Nutritional and environmental factors affecting plasma ghrelin and leptin levels in rats

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Abstract

We examined which factors suppress the rise of ghrelin secretion under hunger in 16-h-starved rats, and compared the responses of plasma ghrelin and leptin levels to various exogenous and endogenous stimuli in intact rats. Although an acute expansion of the stomach by infusion of 6 ml air or 3 ml water in rats starved for 16 h did not change the level of plasma acyl-ghrelin 3 ml corn starch solution, corn oil, or 20% ethanol significantly decreased it. Vagotomy inhibited suppression by nutrients but not by ethanol. Chronic infusion of ethanol into the stomach for 3 weeks in free-feeding rats caused widespread injury of the stomach mucosa, and increased both plasma ghrelin levels and the number of ghrelin cells. In intact rats, low temperature did not change ghrelin levels, but increased leptin levels. On the other hand,

restriction stress decreased plasma ghrelin levels, but had the reverse effect on plasma leptin levels. Although insulin decreased and 20% glucose increased plasma glucose levels, they both decreased plasma ghrelin levels. Insulin elevated plasma leptin levels, but glucose had no effect. These results indicate that 1) acyl-ghrelin secretion from the stomach under fasting condition is suppressed by nutrients but not by mechanical expansion of the stomach; 2) high and low environmental temperature, stress, or administration of insulin reciprocally affect plasma levels of ghrelin and leptin; and 3) an increase of stomach ghrelin cell number and plasma ghrelin levels after chronic ethanol treatment may be involved in restoration of gastric mucosae.

Journal of Endocrinology (2010) **207**, 95–103

Introduction

The peptide ghrelin consisting of 28 amino acids including an *O*-*n*-octanoylated Ser 3 residue has been identified as an endogenous ligand for the GH secretagogue receptor (GHSR; Kojima *et al.* 1999). This *O*-*n*-octanoylated Ser 3 residue is essential for ligand–receptor interaction. GHSR1a is the only receptor specific for acylated ghrelin that has been identified so far (Kojima & Kangawa 2005). Although ghrelin is synthesized in many tissues, including stomach, pancreas, cardiovascular tissue, hypothalamus, pituitary, kidney, and placenta (Mori *et al.* 2000, Gualillo *et al.* 2001, Hattori *et al.* 2001, Korbonsits *et al.* 2001, Date *et al.* 2002b), the main source of plasma ghrelin is X/A-like cells localized in the oxyntic gland in the mucosa of the gastric fundus (Date *et al.* 2000, Hosoda *et al.* 2000). In addition to its GH-stimulating action, many physiological functions of ghrelin have been revealed during the last decade, including the regulation of food intake, energy metabolism, gastrointestinal coordination, cell proliferation, apoptosis, cardiovascular function, and secretion of insulin and gut hormones (Nakazato *et al.* 2001, Kojima & Kangawa 2005, Nakahara *et al.* 2006, Zhang *et al.* 2008).

On the other hand, the peptide leptin consisting of 167 amino acids has been identified as a contributor to obesity in ob/ob mice (Zhang *et al.* 1994, Pelleymounter *et al.* 1995). Leptin is synthesized and released in the systemic fat tissues (white adipose tissue) or the placenta (Pelleymounter *et al.* 1995, Masuzaki *et al.* 1997, Friedman & Halaas 1998), and plays an important role in the regulation of food intake, energy consumption, glucose metabolism, the cardiovascular system, the immune system, and the secretion of insulin and pituitary hormone (Friedman & Halaas 1998, Ahima & Flier 2000).

Although ghrelin and leptin have many functions in common, most of their functions conflict. Ghrelin generally increases body mass by increasing food intake and decreasing energy consumption, while leptin decreases body mass by decreasing food intake and increasing energy consumption (Friedman & Halaas 1998, Kojima & Kangawa 2005). These results suggest that ghrelin and leptin play important roles in maintaining energy balance and the homeostasis of body mass.

