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Enhanced plasma ghrelin levels in rats with streptozotocin-induced diabetes

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Abstract Ghrelin is a novel gastrointestinal peptide that stimulates growth hormone secretion, food intake, and body weight gain. Increased ghrelin secretion has been reported in such negative energy states as starvation and low body weight. We investigated the dynamics of ghrelin in rats with streptozotocin-induced diabetes, because they present reduced body weight and hyperphagia. The plasma ghrelin levels and gastric preproghrelin mRNA expression levels of the diabetic rats increased significantly and their gastric ghrelin levels decreased significantly. Negative energy balance may enhance preproghrelin mRNA expression and ghrelin secretion into the bloodstream.

Key words: Preproghrelin; Ghrelin; Diabetes; Stomach; Growth hormone

1. Introduction

Ghrelin is a novel growth hormone secretagogue recently isolated from human and rat stomach [1]. It possesses unique *n*-octanoyl modification at the third serine residue that is essential to ghrelin's physiological activities. The physiological role of ghrelin is to stimulate growth hormone release, food intake [2], and gastric motility [3], and in both humans and rodents, negative energy balance states, such as starvation or the low body weight in cachexia or anorexia nervosa, have been reported to increase plasma ghrelin levels [4–8]. Many tissues express preproghrelin mRNA [9], but the gastric fundus has the highest levels of expression [10]. A-like cells, which are mainly present in the gastric fundus, have been reported to secrete ghrelin [11].

Streptozotocin (STZ) is an antibiotic that induces diabetes by damaging insulin-secreting cells in the pancreas, and rats with STZ-induced diabetes exhibit reduced body weight, hyperphagia, hyperglycemia, and hypoinsulinemia [12,13]. No studies, however, have addressed the dynamics of ghrelin in rats with STZ-induced insulin-dependent diabetes. The present study investigated the relation between ghrelin dynamics and secretory regulation in rats with STZ-induced diabetes by means of radioimmunoassay (RIA), real-time reverse transcription polymerase chain reaction (RT-PCR), and immunohistochemical techniques.

2. Materials and methods

2.1. Animal procedures

All animal experiments and procedures were approved by the Keio University Animal Research Committee (No. 001022). Diabetes was induced in 8-week-old male Wistar rats by intraperitoneal injection of STZ (60 mg/kg) after an overnight fast ($n = 11$); control rats were injected with physiological saline ($n = 9$). All animals were allowed free access to tap water and a standard pellet rat diet (CE-2, Clea, Tokyo, Japan). Four weeks later, the rats were killed under ether anesthesia after a 16 h fast. The stomachs of the rats were excised, cut along the greater curvature, and rinsed with isotonic saline. The duodenums and ascending colons were also removed. Separately, we performed the same experiment on three animal groups which consisted of control rats ($n = 6$), rats with STZ-induced diabetes ($n = 6$) and insulin-treated diabetic rats ($n = 5$). They were given free access to food. Insulin-treated diabetic rats were given neutral protamine Hagedorn human insulin (7 U/animal) by subcutaneous injection, beginning 1 week after the STZ injection.

2.2. Blood and hormonal assays

Serum glucose values were measured by the glucose dehydrogenase method. Serum insulin, serum insulin-like growth factor-1 (IGF-1), and serum growth hormone (GH) levels were measured with commercial immunoradiometric assays (rat insulin RIA kit, Linco Research, St. Charles, MO, USA; rat IGF-1 RIA kit, Diagnostic Systems Laboratories, Webster, TX, USA; and rat Growth Hormone Assay system, Amersham Biosciences, Piscataway, NJ, USA).

2.3. Ghrelin RIA

The two RIAs of gastric and plasma ghrelin levels were performed as described previously [14,15]. Gastric and plasma ghrelin were measured by RIAs using two polyclonal rabbit antibodies, one raised against the N-terminal [1–11] (Gly1–Lys11) fragment and the other raised against the C-terminal [13–28] (Gln13–Arg28) fragment of rat ghrelin. The anti-ghrelin antisera do not recognize other enteric peptides. Ghrelin values obtained by N-terminal RIA using an anti-ghrelin [1–11] antiserum specifically represent the active, *n*-octanoylated ghrelin. The values obtained by C-terminal RIA using an anti-ghrelin [13–28] antiserum represent the total ghrelin concentration, including the inactive des-acyl ghrelin. The respective intra- and inter-assay coefficients of variation were 3% and 6% for the N-terminal RIA and 6% and 9% for the C-terminal RIA. For peptide extraction, rat stomachs were boiled for 5 min in a 10-fold volume of water to inactivate intrinsic proteases, adjusted with 1 M acetic acid after cooling, and homogenized with a Polytron mixer. The supernatants were lyophilized and resuspended in assay buffer for the ghrelin RIAs. The tissue ghrelin extraction efficiency of this procedure is more than 95%. Whole blood samples were collected with EDTA (1 mg/ml blood) and

