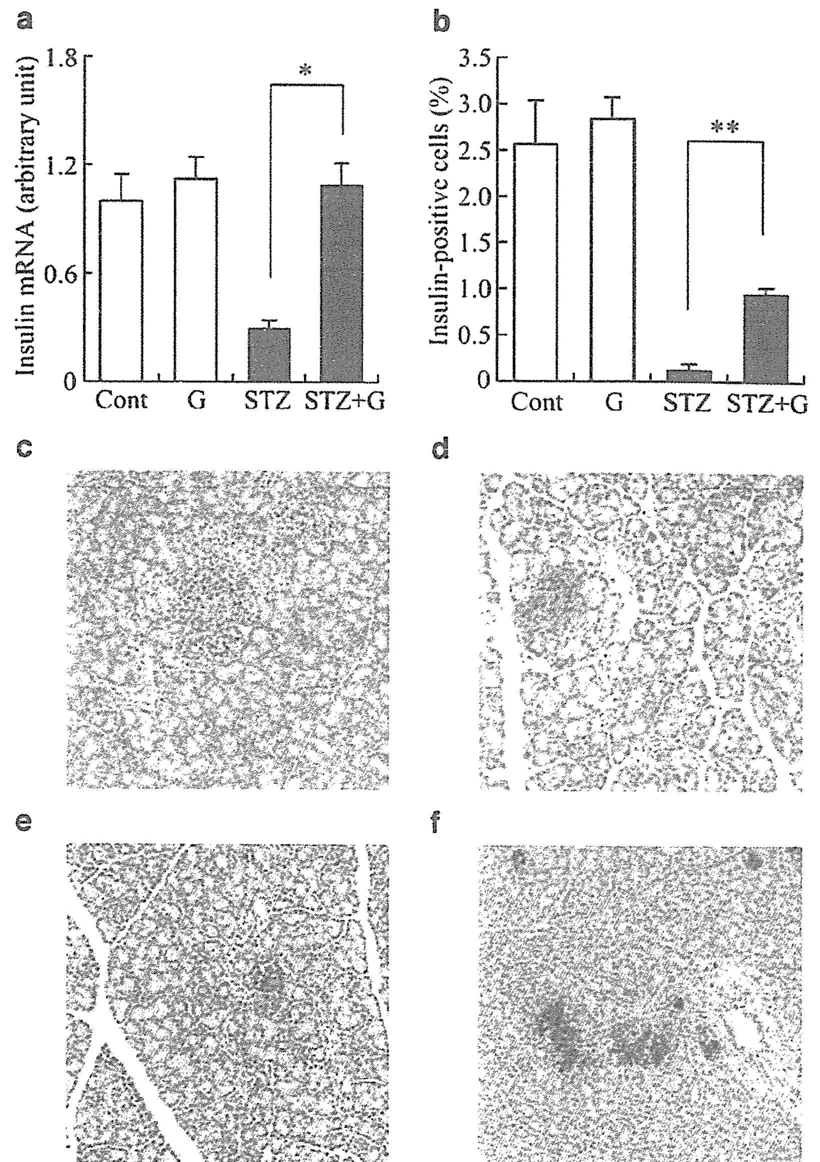


Fig. 1 Gene expression levels (**a**) and immunostaining (**b–f**) of insulin in the pancreases of 21-day-old n0-STZ and n0-STZ/Ghrelin animals. **b** Quantification of insulin-positive cells per unit of total tissue area; **c** Control group; **d** Ghrelin group; **e** n0-STZ group; **f** n0-STZ/Ghrelin group. *Cont* Control group, *G* Ghrelin group, *STZ* n0-STZ group, *STZ+G* n0-STZ/Ghrelin group. Values are expressed as means±SEM for four observations in each group. $^{*}p<0.001$; $^{**}p<0.005$ (n=6–8 rats). In **c–f**, original magnifications were ×100

To explore the mechanism governing the alteration of insulin expression in the pancreas, we examined the expression of Pdx1, one of the major transcriptional factors involved in pancreatic development. *Pdx1* mRNA levels changed in a manner similar to those of insulin except for the Ghrelin group (Fig. 2a–f). The expression levels of the gene encoding Pdx1 in the Ghrelin group was higher than that of the Control (Fig. 2a). *Pdx1* expression levels in the n0-STZ group were less than one-tenth of the Control group levels; those of the n0-STZ/Ghrelin group returned to levels similar to those seen in the Control group. Immunofluorescence staining for Pdx1 within the pancreas exhibited a similar pattern to that of insulin in the n0-STZ and n0-STZ/Ghrelin groups (Fig. 2b–f). Corresponding to the change in *Pdx1* mRNA, the area of Pdx-1-positive cells in the Ghrelin group was significantly increased compared with that in the Control group.

Thus, the n0-STZ group exhibited diminished insulin production at 21 days after birth, although FBG levels achieved a spontaneous remission. The n0-STZ/Ghrelin group showed increased insulin expression in both mRNA and protein levels, Therefore, neonatal ghrelin treatment in this model may improve the reduction of insulin production at 21 days and prevent development of hyperglycaemia at 70 days.

Furthermore, to examine whether beta cell proliferation contributed to ghrelin effects on insulin production and beta cell number in STZ-treated rats or not, we performed phospho-histone H3 immunohistochemical analysis (Fig. 3a). Phospho-histone H3 is a cell proliferation mitosis marker [22, 23]. In 21-day-old rats, ghrelin treatment increased cells double-positive for phospho-histone H3 and insulin approximately 1.7- and 15-fold in Ghrelin and n0-

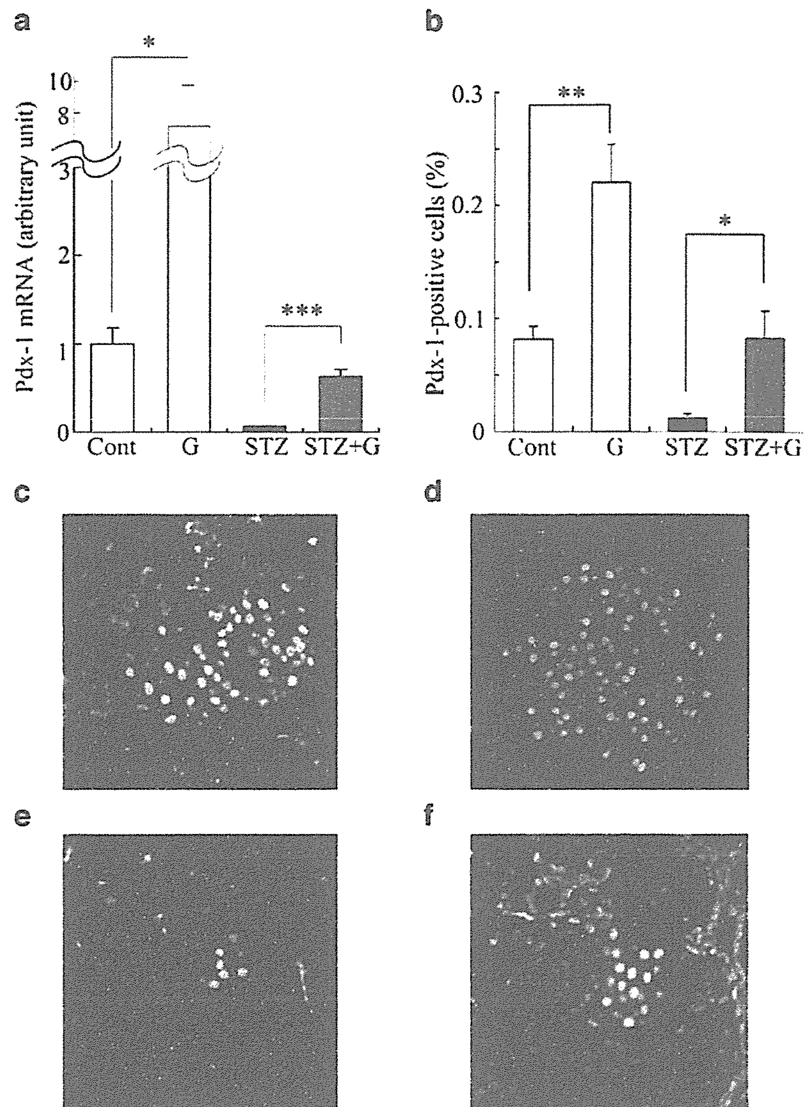


Fig. 2 Gene expression levels (**a**) and immunostaining (**b**–**f**) of Pdx1 in the pancreases of 21-day-old n0-STZ and n0-STZ/Ghrelin animals. **b** Quantification of Pdx1-positive cells per unit of total tissue area; **c** Control group; **d** Ghrelin group; **e** n0-STZ group; **f** n0-STZ/Ghrelin group. *Cont* Control group, *G* Ghrelin group, *STZ* n0-STZ group, *STZ+G* n0-STZ/Ghrelin group. Values are expressed as means±SEM for four observations in each group. In **c**–**f**, original magnifications were ×500. *$p<0.05$; **$p<0.001$; ***$p<0.0001$ (n=6–8 rats)

STZ/Ghrelin groups in comparison with Control and n0-STZ rats, respectively (Fig. 3b).

Long-lasting effects of early treatment with ghrelin: studies at 10 weeks

As the n0-STZ model develops hyperglycaemia after 8–10 weeks [14], we examined the long-term effects of early treatment with ghrelin in this model. The characteristics of Control, Ghrelin, n0-STZ and n0-STZ/Ghrelin animals at 10 weeks are detailed in Table 2. As expected, the n0-STZ group demonstrated reduced body weight and hyperglycaemia. Although the plasma insulin levels of this group were not decreased but tended to be slightly increased, the levels for each rat appeared to be relatively low for elevated glucose levels (Table 2, Fig. 4a). In fact, the pancreatic insulin levels of the n0-STZ group were reduced from those of the Control (Table 2). In contrast, the FBG levels of n0-STZ/Ghrelin group animals, which were not significantly higher than those of the Control, were significantly lower than those of the n0-STZ group. Furthermore, the pancreatic insulin levels of the n0-STZ/Ghrelin group returned to levels similar to those of the Control group. Body weight of the n0-STZ/Ghrelin group was significantly lower than that of the Control group, but not that of the n0-STZ group.