Among many studies of physiological functions of ghrelin and leptin, their relation to obesity, and their molecular mechanisms, few studies have reported which exogenous or endogenous factors influence their secretion. Ghrelin secretion

in the stomach is promoted by hunger and suppressed by food intake (Kojima & Kangawa 2005). Although the effects of oral treatment with macronutrients on the plasma ghrelin levels in fasting rats were investigated, only total ghrelin levels were measured, but not acyl-ghrelin levels (Beck *et al.* 2002, Greenman *et al.* 2004, Heath *et al.* 2004, Bowen *et al.* 2006). Total ghrelin levels may correlate with acyl-ghrelin levels, yet long-term fasting inhibits ghrelin acylation without changing total ghrelin levels (Nass *et al.* 2008). Treatment with gut hormone (such as gastrin or cholecystokinin) and pancreatic hormone (such as glucagon, insulin, or somatostatin) also affects ghrelin secretion (Toshinai *et al.* 2001, Shimada *et al.* 2003, Kojima & Kangawa 2005, Katayama *et al.* 2007). On the other hand, leptin secretion is increased by sympathetic nerve stimulation, food intake, glucocorticoid, tumor necrosis factor- α , interleukin-1, and insulin, and is decreased by starvation (Haynes *et al.* 1997, Trayhurn *et al.* 1999, Olszanecka-Glinianowicz *et al.* 2005, Szkudelski 2007).

Here, we examined which mechanical or nutritional stimuli directly suppressed acyl-ghrelin secretion in the stomach under hunger, and whether the vagus nerve is necessary for its suppression, using both intact and vagotomized rats with implanted gastric cannulae. We also examined the acute and chronic influences of alcohol on ghrelin secretion, since alcohol intake injures the stomach. Next, we investigated how exposure to high and low temperatures affected the plasma ghrelin and leptin levels. To separate the effects of temperature from the effects of stress, we stressed control rats by restricting their activity. Finally, we compared the effects of insulin and blood glucose levels on plasma ghrelin and leptin levels.

Materials and Methods

Animals

Six-week-old adult male Wistar rats were purchased from Charles River Japan Inc. (Shiga, Japan) and maintained under a 12 h light:12 h darkness light regimen (light on at 0700 h) at $23 \pm 1^\circ\text{C}$. Animals were supplied with standard laboratory chow and water made available *ad libitum*. Each rat was handled for 5–10 min every day and was habituated to blood sampling by an incision to the tail tip until the initiation of experiments. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

Experiments

Experiment 1 Five 9-week-old rats were used in two replications of this experiment, 1 week apart. The rats were fitted with a stomach cannula 2 weeks before the experiment: a silicon tube (inside diameter: 1.0 mm and outside diameter: 1.5 mm) was inserted into the cardiac part of the stomach and brought out through the dorsal neck. Before each experiment, the rats were starved for 16 h (1700–0900 h).

To examine which factors suppress or stimulate ghrelin secretion under hunger, we directly infused 6 ml of air or 3 ml of water, 3 ml of 30% corn starch (3.18 kcal/rat; Wako Pure Chemical Industries, Ltd, Osaka, Japan) (to represent carbohydrate), 3 ml of corn oil (27.64 kcal/rat; Nakarai Tesque Inc., Tokyo, Japan) (to represent fat), or 3 ml of 20% ethanol (v/v 5.42 kcal/rat) into the stomach at 0900 h through the cannula. We selected 6 ml air in a preliminary experiment. Although 20% ethanol (about 1.14 g/kg body weight) may be a low dose in rodent alcohol research, we selected it because direct infusion of a higher dose of ethanol into the empty stomach may have induced gastric pain or alcohol intoxication, and thus complicated our interpretation of the change in ghrelin levels after ethanol treatment. Blood samples (60 μl) were collected through a tail tip incision at 20, 60, and 120 min after infusion from the conscious rats. We also examined the effects of corn starch, corn oil, and ethanol on the plasma ghrelin levels in starved rats in which bilateral subdiaphragmatic vagotomy had been performed 4 days earlier. The dosages of corn starch, corn oil, and ethanol administered to intact and vagotomized rats were the same.

