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The Effects of Long-Term Diabetes on Ghrelin Expression in Rat Stomachs*

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article; G – other

Abstract

Background. Ghrelin is a hormone which has effects on the secretion of growth hormone, the gastrointestinal system, the cardiovascular system, cell proliferation and the reproductive system.

Objectives. This study focused on relative ghrelin and GHS-R1a gene expression in the stomach of long-term diabetic rats.

Material and Methods. A total of 36 male Wistar albino rats were divided into four groups: a control group, one-month diabetic rats, two-month diabetic rats and three-month diabetic rats. Diabetes was induced by streptozotocin STZ (40 mg/kg *i.p.*). The rats were decapitated under ketamine anesthesia and their stomach tissues were removed. Tissue ghrelin expression, ghrelin and GHS-R mRNA levels were then compared using immunohistochemistry and qRT-PCR.

Results. After one month of diabetes, the number of ghrelin-immunopositive cells decreased significantly compared to those of the control rats. However, the ghrelin-immunopositive cells increased numerically in the two- and three-month diabetic rats compared to those of the control rats. It was also observed that there were high levels of ghrelin mRNA in the one- and two-month diabetic rats, and a subsequent decrease of ghrelin mRNA levels in the three-month diabetic rats compared to the control rats. However, ghrelin receptor mRNA expression levels decreased in the one-month diabetic rats, and ghrelin levels subsequently increased in the two- and three-month diabetic rats compared to the control rats.

Conclusions. The two- and three-month diabetic rats became cachectic due to the large amount of weight lost. The authors therefore concluded that ghrelin-immunopositive cells increased in these rats due to their cachectic state (Adv Clin Exp Med 2015, 24, 3, 401–407).

Key words: diabetes mellitus, ghrelin, stomach, immunohistochemistry, RT-PCR.

Ghrelin is known to be an endogenous natural ligand and it binds the growth hormone secretagogue receptor (GHS-R). It was recently isolated, first from rat stomachs and afterwards from the human stomach [1]. Studies have shown that ghrelin stimulates growth hormone (GH) secretion from the pituitary. However, ghrelin has been also reported to function in the regulation of energy metabolism, gastric acid secretion and food intake [2–7]. Ghrelin has 28 amino acids, and the

Ser3 residue is known to be n-octanoylated [8, 9]. Acylation is important for ghrelin's binding to the GHS1a receptor, crossing the blood–brain barrier, and for its GH-releasing and other endocrine activities [10].

Ghrelin is mainly found in the stomach [1]. It has already been shown that X/A-like cells in the stomach are responsible for ghrelin production [11]. Ghrelin and GHS-R mRNA have also been found in the gastrointestinal tract, the

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hypothalamic arcuate nucleus, the pituitary gland, the kidney, liver, heart, lung and adipose tissue, as well as in the stomach [12, 13].

Streptozotocin (STZ) is used to induce diabetes in experimental rat models. It acts by disturbing the beta cells in the pancreas. Rats with STZ-induced diabetes have characteristics such as decreased body weight and hyperglycemia. Several studies have pointed out that diabetes is a target of ghrelin function [14]. However, there are conflicting data on ghrelin and gastric preproghrelin mRNA expression levels. Furthermore, so far there have been no studies showing ghrelin expression in STZ-induced insulin-dependent long-term diabetic rat stomachs. This study's aim was to determine if there is any relation between ghrelin and ghrelin receptor regulation in STZ-induced long-term diabetic rats by using RT-PCR and immunochemical methods.

Material and Methods

The Animals

Sexually mature male Wistar rats (obtained from the Hakan Çetinsaya Experimental and Clinical Research Center at Erciyes University, Kayseri, Turkey) were used in this study. They were housed in plastic cages in a well-ventilated rat house, allowed *ad libitum* access to food and water, and were kept on a 12-h light–dark cycle. All the animals received humane care in accordance with standard guidelines. Ethical approval for the study was obtained from the Erciyes University Animal Research Ethics Committee, and ethical regulations were followed in accordance with national and institutional guidelines. The rats were randomly assigned to 4 groups of 9 rats per group. Group 1 served as the control group; Group 2 comprised of 1-month diabetic rats; Group 3 was the 2-month diabetic group; and Group 4 consisted of 3-month diabetic rats.

