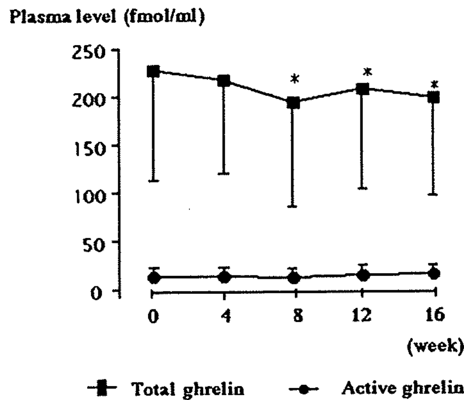

Fig. 2. Changes in total ghrelin and active ghrelin in a 16-week olanzapine treatment in patients with schizophrenia. Values are presented as mean±S.D. *$P<0.0125$ compared to baseline by Wilcoxon test.

patients, there was no significant gender difference in changes of total ghrelin level from baseline to endpoint. Similarly, female patients had higher active ghrelin levels than male patients at both baseline (M: 9.088±5.986 fmol/ml, F: 21.781±10.753 fmol/ml, $P=0.0025$) and endpoint (M: 10.896±5.062 fmol/ml, F: 23.07±11.841, $P=0.0006$). There was a significant negative correlation between total ghrelin levels and age in male, but not female, patients, at both baseline ($P=0.017$) and endpoint ($P=0.05$). Likewise, while plasma active ghrelin levels in male patients were negatively correlated with age at endpoint ($P=0.0161$) and tended to be negatively correlated with age at baseline ($P=0.0792$), there was no significant correlation between active ghrelin levels and age in female patients at either baseline or endpoint. No correlation was found between duration of illness and plasma ghrelin levels either at baseline ($P=0.980$) or at endpoint ($P=0.817$).

Although the mean leptin level at endpoint (8.64±13.89 ng/ml) tended to be increased compared with baseline (5.71±5.76 ng/ml), the difference was not significant ($P=0.0362$) (Table 4). No correlation was found between leptin and ghrelin levels at endpoint ($P=0.203$). Furthermore, the change in ghrelin levels from baseline to endpoint was not correlated with changes in leptin levels ($P=0.220$). In contrast to ghrelin, there was no significant effect of age or gender on the levels of serum leptin at baseline, or at endpoint. The mean GH level at baseline was not different from that at Week 8, or endpoint (Table 4). However, the mean cortisol level significantly decreased from baseline to Week 8 ($P=0.0004$) as well as from baseline to endpoint ($P=0.0010$) (Table 4). No significant correlation was found between ghrelin and cortisol levels at endpoint ($P=0.814$). Furthermore, changes in ghrelin levels from baseline to endpoint were not correlated to changes in cortisol levels ($P=0.550$).

4. Discussion

In this study, we measured the serum levels of leptin, GH, insulin, and cortisol, and plasma levels of total ghrelin and active ghrelin in Japanese hospitalized patients with schizophrenia during a 16-week administration of olanzapine while monitoring body weight, FBS, and lipids. It is surprising that a 16-week administration of olanzapine has no change in weight and BMI, since numerous studies report that long-term administration of olanzapine induces a marked weight gain in patients with schizophrenia. The reason for this controversial result is unknown, but the majority of subjects participating in the previous studies were outpatients or both. Barak et al. (2004) reported that long-term administration of olanzapine had no effect on weight in hospitalized elderly chronic schizophrenia patients. The subjects participating in these studies conducted by Barak et al. had a long history of schizophrenia. Similarly, the mean duration of illness in this study was more than 30 years. In addition, chronic treatment with mood stabilizers, such as lithium or valproic acid, was reported to induce a marked weight gain (Zimmermann et al., 2003). Furthermore, combination treatment with atypical antipsychotics and mood stabilizers also induces a marked weight gain (Kim et al., 2008; Tohen et al., 2002). However, no weight gain was found in 5 patients treated with olanzapine and mood stabilizer in this study. Taking these findings together, long-term exposure to typical antipsychotic drugs and mood stabilizers may already induce a marked weight gain before switching to olanzapine and subsequently induce no weight gain. Thus, it is plausible that the difference in the subject characteristics and treatment settings may be, at least in part, involved in the different responses of weight to olanzapine.

The results of the present study show that while an 8-week or longer administration of olanzapine resulted in a significant decrease in plasma ghrelin levels, there was no change in plasma levels of active ghrelin. To our knowledge, there has been only two other studies that examined changes in the ghrelin levels before and after treatment with olanzapine in schizophrenia (Hosojima et al., 2006; Murashita et al., 2004). Recently, Hosojima et al. (2006) reported that a 4-week administration of olanzapine significantly decreased ghrelin levels, with a significant increase in weight and BMI. On the other hand, Murashita et al. (2004) demonstrated a significant increase in both ghrelin and active ghrelin levels after a 6-month administration of olanzapine, but with no significant change in weight or BMI. The significant decrease in total ghrelin level observed during olanzapine treatment in the study by Hosojima et al. (2006) agrees in many respects with our findings, but in our study, an 8-week administration was required to see a significant decrease. Although the reason for this discrepancy between our study and those by Hosojima et al. (2006) and Murashita et al. (2004) is uncertain, it is plausible that differences in subjects and procedures (e.g., out *vs.* inpatients, duration of olanzapine administration, dose of olanzapine, assessment method of ghrelin) among these 3 studies may be responsible for the controversial results.

While Palik et al. (2005) demonstrated that there was a significant negative correlation between both serum ghrelin level and BMI, and ghrelin level and FBS in patients with schizophrenia treated with atypical antipsychotic drugs, we did not find such a significant negative correlation in the present study. Comparing the BMI levels observed in these two studies, the mean BMI level in Palik's study was significantly higher. In this context, we speculate that since olanzapine was administered over a longer period in Palik's study compared with our study, BMI might induce a different response of ghrelin to long-term administration of olanzapine.

Although the results of studies examining gender differences in plasma ghrelin levels are controversial (Makovey et al., 2007; Purnell et al., 2003; Shiiya et al., 2002; Tschop et al., 2001; Vilarrasa et al., 2005), our finding that plasma ghrelin levels in female patients are higher that those in male patients is in agreement with several previous studies using healthy subjects (Barkan et al., 2003; Greenman et al., 2004; Makovey et al., 2007). The precise reason for the gender difference in this study remains uncertain. However, the mean BMI in female patients (19.6±1.2 kg/m^2) was significantly lower than that in male patients (23.7±0.7 kg/m^2). Several previous studies demonstrated a significant negative correlation between ghrelin levels and BMI (Fargerberg et al., 2003; Greenman et al., 2004; Makovey et al., 2007; Purnell et al., 2003; Shuto et al., 2002). In this context, it is plausible that the difference in BMI between male and female patients may lead to the gender difference in plasma ghrelin levels in this study. In addition, the effect of age on ghrelin levels is also contradictory in healthy controls (Akamizu et al., 2006; Greenman et al., 2004; Purnell et al., 2003; Rigamonti et al., 2002; Shiiya et al., 2002; Sturm et al., 2003). To our knowledge, there is one study examining the effect of age on plasma ghrelin levels in patients with schizophrenia treated with clozapine, which demonstrated no significant effect of age (Theisen et al., 2005). Our observation that there is

a significant negative correlation between plasma ghrelin levels and age in male patients with schizophrenia, does not support the previous findings (Theisen et al., 2005). Recently, Kozakowski et al. (2008) have demonstrated a significant negative correlation between serum ghrelin levels and age in men, suggesting an influence of serum testosterone on serum ghrelin levels. Although we did not measure testosterone levels in this study, it is hypothesized that the age-dependent decline in testosterone in male patients may contribute to the age-dependent decline in ghrelin observed in this study.

It has been revealed that long-term administration of olanzapine significantly increases the levels of leptin in patients with schizophrenia (Atmaca et al., 2003; Melkersson et al., 2000; Haupt et al., 2005). Since it was reported that the administration of leptin inhibited ghrelin secretion from isolated perfused rat stomach (Kamegai et al., 2004), it is hypothesized that the decrease in ghrelin may be due to the upregulation of leptin in response to olanzapine treatment for 16 weeks. However, no significant correlation between the changes in leptin and ghrelin was found in this study. Thus, it is unlikely that the increase in leptin by long-term administration of olanzapine leads to the reduction in ghrelin.

In addition to the decrease in ghrelin plasma levels, long-term administration of olanzapine resulted in a significant decrease in plasma cortisol levels in this study. It was demonstrated that administration of ghrelin significantly stimulated the secretion of ACTH and cortisol in normal subjects (Coiro et al., 2005; Schmid et al., 2005). Since there was no significant correlation between the changes in plasma ghrelin and serum cortisol levels in this study, it is unlikely that the decrease in ghrelin levels in response to olanzapine directly reduced serum cortisol levels. On the other hand, two studies showed that long-term administration of olanzapine significantly reduced cortisol levels in patients with schizophrenia (Mann et al., 2006; Ryan et al., 2004). In this context, the results of the present study are in good agreement with the findings obtained from these previous 2 studies. The precise mechanism by which olanzapine decreases cortisol levels in schizophrenia, is still unknown. Since it was reported that olanzapine administration significantly inhibited *m*-chlorophenylpiperazine-induced cortisol release in schizophrenia (Scheepers et al., 2001), it is conceivable that a potent serotonin$_{2C}$ antagonistic action by olanzapine is, at least in part, involved in olanzapine-mediated inhibition of cortisol release.

There are several limitations in this study that should be taken into consideration. First, a relatively small number of patients participated in this study. Second, there is no comparison of weight and hormones between the groups with and without switching to olanzapine. Third, since we had no medication wash out period in this study, it is unlikely that the findings in the early stage of this study were entirely derived from olanzapine monotherapy. Fourth, 18% of patients received long-term administration of mood stabilizer before the combination treatment with mood stabilizer and olanzapine. So, the involvement of mood stabilizer in the present findings is not completely excluded. Fifth, it has been reported that multi hormonal signals in the stomach such as insulin (Brogilo et al., 2003; Kamegai et al., 2004), leptin (Horvath et al., 2001; Kamegai et al., 2004), glucagons (Kamegai et al., 2004; Kishimoto et al., 2003), and somatostatin (Barkan et al., 2003; Shimada et al., 2003), were involved in ghrelin secretion. Furthermore, adiponectin also plays a role in the regulation of weight. Therefore, additional studies monitoring the levels of these peptides in a large number of participants who are drug-free at baseline, are required to determine whether the treatment with olanzapine significantly affects ghrelin levels in patients with schizophrenia.

5. Conclusion

This study provided evidence that long-term administration of olanzapine did not induce olanzapine-associated weight gain in hospitalized patients with chronic schizophrenia. In addition, a significant decrease in the mean levels of ghrelin, cortisol, FFA, and FBS was found. Based on these findings, it is postulated that the differences in the subject characteristics and treatment settings may affect the responses of weight and hormones to olanzapine in patients with schizophrenia.