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Table 1
Body weight, gastric weight, serum glucose values, serum insulin levels, serum IGF-1 levels and serum GH levels in rats with STZ-induced diabetes

	Body weight (g)	Gastric weight (mg)	Serum glucose (mg/dl)	Serum insulin (ng/ml)	Serum GH (ng/ml)	Serum IGF-1 (ng/ml)
Control rats ($n=9$)	353.6 ± 16.1	2145.9 ± 82.2	136.7 ± 4.2	0.26 ± 0.06	6.64 ± 0.23	1478.9 ± 137.6
Diabetic rats ($n=11$)	208.5 ± 12.6***	2160.8 ± 86.0	451.0 ± 41.6***	0.06 ± 0.03**	47.21 ± 19.14*	449.8 ± 32.6***

Values are given as mean ± S.E.M.
***$P<0.001$, **$P<0.01$, *$P<0.05$ as compared to control value.

aprotinin (70 μg/ml blood), and the plasma passed through a C18 Sep-Pak cartridge (Waters, Milford, MA, USA). The cartridges were washed and eluted with trifluoroacetic acid, acetonitrile and distilled water; and the eluate (a 100 μl equivalent of plasma) was lyophilized and subjected to ghrelin RIAs. The extraction efficiency for plasma ghrelin was approximately 90%.

2.4. Real-time RT-PCR

Total RNA was extracted from the stomach, duodenum, and ascending colon of the rats with the RNeasy Mini Kit (Qiagen, Hilden, Germany). A TaqMan® quantitative real-time RT-PCR was used to detect preproghrelin mRNA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA (Platinum Quantitative RT-PCR ThermoScript One-Step System, Invitrogen, Carlsbad, CA, USA).

The following primers were used to amplify the preproghrelin mRNA: forward primer (ghrelin-F), 5′-GGA ATC CAA GAA GCC ACC AGC-3′; reverse primer (ghrelin-R), 5′-GCT CCT GAC AGC TTG ATG CCA-3′. The following TaqMan probe was used for preproghrelin: 5′-FAM-AAC TGC AGC CAC GAG CTC TGG AAG GC-TAMRA-3′.

As an internal control, the following primers were used to amplify GAPDH mRNA: forward primer (GAPDH-F), 5′-TTC AAC GGC ACA GTC AAG GC-3′; reverse primer (GAPDH-R), 5′-GCC TTC TCC ATG GTG GTG AAG-3′. The following TaqMan probe was used for GAPDH: 5′-FAM-CCC ATC ACC ATC TTC CAG GAG CGA GA-TAMRA-3′.

The PCR cycling parameters were: 30 min at 50°C and 5 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The amplifications were performed with a real-time PCR system (ABI Prism 7700 Sequence Detection System, PE Applied Biosystems, Norwalk, CT, USA). Preproghrelin mRNA expression levels were normalized to the GAPDH mRNA expression levels.

2.5. Immunohistochemistry of ghrelin-immunoreactive cells

Tissue samples were fixed in 10% neutralized formalin and embedded in paraffin. The samples were then sectioned (4–6 μm thick), and the sections were allowed to adhere to slides pretreated with a 0.01% aqueous solution of poly-L-lysine. The sections were deparaffinized by heating for 10 min at 70°C and then hydrated by transferring the slides through the following solutions: xylene (5 min, ×2), 96% ethanol (3 min, ×2), 90% ethanol (3 min, ×1), and double-distilled water (3 min, ×1).