In addition, the levels of *ghrelin* mRNA in the stomach were quantified in 16-h-fasted rats treated with water or 20% ethanol using real-time reverse transcription (RT)-PCR, as described previously (Sato *et al.* 2007). The rats were killed by decapitation 2 h after infusion of water or 20% ethanol into the stomach, and then the stomach was harvested. Total RNA was extracted from each stomach sample using an RNeasy Micro kit (Qiagen) and synthesized into first-strand cDNA using a High Capacity cDNA RT kit (Applied Biosystems, Foster City, CA, USA). A single tissue sample was sufficient for measuring the level of mRNA. An aliquot of first-strand cDNA (equivalent to 40–100 ng tissue) was quantified on a 7300 Real-time PCR system (Applied Biosystems) using TaqMan Gene Expression Master Mix (Applied Biosystems) with primers to amplify β -actin and *ghrelin* specifically. For these two genes, probe/primer kits were purchased from Applied Biosystems (TaqMan Gene Expression Assay ID: Rn00667869_m1, GenBank NM_031144 for β -actin, and Assay ID: Rn01425835_m1, GenBank NM_021669 for *ghrelin*).

Experiment 2 Each of eight 9-week-old rats was used to examine the effect of long-term treatment with ethanol (or water as a control) on plasma ghrelin levels. All the rats were fitted with a stomach cannula, as detailed in Experiment 1, and subsequently infused with ethanol three times a day (at 0730, 1300, and 1830 h) for 3 weeks.

The concentration of ethanol and schedule of treatment were based on a method for inducing fatty liver (Nishimura *et al.* 2001). The dose of ethanol was gradually increased stepwise during 3 weeks. Blood samples were collected at 1300 h on 7, 14, and 21 days after initiation of treatment. Rats were killed on day 21, and each stomach was excised for immunohistochemical analysis of ghrelin.

Experiment 3 To examine the influence of environmental low temperature or stress on ghrelin and leptin secretion, five 8-week-old intact rats per group were used in this experiment. All the experiments were performed twice using new intact rats. For cold exposure, we transferred intact rats from their usual room ($23 \pm 1^\circ\text{C}$) to cold room ($13 \pm 1^\circ\text{C}$). The receiving cages were at the same temperature as the new room. As the rats had been fed *ad libitum* and were transferred at 0900 h, it was likely that they were not hungry. As the rapid change of temperature could stress the rats in addition to the actual temperature treatment, we stressed some of the control rats by inserting them into a 23-cm transparent plastic tube (internal diameter: 5.5 cm) with a wire net flap without a change in the temperature. After the rats were transferred to their new housing, 60 μl of blood were collected at 20, 60, and 120 min.

Experiment 4 To compare the effects of insulin on the plasma levels of ghrelin and leptin, we injected intact rats s.c. with either 200 μl of insulin (2 IU/kg body weight; Eli Lilly K.K. Japan) or 500 μl of 20% glucose solution at 0900 h. Five 8-week-old intact rats per group were used in this experiment. All the experiments were performed twice using new intact rats. Blood (100 μl) was collected at 0 (just before injection), 20, 60, and 120 min after treatment. Control rats were treated with saline solution.

Immunohistochemical analyses for ghrelin

The trimmed glandular part of the stomach was washed in saline and placed in 0.34% formalin for 4 days at 4°C , and then transferred to 0.1 M phosphate buffer containing 20% sucrose. Sections were cut at 18 μm on a cryostat at -20°C . The sections were fixed for 20 min in 40% paraformaldehyde, blocked for 1 h in 5% normal donkey serum in PBST, and then incubated overnight at 4°C with rabbit antiserum against rat ghrelin (Nakahara *et al.* 2006). After washing, they were incubated in a solution of Alexa-488-labeled anti-rabbit IgG antibody for 30 min. Samples were observed under fluorescence microscope (Axioskop 2 Plus, Carl Zeiss GmbH, Jena, Germany). Digital images were adjusted for contrast and color in Adobe Photoshop CS4.