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Ghrelin suppresses cardiac sympathetic activity and prevents early left ventricular remodeling in rats with myocardial infarction

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Soeki T, Kishimoto I, Schwenke DO, Tokudome T, Horio T, Yoshida M, Hosoda H, Kangawa K. Ghrelin suppresses cardiac sympathetic activity and prevents early left ventricular remodeling in rats with myocardial infarction. *Am J Physiol Heart Circ Physiol* 294: H426–H432, 2008. First published November 16, 2007; doi:10.1152/ajpheart.00643.2007.—A recent study suggests that exogenous ghrelin administration might decrease renal sympathetic nerve activity in conscious rabbits. In the present study, we investigated whether ghrelin administration would attenuate left ventricular (LV) remodeling following myocardial infarction (MI) via the suppression of cardiac sympathetic activity. Ghrelin (100 μg/kg sc, twice daily, $n = 15$) or saline ($n = 15$) were administered for 2 wk from the day after MI operation in Sprague-Dawley rats. The effects of ghrelin on cardiac remodeling were evaluated by echocardiographic, hemodynamic, histopathological, and gene analysis. In addition, before and after ghrelin (100 μg/kg sc, $n = 6$) was administered in conscious rats with MI, the autonomic nervous function was investigated by power spectral analysis obtained by a telemetry system. In ghrelin-treated rats, LV enlargement induced by MI was significantly attenuated compared with saline-treated rats. In addition, there was a substantial decrease in LV end-diastolic pressure and increases in the peak rate of the rise and fall of LV pressure in ghrelin-treated MI rats compared with saline-treated MI rats. Furthermore, ghrelin attenuated an increase in morphometrical collagen volume fraction in the noninfarct region, which was accompanied by the suppression of collagen I and III mRNA levels. Importantly, a 2-wk administration of ghrelin dramatically suppressed the MI-induced increase in heart rate and plasma norepinephrine concentration to the similar levels as in sham-operated controls. Moreover, acute administration of ghrelin to MI rats decreased the ratio of the low-to-high frequency spectra of heart rate variability ($P < 0.01$). In conclusion, these data suggest the potential usefulness of ghrelin as a new cardioprotective hormone early after MI.

autonomic nervous function; infarction; peptide hormones

GHRELIN IS A NOVEL growth hormone (GH)-releasing peptide, originally isolated from the stomach, which has been identified as an endogenous ligand for the GH secretagogues receptor (GHS-R) (12). GHS-R mRNA is detected in not only the hypothalamus and pituitary but also the heart and blood vessels (9), and much evidence for a cardiovascular function of ghrelin has been reported. Previous studies revealed that chronic administration of ghrelin improved cardiac performance in rats with chronic heart failure, as indicated by increases in cardiac output and left ventricular (LV) fractional shortening (17) and that intravenous bolus infusion of human ghrelin significantly decreased mean arterial pressure in patients with chronic heart failure (16). However, the precise mechanism of ghrelin actions remains unclear.

On the other hand, LV remodeling after myocardial infarction (MI) is a major cause of subsequent heart failure and death. The sympathetic nervous system is thought to contribute to the post-MI cardiac dysfunction and remodeling, similar to the renin-angiotensin-aldosterone system (21). The β-adrenergic blockade, by the suppression of sympathetic activity, has been shown to attenuate the adverse ventricular remodeling seen in heart failure and to decrease mortality (21, 23). Since recent studies revealed that intracerebroventricular injection of ghrelin decreased renal sympathetic nerve activity in conscious rabbits (15) and suppressed sympathetic nerve activity in brown adipose tissue in rats (24), we hypothesized that ghrelin might decrease the cardiac sympathetic nerve activity and act against the progression of LV remodeling after MI. Therefore, in the present study, we investigated whether peripheral ghrelin administration attenuates LV dysfunction and remodeling after the early stage of MI and whether the underlying mechanisms are associated with the suppression of cardiac sympathetic activity.

MATERIALS AND METHODS

Model of MI. All experimental procedures were performed according to the National Institutes of Health guidelines for the use of experimental animals and the guidelines for animal experimentation of the National Cardiovascular Center. All animal protocols were approved by our Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (Nihon SLC, Hamamatsu, Japan) weighing 180–220 g were anesthetized with pentobarbital sodium (30 mg/kg ip). After a left thoracotomy, the left coronary artery was ligated 2 to 3 mm from its origin using a 6-0 prolene suture. The chest was closed and the rats were allowed to recover. Sham-operated rats underwent the identical surgical procedure without coronary artery ligation.

Administration of ghrelin. From the day after the coronary ligation, the rats with MI were randomly divided into two groups: one to be administered with synthetic rat ghrelin ($n = 15$) and the other with saline as vehicle ($n = 15$). Ghrelin [100 μg/kg twice daily, the dose of which was shown to improve LV function in rats with chronic heart failure (17)] or saline was administered subcutaneously for 2 wk from the day after the MI operation. The duration of ghrelin administration was chosen based on the mean admission period of 3 wk, in patients with acute MI, in our center for future clinical application. The synthetic rat ghrelin was provided by Daiichi Asubio Pharma, (Tokyo, Japan).

Echocardiographic and hemodynamic studies. Echocardiographic studies were performed using an echocardiography system equipped with a 15-MHz phased-array transducer (SONOS 5500, Hewlett-Packard,

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Andover, MA) under anesthesia with pentobarbital sodium (30 mg/kg ip) 1 and 14 days after the experimental MI or sham operation.

After the administration of ghrelin or saline for 2 wk, hemodynamic studies were performed. After anesthesia, a polyethylene catheter (PE-50) was inserted into the aorta through the right carotid artery for the measurement of heart rate and mean arterial pressure, and the catheter was then advanced into the LV to measure LV pressure. These hemodynamic variables were measured with a pressure transducer connected to a physiological recorder (PowerLab system, AD Instruments, Mountain View, CA). After completion of hemodynamic measurements, blood sampling was performed, and the hearts were arrested by the injection of 30 mM potassium chloride through the carotid artery, excised, and weighed.

Histological examination and Northern blot analysis. The heart was divided from apex to base into four transverse sections (2.0 to 2.2 mm thick), identified as *levels 1–4*, respectively. *Levels 1* and *3* were fixed with 4% paraformaldehyde and embedded in paraffin. *Levels 2* and *4* were divided into infarcted (macroscopic connective tissue) and noninfarcted regions and immediately frozen for the measurement of gene expression. Paraffin sections (2 μm) were stained with Masson's trichrome for measurement of infarct size and Sirius red F3BA for determination of collagen volume fraction. The infarct size was expressed as previously described (11). To measure collagen volume fraction, 16 fields in the noninfarcted LV walls per section were scanned and computerized with an Optima 6.5 digital image analyzer (Media Cybernetics, Silver Spring, MD) at a magnification of ×200. The collagen volume fraction was obtained by calculating the mean ratio of connective tissue to the total tissue area of all the measurements of the section. The collagen-positive areas from all sections were determined by a single investigator who was unaware of the experimental groups.

Total RNA (10 μg/lane) was extracted separately from the noninfarcted and infarcted regions of *levels 2* and *4*. Hybridization was carried out with cDNA probes for rat α_1 (type I)-collagen, rat α_1 (type III)-collagen, rat atrial natriuretic peptide, and rat glyceraldehyde-3-phosphate dehydrogenase. The band intensity was estimated by a radioimaging analyzer (BAS-5000, Fuji Film, Tokyo, Japan).

Hormone assays. Serum insulin-like growth factor I (IGF-I) was measured with an enzyme immunoassay kit (Active Rat IGF-I EIA, DSL, Webster, TX). Plasma concentrations of epinephrine, norepinephrine, and dopamine were measured by high-performance liquid chromatography (BML, Tokyo, Japan).

Acute effect of ghrelin on the cardiac sympathetic and parasympathetic nervous activity. In rats at 1 wk after MI or sham operation, the tip of the telemetry transmitter probe (TA11PA-C40, Data Science International, St. Paul, MN) was inserted into the femoral artery. Each rat cage was placed on a signal-receiving board (RLA1020, Data Science International) in the chamber. The pressure signal from conscious and unrestrained rats was continuously recorded by a pressure analyzing system (PowerLab system, AD Instruments). After we recorded the baseline for 0.5 h, ghrelin (100 μg/kg, the same as the one-shot dose of the antiremodeling study, $n = 6$) or saline ($n = 6$) was administered subcutaneously. The signals were recorded for 2.5 h thereafter. Acquisition of the pressure signal data was performed for 20 min before and every 1-h interval after the administration. The autonomic nervous function was investigated by a power spectral analysis of heart rate variability. The heart rate derived from pressure waves was used to generate a power spectral density curve by means of fast-Fourier transform. The range of the low-frequency (LF, 0.04–0.4 Hz) or high-frequency (HF, 0.4–1.5 Hz) component was chosen on the basis of our preliminary study.

Statistical analysis. All values are expressed as means ± SE. Differences among the groups were evaluated by one-way analysis of variance and two-way analysis of variance for repeated measurements, as appropriate. When a statistical difference was detected by analysis of variance, the Bonferroni method of adjusting for multiple pairwise comparisons was used. A value of $P < 0.05$ was considered statistically significant.

RESULTS

The effect of ghrelin on body weight, infarct size, and IGF-I. The body weights of the two MI groups were significantly lower than that of the sham-operated group. However, the decreases in body weights were significantly blunted in rats treated with ghrelin compared with those given the vehicle (Table 1). On the other hand, there was no difference of heart weight between ghrelin- and vehicle-treated MI rats. Therefore, the increase in the heart weight-to-body weight ratio after MI was significantly attenuated in rats treated with ghrelin. As shown in the Table 1, there was no difference of infarct size between the two MI groups.

Although GH is difficult to measure for its instability and the large diurnal change, serum IGF-I, which is secreted from liver in response to GH and more stable than GH in the serum, is readily quantified. Therefore, we measured serum IGF-I concentration instead of GH concentration. Serum IGF-I concentration was lower in the two MI groups than in the sham-operated group. However, there was no difference of IGF-I between the MI groups with the vehicle and ghrelin (Table 1).

The effect of ghrelin on echocardiographic and hemodynamic parameters. Significant thinning of the anterior wall and hypertrophy of the posterior wall were observed in the two MI groups compared with the sham-operated group after MI. There were no significant differences of these parameters between the vehicle and ghrelin groups even after treatment (Fig. 1*A*). Pretreatment, LV diastolic dimension was identical among the three groups, and LV fractional shortening was already smaller in MI rats with the vehicle than in sham-operated rats. In sham-operated rats, these parameters did not change after treatment. In MI rats with the vehicle, the LV enlargement and dysfunction deteriorated progressively during 2 wk. Posttreatment, LV diastolic dimension was significantly smaller in rats treated with ghrelin than in rats treated with the vehicle. Furthermore, LV fractional shortening was significantly greater in rats treated with ghrelin than in rats treated with the vehicle (Fig. 1*B*).

Table 2 shows hemodynamic assessments after the treatment. The important thing to note is that heart rate was increased in MI rats with the vehicle compared with sham-operated rats, but ghrelin significantly decreased heart rate to the same level as in the sham-operated group. LV systolic pressure was lower in the MI groups with the vehicle and ghrelin than in the sham-operated group, but there was no difference in this parameter between the two MI groups. LV end-diastolic pressure was higher in MI rats with the vehicle than in sham-operated rats. Ghrelin significantly decreased LV

Table 1. *Characterization of rats at 2 wk after MI*

	Sham	MI + Vehicle	MI + Ghrelin
n	14	15	15
Body weight, g	302±4	260±3*	272±4*‡
Heart weight, g	0.99±0.02	1.08±0.02*	1.04±0.02
Heart weight/body weight	3.31±0.08	4.16±0.10*	3.82±0.07*§
Infarct size, %		45.5±0.7	44.1±0.9
IGF-I, ng/ml	577±23	473±15*	500±20†

Values are means ± SE; *n*, number of rats. MI, myocardial infarction. *$P < 0.01$ and †$P < 0.05$ compared with the sham-operated group; ‡$P < 0.05$ and §$P < 0.01$ compared with the MI + vehicle group.