After pretreatment with 0.3% hydrogen peroxide and incubation with normal goat serum, all slices were incubated overnight at 4°C with anti-ghrelin [13–28] antiserum (final dilution, 1:10 000) [11]. After washing with phosphate-buffered saline, the slides were incubated overnight at 4°C in the presence of goat biotinylated anti-rabbit IgG (Vectastain, Vector Laboratories, Burlingame, CA, USA) [11]. The slides were then stained for 10 min at room temperature by the avidin–biotin–peroxidase complex method (Vectastain Elite ABC kit, Vector Laboratories) with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (Sigma) and 0.006% hydrogen peroxide in 50 mM Tris–HCl buffer solution (pH 7.2) [11]. After dehydration in a graded alcohol series, the slides were counterstained with hematoxylin.

The stained sections were examined with a light microscope (Eclipse 600, Nikon, Tokyo, Japan) equipped with a 3CCD digital camera

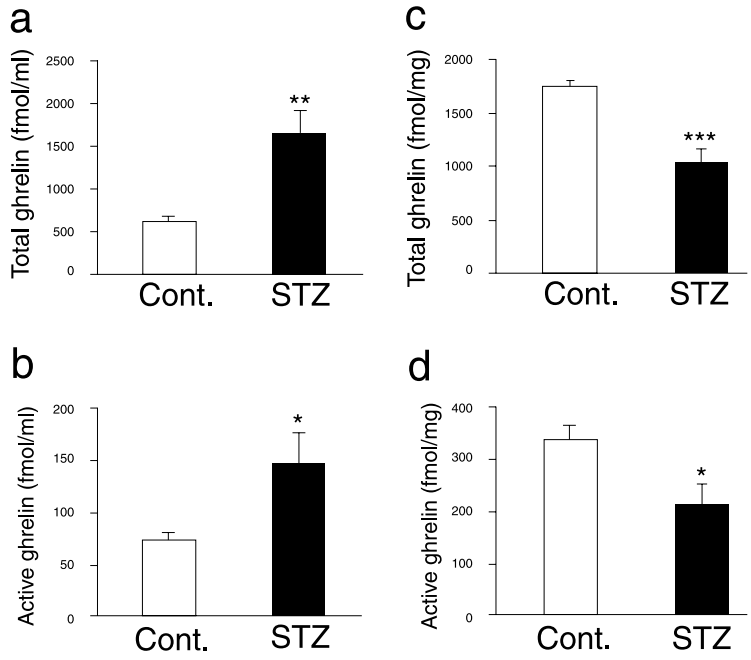

Fig. 1. Plasma ghrelin levels as measured by RIA (a: total ghrelin, b: active ghrelin) in fasted control rats (Cont., open column: $n=9$) and fasted rats with STZ-induced diabetes (STZ, closed column: $n=11$). Total ghrelin was detected by C-terminal RIA with an anti-ghrelin [13–28] antiserum. Active ghrelin was detected by N-terminal RIA with an anti-ghrelin [1–11] antiserum. The plasma total and active ghrelin levels increased significantly in the fasted diabetic rats. Gastric ghrelin levels measured by RIA (c: total ghrelin, d: active ghrelin) in fasted control rats (open column: $n=9$) and fasted diabetic rats (closed column: $n=11$). The gastric total and active ghrelin levels of the fasted diabetic rats decreased significantly. ***$P<0.001$, **$P<0.01$, *$P<0.05$ compared with the fasted control rats.

(C7780, Hamamatsu Photonics, Hamamatsu, Japan), and digital color images were stored as PSD files with Adobe Photoshop 7.0 software. The hematoxylin- and/or diaminobenzidine tetrahydrochloride-stained nuclei in the regions of interest were counted with a particle analysis program (Ultimage Pro. 2.6.4; Alliance Vision, France). The density of the ghrelin-immunoreactive cells ($D_{ghrelin}$) was calculated by using the following formula:

$$D_{ghrelin} = (N_g/N_t) \times 100\ (\%)$$

where N_g represents the number of ghrelin-immunoreactive cells in the region of interest and N_t represents the total number of cells in the region of interest.

2.6. Statistical analysis

All data are expressed as means ± S.E.M. The data were analyzed by one-way analysis of variance followed by Scheffé's multiple comparison tests, and a P value of <0.05 was considered to be statistically significant.

3. Results

The body weight of the diabetic rats decreased significantly ($P<0.001$) and their serum glucose values increased significantly ($P<0.001$), compared to the values in the control rats (Table 1). Their serum insulin levels decreased significantly ($P<0.01$). Although the serum GH levels of the diabetic rats increased significantly ($P<0.05$), their serum IGF-1 levels decreased significantly ($P<0.001$) (Table 1). The wet weight of stomach of the diabetic rats did not differ from that of the controls (Table 1).