Measurement of plasma acyl-ghrelin, leptin, and glucose

Blood samples were collected into microtubes containing 500K IU/ml aprotinin and 2 mg/ml EDTA-2Na, and immediately centrifuged. For ghrelin, but not leptin, plasma samples were acidified with 10% volume of 1 M HCl and stored at -80°C until assay. Acyl-ghrelin concentrations were measured in 25 μl plasma with an active ghrelin ELISA kit (Mitsubishi Kagaku Iatron, Tokyo, Japan), and leptin concentrations were measured in 30 μl plasma with a rat leptin ELISA kit (Yanaiharu Institute Inc., Shizuoka, Japan). The assay ranges of the kits were 2.73–175 fmol/ml ghrelin

Figure 1 Effects of gastric infusion of 6 ml air or 3 ml water, 30% corn starch, corn oil, or 20% ethanol on plasma ghrelin levels in (A) intact and (B) vagotomized rats. Materials were infused directly into the stomach of 16-h-starved rats through an implanted gastric cannula at 0900 h. Samples were collected at 0, 20, 60, and 120 min after infusion. The black bar in (A) represents the plasma ghrelin levels at 0900 h in intact rats ($n=7$) fed *ad libitum*. Symbols and bars represent means, vertical lines represent S.E.M. ($n=10$), and asterisks indicate significant differences ($P < 0.05$ versus control). (C) Effect of a 16-h fast, followed by infusion of water or 20% ethanol, on levels of *ghrelin* mRNA in the stomach. Bars represent means, vertical lines represent S.E.M. ($n=8$), and asterisks indicate significant differences ($P < 0.05$ versus rats fed *ad libitum*).

and 312.5–20 000 pg/ml leptin. The intra- and inter-assay coefficients of variation of the ELISA were 5.5 and 3.2% respectively for ghrelin, and 6.3 and 4.8% for leptin. Plasma glucose concentrations were measured with a DRI-CHEM 3500 analyzer (Fujifilm, Tokyo, Japan).

Data analysis

Control and treated groups were compared by ANOVA with Fisher's *post hoc* test. Differences at $P < 0.05$ were accepted as statistically significant.

Results

In 16-h-starved rats, basal plasma ghrelin levels were increased about four times in comparison with those in free-feeding rats. The direct infusion of 6 ml air or 3 ml water into the stomach had no effect on levels. On the other hand, 3 ml of 30% corn starch, corn oil, or 20% ethanol significantly decreased plasma ghrelin levels (Fig. 1A). At 20 min after infusion, corn starch gave the greatest decrease. Levels that were decreased by corn starch and corn oil, but not by ethanol, tended to recover by 60 min after treatment.

Fasting for 16 h increased the baseline ghrelin level in vagotomized rats as well as in intact rats, but the increase in vagotomized rats was significantly smaller than that in intact rats. The increased baseline ghrelin level at 16 h of fasting in vagotomized rats was approximately the same level as the nadir observed after the administration of corn starch or corn oil in intact rats, as shown in Fig. 1A and B. Differently from intact rats, however, infusion of 30% corn starch or corn oil did not cause a significant decrease in the plasma level of ghrelin in vagotomized rats. On the other hand, the administration of 20% ethanol decreased the level of ghrelin to about 40 fmol/ml, which was almost the same as the basal level in rats fed *ad libitum* (Fig. 1B).

Although the expression of ghrelin mRNA was increased by a 16-h fast, the administration of 20% ethanol did not change ghrelin mRNA expression in the stomach at 2 h after treatment, in comparison with ghrelin mRNA expression in rats administered water (Fig. 1C).

Infusion of ethanol three times a day for 14 and 21 days significantly increased plasma ghrelin levels (Fig. 2B). In water-treated rats, the fundus of the stomach had an intact mucosa, lamina propria mucosae, muscularis mucosae, and gastric gland (Fig. 2C). In ethanol-treated rats, by 21 days the stomach had been injured: the normal mucosa, propria mucosae, and muscularis mucosae had almost disappeared, and widespread propria mucosae including gastric gland cells were observed. Ghrelin-positive cells were distributed in the basal part of the gastric gland in saline-treated rats, but were globally distributed with increased numbers of positive cells in ethanol-treated rats (Fig. 2C).

Exposure to low temperature ($13 \pm 1^\circ\text{C}$) did not change plasma ghrelin levels, but it increased leptin levels at 120 min. On the other hand, restriction stress decreased plasma ghrelin levels and increased leptin levels time dependently (Fig. 3A and B).