The mRNA expression of insulin at the adult stage in the Ghrelin group was lower than that in the Control group (Fig. 4b). In the n0-STZ group, the mRNA expression of insulin at day 70 remained reduced. The n0-STZ/Ghrelin group demonstrated a similar insulin expression level to that of the Control group, as was also observed 21 days after birth (Fig. 1a and Fig. 4b).

The patterns of mRNA expression for Pdx-1 in the four groups except the Ghrelin group were similar to those observed 21 days after birth (Fig. 5a). The expression level

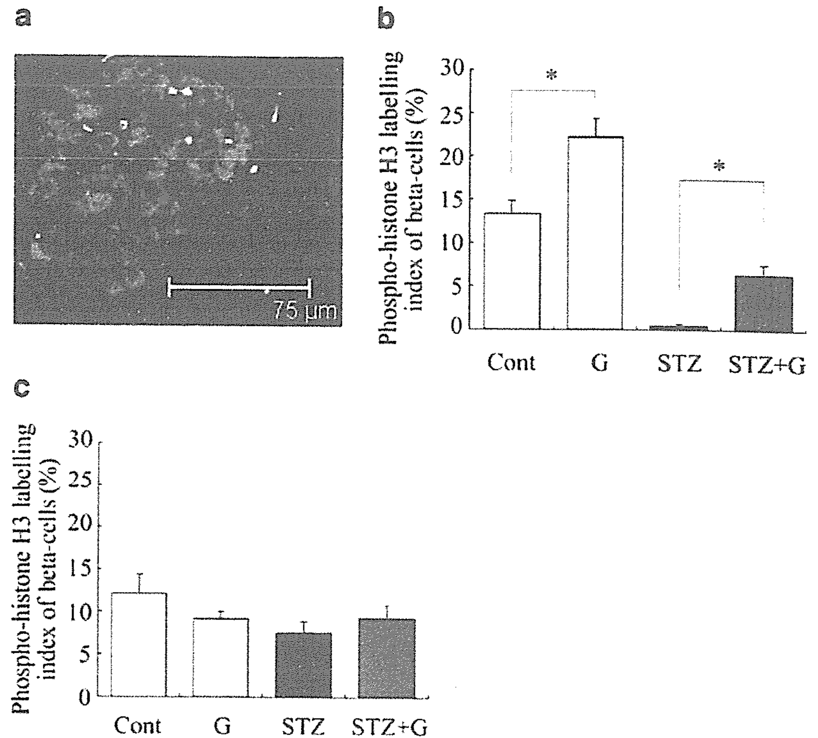


Fig. 3 Examination of beta cell proliferation by phospho-histone H3 staining in rats. **a** A representative confocal image of phospho-histone H3 (*green*) and insulin (*red*) immunostaining in an n0-STZ/Ghrelin rat. Phospho-histone H3 labelling index of the beta cell in 21-day-old (**b**) and 70-day-old (**c**) rats. It was evaluated through quantification of phospho-histone H3-positive cells in the 1,000 insulin-positive cells. *Cont* Control group, *G* Ghrelin group, *STZ* n0-STZ group, *STZ+G* n0-STZ/Ghrelin group. Values are expressed as means±SEM for four observations in each group. Original magnifications were ×400. Bar: 75 μm. $^{*}p<0.01$ (n=5–8 rats)

of *Pdx1* in the Ghrelin group was similar to that of the Control (Fig. 5a). *Pdx1* gene expression in the n0-STZ group was approximately one-third that of the Control, while that in the n0-STZ/Ghrelin group returned to levels close to that of the Control group. In immunofluorescent staining for Pdx-1 in the pancreas, the increase in staining of the n0-STZ/Ghrelin group was not significant compared with the n0-STZ group, although the Ghrelin group exhibited a significant increase compared with the Control group. (Fig. 5a–e).

We performed the experiments of immunostaining for phospho-histone H3 and insulin also in the adult stage. There were no significant differences between the four groups. But in the model of STZ, the ghrelin treatment tended to increase the double-positive cells for phospho-histone H3 and insulin, while in the Control group, the ghrelin treatment tended to decrease those cells (Fig. 3c).

Thus, in this n0-STZ model of diabetes, animals developed reduced insulin production in the pancreas and hyperglycaemia at 10 weeks after birth. Ghrelin treatment may prevent this deterioration through maintenance of insulin-positive cells and insulin production, which mechanistically may involve the increased expression of Pdx1.

Discussion

Neonatal rats treated with STZ exhibit normal glycaemia at 3 weeks after birth by rapid remission, but then deteriorate gradually after 8 weeks [14–16]. This delayed deterioration probably follows from the inability of the insulin production of the pancreas to recover to the levels of control rats [14, 15]. In this study, we observed a similar clinical course, characterised by the reduction of insulin production and content in the pancreases of n0-STZ rats. This neonatal model showed more reduction of pancreatic beta cells compared with other reports, in which the

Table 2 Characteristics (means±SEM) of fasted rats at 70 days

	Control	Ghrelin	n0-STZ	n0-STZ/Ghrelin
Body weight (g)	317±6 (6)	364±22 (8)	285±8 (5)	248±11 (13)*
FBG (mmol/l)	5.7±0.4 (6)	6.9±0.4 (8)	11.8±0.3 (5)**	7.6±0.4 (13)†
Plasma insulin (pmol/l)	181±17 (6)	207±15 (6)	302±68 (5)	160±30 (8)
Pancreas weight (mg)	414±33 (6)	491±16 (6)	520±42 (5)	556±23 (13)
Pancreatic insulin (μg/pancreas)	78.6±7.8 (6)	99.6±6.0 (6)	39.6±14.1 (5)	92.6±2.4 (8)††

Statistical analysis (Mann–Whitney test) was carried out between all groups
$^{*}p<0.05$, $^{**}p<0.0005$ (vs. Control)
$^{†}p<0.0005$, $^{††}p<0.05$ (vs. n0-STZ)

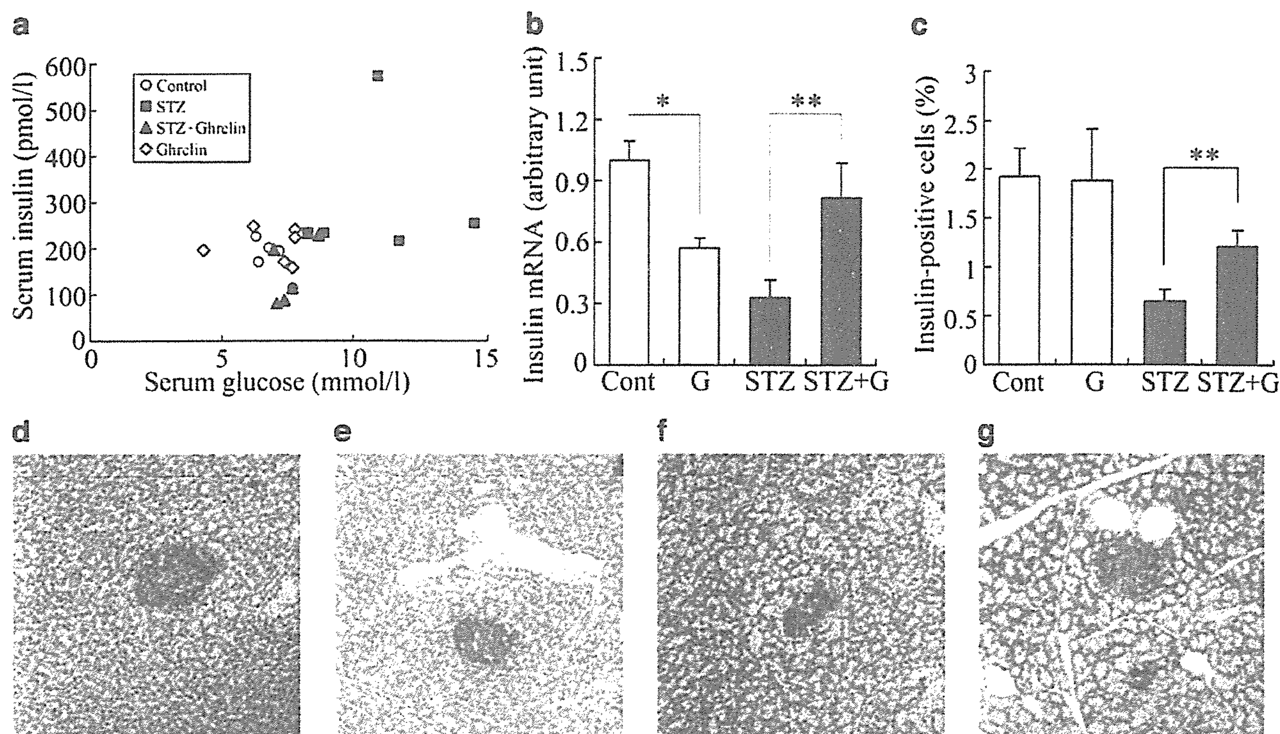


Fig. 4 **a** Relationship between serum glucose and insulin concentrations in adult animals following fasting. Gene expression levels (**b**) and immunostaining (**c**–**g**) of insulin in the pancreases of adult rats. **c** Quantification of insulin-positive cells, as performed in Fig. 1b; **d** Control group; **e** Ghrelin group; **f** n0-STZ group; **g** n0-STZ/Ghrelin group. *Cont* Control group, *G* Ghrelin group, *STZ* n0-STZ group, *STZ+G* n0-STZ/Ghrelin group. Values are expressed as means±SEM for four observations in each group. $^{*}p<0.005$; $^{**}p<0.05$ (n=5–8 rats). In **d**–**g**, original magnifications were ×100

reduction was approximately 50% that of controls [15, 16, 24]. This may be due to the different timing of STZ injection, which is known to result in type 2 diabetes models with different severity [14]. In fact, we injected STZ at 24 h after birth, while others injected within 12 h of birth [15, 16, 24].