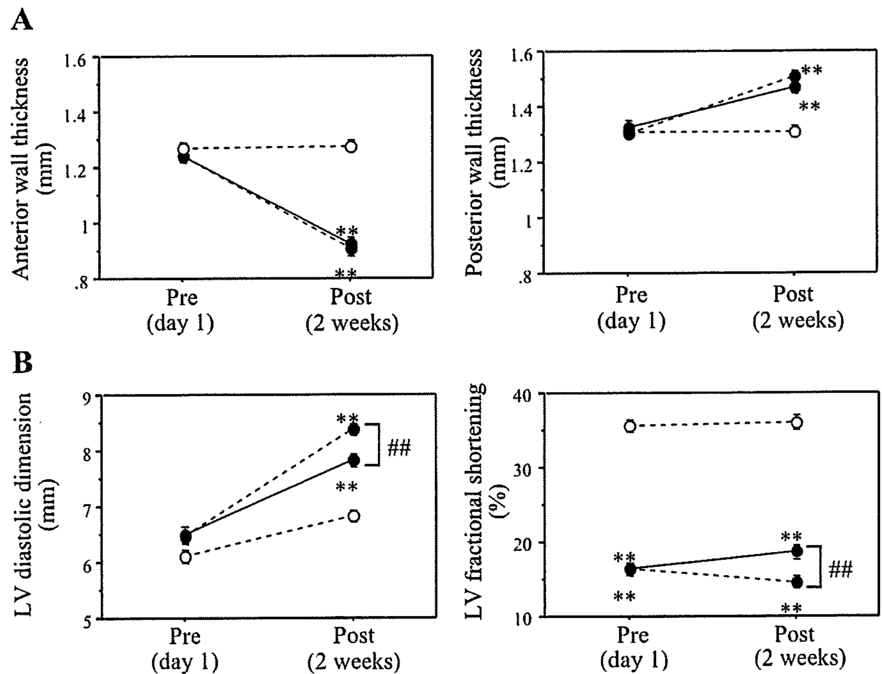

Fig. 1. Echocardiographic parameters before and after ghrelin treatment in rats with experimental myocardial infarction (MI). The parameters were examined using echocardiography 1 day (Pre) and 2 wk (Post) after MI. *A*: significant thinning of anterior wall and hypertrophy of posterior wall were observed in 2 MI groups (●) compared with the sham-operated group after MI (○). There were no significant differences of these parameters between vehicle (dotted line) and ghrelin (solid line) groups even after treatment. *B*: in contrast, left ventricular (LV) end-diastolic dimension and LV fractional shortening were significantly improved in ghrelin-treated MI group compared with vehicle-treated MI group. $**P < 0.01$ compared with sham-operated group; $\#\#P < 0.01$ compared with control (vehicle treated) MI group.

end-diastolic pressure. The peak rate of the rise and fall of LV pressure ($dP/dt_{max/min}$) was lower in MI rats with the vehicle than in sham-operated rats. The MI-induced systolic and diastolic LV dysfunction was significantly improved by ghrelin.

The effect of ghrelin on cardiac collagen volume and gene expression. To clarify the mechanism of improved cardiac performance caused by ghrelin, we examined the effects of ghrelin treatment on collagen volume in the noninfarcted LV region. Ghrelin markedly attenuated an increase in morphometrical collagen volume fraction in the noninfarcted region in rats with MI (Fig. 2). To confirm the effects of ghrelin on cardiac remodeling, we examined the expression of several mRNAs associated with heart failure and fibrosis in the noninfarcted LV region. As shown in Fig. 3, the increased mRNA expressions of atrial natriuretic peptide and collagen type I and III in MI rats were significantly suppressed by treatment with ghrelin.

The effect of ghrelin on plasma catecholamine concentrations. To further clarify the mechanism of improved cardiac performance caused by ghrelin, we examined the effects of ghrelin on plasma catecholamine concentrations, which reflect sympathetic activation and spillover from nerve endings into circulation. Figure 4 shows the effects of ghrelin on plasma concentrations of catecholamines. Plasma norepinephrine concentration was significantly increased in MI rats with the vehicle compared with sham-operated rats. Importantly, ghrelin markedly decreased plasma concentration of norepinephrine to the same level as in the sham-operated group. Although there were no significant differences of epinephrine and dopamine concentrations between the three groups, ghrelin tended to decrease these hormone levels.

Acute response of heart rate variability to ghrelin. As shown in Fig. 5, we next examined the acute effect of ghrelin on the heart rate variability, which has been used to investigate the cardiac autonomic activity separately for the sympathetic and parasympathetic nerves in human and rats. In conscious rats, 7 days after MI, heart rate, the LF power, and the LF power-to-HF power ratio (LF/HF) were higher and mean arterial pressure was lower than that in sham-operated rats. On the other hand, there was no difference of the HF power between rats with MI and sham operation.

Acute administration of ghrelin significantly decreased heart rate in rats with MI, whereas ghrelin did not affect the heart rate in sham-operated rats. Ghrelin also tended to decrease mean arterial pressure in rats with and without MI. In conscious rats after MI, an acute administration of ghrelin decreased the LF power and tended to increase HF power obtained by a telemetry system. Therefore, ghrelin significantly decreased the LF/HF ratio (i.e., cardiac sympathetic activity) in MI rats (Fig. 5). In sham-operated rats, ghrelin had no substantial effect on heart rate variability. Administration of saline (vehicle) instead of ghrelin did not affect the heart rate variability in rats with MI.

Table 2. *Hemodynamic parameters*

	Sham	MI + Vehicle	MI + Ghrelin
Heart rate, beats/min	427±7	449±8†	422±9‡
MAP, mmHg	122±4	108±2*	108±3*
LVSP, mmHg	140±4	121±3*	123±3*
LVEDP, mmHg	7±1	21±2*	15±2*‡
LV dP/dt_{max}, mmHg/s	7,544±238	5,391±282*	6,274±304*‡
LV dP/dt_{min}, mmHg/s	−5,664±182	−4,108±184*	−4,902±255†‡

Values are means ± SE. MAP, mean arterial pressure; LVSP, left ventricular (LV) systolic pressure; LVEDP, LV end-diastolic pressure; LV dP/dt_{max} or dP/dt_{min}, peak rate of LV pressure rise or fall, respectively. $*P < 0.01$ and $†P < 0.05$ compared with the sham-operated group; $‡P < 0.05$ compared with the MI + vehicle group.

DISCUSSION

The main novel findings of the present study are that a continuous administration of ghrelin improved LV dysfunc-

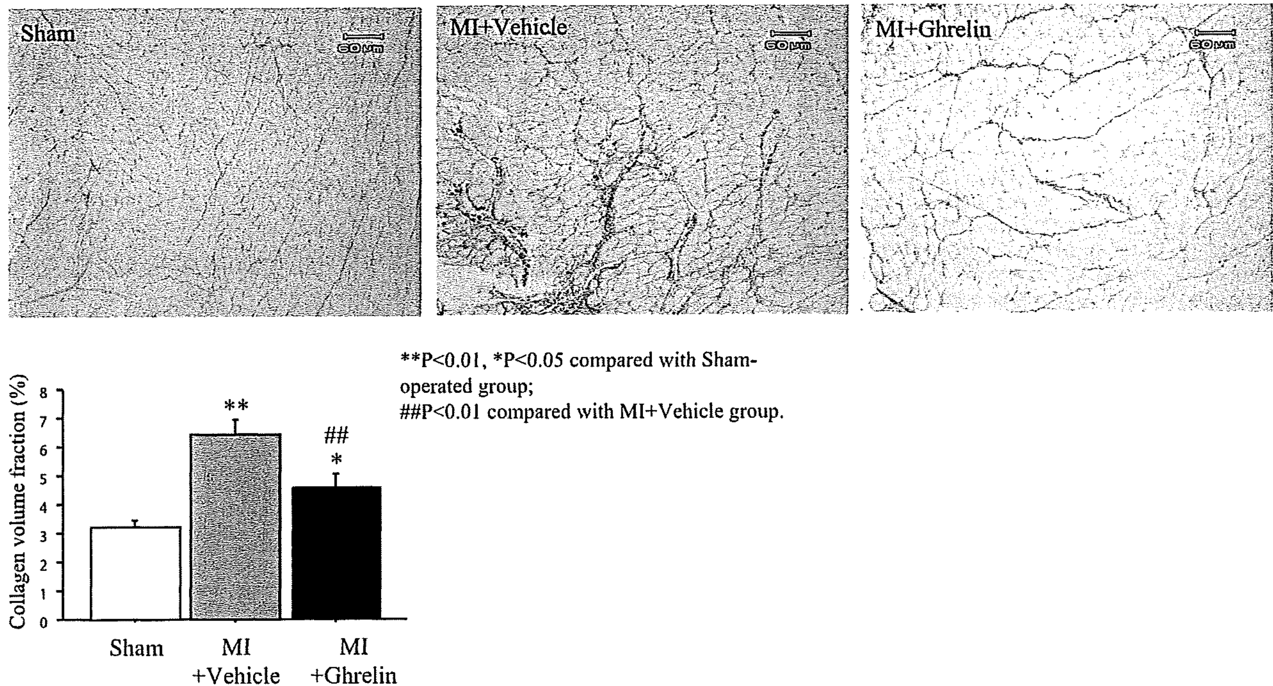

Fig. 2. Effects of ghrelin treatment on collagen volume in the noninfarcted LV region. After the administration of ghrelin for 2 wk in rats with MI, the LV sections were stained with Sirius red. Representative photomicrographs of collagen volume (*top*) and quantitative morphometric analysis (*bottom*) were shown. Ghrelin markedly attenuated an increase in morphometrical collagen volume fraction in the noninfarcted LV region in rats with MI. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with sham-operated group; $^{\#\#}P < 0.01$ compared with MI + vehicle group.

tion and attenuated early cardiac remodeling after acute MI. The beneficial effects of ghrelin were accompanied by the suppression of MI-induced increase of heart rate and plasma norepinephrine concentration. In addition, in conscious rats after MI, an acute administration of ghrelin decreased the cardiac sympathetic nerve activity, which was examined by heart rate variability using a telemetry system. Taken together, the cardioprotective effects of ghrelin could be mediated by the suppression of cardiac sympathetic nerve activity.