Figure 2 (A) The schedule of ethanol administration. The dose of ethanol was gradually increased stepwise during 3 weeks. Although ethanol was infused into the stomach three times a day (at 0730, 1300, and 1830 h), the dose was represented by the total ethanol weight (g)/kg bodyweight/day. (B and C) Effects of chronic treatment with ethanol on (B) plasma ghrelin levels and (C) stomach integrity. Ethanol (filled circles) and water (clear circles) were infused directly into the stomach through an implanted gastric cannula three times a day for 3 weeks. Blood samples were collected 7, 14, and 21 days after initiation of treatment. Symbols represent means, vertical lines represent S.E.M. ($n=8$), and asterisks indicate significant differences ($P < 0.05$ versus water treatment). Image in (C) shows immunofluorescence staining for acyl-ghrelin in stomach section. Bar scales are 100 μm . The white arrow with same scale shows normal mucosa (control) and injured mucosa (ethanol treatment). Full colour version of this figure available via <http://dx.doi.org/10.1677/JOE-10-0062>.

Figure 3 Effects of environmental temperature and restriction stress on (A) plasma ghrelin and (B) leptin levels. Intact rats fed *ad libitum* were transferred from their usual room ($23 \pm 1^\circ\text{C}$) to a cold room ($13 \pm 1^\circ\text{C}$) at 0900 h. To separate the effects of stress from the effects of temperature, some control rats were stressed by insertion into plastic tubes. Plasma samples were collected at 0, 20, 60, and 120 min. Symbols represent means, vertical lines represent S.E.M. ($n=10$), and asterisks indicate significant differences ($P<0.05$ versus control).

A single injection of 20% glucose increased and insulin decreased blood glucose levels in free-feeding rats (Fig. 4A). Each injection significantly decreased plasma ghrelin levels 20 and 60 min after treatment, and levels returned to normal at 120 min (Fig. 4B). On the other hand, insulin increased plasma leptin levels at 120 min, but glucose had no significant effect (Fig. 4C). In saline-treated rats, basal plasma leptin levels decreased 120 min after treatment, which might be a diurnal change (Haynes *et al.* 1997, Trayhurn *et al.* 1999).

Discussion

Basal plasma ghrelin levels and stomach *ghrelin* mRNA levels were increased after 16-h overnight starvation, supporting the view that ghrelin secretion is promoted by hunger. Under hunger, direct infusion of a carbohydrate or fat into the stomach significantly decreased the high plasma ghrelin levels, suggesting that they alleviated hunger. These observations agree with previous observations in rat and human (Beck *et al.* 2002, Greenman *et al.* 2004, Bowen *et al.* 2006, Foster-Schubert *et al.* 2008). As the half-life of plasma ghrelin is about 10 min (Hosoda *et al.* 2000, Kojima & Kangawa 2005), the fact that the plasma ghrelin levels decreased to half levels at 20 min after the administration of 30% corn starch, corn oil, and 20% ethanol suggests that *de novo* secretion of ghrelin in the stomach was halted after those administration. In contrast, the infusion of air or water did not change the plasma ghrelin level. Although water, 30% corn starch, and corn oil pass from the stomach to the duodenum at different rates, making it difficult to compare their effects on the stomach, these results together with previous reports suggest that mechanical stimulation of the stomach is not effective at provoking ghrelin secretion under hunger (Kojima & Kangawa 2005). In addition, both the acute (<20 min) and long-term suppression (>1 h) of plasma ghrelin levels by 30% corn starch, corn oil, and 20% ethanol suggest that suppression depends on both neuronal (acute) and humoral (long term) action. Although the amount of calories administered differed among 30% corn starch, corn oil, and 20% ethanol, the strength of suppression was almost the same at 20 and 60 min after these treatments. Therefore, the suppression may not have been due to nutritional calories, but may be due to chemical stimulation by the carbohydrate, protein, and ethanol. The most long-term suppression was observed after the administration of 20% ethanol. As the expression of *ghrelin* mRNA in the stomach was not changed by 20% ethanol at 2 h after administration, this long-term suppression did not appear to be due to a decrease in ghrelin synthesis.