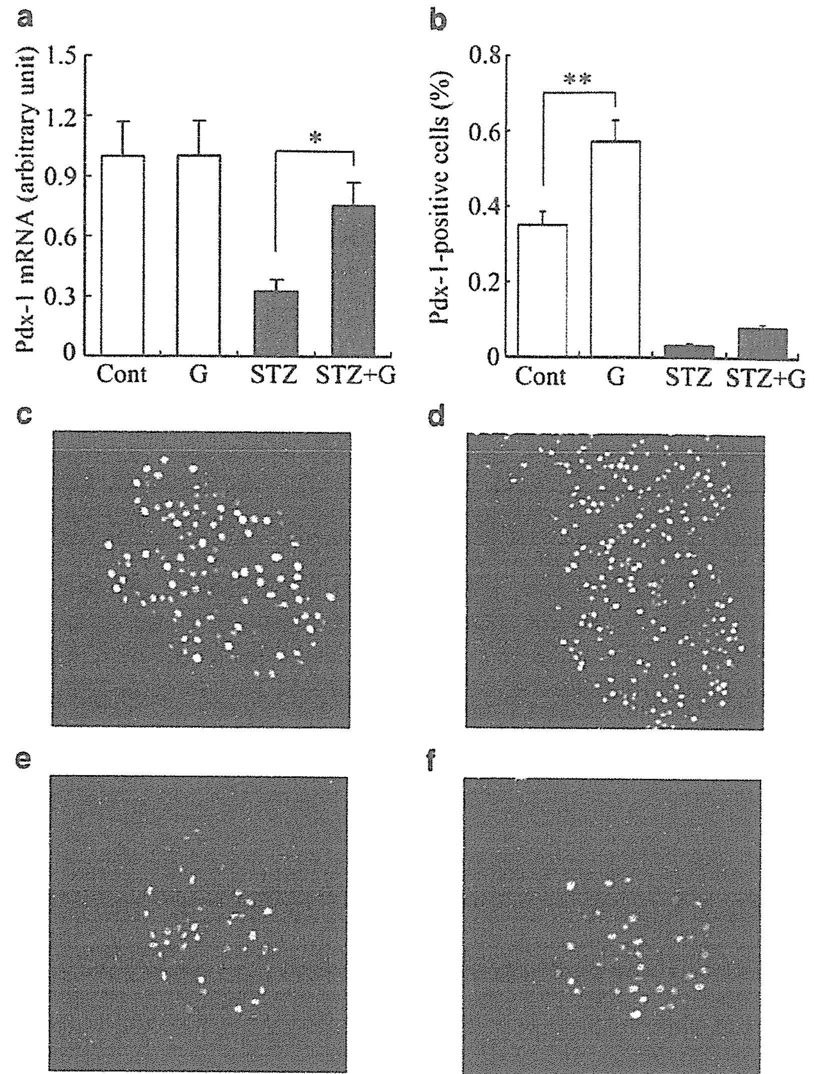
Moreover, we first demonstrated that ghrelin treatment prevented the development of hyperglycaemia in n0-STZ rats at adult ages. In n0-STZ/Ghrelin rats, both the transcription and translation of insulin in the pancreas were significantly increased at 21 and at 70 days. In addition, Pdx1 gene and protein expression levels at 21 days were restored to levels similar to those of the Control group. These findings strongly suggest that beta cells damaged by STZ administration during the neonatal stage can be regenerated or replicated following ghrelin treatment. Indeed, the immunohistochemical analysis for phospho-histone H3, which is a proliferation marker, demonstrated that ghrelin treatment significantly increased the staining in both Control and STZ-treated rats at the age of 21 days.

Similar findings have been reported for glucagon-like peptide-1 and exendin-4 by Tourrel et al. [13]. They demonstrated that glucagon-like peptide-1 and exendin-4 stimulated beta cell neogenesis or regeneration in STZ-treated newborn rats. While these n0-STZ rats ameliorated plasma glucose levels at adult stages, such treatment could not improve insulin secretion during a glucose-tolerance test. In future, it will be necessary to examine the effects of ghrelin treatment on insulin secretion in this n0-STZ/ghrelin model in detail, including an examination of glucose tolerance. In addition, a possibility that ghrelin treatment might modulate STZ toxic effects should be considered, because ghrelin administration was performed shortly after STZ treatment. Finally, another possibility that STZ may have affected hepatic metabolism and/or insulin sensitivity in peripheral tissues should be taken in account. These effects could explain some of the discrepancies between pancreas and plasma insulin levels.

Several reports have shown that both ghrelin and GHS-R are expressed in the pancreas [8, 9, 25–27]. Ghrelin is expressed in both the alpha [28] and beta [9, 29] cells of the pancreas. Recently, ghrelin was also identified in epsilon cells [30]. The pancreas is one of the organs in which GHS-R expression has been detected by RNA protection assay [8]. In addition, ghrelin gene expression is higher in the fetal pancreas than in fetal stomach [10], while expression is highest in the stomach in adults. Thus, ghrelin may act on beta cells in a paracrine or autocrine manner, potentially functioning in the differentiation, proliferation or development of beta cells.

Ghrelin has been shown to induce the proliferation of neoplastic cells in certain systems [31], while other studies have demonstrated [2] anti-proliferative or anti-apoptotic effects against neoplastic cells [32]. Ghrelin treatment of pre-adipocytes or motor neurons induced cellular prolifer-

Fig. 5 Gene expression levels (a) and immunostaining (b–e) of Pdx1 in the pancreases of adult rats. b Quantification of Pdx1 positive cells per unit of total tissue area; c Control group; d Ghrelin group; e n0-STZ group; f n0-STZ/Ghrelin group. *Cont* Control group, *G* Ghrelin group, *STZ* n0-STZ group, *STZ+G* n0-STZ/Ghrelin group. Values are expressed as means±SEM for four observations in each group. In c–f, original magnifications were ×500. $*p<0.05$; $**p<0.001$ (n=5–8 rats)



ation and differentiation into mature adipocytes or neurons [11, 12, 33]. While these findings suggest ghrelin may influence cell differentiation, proliferation, development or apoptosis, the mechanism by which ghrelin treatment exerts these effects is not clear. In the present study, expression of Pdx1, insulin and phospho-histone H3 in n0-STZ/Ghrelin rats was markedly increased in comparison with the level observed in 21-day-old n0-STZ rats. These findings strongly favour the conclusion that ghrelin is stimulating beta cell regeneration or replication in STZ-treated newborn rats, although further studies are necessary to clarify the mechanism including the contribution of apoptosis. The values obtained with phospho-histone H3 were higher than those obtained with 5-bromodeoxyuridine (BrdU) staining of beta cells at 21 days in Wistar rats (approximately 0.5–1.5%) [16, 24]. At this moment, we do not clearly know why we obtained higher phospho-histone H3 values in Sprague–Dawley rats of the same age and at 70 days, compared with the BrdU values. Although the correlation between anti-BrdU and -histone labelling indices was statistically significant, variation was noted in the percentage of the BrdU-positive cells double-labelled with phospho-histone H3, depending on cell type and length of BrdU incubation [34, 35]. Because, as far as we know, no phospho-histone H3 staining of pancreatic beta cells has been performed, it is difficult to directly compare our results with others. Nonetheless, ghrelin treatment increased the expression level of phospho-histone H3 at the age of 21 days but not 70 days. Finally, neonatal STZ treatment in earlier studies resulted in a slight increase of beta cell proliferation measured by the BrdU labelling index, in contrast to marked decrease in the present study. This discrepancy will be due to the fact that this neonatal model showed more reduction of pancreatic beta cells compared with other reports, as already discussed.

In addition, it was observed that ghrelin treatment in control rats appeared to result in different alterations from that in STZ-treated rats, for example, regarding body weight and FBG at day 21 and insulin gene expression

level at day 70. This is probably because physiological and pathological responses to ghrelin treatment are different between healthy control and diabetic STZ-treated rats. Although it is hard to explain these apparent discrepancies at this time, it is necessary to study ghrelin action in healthy controls separately from morbid subjects. Although ghrelin treatment increased body weight in the Ghrelin group compared with the Control group in this study, such a significant difference was not observed in a previous report by Hayashida et al. [36]. However, they also observed that body weight tended to increase after ghrelin treatment, albeit not statistically significantly (personal communication with N. Murakami, Miyazaki University, Miyazaki, Japan). They speculate that the increase in body weight might have reached statistically significance if the number of rats was increased.

From a clinical standpoint, results of the present study suggest that the n0-STZ/Ghrelin rat may be a valuable model to investigate novel treatment strategies for diabetes mellitus. In addition, ghrelin may be a useful treatment for diabetes. Recently, circulating ghrelin levels were discovered to be decreased in patients with type 2 diabetes [37]. Ghrelin activity is involved in insulin resistance as well as the maintenance of energy balance [37]. As multiple studies have demonstrated the importance of ghrelin in glucose and insulin metabolism [2, 3], ghrelin will be an important molecule to investigate the pathophysiology of diabetes.

In conclusion, we have provided evidence that ghrelin may assist the regeneration of beta cells, using STZ-treated newborn rats as a model of diabetes mellitus. In these rats, ghrelin treatment increased pancreatic insulin expression and resulted in the improvement of plasma glucose levels with normal plasma insulin levels. These findings suggest that early administration of ghrelin may prevent the development of diabetes in disease-prone subjects after beta cell destruction.