Diabetes was induced in the 27 rats in Groups 2–4 by intraperitoneal injection of STZ (40 mg/kg, Sc-200719, Santa Cruz Biotechnology, CA, USA); a physiological saline injection was administered to the control rats ($n = 9$). The rats in Groups 2–4 were confirmed for hyperglycemia 72 h after the streptozotocin injection by assessing glucose levels. Those with average blood glucose levels higher than 250 mg/dL were considered diabetic. Glycemia was also checked at the time the rats were sacrificed, 4, 8 or 12 weeks after the streptozotocin injection.

At the end of the experimental period, the rats were killed by decapitation under intraperitoneal

ketamine (75 mg/kg) + xylazine (10 mg/kg) anesthesia. After decapitation, the stomach tissues were quickly removed. Some of the stomach tissues were used for RT-PCR analyses and the other tissues were used for histological procedures.

Immunohistochemistry

The expression of rabbit polyclonal antibody against ghrelin (Sc-50297, Santa Cruz Biotechnology, CA, USA) was detected immunohistochemically in the stomach. Immunohistochemistry was performed using the streptavidin–biotin peroxidase technique (Immunocruz LSAB staining system, Sc-2051, Santa Cruz Biotechnology, CA, USA). Paraffin sections (5 μ m) were deparaffinized in xylene. The sections were rehydrated, rinsed in deionized water, and antigen retrieval was carried out by microwave treatment in 0.01 M sodium citrate buffer (pH 6.0) at 95°C for 5 min, after which the slides were cooled rapidly at room temperature for 20 min. After the sections were washed with phosphate-buffered saline (PBS), endogenous peroxidase activity was inhibited by placing them in 1% H_2O_2 in methanol for 10 min. A 5% blocking solution was used for the nonspecific staining. The histological sections were then incubated with the polyclonal antibody for ghrelin at a dilution of 5 μ g/mL in 5% serum and blocked overnight at 4°C. After serial washes with PBS, the sections were incubated using secondary antibodies for 30 min. Then the immunoreaction was amplified with streptavidin–avidin–peroxidase complex, and the sections were visualized by using 3,3P-diaminobenzidine tetrahydrochloride (DAB) and lightly counterstained with hematoxylin. Negative controls where incubation with the primary antisera was omitted were completely unlabelled.

The sections were examined using a light microscope (BX-51, Olympus, Tokyo, Japan) at $\times 400$ magnification (visual field diameter 1 mm). Transverse sections of oxyntic mucosa were assessed by the density of immunoreactive cells (number of cells per mm of mucosal length, horizontal dimension). The number of immunoreactive cells (with visible nuclei) was counted in 4–5 fields on at least 5 sections from each specimen. Ghrelin-immunopositive cells were counted using an eyepiece graticule (1 $\times$ 1 mm).

qRT-PCR

Total RNA was isolated from 20–30 mg stomach tissue specimens using an MO Bio kit (MO Bio, Solana Beach, CA, USA, cat.: 15000-50), following the manufacturer's protocol. Reverse transcription was performed using hexamer and

Table 1. Sequences of primers and ID numbers of UPL probes used for qRT-PCR

Organism	Gene symbol	Gene description	Assay ID	Forward primer sequence	Reverse primer sequence	UPL probe no
<i>R. norvegicus</i>	GapdH	glyceraldehyde-3-phosphate dehydrogenase	502303	AAAGCTGTGGCGTGATGG	TTCAGCTCTGGGATGACCTT	26
<i>R. norvegicus</i>	Ghrl	Appetite-regulating hormone precursor (growth hormone secretagogue)	503935	GGAGGAGCTGGAAATCAGGT	GCTGGTACTGAGCTCCTGACA	148
<i>R. norvegicus</i>	Ghsr	Growth hormone secretagogue receptor type 1 (GHS-R)	503831	CCAGAACCACAAGCAGACAG	CGAAGGACTTGGAAAAGAGGT	71