Vagotomy partially inhibited the baseline increase caused by 16 h of fasting, and completely inhibited the decrease of ghrelin secretion induced by administration of corn starch or corn oil. The increased baseline ghrelin level at 16 h of fasting in vagotomized rats was approximately the same level as the nadir observed after the administration of corn starch or corn oil in intact rats. These results suggest that the central nervous system (CNS) in vagotomized rats might not adequately perceive the metabolic state of starvation, suggesting that the underlying pathophysiology may involve impairment of afferent, rather than of efferent, innervations of the stomach. We previously reported that electrical stimulation of the vagus nerve increased the plasma ghrelin level in rat (Murakami *et al.* 2002). On the other hand, vagus stimulation exaggerates the inhibitory ghrelin response to oral fat in humans, probably through activation of the vagal afferent impulse (Heath *et al.* 2004). Although the reason for the discrepancy between the

Figure 4 Effect of glucose and insulin in intact rats fed *ad libitum* on (A) plasma glucose, (B) ghrelin, and (C) leptin levels. Either 200 μ l of insulin or 500 μ l of 20% glucose solution was injected s.c. at 0900 h. Blood was collected at 0, 20, 60, and 120 min after treatment. Control rats were treated with saline. Symbols represent means, vertical lines represent s.e.m. ($n=10$), and asterisks indicate significant differences ($P<0.05$ versus control).

stimulatory and inhibitory effects evoked by vagal stimulation is unknown, these results suggest that the vagus nerve may play an important role in ghrelin secretion by transmitting nutrient information to the CNS as an afferent impulse, or CNS information to the stomach as an efferent impulse.

Acute infusion of ethanol into the stomach strongly suppressed ghrelin secretion. This remains to be understood because of the complexity of peripheral or central action of alcohol. However, vagotomy did not prevent this alcohol-induced suppression of ghrelin secretion, suggesting that alcohol acts directly. In human, ghrelin declined significantly within 15 min after alcohol intake, fell to a minimum of 66% of baseline at 75 min, and remained at that level until the last sample at 120 min (Zimmerman *et al.* 2007). Our results show that chronic ethanol treatment caused major injury in the stomach, eroding the mucosa, propria mucosae, and muscularis mucosae, and revealing widespread propria mucosae including gastric gland cells. Interestingly, and in contrast, ghrelin-positive cells were globally distributed with increased numbers in alcohol-treated rats, and plasma ghrelin levels were subsequently increased. From this observation, we speculate that ghrelin plays an important role in the restoration of stomach mucosa injured by alcohol (Konturek *et al.* 2004, 2009). Sibilia *et al.* (2003) reported that i.c.v. or i.p. injection of ghrelin at 30 min before oral treatment with 1 ml of 50% alcohol reduced the incidence of alcohol-induced gastric ulcers in rats by mediating endogenous nitric oxide release and stimulating the vagus nerve. On the other hand, Konturek *et al.* (2004) reported that i.p. injection of ghrelin at 30 min before intragastric administration of 1.5 ml of 75% ethanol decreased the incidence of acute ethanol-induced gastric lesions by mediating prostaglandins. In addition, ghrelin stimulates cell proliferation (Nakahara *et al.* 2006). Our results demonstrate, therefore, that an increase of ghrelin-positive cells in stomach injured by chronic ethanol treatment may be an attempt to restore the mucosa. Further study is required to examine what increases the number of ghrelin-positive cells.

Exposure to low temperature increased plasma leptin levels but had no effect on ghrelin levels. Restriction stress decreased plasma ghrelin levels and increased leptin levels. These results suggest that the changes in levels in response to temperature were not due to stress, and that low temperature-induced increase in plasma leptin may play important roles in thermoregulation. Several observations of the relationship between plasma ghrelin and leptin levels in hibernators and environmental temperature are reported (Korhonen *et al.* 2008, Zhang *et al.* 2009). However, the responses in hibernators are responses to seasonal or long-term changes in temperature, and are not acute responses as presented here. Thermogenesis in brown adipose tissue (BAT) depends on activation and increased expression of β 3-adrenergic receptors and the consequent up-regulation of uncoupling protein 1 (UCP1; Cannon & Nedergaard 2004). This activation is partially regulated by the sympathetic nerves, and i.c.v. administration of ghrelin and leptin respectively

suppresses and stimulates sympathetic efferent signaling to BAT (Friedman & Halaas 1998, Ahima & Flier 2000, Yasuda *et al.* 2003, Mano-Otagiri *et al.* 2009). In addition, leptin and ghrelin respectively increased and decreased the expression of *UCP1* mRNA in BAT (Pelleymounter *et al.* 1995, Scarpace & Matheny 1998, Theander-Carrillo *et al.* 2006, Mano-Otagiri *et al.* 2010). Therefore, leptin, but not ghrelin, release under low temperature may promote thermogenesis and increase body temperature (Asakuma *et al.* 2003).