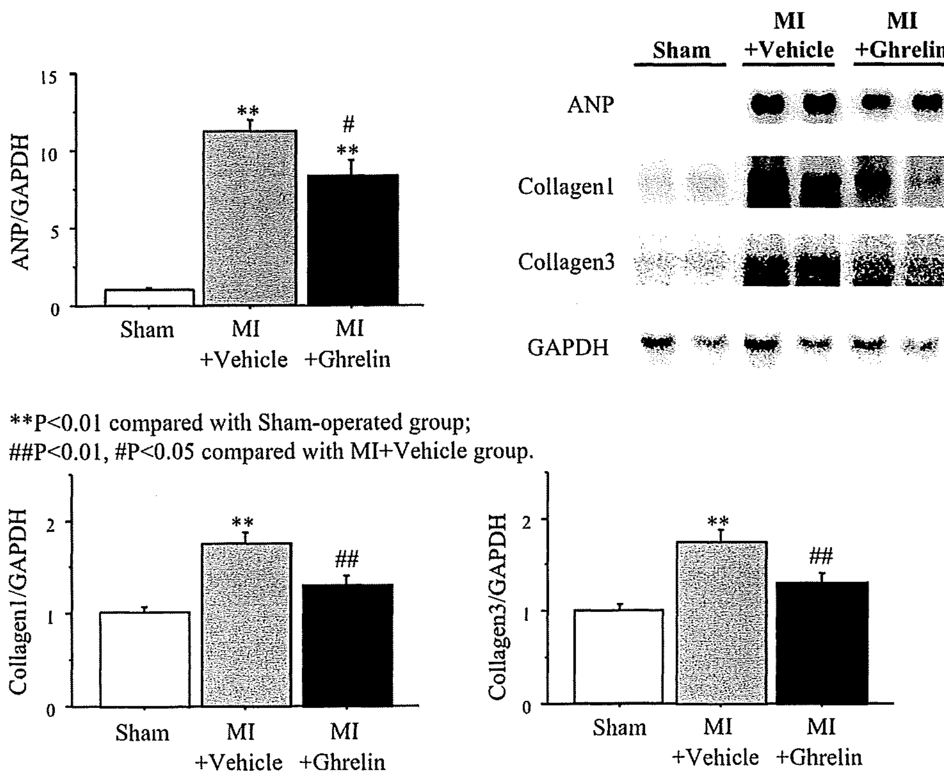

Fig. 3. Expression of genes associated with heart failure and fibrosis in noninfarcted LV region. After the administration of ghrelin for 2 wk in rats with MI, RNAs were extracted from the noninfarcted portion of LV. As shown, the increased mRNA expressions of atrial natriuretic peptide (ANP) and collagen type I and III in MI rats were significantly suppressed by treatment with ghrelin. Each mRNA expression was corrected by the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). $^{**}P < 0.01$ compared with sham-operated group; $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with MI + vehicle group.

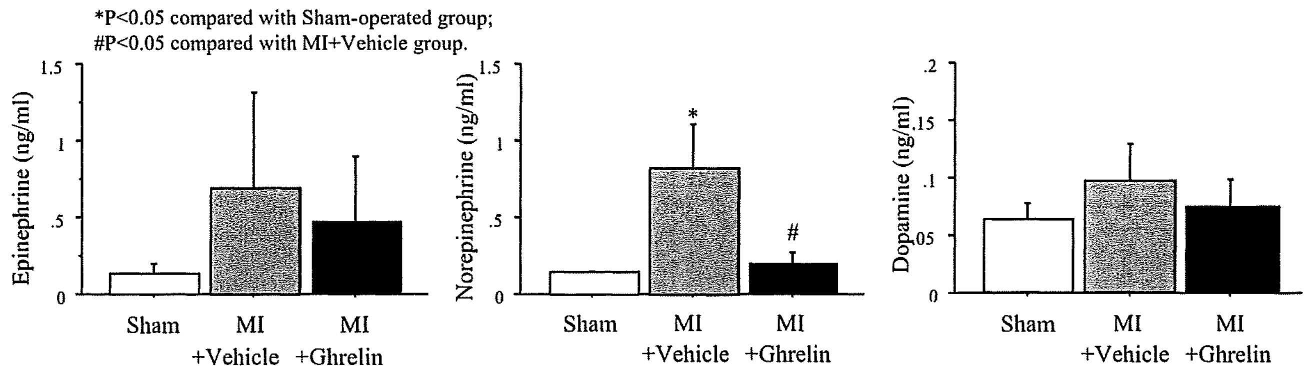

Fig. 4. Effect of ghrelin on plasma concentrations of catecholamines. After the administration of ghrelin for 2 wk in rats with MI, plasma catecholamines were measured. Plasma norepinephrine concentration was significantly increased in vehicle-treated MI rats compared with sham-operated rats. Ghrelin markedly decreased plasma norepinephrine level almost to the same level as seen in sham-operated group. There was a tendency that ghrelin decreases epinephrine and dopamine levels compared with vehicle, although it did not reach statistical significance. $*P < 0.05$ compared with sham-operated group; $\#P < 0.05$ compared with MI + vehicle group.

In the present study, LV enlargement induced by MI was significantly attenuated by ghrelin treatment. Moreover, there was a substantial decrease in LV end-diastolic pressure, and there were increases in $dP/dt_{max/min}$ in ghrelin-treated MI rats compared with saline-treated MI rats. We have previously reported that subcutaneous administration of ghrelin improves LV dysfunction and attenuates the development of LV remodeling in rats with chronic heart failure (17). In the study, ghrelin apparently stimulates the GH/IGF-I axis, which could induce myocardial growth (1), and, therefore, the beneficial effects of ghrelin could be mediated by the activation of the GH/IGF-I pathway. In the present study, serum IGF-I concentration did not increase in MI rats treated with ghrelin, and there was no difference of heart weight between MI rats with and without ghrelin. The discrepancy between the present study and the previous study using same daily dose of ghrelin might be due to the different study period (acute phase and chronic phase) after MI. In the early phase of MI, serum IGF-I levels were shown to decrease (5), which is compatible with our results that serum IGF-I concentration was lower in the two MI groups than in the sham-operated group. The neurohumoral changes following MI, which include elevated interleukin-1 and tumor necrosis factor-α or reduced IGF-binding proteins, might contribute to the sustained decrease in IGF-I (7, 8). The suppressive effects of these factors on IGF-I might be stronger than the stimulatory effect of exogenous ghrelin on the GH/IGF-I axis. Furthermore, several previous studies suggested that ghrelin has cardioprotective and vasodilatory effects not mediated by GH, because the synthetic GHS-R ligand hexarelin prevented cardiac damage after ischemia-reperfusion even in hypophysectomized rats (14), and vasodilatory effects of ghrelin were not affected by GH release inhibitors (19). Taken together, we suggest that ghrelin has

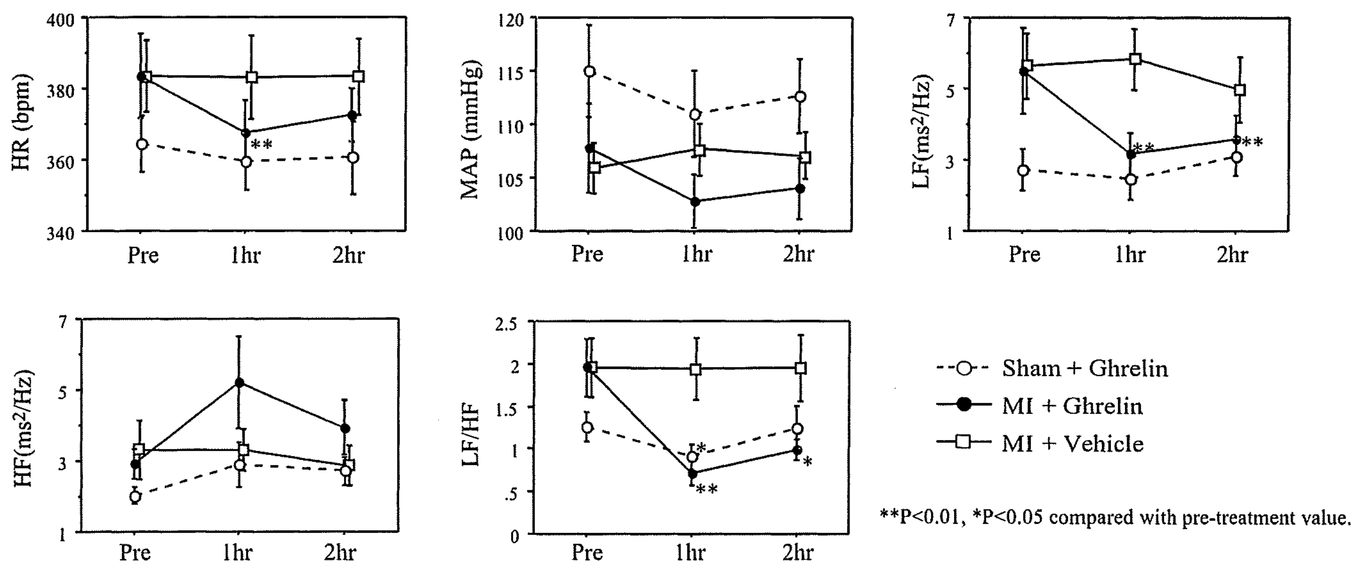

Fig. 5. Effect of ghrelin on heart rate (HR) variability. At 1 wk after MI (●) or sham operation (○), the autonomic nervous function was investigated by power spectral analysis of HR variability, before (Pre), and 1 to 2 h after ghrelin (100 μg/kg) or saline subcutaneous injection. In conscious rats, after MI, acute administration of ghrelin decreased the low-frequency power (LF) and tended to increase high-frequency power (HF), which was accompanied by the significant decrease in HR. In sham-operated rats, ghrelin had no substantial effect on LF or HF. Ghrelin did not have a significant effect on mean arterial pressure (MAP) in rats with or without MI. Administration of saline (vehicle) instead of ghrelin did not affect the HR variability in rats with MI. $**P < 0.01$ and $*P < 0.05$ compared with pretreatment values.

beneficial effects on early cardiac remodeling and dysfunction after acute MI through a GH/IGF-I-independent mechanism.

As shown in RESULTS, ghrelin decreased heart rate to the same level as in the sham-operated group without affecting the arterial pressure in MI rats. In accordance with this result, a chronic administration of ghrelin markedly decreased the plasma norepinephrine level to the similar level as in the sham-operated group. In addition, in the present study, we found that chronic ghrelin administration significantly attenuated an increase in morphometrical collagen volume fraction in the noninfarcted LV and that the mRNA levels of collagen type I and III in the noninfarcted LV were suppressed by treatment with ghrelin. It may be due in part to the suppression of sympathetic nerve activity by ghrelin, because it has been suggested that norepinephrine regulates synthesis of myocardial type 1 collagen via an indirect effect (3). Since these results suggest the suppression of the sympathetic nervous system in ghrelin-treated MI animals, we next examined the effect of ghrelin on the autonomic nerve activity by the heart rate variability spectra using the telemetry system. In the present study, we have shown for the first time that, in conscious rats after MI, an acute administration of ghrelin decreased the activated LF and LF/HF ratio, reflecting sympathetic activity. In contrast, in sham-operated rats, the LF, LF/HF ratio, and heart rate were substantially not affected by ghrelin administration. Thus ghrelin may have stronger effect on the activated sympathetic nervous system than on the nonactivated system. This hypothesis is supported by our preliminary study that, in sham-operated rats, ghrelin had no significant effects on the body weight, heart weight, and serum IGF-I concentration (sham + ghrelin: body weight, 305 ± 4 g; heart weight, 1.04 ± 0.02 g, heart weight/body weight, 3.42 ± 0.08, and IGF-I, 602 ± 17 mg/ml). Since a previous study reported that higher LF and total power were associated with the subsequent LV dilatation in patients with first MI (18), the suppressive effect of ghrelin on the sympathetic activity could lead to the attenuated LV remodeling in rats with MI.