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REVIEW

Translational Research on the Clinical Applications of Ghrelin

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Key words: Cachexia, Anorexia nervosa, GH deficiency, Clinical trial, Ghrelin

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GHRELIN is a peptide hormone that was discovered in 1999 as an endogenous ligand for the growth hormone (GH)-secretagogue receptor (GHS-R) [1, 2]. Ghrelin, a 28-amino-acid peptide, possesses a unique fatty acid modification, an *n*-octanoylation, at Ser 3. Of the two circulating forms of ghrelin, acylated and unacylated (desacyl), the acylated form is thought to be essential for ghrelin's biological activity through GHS-R. Recently, however, desacyl ghrelin was reported to influence both cell proliferation and adipogenesis through an unknown receptor different from GHS-R [2–5]. Ghrelin is mainly produced in the stomach and circulates in the blood at a considerable plasma concentration. Expression of ghrelin is also detectable in the hypothalamus, intestine, pituitary, placenta and other tissues [1, 3–5]. Ghrelin is now known to play a role in a number of different physiological processes. For example, ghrelin increases GH secretion, feeding, and body weight when administered centrally or peripherally (Fig. 1) [1, 6–15].

These unique effects of ghrelin and GHS should be invaluable for the development of novel treatments and disease diagnostic techniques [16–18]. Clinical trials have already been performed to assess the utility of GHS for the treatment of short stature [19], GH deficiency [19, 20], obesity [21] and catabolic conditions [22]. Several preliminary studies have also been performed to assess the possible benefits of ghrelin administration to humans [9–15, 23–26]. Because many excellent reviews concerning basic and clinical researches on ghrelin have already been published, we will summarize and discuss recent clinical trials of ghrelin in this work.

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Phase I studies

In 2001, Nagaya *et al.* gave six healthy men either an intravenous bolus of human ghrelin (acylated form) (10 μg/kg) or placebo and then the opposite injection 1–2 wks later in a randomized fashion [12]. They found that the plasma total (acylated and desacyl) ghrelin level dropped with a half life of 10 min and that all study subjects tolerated this study protocol well, although ghrelin caused a slight warm feeling and drowsiness in four subjects. In 2003, we conducted a larger double-blinded, randomized, placebo-controlled trial in which the pharmacokinetics of both total and acylated ghrelin were analyzed simultaneously [27]. Eighteen male volunteers were randomly assigned into three groups of six subjects: members of the low- and high-dose ghrelin groups received intravenous injections of 1 and 5 μg/kg ghrelin (acylated form), respectively, and those in the placebo group were injected with mannitol instead of ghrelin. Acylated ghrelin disappeared more rapidly from the plasma than did total ghrelin, with elimination half-lives of 9–13 and 27–31 min, respectively. The number of subjects that experienced adverse effects did not significantly differ among the three groups, and all adverse effects were transient and well tolerated.

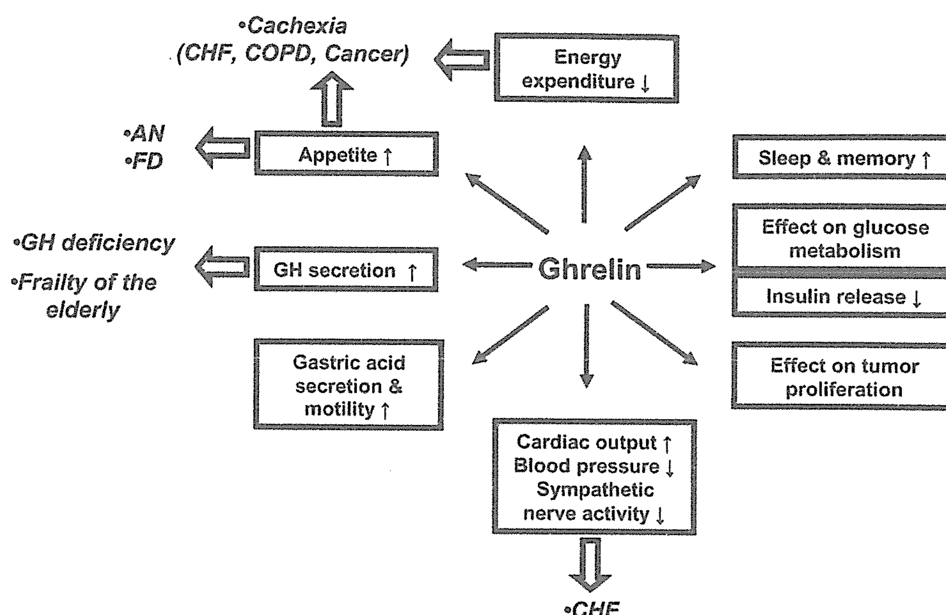

Fig. 1. Examples of the multiple actions and potential clinical applications (in italics) of ghrelin. CHF, congestive heart failure; COPD, chronic obstructive pulmonary disease; AN, anorexia nervosa; FD, functional dyspepsia, GH, growth hormone.

Among all studies analyzed, more than one hundred subjects have received ghrelin injections, and only mild adverse effects have been reported, including sensations of bowel movement, feelings of warmth, huger, somnolence, and hyperhidrosis [9–15, 23–26].

Phase II studies

1. Appetite-related disorders

Ghrelin is the first circulating hormone demonstrated to stimulate appetite in humans. Wren *et al.* showed that a 17 ng/kg/min intravenous infusion of ghrelin for 270 min enhanced appetite and food intake in humans [15]. The total amount of ghrelin infused in this study was 4.5 μg/kg. Similarly, we found that ghrelin administration tended to increase the sensation of hunger in a dose-dependent manner, particularly in the early phase after injection, although the difference between groups was not statistically significant. In addition, whereas two of the six subjects in the placebo group did not show any change in the hunger score, all of the subjects in both the low- and high-dose groups reported increases. To confirm the orexigenic effect of ghrelin, either a crossover study or one analyzing larger groups will be necessary. In addition, because tests for appetite are subjective and variable among subjects, only double-blinded studies such as that described here will yield reliable findings.

1) Cachexia

Cachexia manifests with excessive weight loss in the setting of ongoing disease including congestive heart failure (CHF), cancer, chronic obstructive pulmonary disease (COPD) and severe inflammation [28]. Anorexia is among the major causes of weight loss in cachexia. Loss of appetite and loss of weight are major causes of morbidity and mortality affecting many patients with anorexia-cachexia syndrome. There is a strong and immediate need for more effective and better-tolerated appetite stimulatory treatments. Several trials have already been performed to explore the utility of ghrelin in the treatment of cachexia. Circulating ghrelin levels are elevated in patients with cachexia, compared with normal-weight control subjects, reflecting the state of negative energy balance [29–32].

Nagaya *et al.* investigated the effects of ghrelin on cardiac cachexia in patients with CHF [33]. A three-week administration of ghrelin (2 μg/kg twice a day) significantly increased food intake and tended to increase body weight. Moreover, they demonstrated improvement in exercise capacity, muscle wasting and

left ventricular function. Ghrelin treatment resulted in a significant decrease in plasma norepinephrine. Although this study was neither randomized nor placebo-controlled, eight patients with CHF who did not receive ghrelin (control group) were studied to exclude time-course effects during hospitalization. All of the aforementioned parameters remained unchanged in the patients with CHF who did not receive ghrelin therapy.

Anorexia is frequently encountered in cancer patients, and is one of the major causes of malnutrition and eventually cachexia. Nearry *et al.* performed an acute, randomized, placebo-controlled, cross-over clinical trial to determine whether ghrelin stimulates appetite in seven cancer patients with anorexia [34]. A marked increase in energy intake (31 +/− 7%; P = 0.005) was observed with ghrelin infusion (17 ng/kg/min intravenous infusion of ghrelin for 270 min) compared with saline control, and every patient in the study increased food consumption. The meal appreciation score was higher by 28 +/− 8% (P = 0.02) in ghrelin-treated individuals.

The ability of ghrelin to improve cachexia and functional capacity in patients with COPD has been studied in an open-label pilot study in which ghrelin (2 μg/kg bid) was administered intravenously to seven cachectic patients with COPD for three weeks [35]. Repeated administration of ghrelin resulted in a significant increase in food intake, body weight, lean body mass and peripheral and respiratory muscle strength. Ghrelin attenuated the exaggerated sympathetic nerve activity, as indicated by a marked decrease in plasma norepinephrine level. Thus, treatment with ghrelin improved appetite, body composition, muscle wasting, functional capacity, and sympathetic augmentation in cachectic patients with COPD.

Finally, ghrelin may have a beneficial effect on anorexia in sepsis. In an animal model of excessive inflammation, septic shock, and wasting syndrome, repeated ghrelin treatment (twice daily for five days) increased food intake and body weight [36]. Although no clinical trial has been attempted in patients with sepsis, these findings suggest the therapeutic potential of the anti-wasting effects of ghrelin.

2) Anorexia nervosa (AN) and its related disorders

Anorexia nervosa (AN) is an eating disorder characterized by chronically decreased caloric intake, resulting in self-induced starvation. Plasma ghrelin levels are elevated in lean patients with anorexia nervosa, consistent with a state of negative energy balance [37–39]. Only a few preliminary studies have been performed to examine the effects of ghrelin in individuals with AN. Miljic *et al.* infused ghrelin (300-min intravenous infusion of 5 pmol/kg/min ghrelin) into nine AN patients with very low body weights six AN patients who had partially recovered their body weight but were still amenorrheic, and ten constitutionally thin female subjects [40]. The fifteen AN patients felt significantly less hungry compared with the constitutionally thin subjects, suggesting that AN patients are less sensitive to the orexigenic effects of ghrelin compared with healthy controls. In another paper, however, six of nine patients with restrictive AN were reported to be hungry after ghrelin administration (1.0 μg/kg as an intravenous bolus) which was a similar ratio to that seen in normal subjects (five of seven) [41]. Clearly, further studies, including randomized controlled trials, are needed to determine whether ghrelin is useful for the treatment of AN.