The plasma total (Fig. 1a) and active (Fig. 1b) ghrelin levels of the diabetic rats increased significantly (total ghrelin, $P<0.01$; active ghrelin, $P<0.05$), compared with those of the control rats. The plasma total ghrelin levels of the diabetic rats correlated significantly with their serum GH levels ($r=0.81$, $P<0.01$) and with their serum IGF-1 levels ($r=-0.58$, $P<0.01$).

The gastric total (Fig. 1c) and active (Fig. 1d) ghrelin levels of the diabetic rats decreased significantly (total ghrelin, $P<0.001$; active ghrelin, $P<0.05$), compared with the levels in the controls, and their gastric total ghrelin levels correlated significantly with their serum IGF-1 levels. ($r=0.65$, $P<0.01$).

The preproghrelin mRNA expression levels in the stomach increased significantly ($P<0.001$) in the diabetic rats compared with the controls. The preproghrelin mRNA expression levels in the duodenum and colon of the diabetic rats were not significantly different from those in the controls (Fig. 2).

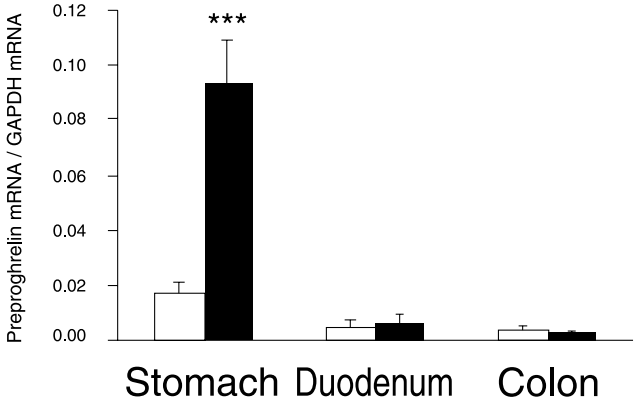

Fig. 2. Preproghrelin mRNA expression levels in the gastrointestinal tract of the fasted control rats (open column: $n=9$) and the fasted rats with STZ-induced diabetes (closed column: $n=11$), normalized to the GAPDH mRNA expression level measured using real-time RT-PCR. ***$P<0.001$ compared with the fasted control rats.

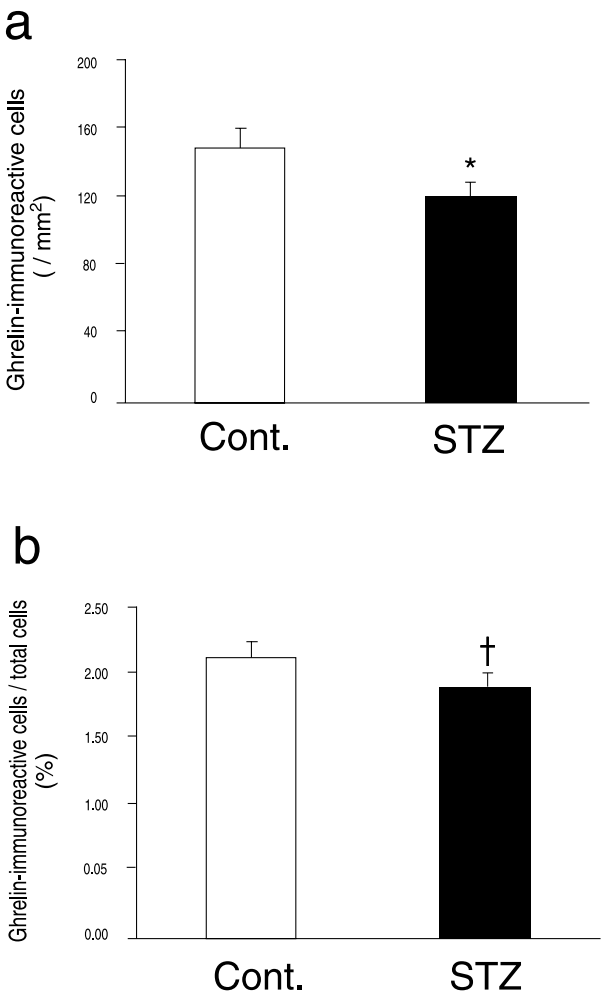

Fig. 3. Analysis of ghrelin-immunoreactive cells in the gastric fundus; ghrelin-immunoreactive cells were detected with an antiserum for the C-terminal of ghrelin. Number of ghrelin-immunoreactive cells (a) and density of ghrelin-immunoreactive cells (b) in fasted control rats (Cont., open column: $n=9$) and fasted rats with STZ-induced diabetes (STZ, closed column: $n=11$). *$P<0.05$, compared with the control rats; †$P=0.054$ compared with the fasted control rats.