oligo-dT primers and Transcriptor High Fidelity Reverse Transcriptase (Roche, REF: 05081955001; v. 6.0), following the manufacturer's protocol (1 µg of isolated RNA per reaction). The acquired cDNA was subjected to quantitative RT-PCR reaction using a LightCycler® 480 Probes Master (Roche, REF: 04707494001; v. 09) and detected with Universal ProbeLibrary Probes (Roche, Applied Sciences, Mannheim, Germany) for the ghrelin, ghrelin receptor and housekeeping genes (Table 1). As Dietrich et al. described: "UPL probes are labeled at the 5' end with fluorescein (FAM) and at the 3' end with a dark quencher dye. UPL assays are compatible with all real-time PCR instruments capable of detecting fluorescein (FAM) or SYBR Green I" [15]. The probes for ghrelin and ghrelin receptor were labeled with FAM dye. Amplification of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping/reference gene for the quality and endogenous normalization of the RNA samples investigated. The GAPDH was labeled with FAM dye so that the amplifications were carried out in the same reactions as ghrelin or GHSR amplification. The reaction was conducted on a LightCycler® 480 II (Roche, Germany) in 45 cycles and the reaction results were analyzed using the efficiency-corrected Advanced Relative Quantification algorithm on LightCycler® 480 SW 1.5 software. The conditions of the reaction were as follows: denaturation 95°C, 5 min; annealing of the primers 50°C, 15 s; elongation 72°C, 10 s. All samples were tested in duplicate.

Statistical Analysis

Statistical analyses were done using ANOVA and *post hoc* Tukey tests. All results are given as mean ± SEM. Values were considered statistically

significant if $p < 0.05$. The SPSS/PC program (v. 15.0; SPSS, Chicago, IL, USA) was used for the statistical analysis.

Results

The final blood glucose levels in the STZ-treated rats were significantly higher than in the control rats (Table 2, $p < 0.05$). The initial body weights were similar in all groups. However, at the end of the experiment the body weights of two- and three-month diabetic rats were significantly decreased compared to the control rats (Table 2, $p < 0.05$).

Ghrelin-containing cells were found in the mucosal layer of the stomach, but not in the submucosal or muscular layers. Most of the ghrelin-immunoreactive cells were observed in the glandular base of the fundic glands, and a few ghrelin-immunopositive cells were observed in the glandular neck, as seen in Fig. 1. The labeled cells were small, round or oval shaped (without visible luminal contact), as seen in Fig. 1.

The mean (± standard deviation) number of ghrelin-immunopositive cells following diabetes in

Table 2. Body weight and blood glucose levels of control rats and streptozotocin (STZ)-induced diabetic rats

	Body weight (g)	Blood glucose (mg/dL)
Control	293.87 ± 11.81	110.22 ± 2.90
One month	277.55 ± 16.77	499.55 ± 37.33*
Two months	170.11 ± 17.35*	504.55 ± 36.38*
Three months	202.33 ± 16.22*	464.33 ± 33.97*

Values are expressed as mean ± SE.

* $p < 0.05$ compared to the control group.

Fig. 1. Ghrelin-immunopositive cells can be seen in the glandular mucosa of the stomach (a) control, (b) one-month diabetes, (c) two-month diabetes, (d) three-month diabetes

the different rat groups is summarized in Fig. 2A. The number of ghrelin-immunopositive cells in the control rats was 6.94 ± 0.20 . In the rats that had been diabetic for one month, the ghrelin-immunopositive cells decreased significantly to 5.14 ± 0.25 labeled cells ($p < 0.05$). However, the ghrelin-immunopositive cells increased in two- and three-month diabetic rats compared to the controls.

Ghrelin and ghrelin receptor mRNA expression levels in the stomach were quantified using RT-PCR. Higher levels of ghrelin were observed in the one- and two-month diabetic rats, and there was a subsequent decrease in ghrelin levels in the three-month diabetic rats compared to the control rats. However, ghrelin receptor mRNA expression levels decreased numerically in the one-month diabetic rats, and a subsequent increase in ghrelin levels was observed in the two- and three-month diabetic rats compared to the control rats. Although there was a difference in the expression levels of both genes in the different groups, the differences were not significant (Fig. 2).

Discussion

Ghrelin has an important stimulatory effect on GH secretion, metabolism and energy balance [16, 17]. Ghrelin affects short-term energy homeostasis, and for this reason it increases hunger and, accordingly, food intake. This action occurs due to the activation of hypothalamic neuropeptides NPY/AgRP neurons [18]. In addition, ghrelin is known to be involved in long-term energy balance by increasing weight gain and also adiposity [19, 20]. Therefore, ghrelin has a role in insulin secretion and glucose metabolism, as has already been shown in humans as well as in rodents [16, 21–23]. Several studies have pointed to the role of ghrelin

in the release of insulin from the pancreatic islets. It has been shown that circulating ghrelin destroys glucose tolerance and glucose-stimulated insulin secretion in healthy subjects [24]. These findings show that ghrelin plays an active role in physiologic insulin secretion, and accordingly ghrelin antagonists could improve beta-cell function [24, 25].