Functional dyspepsia (FD) is a disorder characterized by the presence of chronic or recurrent symptoms of upper abdominal pain or discomfort [42]. Although no known specific organic abnormalities are present in FD, abnormalities in gastrointestinal motility and sensitivity are thought to play a role in a substantial subgroup of patients. In addition, some patients also suffer from anorexia and body-weight loss. Therefore, we are currently examining whether repeated ghrelin administration increases food intake in FD patients. We found that plasma acylated, but not desacyl ghrelin, levels were correlated with a subjective symptom score in FD patients, suggesting that acylated ghrelin may play a role in the pathophysiology of FD [43].

2. GH deficiency-related disorders

Strong stimulation of GH secretion by ghrelin has been well demonstrated in humans [9–13, 17, 27]. As with GHS, ghrelin may be useful for the diagnosis and treatment of short stature and GH deficiency. Elderly individuals would be particularly suitable candidates for ghrelin treatment. Aging is associated with progressive decreases in GH secretion, appetite and energy intake [44–47]. This reduced GH secretion is called "somatopause" and may be a cause of age-related metabolic and physiologic changes including reduced lean body mass and expansion of adipose

mass. Sarcopenia is associated with functional decline and death. Altered blood lipid profiles also favor the development of vascular diseases that may increase overall mortality. The age-related reduction in energy intake has been termed "the anorexia of aging" and predisposes to the development of under-nutrition, which has been implicated in the development and progression of chronic diseases commonly affecting the elderly, as well as in increasing mortality. Growth hormone therapy increases IGF-I levels, promotes anabolism and increases muscle strength in healthy elderly individuals, as well as in selected patient groups [48–50]. Therefore, ghrelin and GHS may also have therapeutic potential to assist in the recovery of frail patients who require nutritional support and conventional rehabilitation [51]. Indeed, we are currently evaluating whether repeated ghrelin administration during the perioperative period would promote functional recovery in elderly patients undergoing elective total hip replacement.

We found that plasma levels of acylated ghrelin in healthy elderly female subjects tended to be low and were correlated positively with IGF-1 levels, suggesting that the negative feedback mechanism does not function properly in elderly subjects [52]. Further, acylated ghrelin concentrations in elderly females correlated with both systolic blood pressure and the frequency of bowel movements. These findings suggest that, in elderly females, acylated ghrelin may play a role in the regulation of the GH/IGF-1 axis, blood pressure and bowel movements.

3. Other disorders

Reflecting the wide expression patterns of both ghrelin and its receptor, this peptide is now known to play a role in a number of different physiological processes including cardiovascular function, cellular proliferation and differentiation, gastric motility and acid secretion, pancreatic exocrine and endocrine function as well as on glucose metabolism, sleep and behavior, and immune regulation. For example, as we mentioned above, repeated administration of ghrelin in patients with CHF significantly improved left ventricular function as well as food intake. A large number of studies have been vigorously performed to elucidate the various activities of ghrelin by many investigators all over the world. We believe that some of these may lend support to the development of novel clinical applications of ghrelin in the future.

Epilogue

Since the discovery of ghrelin, more than six years have passed and abundant evidence supporting its variety of functions has accumulated. In parallel, clinical trials have begun and proceeded to exploit these activities in the treatment and diagnosis of human disease. There are several characteristic features of the clinical applications of ghrelin; 1) the multiplicity and uniqueness of its function, 2) the unique structure with fatty acid modification, and 3) the paucity of severe adverse effects. These characteristics should allow us to develop novel and unique therapies for a variety of disorders, including many currently intractable and serious diseases. Indeed, translational research into the clinical applications of ghrelin is a challenging and potentially rewarding avenue for the future.

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Molecular Forms of Hypothalamic Ghrelin and Its Regulation by Fasting and 2-Deoxy-D-Glucose Administration

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Ghrelin, an endogenous ligand for the GH secretagogue receptor, is a hormone expressed in stomach and other tissues, such as hypothalamus, testis, and placenta. This hormone acts at a central level to stimulate GH secretion and food intake. Little is known, however, about the molecular forms and physiological roles of ghrelin within the hypothalamus. In this report, we detail the molecular forms, mRNA expression patterns, and peptide contents of ghrelin within the rat hypothalamus. Using the combination of reverse-phase HPLC and ghrelin-specific RIA, we determined that the rat hypothalamus contains both *n*-octanoyl-modified and des-acyl ghrelins. Fasting for 24 and 48 h significantly decreased ghrelin mRNA expression in the hypothalamus to 24% and 28% of control values, respectively. Both *n*-octanoyl-modified and des-acyl ghrelin content in the hypothalamus decreased after 24 and 48 h of fasting. These results contrast the changes in gastric ghrelin after fasting, which decreased in content despite increased mRNA expression. Two hours after injection of 2-deoxy-D-glucose (2-DG), a selective blocker of carbohydrate metabolism, ghrelin peptide levels also decreased. Thus, induction of glucoprivic states, such as fasting and 2-DG treatment, decreased ghrelin gene expression and peptide content within the hypothalamus. (*Endocrinology* 146: 2510–2516, 2005)

GHRELIN, ORIGINALLY purified from rat stomach, is an endogenous ligand of the GH secretagogue receptor (GHS-R) (1). Ghrelin is a 28-amino acid peptide existing in two major forms: *n*-octanoyl-modified ghrelin, which possesses an *n*-octanoyl modification on serine-3, and des-acyl ghrelin (2). Lipid modification of ghrelin is essential for ghrelin-induced GH release from the pituitary (3) and appetite stimulation (4–7). Thus, the posttranscriptional regulation of octanoyl modification is an important step controlling the biological activity of ghrelin.

In addition to the stomach, ghrelin is localized to the hypothalamic arcuate nucleus (ARC) of rats and mice (1, 8, 9). The hypothalamus, especially the ARC, plays a central role in the integration of different metabolic signals and in appetite regulation. In the hypothalamus, ghrelin neurons contact the cell bodies and dendrites of neuropeptide Y (NPY)/agouti-related protein (AgRP) and proopiomelanocortin neurons, which produce orexigenic peptides and anorexigenic peptide, respectively (9). Intracerebroventricular injection of ghrelin potently promotes food intake in rats and mice (5, 10, 11), suggesting that hypothalamic ghrelin acts as an orexigen.

The molecular composition of hypothalamic ghrelin and its functions in that site, however, remains unclear. In this study, we investigated the molecular forms of hypothalamic ghrelin and the changes in hypothalamic ghrelin mRNA and peptide levels after fasting and 2-DG treatment.

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Abbreviations: ABC, Avidin-biotinylated-peroxidase complex; AgRP, agouti-related protein; ARC, arcuate nucleus; C-RIA, carboxyl-terminal RIA; 2-DG, 2-deoxy-D-glucose; GHS-R, GH secretagogue receptor; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; N-RIA, amino-terminal RIA; RP-HPLC, reverse phase HPLC; SP, sulphopropyl; TFA, trifluoroacetic acid.

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Materials and Methods

Animals

Sprague Dawley male rats (CREA, Tokyo, Japan) were individually housed in a room maintained at a constant ambient temperature of 25 C with a 12-h light, 12-h dark cycle of 12 h (lights on at 0700 h, lights off at 1900 h). Animals were fed standard rodent chow pellets with water *ad libitum*. Body weight was approximately 310 g at the beginning of the experiments. All animal procedures were performed in accordance with the National Institutes of Health guidelines for the human care of laboratory animals and approved by the Institutional Animal Care Committee.

Fasting experiment

Rats were divided into three groups (12 rats per group) and subjected to experimental fasting for 0, 24, and 48 h. Samples from half of the animals in each group were used for ELISAs, whereas the other half were used for mRNA analysis.

2-DG injection

The rats were divided into two groups (16 rats per group). Rats were injected with either 2-DG (600 mg/kg, ip; Sigma-Aldrich, Tokyo, Japan) or saline (0.9% NaCl, ip). We collected the hypothalami from rats 2 h after injection. Half of the animals in each group were used for ELISAs, whereas the other half were used for mRNA analysis.

Quantification and molecular forms of immunoreactive ghrelin in rat hypothalamus

Fresh rat hypothalami (18 g) were diced and boiled for 5 min in 10 vol of water to inactivate intrinsic proteases. The solution was then adjusted to a final concentration of 1 M acetic acid (AcOH) and 20 mM HCl. The boiled hypothalami were homogenized with a Polytron mixer. The supernatants of the homogenized samples, obtained after centrifugation at 15,000 rpm (18,000 × *g*) for 40 min, was concentrated to

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approximately 20 ml in an evaporator. The residual concentrate was subjected to acetone precipitation in a concentration of 66% acetone. After removal of the precipitates, the acetone supernatant was evaporated. The sample was loaded onto a 10-g Sep-Pak C18 cartridge (Waters, Milford, MA) and washed with 10% CH_3CN/0.1% trifluoroacetic acid (TFA). After elution with 60% CH_3CN/0.1% TFA, samples were evaporated and lyophilized. The residual materials were redissolved in 1 M AcOH and adsorbed on an SP (sulphopropyl)-Sephadex C-25 (H^+ form) column that had been preequilibrated in 1 M AcOH. Successive elution with 1 M AcOH, 2 M pyridine, and 2 M pyridine-AcOH (pH 5.0) provided three fractions, designated SP-I, SP-II, and SP-III. The lyophilized SP-III fraction was separated by reverse-phase HPLC (RP-HPLC) using a μBondasphere C18 (3.9 × 150 mm; Waters) column. A linear gradient of CH_3CN from 10–60% in 0.1% TFA for 40 min served as the solvent system using a flow rate of 1 ml/min. Each fraction (0.5 ml) was lyophilized and subjected to RIAs specific for ghrelin.