I.c.v. injection of ghrelin increased core temperature in rats (Jászberényi *et al.* 2006). Although we do not know the reason for this discrepancy between the effects of ghrelin on body temperature, the effects of central and peripheral ghrelin on thermoregulation may be different, since peripheral ghrelin acts on the CNS via the vagus nerve (Date *et al.* 2002a). Further study is required to elucidate how ghrelin controls body temperature.

Leptin and ghrelin are involved in hypothalamo-pituitary-adrenal (HPA) responses to stressful stimuli (Spinedi & Gaillard 1998, Kristensson *et al.* 2006, Patterson-Buckendahl *et al.* 2007, Ochi *et al.* 2008). Here, restriction stress decreased plasma ghrelin levels and increased leptin levels. Although the mechanism of these reciprocal responses to stress is not clear, endogenous ghrelin and leptin may play important roles in stress responses. Plasma ghrelin levels decreased significantly from 20 min onward during stress treatments. On the other hand, acute psychological stress raises them (Kristensson *et al.* 2006). In addition, Zimmermann *et al.* (2007) have reported that psychological stress in humans does not affect ghrelin secretion. Although we do not know the reason for this discrepancy, the ghrelin response to stress may be different depending on exogenous (such as mechanical or environmental) and endogenous (such as psychological) stresses. Plasma leptin levels showed a significant increase at 120 min, but not at 20 or 60 min, suggesting that leptin responds more slowly to stress than ghrelin. An i.c.v. injection of leptin attenuated the increases in hypothalamic noradrenaline release and plasma ACTH concentrations after electrical foot-shock stress (Kawakami *et al.* 2008). On the other hand, leptin activated the HPA axis (van Dijk *et al.* 1997). It is not likely that the slow response of leptin shown here activates the HPA axis.

It has been well documented that insulin interacts with ghrelin and leptin (Adeghate & Ponery 2002, Date *et al.* 2002b, Lee *et al.* 2002, Saad *et al.* 2002, Kapica *et al.* 2008). The pancreatic β -cells have receptors for both ghrelin and leptin (Kieffer *et al.* 1996, Date *et al.* 2002b). Exogenous administration of leptin decreases insulin secretion (Fehmann *et al.* 1997, Kulkarni *et al.* 1997, Poitout *et al.* 1998), but ghrelin stimulates or inhibits insulin secretion depending on dose or experimental conditions (Date *et al.* 2002b). The exogenous administration of insulin inhibited ghrelin secretion and stimulated leptin secretion (Nakagawa *et al.* 2002, Saad *et al.* 2002, Seufert 2004). Our results clearly support those observations. However, the time course of the responses to insulin and glucose differed between ghrelin and leptin. Plasma ghrelin decreased significantly by 20 min after

insulin treatment and returned to basal levels by 120 min. Although the plasma glucose levels showed opposite responses to insulin and 20% glucose solution, plasma ghrelin levels decreased in response to both. These results suggest that insulin and glucose independently decreased ghrelin secretion. However, the possibility that the ghrelin suppression after glucose was brought about by glucose-induced insulin secretion remains.

Plasma leptin levels showed a significant increase by 120 min after treatment with insulin, but no significant change after glucose. Hypoglycemia induced by a glucose clamp technique increased plasma leptin levels in women (Ludwig *et al.* 2007). Therefore, the significant increase of leptin may be due to either the direct action of insulin or the insulin-induced hypoglycemia.

In conclusion, our results demonstrate that not only exogenous stimuli, such as temperature and stress, but also endogenous factors, such as nutrition, hyperglycemia, hypoglycemia, and insulin, affect the plasma levels of ghrelin and leptin in almost reciprocal fashion. This acute response may maintain homeostasis for many physiological functions.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported in part by grants-in-aid from the Ministry of Education, Science, Sports and Culture of Japan and by the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN).

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Received in final form 10 June 2010

Accepted 14 July 2010

Made available online as an Accepted Preprint

14 July 2010