The nucleus of the solitary tract, where baroreceptor and chemoreceptor afferent terminate, is one of the most important brain regions to regulate blood pressure and the sympathetic nervous system (20). A previous study demonstrated that microinjection of ghrelin into the nucleus of the solitary tract elicited dose-related decreases in heart rate and mean arterial pressure and that the GHS-R were predominantly present in the nucleus of the solitary tract (13). A recent study suggested that the effects of ghrelin on renal sympathetic nerve activity and blood pressure might be caused via the histaminergic system connecting to the brain stem, including the nucleus of the solitary tract (22). In addition, it has been shown that ghrelin produced in the stomach stimulates the gastric vagal afferent nerve and influenced the neuronal activity in the nucleus of the solitary tract (6), resulting in an increase in feeding behavior. Taken together, the present study suggests that peripheral ghrelin might act on the cardiac vagal afferent nerve, which sends projection to the nucleus of the solitary tract, resulting in a decrease in sympathetic activity and heart rate in rats with MI.

Another possibility is that ghrelin has a direct effect on cardiomyocytes not through GH or autonomic nervous system. Some in vitro studies support the direct action of ghrelin on cardiomyocytes. Ghrelin was shown to reduce the doxorubicin-induced mortality of H9c2 cardiomyocytes and endothelial cells (2) and the Ara C-induced mortality of HL-1 cardiomyocytes (10). In addition, a recent ex vivo study has shown that the administration of ghrelin during ischemia-reperfusion protects against myocardial injury, and this effect involves binding to cardiovascular receptors, a process that is upregulated during ischemia-reperfusion (4). Unfortunately, we could not exclude these possible mechanisms of the action of ghrelin in the present study. Further studies are necessary to establish the precise mechanism of ghrelin on the in vivo cardiovascular system.

In conclusion, the present study demonstrated that subcutaneous administration of ghrelin improved LV dysfunction and attenuated early cardiac remodeling after MI. These beneficial effects of ghrelin might be mediated by the suppression of cardiac sympathetic nerve activity. These data suggest the potential usefulness of ghrelin as a new therapeutic agent after MI.

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GRANTS

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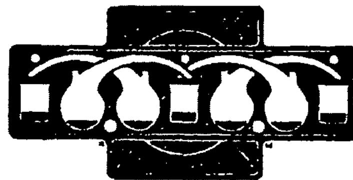

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The autonomic nervous system regulates gastric ghrelin secretion in rats

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Abstract

Plasma ghrelin levels are responsive to short- and long-term nutrient fluctuation, but the mechanisms of its regulation are largely unknown. To explore the role of the autonomic nervous system in the regulation of ghrelin secretion, we measured plasma ghrelin levels after administration of cholinergic and adrenergic agents in rats under normally fed and 48-h fasting conditions. To assess the short- and long-term effects of vagotomy on ghrelin secretion, plasma ghrelin levels and stomach ghrelin levels and gene expressions were measured in rats subjected to fed or fasting. Additionally, we investigated whether plasma ghrelin levels were affected by the anorexigenic gastrointestinal peptides cholecystokinin and somatostatin. In the pharmacological study, plasma ghrelin levels were increased by a muscarinic agonist, an α-adrenergic antagonist, and a β-adrenergic agonist, and decreased by a muscarinic antagonist and an α-adrenergic agonist. Vagotomy inhibited ghrelin secretion acutely, but promoted ghrelin release from the stomach at later time points. Stomach ghrelin mRNA levels were unchanged after fasting, but were significantly upregulated in vagotomized rats. The change of plasma ghrelin levels in nutrient fluctuation was independent of the endogenous effects of cholecystokinin and somatostatin. This study demonstrates that stomach ghrelin secretion is modulated by both the cholinergic and adrenergic arms of the autonomic nervous system. The dissociation between the short- and long-term effects of vagotomy on plasma ghrelin level indicates that an additional neural control mechanism might be involved in the regulation of ghrelin secretion.
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Keywords: Ghrelin; Autonomic nervous system; Vagus nerve; Stomach; Ghrelin secretion

1. Introduction

Ghrelin, an acylated 28-amino acid peptide originally isolated from human and rat stomach, is an endogenous ligand for the growth-hormone-secretagogue receptor (GHS-R) [1,2]. It is produced predominantly in the stomach and in smaller amounts by many other endocrine and non-endocrine tissues [3–5]. The majority of circulating ghrelin is produced in the stomach, as demonstrated by its reduction after gastrectomy [5,6]. Ghrelin secretion is upregulated under negative energy conditions such as anorexia and cachexia, downregulated under positive energy conditions like obesity, and normalized upon recovery of ideal body weight [5,7–9]. The mechanisms that underlie the reduction and elevation of plasma ghrelin levels in the presence and absence of nutrients have not been identified.

In both humans and other animals, the cholinergic system plays a major role in regulating gastro-entero-pancreatic functions including insulin, glucagon, and somatostatin secretion, as well as activity of the hypothalamus–pituitary axis such as GH secretion [10,11]. Previous studies demonstrated that the cholinergic nervous system also mediates ghrelin secretion. Ghrelin secretion is under cholinergic, specifically muscarinic, control in humans [12], and the elevation of plasma ghrelin in fasting rats was substantially reduced by atropine treatment [13], raising the possibility that the vagus nerve mediates nutrient-related changes in ghrelin levels. In addition, it is important to consider the role of sympathetic communication in the regulation of the autonomic nervous system. The effector molecules of the sympathetic nervous system, especially noradrenaline, play a key role in the regulation of appetite, energy expenditure, and the secretion of adipokines such as leptin [14]. Circulating ghrelin levels are also affected by nutrient-regulating peptides such as cholecystokinin (CCK) and somatostatin (SST) [15,16]. Recent studies demonstrate that

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peripheral peptidergic signals contribute to the regulation of food intake and body weight, as well as the control of islet function and gastric-acid secretion [17].

In the present study, to explore the effect of the autonomic nervous system on stomach ghrelin secretion, we measured plasma ghrelin levels in fed and fasted rats treated with drugs that modulate the cholinergic and adrenergic nervous systems. We further evaluated the effects of vagal activity on ghrelin secretion by examining rats that had been vagotomized subdiaphragmatically after 48 h of continuous fasting or feeding. In these rats, the gene expression of stomach ghrelin was also examined. Finally, we investigated whether stomach ghrelin secretion is mediated by endogenous CCK and somatostatin, both of which are affected by fluctuating nutritional states.

2. Materials and methods

2.1. Animals and materials

Male Sprague–Dawley (S–D) rats weighing 300–350 g (SLC Japan, Shizuoka, Japan) were used in these experiments. OLETF (Otsuka Long-Evans Tokushima Fatty) rats, which harbor a CCK-A receptor mutation, and their LETO (Long-Evans Tokushima) littermates were kindly provided by Otsuka Pharmaceutical Co. (Tokushima, Japan) and were used at eight weeks of age [18]. All rats were housed individually under standardized environmental conditions (temperature of 24–25 °C, artificial lighting 0700–1900). Tap water and standard laboratory chow were freely available. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. All drugs administrated to rats were purchased from Sigma Chemical Co. (St. Louis, MO).

2.2. Vagotomy

To transect the trunks of the subdiaphragmatic vagal nerves, rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (50 mg/kg, Dainippon Pharmaceutical Co., Osaka, Japan) or urethane (1.5 mg/kg, Tokyo Kasei Kogyo Co., Tokyo, Japan). A left subcostal incision was made, and the dorsal and ventral branches of the vagal nerves were dissected from the esophagus just under the diaphragm. Each branch of the nerve was tied with surgical sutures at two points and then cauterized between the sutures. Sham operations were also performed in which each trunk of the nerve was exposed but not tied or cauterized.

2.3. Preparation of plasma samples

Blood samples were collected from the inferior vena cava in presence of EDTA-2Na (2 mg/ml) and aprotinin (500 KIU/ml). Each plasma sample was added to 10% volume of 1 M hydrogen chloride, acidifying it to a pH of about 4, and then passed through Sep-Pak C18 cartridges (Waters, Milford, MA) [19]. The eluate (100-μl equivalent of plasma) was lyophilized and subjected to ghrelin RIA [4].

2.4. Preparation of stomach tissue

Fresh glandular stomach tissue was divided into anterior- and posterior-wall sections, the first for measurement of ghrelin content and the latter for analysis of ghrelin gene expression. The tissues were quickly diced, frozen, and stored at −80 °C until use. Each tissue sample was boiled for 10 min in a five-fold volume of water to inactivate intrinsic proteases. The solution was adjusted to 1 M acetic acid after cooling, and the tissue was homogenized with a Polytron mixer. After centrifugation, the supernatant was lyophilized and subjected to ghrelin RIA [4].

2.5. Ghrelin measurement

Plasma and stomach levels of active ghrelin were measured with a specific RIA system using polyclonal rabbit antibodies raised against the N-terminal [1–11] (Gly1-Lys11) fragments of rat ghrelin [4].

2.6. Analysis of gene expression by real-time quantitative PCR

Total RNA was extracted from stomach tissue using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). Residual DNA was removed by treatment with DNase I (Invitrogen). The RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen). The RNA concentration was adjusted to 0.1 mg/ml for PCR amplification. Real-time PCR was performed using a LightCycler system (Roche Diagnostics Ltd, Indianapolis, IN). A 20 μl-reaction mixture was prepared with a Lightcycler-FastStart DNA Master SYBR Green I kit (Roche) containing 2 μl cDNA and gene-specific primers. At the end of the PCR, melting-curve analysis was performed to verify product specificity. The predicted length of each product was confirmed by agarose gel electrophoresis. Expression levels for each gene are expressed as means of triplicate amplification reactions normalized against 28S ribosomal RNA (r28S) expression. We used the following primers for amplification: ghrelin, 5′-ACCAGAAAGCCCAGCAGAGAAAGG-3′ and 5′-ACTGAGCTCCTGACAGCTTGATGC-3′; and r28S, 5′-AGGATTCCCTCAGTAACGGCGAGTG-3′ and 5′-GCTGCATTCCCAAGCAACCCGACTC-3′.

2.7. Study protocols

2.7.1. Experiment 1

The effect of the autonomic nervous system on stomach ghrelin secretion in fed and fasted rats was assessed using standard pharmacological treatments. After 48 h of starvation or normal feeding, blood samples were obtained before and 15, 30 and 60 min after subcutaneous administration of bethanechol (1 mg/kg), a muscarinic receptor agonist; atropine (1 mg/kg), a muscarinic receptor antagonist; pirenzepine (20 mg/kg), a selective m-1 muscarinic receptor antagonist; phenylephrine (5 mg/kg), an α-adrenergic receptor agonist; phentolamine (5 mg/kg), an α-adrenergic receptor antagonist; isoproterenol (0.2 mg/kg), a β-adrenergic receptor agonist; propranolol (3 mg/kg), a β-adrenergic receptor antagonist; or 0.9% saline as a control. Each animal was used for only one drug test. In this

pharmacological experiment, we used sufficient quantity of the drugs in order to activate the autonomic nervous system [20–24].