The number of ghrelin-immunoreactive cells in the gastric fundus of the diabetic rats decreased significantly compared with the control rats (Fig. 3a), suggesting an increase of empty cells in the stomachs of the diabetic rats. The ratio of ghrelin-immunoreactive cells to the total number of cells in the tissue samples from the gastric fundus of the diabetic rats tended to be smaller, but the difference was not statistically significant compared to the control rats ($P=0.054$; Fig. 3b).

In the experiments with rats allowed to feed ad lib, the plasma total ghrelin levels increased significantly in diabetic rats ($P<0.05$), but insulin treatment tended to attenuate the increase ($P=0.05$; Fig. 4a). Although the gastric total ghrelin levels decreased in the diabetic rats compared with the controls ($P<0.01$), insulin treatment significantly attenuated the decrease in these levels in the diabetic rats ($P<0.001$; Fig.

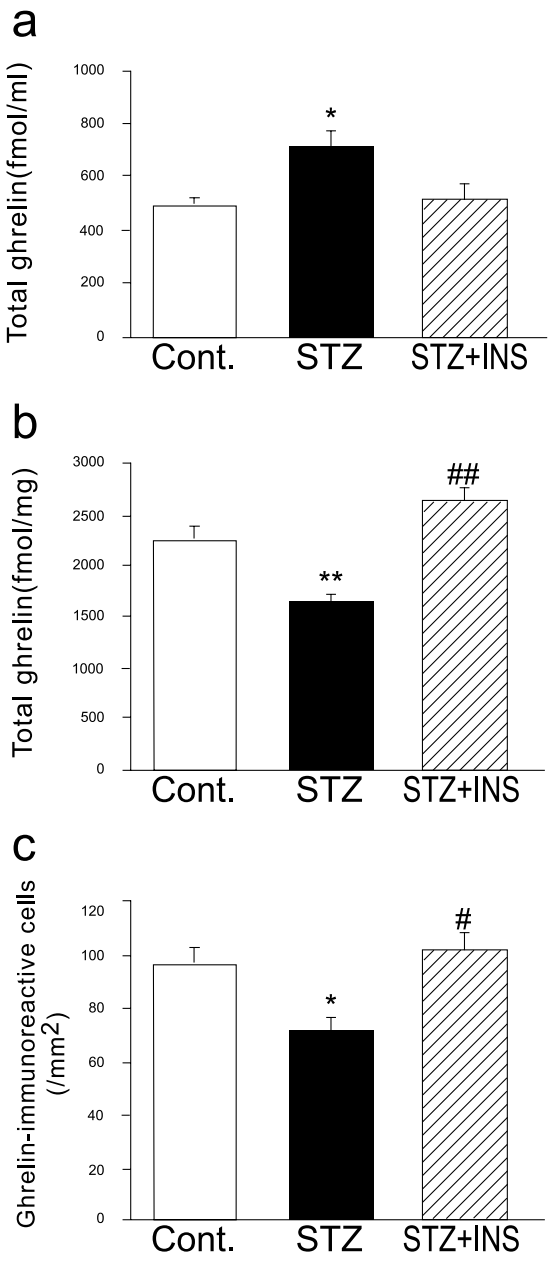

Fig. 4. Plasma total ghrelin levels measured by RIA in fed control rats (Cont., open column: $n=6$), fed STZ-induced diabetic rats (STZ, closed column: $n=6$), and fed insulin-treated diabetic rats (STZ+INS, striped column: $n=5$) (a). Total ghrelin was detected by C-terminal RIA with an anti-ghrelin [13–28] antiserum. Although the plasma total ghrelin levels increased significantly in the diabetic rats, insulin treatment tended to attenuate ($P=0.05$) the increase. Gastric total ghrelin levels measured by RIA in fed control rats (open column: $n=6$), fed diabetic rats (closed column: $n=6$), and fed insulin-treated diabetic rats (striped column: $n=5$) (b). Although the gastric total ghrelin levels decreased in the fed diabetic rats as compared with the controls, insulin treatment significantly attenuated the decrease. Ghrelin-immunoreactive cells in the gastric fundus were detected with an antiserum for the C-terminus of ghrelin. The number of ghrelin-immunoreactive cells (c), in fed control rats (open column: $n=6$), fed diabetic rats (closed column: $n=6$), and fed insulin-treated diabetic rats (striped column: $n=5$). The number of ghrelin-immunoreactive cells in the fed STZ-induced diabetic rats decreased significantly. $^{*}P<0.05$, compared with the fed control rats; $^{\#\#}P<0.001$, compared with the fed diabetic rats; $^{\#}P<0.05$, compared with the fed insulin-treated diabetic rats.

4b). The ratio of gastric active and total ghrelin was unaltered across these three cohorts (data not shown). Although the number of ghrelin-immunoreactive cells in the gastric fundus of the diabetic rats decreased significantly as compared with their controls ($P<0.05$), insulin treatment significantly attenuated ($P<0.01$) the decrease in the ghrelin-immunoreactive cells observed in the gastric fundic specimens of the diabetic rats (Fig. 4c).

4. Discussion

The body weight and serum insulin levels of the STZ-treated rats decreased significantly and their blood glucose values markedly increased. These findings agree with those in a previous report [13] and suggest that the STZ injection had been successful in inducing diabetes mellitus in our cohort of rats.

