054); Open Topic of the Beijing Key Laboratory (Project 2007TY021) and Australian NHMRC.

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