2.7.2. Experiment 2

First, to assess the acute effect of vagotomy on ghrelin secretion, rats that had fasted for 48 h were anesthetized with urethane and inserted catheter into the right jugular vein for blood sampling. After vagotomy or sham operation, blood sampling was repeated at 0 (immediately before vagotomy), 15, 30 and 60 min. Second, the chronic effect of vagotomy on plasma and stomach ghrelin levels, as well as stomach ghrelin gene expression in response to food deprivation, was assessed. This experiment was performed 7 days after vagotomy or sham operation. Each group was divided into two subgroups: animals maintained under 48-h starvation or fed conditions. Blood and stomach samples were obtained from both groups.

2.7.3. Experiment 3

The effects of endogenous CCK or SST on plasma ghrelin levels were determined in order to assess their roles in mediating ghrelin levels during fluctuating nutritional states. CCK stimulates the CCK-A receptor at afferent vagal nerve terminals and controls food intake and various gastro-entero-pancreatic functions [25]. We measured plasma ghrelin levels in OLETF and LETO rats after 48 h of starvation or free feeding. In order to assess the role of SST, we used cyclosomatostatin (cyclo-SST), a somatostatin antagonist that increases GH, insulin, and glucagon release by blocking the inhibitory effects of endogenous SST [26]. Cyclo-SST (0.1 μmol/kg) or saline was injected subcutaneously into 48-h fasting S–D rats 30 min prior to an intraperitoneal injection of glucose solution (5 g/kg) or vehicle. Sixty min later, we measured plasma ghrelin levels.

2.8. Statistical analysis

All data are expressed as means±SE. Comparisons of parameters between groups were made with two-way ANOVA followed by Fisher's protected least significant difference (PLSD) test. Comparisons of plasma ghrelin levels at extended time points following vagotomy were made by two-way ANOVA for repeated measures. A value of $P<0.05$ was considered statistically significant.

3. Results

3.1. Experiment 1: pharmacological study of ghrelin secretion in the rat after 48 h of feeding or fasting

Representative data in time course experiments were shown for the changes of plasma active ghrelin levels after administration of pharmacological agents (Fig. 1a,b). Isoprotelenol and phenylephrine treatments led to the changes of plasma ghrelin levels those were already statistically significant at time point 15 min and further induced the greatly changes at 30 min.

In normally fed rats (Fig. 1c), phentolamine and isoprotelenol administration induced significant increases in plasma ghrelin levels (105.1±6.0 fmol/ml and 99.2±9.3, respectively, $P<0.005$) at time point 30 min compared with saline control (63.5±6.6). Plasma ghrelin was also elevated by bethanechol treatment (76.2±7.0 fmol/ml, $P<0.05$). Neither the muscarinic receptor antagonists atropine and pirenzepine, nor the adrenergic modulators phenylephrine and propranolol significantly modified ghrelin levels. Plasma ghrelin level in saline-treated rats that had fasted for 48 h was two times as high as in normally fed rats (136.7±9.8 fmol/ml, $P<0.005$) (Fig. 1d). Atropine and pirenzepine administration significantly decreased plasma ghrelin levels (80.9±9.4 fmol/ml and 47.5±7.6, respectively, $P<0.005$) in the fasted rats. Phenylephrine also decreased ghrelin levels (58.1±8.4 fmol/ml, $P<0.005$). No significant changes in ghrelin levels were observed in bethanechol-, phentolamine-, isoproterenol-, or propranolol-treated rats compared with saline-treated controls.

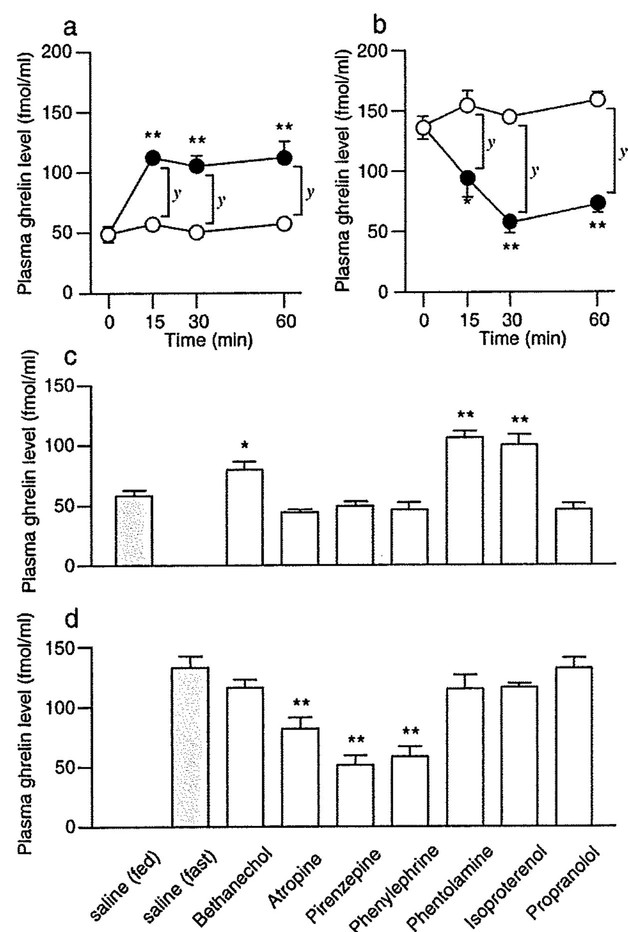

Fig. 1. Pharmacological analysis of the effect of the autonomic nervous system on ghrelin secretion in conscious rats. The time courses of plasma ghrelin level in normally fed rats injected with isoproterenol (*black circles*) or saline (*open circles*) (a), and after 48 h of food deprivation in rats injected with phenylephrine (*black circles*) or saline (*open circles*) (b). *, $P<0.05$; **, $P<0.005$ *vs.* basal level (0 min). *y*, $P<0.005$ *vs.* saline control. In normally fed (c) and 48-h fasting (d) rats, plasma ghrelin levels 30 min after subcutaneous administration of bethanechol, atropine, pirenzepine, phenylephrine, phentolamine, isoproterenol, propranolol, or saline control are shown. Data are means±SE (n=5–7). *, $P<0.05$; **, $P<0.005$ *vs.* saline control.

3.2. Experiment: effect of vagotomy on stomach ghrelin biosynthesis and secretion

Vagotomy in urethane-anesthetized rats led to a decrease in plasma levels of active ghrelin that was already significant after

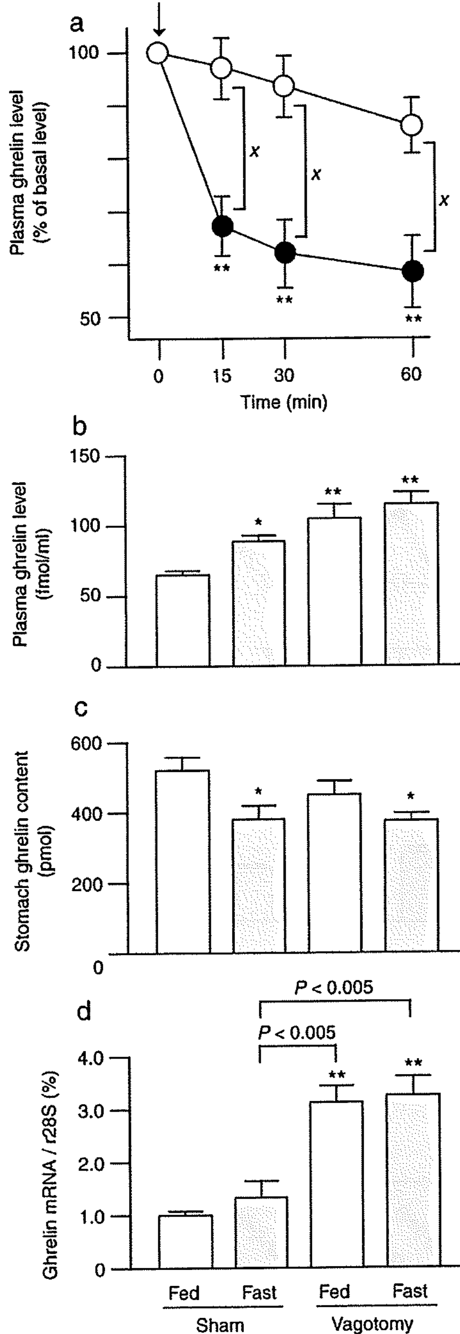

Fig. 2. Acute effect of vagotomy on ghrelin secretion in urethane-anesthetized rats after 48-h fasting (a). The time courses of plasma ghrelin level after vagotomy (*black circles*) or sham operation (*open circles*) are shown. Data are means±SE (n=7). **, P<0.005 *vs.* basal level (0 min). x, P<0.05 *vs.* saline control. The chronic effects of vagotomy on stomach ghrelin secretion and gene expression in rats after 48 h of starvation or free feeding are shown (b–d). Plasma ghrelin (b), stomach ghrelin (c), and ghrelin gene expression (d) were measured. Data are means±SE (n=7–9). *, P<0.05; **, P<0.005 *vs.* sham-operated and fed rats.

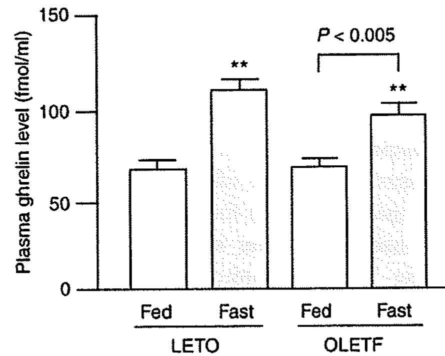

Fig. 3. Comparison of plasma ghrelin levels after 48 h of starvation or free feeding in OLETO and LETO rats. Data are means±SE (n=7–8). **, P<0.005 *vs.* fed LETO rats.

15 min and that decreased further to a minimum at 60 min as compared with sham-controls (Fig. 2a). The results of Experiment 1 indicated that ghrelin secretion is increased by muscarinic agonists and decreased by muscarinic antagonists in normally fed and fasted rats, respectively. These observations were compatible with the hypothesis that muscarinic activity upregulates ghrelin secretion. Unexpectedly, longer-term time points revealed a chronic effect of vagotomy in which plasma ghrelin levels were increased, even in normally fed rats as well as fasted rats (104.8±11.2 and 115.1±9.2 fmol/ml, respectively, P<0.005 *vs.* sham-operated and fed rats) (Fig. 2b). In sham-operated rats, plasma ghrelin levels increased significantly after a 48-h fast (88.4±4.4 fmol/ml, P<0.05), compared with fed controls (65.7±3.5). Stomach ghrelin content was lower in sham-operated and fasted rats (381.2±41.7 pmol, P<0.05) than in fed controls (519.2±38.0) (Fig. 2c). Likewise, stomach ghrelin was decreased in vagotomized rats that were fed (449.2±41.0 pmol, not significant) and in those that had fasted (377.4±23.8, P<0.05). These results indicate that vagotomy as well as food deprivation induces the release of ghrelin from the stomach. Stomach ghrelin mRNA levels were unchanged after fasting, but were significantly upregulated in vagotomized rats, as compared to normally fed controls (Fig. 2d).