Exogenous ghrelin administration has been reported to increase the serum GH levels of rats [16], and the increased serum GH levels correlated well with the plasma ghrelin levels in the rats with STZ-induced diabetes (Table 1), the same as reported in the cachexia associated with chronic heart failure [6].

The results of the present study in rats with STZ-induced diabetes clearly demonstrate that the plasma ghrelin levels and the gastric preproghrelin mRNA expression levels in the stomachs of the diabetic rats increased significantly and their gastric ghrelin levels and numbers of ghrelin-immunoreactive cells in the gastric fundus decreased significantly.

No significant differences in preproghrelin mRNA expression levels were observed in the colon and duodenum in the present study, although the second highest levels of preproghrelin mRNA expression, ghrelin peptide concentration and number of ghrelin-immunoreactive cells were found in the duodenum [11,17,18]. This suggests that the ghrelin-producing cells in the stomach, not in the duodenum and colon, play a major role in the increase in plasma ghrelin levels observed in STZ-induced diabetic rats.

The reversibility of the decreased number of ghrelin-immunoreactive cells in the gastric fundus in response to insulin treatment indicates that the decrease in the labeled cells reflects a decrease in ghrelin content in A-like cells in the stomach, not a decrease of ghrelin-producing A-like cells. The increased plasma ghrelin levels, the decreased gastric ghrelin levels and the numbers of ghrelin-immunoreactive cells in the diabetic rats may be due to an increase in ghrelin release by the stomach into the bloodstream. The content of A-like cells (ghrelin) might become empty.

Since the body weight of the diabetic rats decreased significantly, and the changes in ghrelin dynamics (plasma ghrelin levels, gastric ghrelin levels and ghrelin-immunoreactive cell number in the fundic mucosa) in the diabetic rats are reversible in response to insulin treatment, these changes could be attributable to negative energy balance states such as low body weight, not to direct irreversible toxicity of STZ on the gastric mucosa. Although the mechanism of the signal pathway that stimulates ghrelin synthesis or secretion remains unknown, decreased serum IGF-1 levels may play a role in it since serum IGF-1 levels correlated significantly with plasma and gastric total ghrelin.

Negative energy balance might induce a compensatory signal to up-regulate the levels of preproghrelin mRNA expres-

sion in the stomach and to increase ghrelin generation and secretion.

In conclusion, ghrelin secretion by the stomach into the bloodstream increased significantly in the rats with STZ-induced diabetes suggesting that the negative energy balance in the STZ-treated rats may have induced the signal to up-regulate the preproghrelin mRNA expression levels and stimulated ghrelin synthesis as well as secretion by the ghrelin-producing cells in the stomach.

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