It has previously been shown that in totally gastrectomized patients, plasma ghrelin levels were decreased by almost 65%, showing that the stomach is the major source of circulating ghrelin [26]. Ghrelin-immunopositive cells were found in the mucosal layer but not in the myenteric plexus in the stomach, and most of the ghrelin-immunopositive cells were observed in the glandular base of the fundic gland, with a few ghrelin-immunopositive cells noted in the glandular neck [11]. The present study examined ghrelin immunolocalization as well as ghrelin mRNA and ghrelin receptor mRNA levels in the stomachs of diabetic rats. Ghrelin-immunopositive cells were found only in the mucosal layer of the stomach. Most of the ghrelin-immunopositive cells were found in the glandular base, and a few were found in the glandular neck. The ghrelin-immunopositive cells were small and round shaped. These findings agree with distribution patterns reported previously in the rat stomach [11, 27, 28].

There are not many studies showing the effects of diabetes on the number of ghrelin-immunopositive cells in the stomach. Rauma et al. [29] recently demonstrated a decrease in the number of ghrelin-immunopositive cells in the upper segment of the gastrointestinal tract in diabetic mice. Similarly, Masaoka et al. [14] showed a diminished number of ghrelin-positive cells in diabetic rats at 4 weeks as compared to control rats. Another study also showed that ghrelin-immunopositive cells were diminished in one-month diabetic rats [30].

Fig. 2. (A) The number of ghrelin-immunopositive cells, (B) ghrelin receptor mRNA and (C) ghrelin mRNA expression levels in the stomachs of control rats and streptozotocin-induced diabetic rats

However, Dong et al. [31] found an increase in the number of ghrelin-positive cells in the stomachs of diabetic rats. It seems clear that the contradictory results among these studies are related to the duration of the diabetes. In the present study, when the rats were diabetic for one month, ghrelin-immunopositive cells decreased significantly compared to the controls. In the gastric fundus, the diminished number of ghrelin-immunoreactive cells

in the one-month diabetic rats showed a decrease in ghrelin content in A-like cells in the stomach. These observations are in agreement with previous studies. However, ghrelin-immunopositive cells increased in the two- and three-month diabetic rats compared to the controls. This study clearly showed that two- and three-month diabetic rats became cachectic due to large weight losses. As is known, plasma ghrelin levels are elevated in cachexia and are inversely associated with BMI [32].

There have been many studies on the effect of diabetes on serum and tissue levels of ghrelin. However, in diabetic rats, changes in ghrelin levels vary in different tissues. Masaoka et al. [14] observed an increase in preproghrelin mRNA levels in the stomachs of diabetic rats; on the other hand, they did not observe any difference in the duodenum or the colon. Ishii et al. [30] showed a non-significant increase in ghrelin levels in the stomachs of diabetic rats. However, there have been no studies on the effects of long-term diabetes on ghrelin expression in the stomach. In the present study, high levels of ghrelin mRNA were observed in one- and two-month diabetic rats, and a subsequent decrease in ghrelin mRNA levels was observed in three-month diabetic rats compared to the control rats. However, ghrelin receptor mRNA expression levels decreased in one-month diabetic rats, and a subsequent increase in ghrelin receptor mRNA levels was observed in two- and three-month diabetic rats compared to the control rats. The increase in gastric ghrelin mRNA expression observed during the first month was compatible with the results of previous studies [14, 30]. These elevations are a compensatory response reflecting the negative energy balance state. However, ghrelin mRNA expression levels decreased and ghrelin receptor mRNA expression levels increased in three-month diabetic rats compared to the control rats. In previous studies, a negative correlation was shown between gastric ghrelin mRNA expression and ghrelin-immunopositive cells [14, 30]. Therefore, long-term diabetes, which causes cachexia and accordingly increases the ghrelin positive cells, could be a possible reason for the reduction in the quantity of ghrelin mRNA.

In conclusion, as stated by Gelling et al., "STZ administration provides a highly reproducible rodent model of uncontrolled, insulin-deficient diabetes mellitus and is characterized by weight loss and hyperglycemia" [33]. In the present study, two- and three-month diabetic rats became cachectic due to the large amount of weight lost. Therefore, it was concluded that ghrelin-immunopositive cells increased due to the cachectic state of two- and three-month diabetic rats.

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