RIAs for rat ghrelin

To characterize the molecular forms of immunoreactive ghrelin, we employed two polyclonal antibodies [no. 6-6 for amino-terminal RIA (N-RIA), and no. 1-7 for carboxyl-terminal RIA (C-RIA)] raised against the C-RIA (Gln13-Arg28) and amino- (Gly1-Lys11 with *O-n*-octanoylation at Ser3) terminal fragments of rat ghrelin (2). Each RIA incubation mixture contained 100 μl of either a ghrelin standard or an unknown sample and 200 μl of antiserum diluted in RIA buffer [50 mM PBS (pH 7.4), 0.5% BSA, 0.5% Triton X-100, 80 mM NaCl, 25 mM EDTA-2Na, and 0.05% NaN_3] containing 0.5% normal rabbit serum. Two antisera were added at final dilutions of 1:12,000 (C-RIA) and 1:2,500,000 (N-RIA) and incubated for 12 h. Samples were then incubated with 100 μl of ^{125}I-labeled ghrelin (20,000 cpm/tube) for 36 h. Next, 100 μl of antirabbit IgG goat serum was incubated with the samples for 24 h. Free and bound tracers were separated by centrifugation at 3,000 rpm for 30 min. Radioactivity in the pellet was quantitated with a γ counter (ARC-1000M, Aloka, Tokyo, Japan).

Immunohistochemistry

Porcine hypothalamus was immersed in 4% PFA solution, then immersed in a series of 10%, 20%, and 30% sucrose solutions with 10% alabia gum. Tissues were then embedded in OCT compound (Tissue-Tek Miles, Elkhart, IN). Sections were cut at a thickness of 20 μm using a cryostat (CM 3050S; Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland) and mounted on Matsunami adhesive slide-coated slides (Matsunami, Osaka, Japan). Ghrelin immunohistochemical staining was performed by the avidin-biotinylated-peroxidase complex (ABC) system using a VECTASTAIN ABC-PO kit (Vector Laboratories Inc., Burlingame, CA) as previously described (12). Briefly, sections were dried at 37 C for 30 min, washed in 10 mM PBS (pH 7.4), and pretreated with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity. Sections were treated with 0.01% saponin in PBS for 20 min. After rinsing with PBS, sections were treated with 3% normal goat serum for 1 h, then incubated in polyclonal rabbit antighrelin antibody (no. 6-6; diluted 1:80,000) for 16 h in 4 C. Sections were rinsed with PBS, then incubated with biotinylated antirabbit IgG for 40 min. After washing in PBS, sections were incubated with VECTASTAIN ABC Reagent for 1 h. Samples were visualized in 3,3′-diaminobenzidine using a Dako liquid diethylaminobenzidine substrate-chromogen system (Dako, Kyoto, Japan). The specificity of the antibodies was demonstrated by immunoabsorption and by the total loss of staining when primary antibodies were omitted. For an immunoabsorption test, we examined porcine ghrelin antigen at concentrations of 0, 0.01, 0.1, 1, and 10 (μg/ml).

Preparation of tissue and plasma samples

To prepare hypothalamus and stomach samples, rat tissues were quickly removed after the rats were killed. Each tissue was diced and boiled for 5 min in a 10-fold volume of water to inactivate intrinsic proteases. The solutions were adjusted to a final concentration of 1 M AcOH and 20 mM HCl after cooling. Tissues were then homogenized with a Polytron mixer and after centrifugation at 15,000 rpm for 10 min supernatants were obtained as tissue samples. To prepare plasma samples, whole blood was mixed with EDTA-2Na (2 mg/ml) and aprotinin (500 kIU/ml). Plasma was collected by centrifugation at 4 C. Tissues and plasma samples were loaded onto Sep-Pak C18 cartridges (Waters). The cartridges were washed in 0.9% NaCl and 10% CH_3CN/0.1% TFA. Bound protein was eluted with 60% CH_3CN/0.1% TFA. The eluate was lyophilized and subjected to ghrelin-specific ELISA.

ELISA

To quantify hypothalamus, stomach, and plasma ghrelin levels, we used an Active Ghrelin ELISA Kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) to assess *n*-octanoyl modified ghrelin and a Desacyl-Ghrelin ELISA Kit (Mitsubishi Kagaku Iatron, Inc.) to measure des-acyl ghrelin according to the manufacturer's instructions.

Real-time PCR of rat ghrelin, NPY, AgRP, and melanin-concentrating hormone (MCH)

Total RNA was extracted from frozen rat stomach and hypothalamus samples using TRIzol (Invitrogen, Tokyo, Japan). Poly(A)$^+$ RNA was purified from 75 μg total hypothalamic RNA using Oligotex-dT30 <Super> (Roche, Tokyo, Japan), according to the manufacturer's instructions. To synthesize cDNA, poly(A)$^+$ RNA (0.4 μg/animal) derived from the hypothalamus and total RNA (1 μg/animal) from the stomach were used. Reaction mixtures were incubated at 37 C for 60 min. Reactions were stopped by incubation at 70 C for 15 min.

Real-time PCR was performed using a PE Applied Biosystems PRISM 7000 Sequence Detection System (PE Applied Biosystems, Foster City, CA). We measured the expression levels of the ghrelin, NPY, AgRP, and MCH genes in hypothalamus and the ghrelin gene in the stomach of rats. cDNA amplification was performed using SYBR Green PCR Core Reagents (PE Applied Biosystems) and uracil-N-glycosylase (Invitrogen) to prevent contamination with carried-over PCR products, as suggested by the manufacturer. All samples were amplified in a single MicroAmp Optical 96-well reaction plate (PE Applied Biosystems). Results reflect duplicate runs of at least two independent experiments. Primer pairs for each gene were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The gene names, forward and reverse primer sequences, and amplicon sizes are listed in Table 1. PCR cycling conditions were initiated by a 2-min incubation at 50 C to eliminate any deoxyuridine triphosphate-containing PCR products resulting from carryover contamination. After a 15-min period at 95 C to activate HotStarTaq DNA polymerase, PCR fragments were amplified by 40 cycles of 95 C for 30 sec, 60 C for 30 sec, and 1 min at 72 C. Each standard well contained the pGEM-T Easy vector, containing the standard cDNA fragment. The concentration of the standards covered at least 6 orders of magnitude. We also included no template controls on each plate. Experimental samples with a threshold cycle value within 2 SD of the mean threshold cycle value for the no template controls were considered to be below the limits of detection. The relative levels of mRNA were standardized to a housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, to correct for any bias among the samples caused by RNA isolation, RNA degradation, or the efficiencies of the RT. After amplification, PCR products were analyzed by melting curve to confirm amplification specificity. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis.

TABLE 1. Primer sequences used for real-time PCR analysis

cDNA		Sequence (5′–3′)	Product size (bp)
Ghrelin	S	GAAGCCACCAGCTAAACTGC	155
	AS	GCTGCTGGTACTGAGCTCCT	
NPY	S	CCCCAGAACAAGGCTTGAAG	150
	AS	GAATCCAGCCTGGTGGTGG	
AgRP	S	AAGCTTTGGCAGAGGTGCTA	142
	AS	GTCTTGAAGAAGCGGCAGTAG	
MCH	S	GACCAGCAGTCTTCCCAGCT	151
	AS	CGGTAGACTCGTCCCAGCAT	
GAPDH	S	CGGCAAGTTCAATGGCACA	147
	AS	AAGACGCCAGTAGACTCCACGA	

S, Sense primer; AS, antisense primer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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Statistical analysis

Results are presented as mean ± SEM for each group. Data from the fasting experiment were analyzed with a Kluskal-Wallis nonparametric ANOVA followed by *post hoc* Fisher tests; and data from the experiment of 2-DG injection were analyzed with Student's *t* test. $P < 0.05$ was accepted as statistically significance.

Results

Identification and molecular forms of ghrelin in hypothalamus

Ghrelin has been found in the hypothalamic ARC (1, 8, 9), an important region in the control of appetite. A recent study reported the presence of ghrelin in previously uncharacterized hypothalamic neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei (9). It remains unclear, however, whether hypothalamic ghrelin is modified by an octanoic acid in a similar manner as gastric ghrelin.

To characterize the molecular forms of hypothalamic ghrelin, we analyzed peptide extracts from rat hypothalami by using RP-HPLC and ghrelin-specific RIAs. Two major ghrelin peaks were detected by ghrelin C-RIA (Fig. 1), which can recognize both *n*-octanoyl-modified and des-acyl ghrelins. One of the two major peaks eluted at a retention time of 21 min by HPLC (Fig. 1B, *arrow d*), the same position as that of *n*-octanoyl-modified ghrelin (Fig. 1A, *arrow b*). This major peak was detected by both ghrelin C-RIA and ghrelin N-RIA (Fig. 1C, *arrow e*), which specifically recognizes *n*-octanoyl-modified ghrelin. These results confirm the identity of the peak eluting at 21 min as *n*-octanoyl modified ghrelin, the major active form of ghrelin molecule.

An additional peak (Fig. 1B, *arrow c*), eluted at 12 min in HPLC, was only detected by ghrelin C-RIA. This elution position was identical with that of des-acyl ghrelin (Fig. 1A, *arrow a*). These results confirm identity of the peak as des-acyl ghrelin. Thus, in a manner similar to ghrelin in the stomach, the two major forms of ghrelin, *n*-octanoyl-modified and des-acyl ghrelins, also exist in the hypothalamus.