3.3. Experiment 3: effects of endogenous CCK and SST on ghrelin secretion

As shown in Fig. 3, plasma ghrelin levels were clearly increased after 48-h fasting in OLETF rats as well as LETO rats compared with fasting controls in each group. Baseline ghrelin levels were not affected by cyclo-SST administration (Fig. 4). In addition, comparable glucose load-related reductions in plasma ghrelin levels were observed in both control and cyclo-SST-pretreated rats.

4. Discussion

The results obtained from this pharmacological study demonstrate that stomach ghrelin secretion in rats is mediated by both the cholinergic and adrenergic branches of the autonomic nervous system in a nutrition-dependent manner.

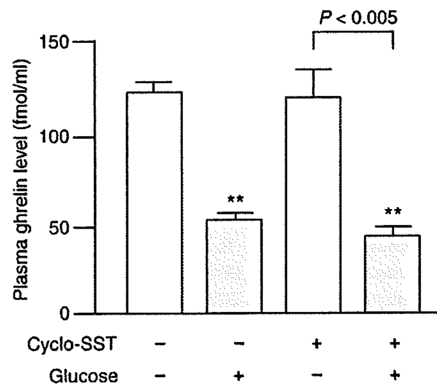

Fig. 4. Effect of cyclosomatostatin, a somatostatin antagonist, on the changes in plasma ghrelin after glucose injection in 48-h fasting rats. Data are means±SE (n=5–6). **, P<0.005 *vs.* saline control.

We found that in rats, ghrelin secretion is stimulated by muscarinic agonists, α-adrenergic antagonists, and β-adrenergic agonists, and inhibited by muscarinic antagonists and α-adrenergic agonists. The gastrointestinal tract and pancreatic islets are richly innervated with autonomic fibers, which affect the regulation of gastropancreatic hormone secretion [27]. For example, insulin secretion is stimulated by muscarinic and β-adrenergic activity and inhibited by α-adrenergic blockade, and meal-related insulin secretion is possibly mediated by both branches of the autonomic nervous system [28]. Ghrelin is produced mainly in X/A-like gastric endocrine cells [29], suggesting that the autonomic nervous system may modulate the regulation of ghrelin secretion. Previous reports demonstrated that the cholinergic nervous system is involved in the regulation of ghrelin secretion in humans and other animals [12, 14, 30]. Vagal efferent activity has been implicated in the starvation-induced elevation of plasma ghrelin. In agreement with these reports, the present study shows that pharmacological inhibition with the muscarinic receptor antagonists atropine and pirenzepine induces decreases in plasma ghrelin levels in starved rats. Conversely, muscarinic receptor activation by bethanechol leads to elevation of plasma ghrelin. We also determined the effect of adrenergic regulation on stomach ghrelin secretion. In fed rats, α-adrenergic inhibition of ghrelin levels was less pronounced, perhaps because ghrelin levels were already at minimal values at baseline. Likewise, the attenuated elevation of ghrelin after administration of α-adrenergic antagonist or β-adrenergic agonist may be due to pre-saturation of ghrelin levels under fasting conditions.

Vagotomy led immediately to an inhibition of stomach ghrelin secretion, which is in accord with the muscarinic effects seen in the pharmacological study. It is noteworthy that, in contrast to its acute response, the long-term effect of vagotomy was the activation of ghrelin secretion from the stomach in both fed and fasted rats. We found that plasma ghrelin levels were decreased within one day after vagotomy, and thereafter increased gradually (data not shown). Vagotomy appeared to result in uncontrolled release of stomach ghrelin in response to cholinergic stimulation. One prior study showed that vagotomy substantially elevated circulating ghrelin [31], whereas another showed that vagotomy affected neither baseline ghrelin levels nor the suppression of ghrelin by a nutrient load [13]. These results raise some possible explanations for the striking discrepancy between the short- and long-term effects of vagotomy on ghrelin secretion. The results of our pharmacological manipulation would suggest that the increase in plasma ghrelin at later time points after vagotomy may arise from counteracting forces: that is, adrenergic modulation, which may occur *via* β-adrenergic stimulation, probably masks the initial inhibition of ghrelin secretion caused acutely by the vagotomy. Alternatively, the regulation of ghrelin secretion can be modulated by the balance between cholinergic and adrenergic tone that governs the enteric nervous system [32,33]. To understand the interaction between the autonomic nervous system and intrinsic elements of the enteric nervous system, it is necessary to know the locations of the vagal efferent termini within the enteric plexuses. The autonomic nervous system, including the enteric nervous system, has been shown in the stomach to comprise many neural fiber types, containing those whose transmission is mediated by neurotransmitters, peptides, opioids, gamma-amino-butyric acid (GABA), and nitric oxide [27,34,35], but little is known about the involvement of these transmitters in the control of ghrelin secretion. Exogenous CCK and SST mediate the secretion of stomach ghrelin [15,16]. Unexpectedly, plasma ghrelin was unaffected by endogenous effects in CCK and SST in fluctuating nutritional status; thus, these peptides probably have little direct effect on the regulation of stomach ghrelin secretion during nutritional starvation. Taken together, these results indicate that vagal and sympathetic components of the nervous system make up the major link between the brain and the stomach, at least in terms of controlling ghrelin function (Fig. 5).

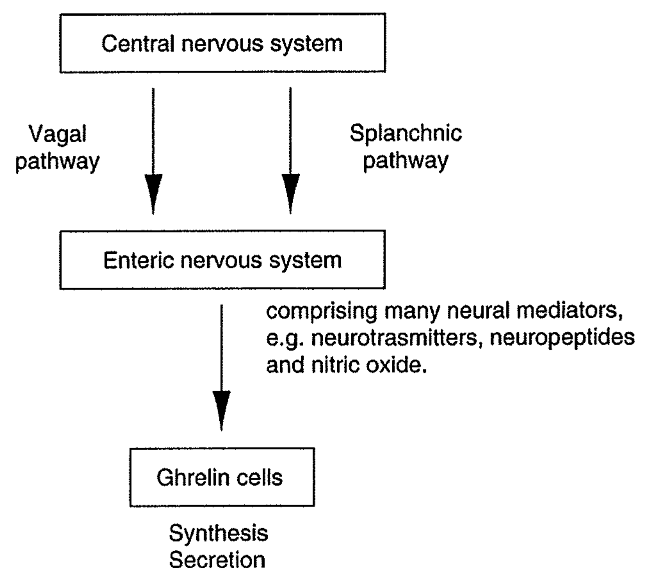

Fig. 5. Proposed pathway for the regulation of stomach ghrelin function by the autonomic nervous system, incorporating a two-way communication circuit between the central and enteric nervous systems that comprises the vagal and splanchnic pathways. The enteric nervous system may integrate information not only from the central nervous system but also from the enteric plexus neurons, and send signals to ghrelin-producing cells in the gastric mucosa.

In this study, we measured ghrelin levels in plasma and stomach to evaluate the role of the autonomic nervous system in stomach ghrelin secretion. We previously reported that the two major forms of the ghrelin peptide, ghrelin and des-acyl ghrelin, exist in rat plasma and gastrointestinal tract [4]. Since only the active form of ghrelin is biologically relevant for its effects on GH secretion and appetite stimulation, only active ghrelin was detected and reported in this study. Our measurements of total ghrelin largely corroborated the results obtained for active ghrelin alone (data not shown).

Stomach ghrelin mRNA production is upregulated by vagotomy in both fed and fasted rats, whereas it is not affected by fasting in sham-operated rats. Analysis of gut hormone mRNA expression is greatly influenced by the choice of housekeeping genes used as internal standards: in particular, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression is reduced in fasted rats compared to fed rats [36]. Previous data showed that stomach ghrelin mRNA level, relative to that of GADPH, was increased by a 48-h fast [37]. Consequently, in fasted rats, stomach ghrelin mRNA expression might also appear to be elevated relative to GAPDH expression. It is likely preferable, then, to use the r28S gene, whose expression is not affected by nutrient deprivation, as an internal control in future studies.

5. Conclusion

This study demonstrates that stomach ghrelin secretion responds to cholinergic and adrenergic manipulation. Plasma ghrelin levels in rats are increased by a muscarinic agonist, an α-adrenergic antagonist, and a β-adrenergic agonist, and are decreased by a muscarinic antagonist and an α-adrenergic agonist. Vagotomy inhibited ghrelin secretion in the short term and promoted it in the long term, implying a complicated relationship between the regulation of ghrelin secretion and the balance of cholinergic and adrenergic tone. Further work is required to clarify the non-cholinergic and non-adrenergic mechanisms involved in the relationship between ghrelin secretion and nutritional status.

Acknowledgments

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GHRP-2, a GHS-R agonist, directly acts on myocytes to attenuate the dexamethasone-induced expressions of muscle-specific ubiquitin ligases, Atrogin-1 and MuRF1

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Abstract

Recent reports suggest that Atrogin-1 and MuRF1, E3 ubiquitin ligases, play a pivotal role in muscle atrophy. In the present study, effect of Growth Hormone Releasing Peptide-2 (GHRP-2), a GH secretagogue receptor (GHS-R) agonist, on the expressions of Atrogin-1 and MuRF1 in vivo rat muscles was examined. Dexamethasone administration increased Atrogin-1 mRNA level in rat soleus muscle. The increased mRNA level of Atrogin-1 was significantly attenuated by GHRP-2. In addition, GHRP-2 decreased MuRF1 mRNA level irrespective of the presence of dexamethasone. Although IGF-I is a well-known protective factor for muscle atrophy, GHRP-2 did not influence plasma IGF-I levels and IGF-I mRNA levels in muscles. To clarify a direct effect of GHRP-2, differentiated C2C12 myocytes were used. Ten micrometer dexamethasone increased both Atrogin-1 and MuRF1 mRNA levels in C2C12 cells. GHRP-2 attenuated dexamethasone-induced expression of them dose-dependently and decreased the basal level of MuRF1 mRNA. The suppressive effect on the expressions of Atrogin-1 and MuRF1 by GHRP-2 was blocked by [D-Lys^3]-GHRP-6, a GHS-R1a blocker, suggesting the effect of GHRP-2 was mediated through GHS-R1a. Taken together, GHRP-2 directly attenuates Atrogin-1 and MuRF1 mRNA levels through ghrelin receptors in myocytes.

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Keywords: GHRP-2; Muscle-specific ubiquitin ligases; Atrogin-1; MuRF1; IGF-I

Introduction

A variety of diseases and conditions, including sepsis, cancer, renal failure, excess of glucocorticoid, denervation and disuse of muscle, can cause muscle atrophy. In these diverse conditions, the atrophying muscles show increased protein degradation through activation of the ubiquitin (Ub)-proteasome pathway (Baracos et al., 1995; Kayali et al., 1987; Price et al., 1996; Tiao et al., 1997; Tischler et al., 1990). It is recently reported that the expressions of Atrogin-1 and MuRF1, both of which are muscle-specific Ub-ligases, are involved in protein degradation in muscle and increased in these diverse conditions causing muscle atrophy (Bodine et al., 2001; Gomes et al., 2001; Lecker et al., 2004). Atrogin-1 is a muscle-specific F-box type E3 ligase and reported to be induced 8 to 40 fold in muscle atrophy during fasting, diabetes, cancer and renal failure (Bodine et al., 2001), up to 3 fold in hind limb suspension, immobilization and denervation, and up to 10 fold in cachetic or dexamethasone administration model (Gomes et al., 2001). MuRF1 is a Ring Finger type

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muscle-specific E3 ligase that is initially found in association with the myofibril (Kandarian and Jackman, 2006) and suggested to play an important role in the myofibrillar proteins breakdown. Both muscle-specific E3 ligases are considered to play a pivotal role in muscle atrophy because knockout mice lacking these E3 ligases are prevented from muscle atrophy (56% sparing for atrogin-$1^{-/-}$ and 36% for MuRF$1^{-/-}$) (Bodine et al., 2001).