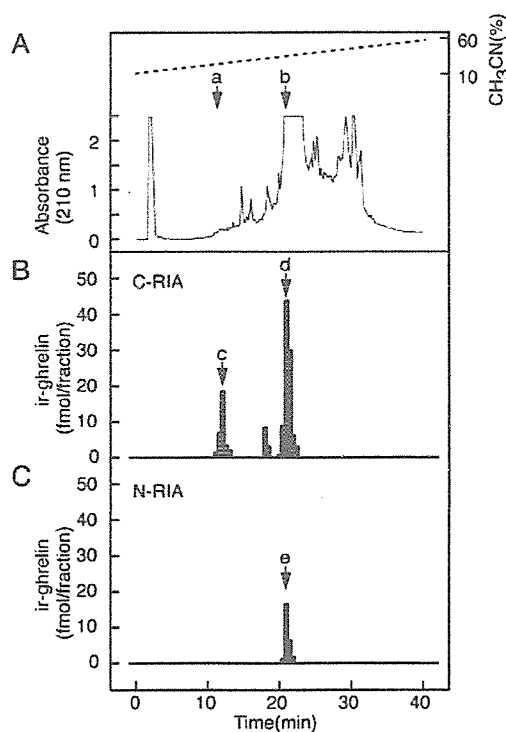

FIG. 1. Representative RP-HPLC profiles of ghrelin immunoreactivity in the rat hypothalamus. A linear gradient of 10–60% CH_3CN containing 0.1% TFA was run for 40 min at 1.0 ml/min. A, Chromatograph of rat hypothalamic extract. RP-HPLC of rat hypothalamus was monitored by C-RIA (B) and N-RIA (C) for ghrelin using fraction volumes of 0.5 ml. The arrows indicate the elution points of des-acyl rat ghrelin-(1–28) (*arrow a*) and *n*-octanoylated rat ghrelin-(1–28) (*arrow b*). The two major peaks observed were consistent with the elution points of des-acyl rat ghrelin-(1–28) (*arrow c*) and *n*-octanoylated rat ghrelin-(1–28) (*arrows d* and *e*).

Immunohistochemical analysis of porcine ghrelin neurons

Although hypothalamic ghrelin was previously observed in the rat and mouse hypothalamus (1, 8, 9), there is little information concerning hypothalamic ghrelin in other mammals. We, therefore, confirmed the existence of ghrelin in the porcine hypothalamus by immunohistochemistry using an antibody that recognizes *n*-octanoyl-modified ghrelin. Our data indicated that ghrelin neurons were present in the porcine hypothalamus (Fig. 2), similar to the ghrelin neurons in the rat hypothalamus (1, 8, 9). As in rats and mice, porcine ghrelin-positive neurons were distributed in the ARC and periventricular areas; the fibers and terminals of these neurons projected onto other ghrelin-containing neurons in these areas. In the pig, multiple ghrelin-positive neurons were localized to the paraventricular nucleus (Fig. 2A). The cell shapes of porcine ghrelin-positive neurons were variable (Fig. 2B). The processes of a subset of these neurons projected onto both ghrelin-containing neurons (Fig. 2C) and ghrelin-negative neurons (Fig. 2D), suggesting contact regulation among ghrelin-positive neurons in the hypothalamus. Thus, we confirmed the presence of ghrelin-containing neurons in the porcine hypothalamus, indicating the general existence of *n*-octanoyl-modified ghrelin in the hypothalami of mammals.

mRNA expression of ghrelin in the hypothalamus and stomach under fasting condition

The most important influence on the regulation of ghrelin secretion is feeding (13, 14). Plasma ghrelin concentrations increase during fasting and decrease after food intake (13). To examine the role of hypothalamic ghrelin in feeding regulation, we investigated the changes in ghrelin gene expression and ghrelin content in the hypothalamus after fasting.

After 24 and 48 h of fasting, body weight decreased and blood glucose levels were lower than those in fed animals (Fig. 3A), indicating that the fasting experiment was performed properly.

Ghrelin mRNA expression in the hypothalamus was significantly decreased by 24% and 28% compared with those of control (*ad libitum* fed) when fasted for 24 and 48 h, respectively (Fig. 3B). As predicted by previous reports, ghrelin mRNA expression in the stomach increased by 75% for 48 h fasting (Fig. 3B). Thus, fasting for 24 and 48 h decreased ghrelin mRNA levels in the hypothalamus, but

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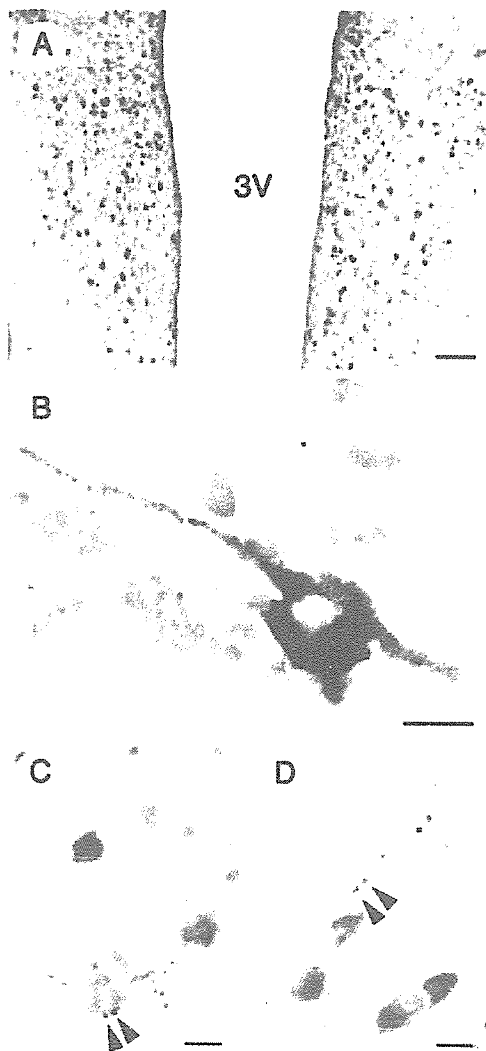

FIG. 2. Localization of ghrelin-immunopositive neurons in the porcine hypothalamus. A, Ghrelin neurons distribution in the paraventricular nucleus. B, A ghrelin-producing neuron in paraventricular nucleus. A subset of ghrelin-positive neurons projected to cell bodies of either additional ghrelin-positive neurons (C, *arrowheads*) or ghrelin-negative neurons (D, *arrowheads*). 3V, Third ventricle. *Bar*, 200 μm (A), 20 μm (B–D).

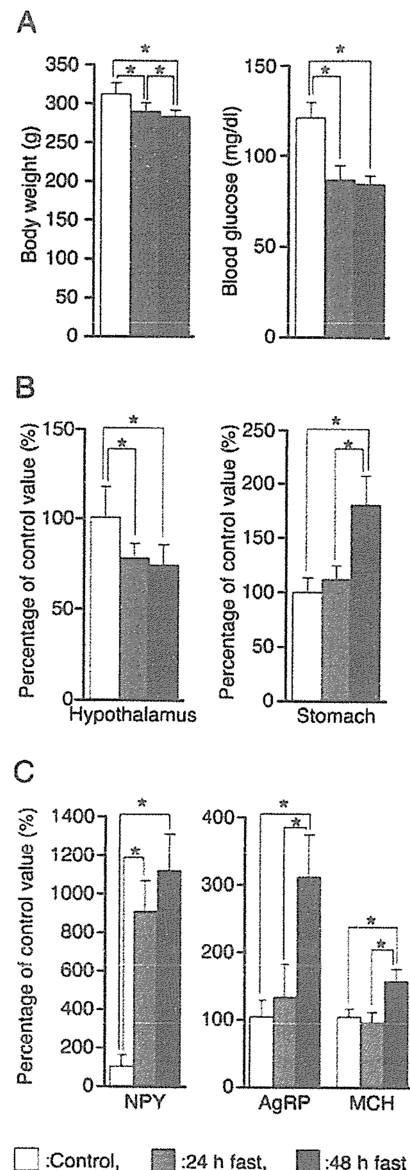

FIG. 3. A, Body weights (*left panel*) and blood glucose concentrations (*right panel*) of rats after fasting. B, Ghrelin mRNA levels in the hypothalamus and stomach. C, NPY, AgRP, and MCH mRNA levels in the hypothalamus of rats fed *ad libitum* (control) or animals that fasted for 24 or 48 h. Note that the values of the longitudinal axes are different in each graph. *Asterisks* indicate the differences between each *bar* ($P < 0.05$).

increased the levels present in the stomach. As the expression levels of hypothalamic appetite-regulating peptides, including NPY, AgRP, and MCH, increased upon fasting as expected (Fig. 3C) (15), the hypothalamic samples were processed and analyzed correctly. These results indicate that the regulatory mechanism(s) governing ghrelin secretion in the hypothalamus differ from that in the stomach.

Concentration of ghrelin in the hypothalamus and stomach under fasting condition

We next investigated the concentrations of ghrelin under fasting conditions. As seen at the mRNA level, ghrelin content in the hypothalamus also decreased after 48 h fasting (*n*-octanoyl-modified ghrelin: −64% and des-acyl ghrelin: −78%) (Fig. 4A). Whereas ghrelin concentrations in the stomach also decreased after 48 h of fasting, plasma ghrelin concentrations increased as previously reported (Fig. 4, B and C) (13). These results indicate that fasting stimulates the release of ghrelin from the stomach into the blood. In a similar fashion, hypothalamic ghrelin may be released from ghrelin-producing neurons in a fasting-dependent manner. The ratio of *n*-octanoyl-modified ghrelin to des-acyl ghrelin did not changed in either the stomach, plasma, or hypothalamus.

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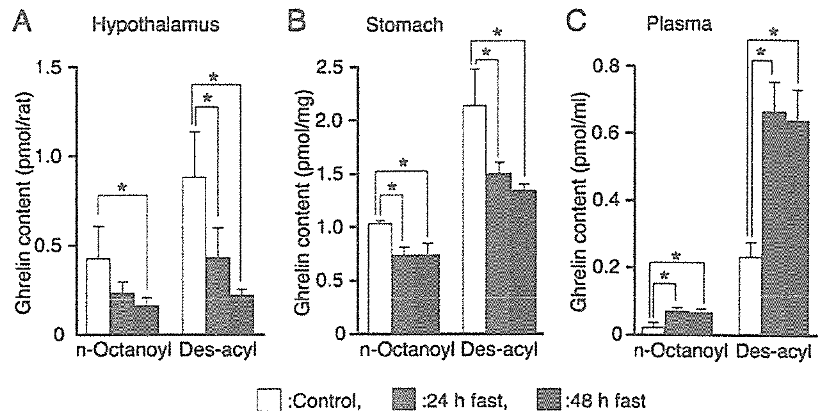

FIG. 4. Concentrations of n-octanoyl-modified and des-acyl ghrelin in the hypothalamus (A), stomach (B), and plasma (C) after fasting for 24 or 48 h. Note that the values of the longitudinal axes are different in each graph. *Asterisks* indicate the differences between each *bar* ($P < 0.05$).

mRNA expression in the hypothalamus and stomach after 2-DG treatment

Blood glucose levels are important factors for release of ghrelin; both oral and iv administration of glucose decreases plasma ghrelin concentrations (16). Because injection of 2-DG stimulates food intake by antagonizing glucose utilization (17), we investigated the effect of 2-DG on the ghrelin mRNA expression and concentration in the hypothalamus.

After administration of 2-DG, mean food intake for 2 h by rats was dramatically increased in comparison with food intake after saline administration (saline group: 0.4 ± 0.2 g, 2-DG group: 5.1 ± 1.1 g) (Fig. 5A), indicating the effectiveness of 2-DG administration. In rats, 2-DG treatment produced a significant decrease in ghrelin mRNA expression within the hypothalamus only (−50.2%); 2-DG did not alter ghrelin mRNA levels in the stomach (Fig. 5B). In contrast, additional orexigenic peptides produced by the rat hypothalamus, including NPY, AgRP, and MCH, increased after 2-DG treatment as reported (Fig. 5C) (17).

Concentration of ghrelin in the hypothalamus and stomach after 2-DG treatment

Ghrelin peptide levels in the hypothalamus were also decreased by 2-DG treatment (Fig. 6A). Both n-octanoyl-modified and des-acyl ghrelin decreased by 57% and 44% in comparison with control values (saline-treated group) values, respectively. There was no change in ghrelin peptide levels, however, in either the stomach or the plasma (Fig. 6, B and C). The ratio of n-octanoyl-modified ghrelin to des-acyl ghrelin in the hypothalamus was not changed by 2-DG treatment. Thus, antagonism of glucose utilization by 2-DG decreases ghrelin mRNA expression and protein levels in the hypothalamus only.

Discussion

The hypothalamus is one of the target tissues of ghrelin, a potent appetite-stimulating hormone. Previous studies have reported that ghrelin and its receptor are expressed within the hypothalamic ARC (1, 8, 9, 18) to function in appetite regulation (4–7). The molecular forms of hypothalamic ghrelin have remained unclear; it was previously unknown whether hypothalamic ghrelin is also modified with an octanoic acid. Moreover, the regulation of hypothalamic ghrelin expression and concentrations remains to be elucidated.

In this study, we examined the molecular composition of hypothalamic ghrelin by HPLC and ghrelin RIAs. We identified two molecular forms of hypothalamic ghrelin, n-octanoyl-modified ghrelin and des-acyl ghrelin, as seen for ghrelin within the stomach (2). In the stomach, additional minor molecular forms of ghrelin, such as hexanoyl-, decenoyl-, and decanoyl-modified ghrelins, also exist at limited concentrations (19). Due to the low content of ghrelin in

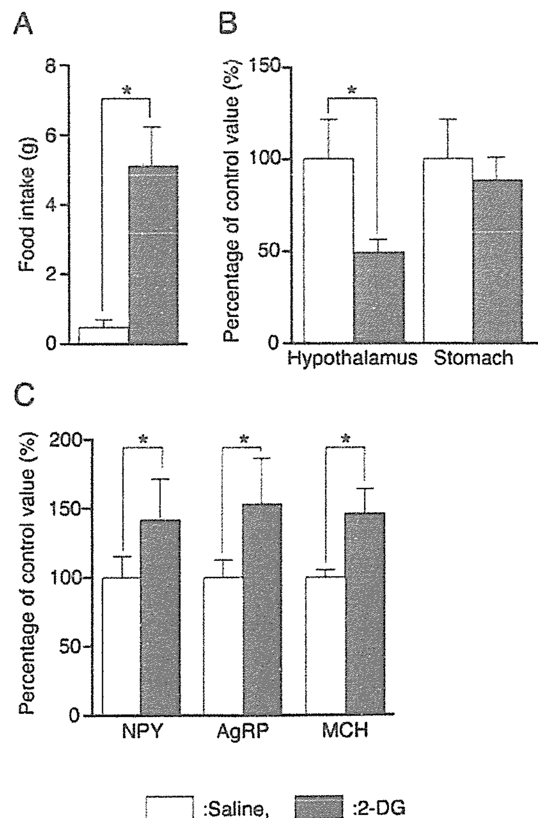

FIG. 5. A, Food intake in the 2 h after 2-DG injection. B, Ghrelin mRNA levels in the hypothalamus and stomach. C, NPY, AgRP, and MCH mRNA levels in the hypothalami of rats treated with 2-DG for 2 h. *Asterisks* indicate the differences between each *bar* ($P < 0.05$).

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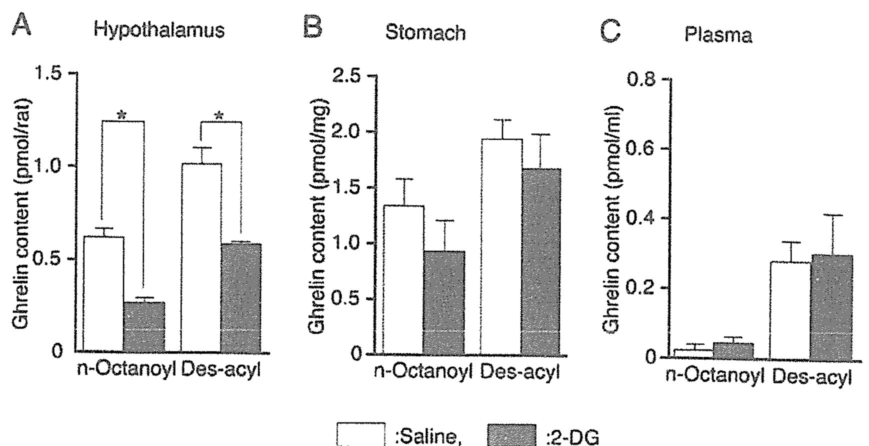

FIG. 6. *n*-Octanoyl and des-acyl ghrelin peptide content in the hypothalamus (A), stomach (B), and plasma (C) of rats treated with 2-DG for 2 h. Note that the values of the longitudinal axes differs in every graph. *Asterisks* indicate the differences between each *bar* ($P < 0.05$).

the hypothalamus, we could not detect these minor forms. Thus, the main active form of hypothalamic ghrelin is *n*-octanoyl-modified ghrelin, which is also the primary active form of ghrelin present in the stomach.

We next examined the changes in ghrelin mRNA expression levels and peptide concentrations in the hypothalamus after either fasting or treatment with 2-DG, an antagonist of glucose utilization. The results demonstrate that both ghrelin mRNA expression and peptide content in the rat hypothalamus decreased after fasting. These changes in the hypothalamus do not correlate with those previously seen in the stomach. Ghrelin mRNA expression in the stomach increases after fasting (Fig. 3), whereas peptide concentrations of ghrelin in the stomach are decreased. Because plasma ghrelin concentrations increase after fasting, fasting may induce the excessive secretion of ghrelin from the stomach into the blood, resulting in a decrease of ghrelin peptide content in the stomach. Thus, fasting likely also stimulates ghrelin release from the hypothalamus, resulting in a decrease in hypothalamic ghrelin concentrations. 2-DG treatment reduced hypothalamic ghrelin concentrations in the absence of any changes in the peptide content of either the stomach or plasma. Because 2-DG stimulates feeding by exerting central metabolic influences (20), hypothalamic ghrelin secretion should also be centrally regulated. Therefore, we think that glucoprivic states in hypothalamus, such as fasting or 2-DG treatment, promote hypothalamic ghrelin secretion.

In contrast to the increases in gastric ghrelin mRNA during fasting conditions, hypothalamic ghrelin mRNA decreased until 48 h of fasting. Although we cannot clearly explain this phenomenon as yet, one possibility is that ghrelin gene expression levels in the hypothalamus are suppressed after ghrelin release to prevent excessive ghrelin secretion. Because in case of long-term starvation, excessive food-exploratory behavior induced by orexigenic peptides may result in exhaustion and death. Thus, hypothalamic ghrelin might control effectual feeding behavior in response to a nutritional state.

Starvation in goldfish induces increases in hypothalamic ghrelin mRNA on d 7, whereas serum ghrelin levels increased at d 3 and 5 and returned on d 7 to the level of d 1 (21). These data for goldfish ghrelin are quite similar to our results. Thus, hypothalamic ghrelin gene expression may be regulated by multiple additional factors. Further studies will be necessary to understand the induction of hypothalamic ghrelin secretion by fasting and 2-DG.

In summary, we revealed that hypothalamic ghrelin exists as two major forms: the *n*-octanoyl-modified and des-acyl ghrelin peptides. The glucoprivic state of the hypothalamus, induced by fasting and 2-DG treatment, stimulates ghrelin secretion from ghrelin-producing neurons. Moreover, the contradictory expression patterns of ghrelin mRNA expression in the hypothalamus and the stomach after fasting imply that the mechanism of ghrelin synthesis differs between these two locations.

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