On the other hand, several protective factors for muscle atrophy have been reported. One of the potent protective factors is IGF-I. IGF-I prevents muscle atrophy induced by glucocorticoid (Kanda et al., 1999; Schakman et al., 2005), disuse (Alzghoul et al., 2004) and denervation (Day et al., 2002). IGF-I has a potency to inhibit Atrogin-1 and MuRF1 expressions in atrophying muscle (Sacheck et al., 2004; Stitt et al., 2004). The protective effect of IGF-I for muscle atrophy, at least partly, is exerted by this mechanism (Bodine et al., 2001; Lecker et al., 2004).

Ghrelin stimulates GH release from the pituitary through the GH secretagogue receptor (GHS-R) (Kojima et al., 1999). Also, Growth Hormone Releasing Peptide-2 (GHRP-2), a synthetic ligand for GHS-R, stimulates GH release from the pituitary (Wu et al., 1996). GHRP-2 administration increases plasma GH levels in rats (Sawada et al., 1994) and humans (Pihoker et al., 1995). As a result, plasma IGF-I levels are reported to increase in some studies (Bowers et al., 2004). Thus, GHRP-2 is expected to have a protective action against muscle atrophy via IGF-I. Indeed, a recent report suggested that GHRP-2 was able to prevent arthritis-induced increase in Atrogin-1 and MuRF1 expressions in rat muscle (Granado et al., 2005).

On the other hand, there are reports suggesting the presence of GHS-R in muscle (Papotti et al., 2000; Pierno et al., 2003) and the signal transduction mechanism of ghrelin is partly similar to those of IGF-I and insulin (Murata et al., 2002). Hence GHS-R ligands may play a role in the process of muscle atrophy.

In the present study, we have examined the effect of GHRP-2 on Atrogin-1 and MuRF1 mRNA levels in dexamethasone-induced muscle atrophy in the rats, as a model of muscle atrophy that is often observed during steroid hormone-treatment in human. We have further tested whether the effect is a direct action on myocytes through GHS-R and found for the first time that GHRP-2 directly acted on myocytes to suppress Atrogin-1 and MuRF1 mRNA levels.

Materials and methods

Animals

All experiments were performed using 6-week-old male Sprague–Dawley rats weighing 170–190 g. Animals were maintained in cages at 25 °C under a 12 h light/12 h dark cycle and were allowed free access to food and water. All the animal protocols were approved by the Committee on Animal Experimentation, Kobe University School of Medicine.

In vivo experiments

Twenty-four rats were divided into four groups of six animals each. One group was used as a glucocorticoid-induced skeletal muscle atrophy group and given dexamethasone. The second group received dexamethasone and an additional injection of GHRP-2. The third group received only GHRP-2. The fourth, control group was given an equivalent volume of saline (0.9% NaCl). Dexamethasone (600 μg/kg body weight) was injected intraperitoneally once a day. This dose was determined from a previous result (Ma et al., 2003). GHRP-2 (100 μg/kg body weight) was injected subcutaneously twice a day. Body weights of rats were measured everyday at 1000 h. After 5 days treatment with dexamethasone, GHRP-2 or both, soleus muscles and venous blood were collected for analysis under the pentobarbital anesthesia.

Cell culture and in vitro experiments

Fetal bovine serum (FBS) was obtained from ICN Biomedicals. Horse serum (HS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Invitrogen. C2C12 cells, a mouse myoblast cell line, which were obtained from the Riken Cell Bank (Cell No. RCB0987), were maintained in DMEM with 100 μg/ml penicillin G, 15.5 μg/ml kanamycin and 10% FBS. The undifferentiated C2C12 cells were plated onto 6-well-plate and cultured in DMEM containing 10% FBS until the cells reached 100% confluence. Then the medium was replaced with DMEM containing 2% HS and incubated for further five days to induce myogenic differentiation. After differentiation, the cells were washed and incubated for 6 h in serum-free DMEM and then treated with DMEM containing 10 μM dexamethasone, GHRP-2 (1 nM, 1 μM) or both for 24 h. In another experiment examining the involvement of GHS-R1a, 100 μM [D-Lys^3]-GHRP-6, a GHS-R1a antagonist (Cheng et al., 1989; Kojima et al., 1999; Smith et al., 1993), was added 30 min before the stimulation with 10 μM dexamethasone, 1 μM GHRP-2 or both.

Total RNA extraction

Total RNA was extracted from soleus muscle using RNeasy Fibrous Tissue Midi Kit (QIAGEN) according to the manufacture's instruction. Total RNA was isolated from the undifferentiated and differentiated C2C12 cells using TRIzol reagent (Invitrogen) according to the manufacturer's protocol.

Reverse transcription (RT)

RT reaction was performed at 42 °C for 60 min with 2 μg of total RNA from the C2C12 cells or rat soleus muscle in 25 μl reaction volume. The cDNA obtained by RT was diluted 1:30 and 3 μl of the diluted solution was used as template in the next real-time PCR and conventional PCR testing the presence of GHS-R1a.

Primer design

All primers used in the present study were designed using the Primer 3 software. Primer sequences and expected size of PCR products are listed in Table 1.

Table 1
Forward (-F) and reverse (-R) primer sequences, amplification products length

Primer	Sequence (5′=>3′)	Length (bp)
Atrogin-1-F	ATGCACACTGGTGCAGAGAG	170
Atrogin-1-R	TGTAAGCACACAGGCAGGTC	
MuRF1-F	ACATCTTCCAGGCTGCCAAT	154
MuRF1-R	GTTCTCCACCAGCAGGTTCC	
IGF-I-F	TGCAAAGGAGAAGGAAAGGA	153
IGF-I-R	TGTTTTGCAGGTTGCTCAAG	
GHS-R1a-F	ACTGCAACCTGGTGTCCTTT	165
GHS-R1a-R	CGGGAACTCTCATCCTTCAG	
GAPDH-F	AACGACCCCTTCATTGAC	193
GAPDH-R	TCCACGACATACTCAGCAC	

Real-time PCR

Real-time quantitative PCR analysis (QuantiTect SYBR Green PCR kit; QIAGEN) was carried out using ABI PRISM 7700 Sequence Detection Systems (Applied Biosystems). The real-time PCR parameters were 15 min at 95 °C followed by 40 cycles of 15 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C. PCR products of each assay were subjected to agarose gel electrophoresis to confirm amplification specificity. All measurements were performed in triplicate and a series of experiments was repeated twice independently. All specific quantities were corrected for the amount of GAPDH amplification.

Quantification

For each sample, the threshold cycle (Ct) was calculated based on the cycle at which the fluorescence increased above a threshold level. The ΔCt values were calculated in every sample for target gene as follow: Ct (target gene) − Ct (internal control gene), with GAPDH as internal control gene. Relative expression level for one target gene (ΔΔCt) was calculated by the subtraction of mean ΔCt of control group from the ΔCt of each sample of treated groups. Finally, relative expression value, normalized to an endogenous reference, was given by: $2^{-\Delta\Delta Ct}$.

Conventional PCR

Conventional PCR was carried out in a 50 μl reaction mixture containing cDNA from undifferentiated and differentiated C2C12 cells, 20 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol each primer and 1.25 U Taq DNA polymerase (TOYOBO). The PCR involved 35 cycles consisting of 30 s of denaturation at 94 °C, 30 s of annealing at 57 °C and 60 s of extension at 72 °C. These PCR products were verified with 2% agarose gel electrophoresis.

IGF-I assay

Plasma IGF-I levels were measured using the DSL-10-2900 ACTIVE Mouse/Rat IGF-I Enzyme Immunoassay Kit (Diagnostic Systems Laboratories) according to the manufacturer's instructions.

Statistical analysis

Results are expressed as mean±SEM. Differences were determined by ANOVA followed by the Tukey–Kramer test. $P<0.05$ was considered as significant.

Results

In vivo experiments

During the 5-day treatment, body weight was decreased in rats treated with dexamethasone (Fig. 1A). On the other hand, GHRP-2 administration had no effect in body weight (Fig. 1A). GHRP-2 and dexamethasone did not show significant influence on the soleus muscle weight, although dexamethasone had a tendency to reduce (Fig. 1B). Administration of dexamethasone for 5 days increased Atrogin-1 mRNA level in soleus muscles of rats in vivo (Fig. 2A). Dexamethasone also showed a tendency to increase MuRF1 mRNA level (Fig. 2B). The increased Atrogin-1 mRNA level was significantly attenuated by GHRP-2 (Fig. 2A). In addition, GHRP-2 significantly attenuated MuRF1 mRNA level

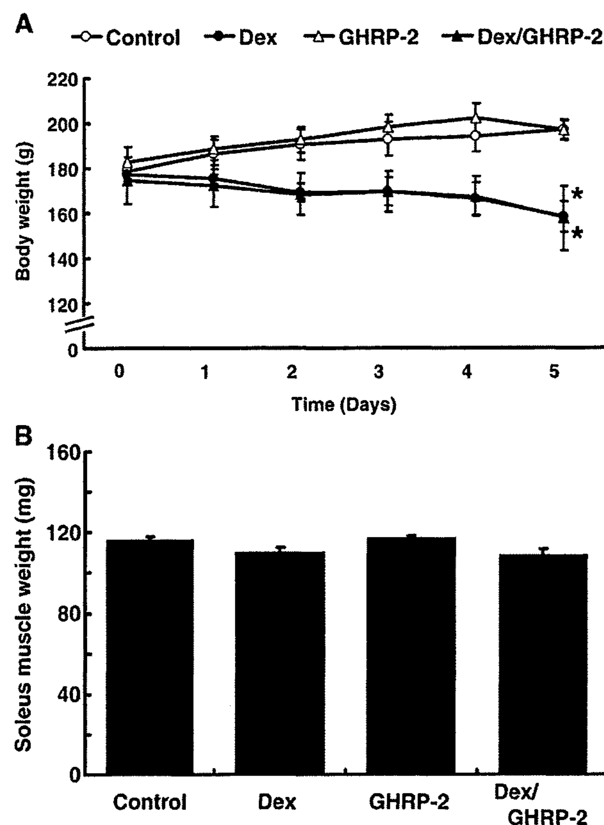

Fig. 1. GHRP-2 did not influence body weight gain whereas dexamethasone reduced it. Dexamethasone (Dex, 600 μg/kg) was intraperitoneally injected to 6-week-old male rats once a day for 5 days. A; GHRP-2 (100 μg/kg) was subcutaneously injected twice a day for 5 days. The effect of 5 days injections of Dex (filled circle), GHRP-2 (open triangle) or both (filled triangle) on body weight is shown. GHRP-2 did not affect, but Dex decreased body weight compared with saline control (open circle) on the fifth day of the treatment. B; GHRP-2 and Dex did not show significant influence on soleus muscle weight, although Dex had a tendency to reduce, * $P<0.05$ vs. control group.