METABOLISM CLINICAL AND EXPERIMENTAL 61 (2012) 491–495

Available online at www.sciencedirect.com

Metabolism

www.metabolismjournal.com

Increased production of active ghrelin is relevant to hyperphagia in nonobese spontaneously diabetic Torii rats

Hiroharu Mifune[a], *Yoshihiro Nishi*[b], *Yuji Tajiri*[c,*], *Taku Masuyama*[d], *Hiroshi Hosoda*[e], *Kenji Kangawa*[e], *Masayasu Kojima*[f]

[a] *Institute of Animal Experimentation, Kurume University School of Medicine, Kurume 830-0011, Japan*
[b] *Department of Physiology, Kurume University School of Medicine, Kurume 830-0011, Japan*
[c] *Division of Endocrinology and Metabolism, Kurume University School of Medicine, Kurume 830-0011, Japan*
[d] *Toxicology Research Laboratories, Central Pharmaceutical Institute, Japan Tobacco Inc, Hadano 257-0024, Japan*
[e] *Department of Biochemistry, National Cardiovascular Center Research Institute, Suita 565-8565, Japan*
[f] *Molecular Genetics, Life Science Institute, Kurume University, Kurume 839-0864, Japan*

ARTICLE INFO

Article history:
Received 18 April 2011
Accepted 6 September 2011

ABSTRACT

An abnormal eating behavior is often associated with diabetes mellitus in individuals. In the present study, we investigated the mechanisms underlying the relationship among uncontrolled diabetes, food intake, and the production of ghrelin, an orexigenic hormone, in spontaneous diabetic Torii (SDT) rats. Male SDT rats and age-matched control Sprague-Dawley (SD) rats were housed from 8 to 38 weeks of age. Body weight and daily food intake were measured weekly, whereas blood and whole stomach samples were obtained at the age of 8, 25, and 38 weeks in both SDT and SD rats. The SDT rats at both 25 and 38 weeks of age demonstrated significantly lower body weights despite almost doubled food consumption compared with the SD rats of the same age. The SDT rats showed overt hyperglycemia at 25 and 38 weeks of age with concomitant hypoinsulinemia. The plasma active ghrelin levels and the ratio to total ghrelin levels of SDT rats at 38 weeks of age were significantly higher than those of SD rats of the same age. Stomach ghrelin and ghrelin *O*-acyltransferase messenger RNA expression levels were higher in SDT rats than in SD rats after the induction of diabetes, with a concomitant decrease of stomach ghrelin-immunopositive cell numbers in SDT rats at 38 weeks of age. The SDT rats with uncontrolled hyperglycemia show hyperphagia with a concomitant increase of plasma active ghrelin concentration. This report is the first to clarify the relevance of ghrelin to hyperphagia in diabetic state over an extended period.

Authors' contributions: H Mifune and Y Nishi contributed to the maintenance of Sprague-Dawley and spontaneous diabetic Torii rats in the animal department. Y Nishi, H Hosoda, and K Kangawa contributed to radioimmunoassay of ghrelin. T Masuyama supervised the maintenance of spontaneous diabetic Torii rats. Y Nishi and Y Tajiri contributed to the reverse transcriptase polymerase chain reaction study of ghrelin and ghrelin *O*-acyltransferase. Y Tajiri contributed to writing the manuscript and supervised the work together with M Kojima. H Mifune and Y Nishi contributed to this work equally.

* *Corresponding author*. Tel.: +81 942 31 7563; fax: +81 942 35 8943.
E-mail address: tajiriy@med.kurume-u.ac.jp (Y. Tajiri).

0026-0495/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2011.09.001

1. Introduction

Diabetic hyperphagia has the potential to hinder the success of following dietary advice. A modest number of patients with type 2 diabetes mellitus, from 5% to 21%, are estimated to have an abnormal eating behavior [1,2]. However, to date, the mechanisms relating how and the reason why hyperphagia is relevant to patients with diabetes mellitus have not been fully elucidated.

Ghrelin, originally identified as a growth hormone secretagogue, is an orexigenic gut hormone. It is a 28-amino-acid peptide produced by the X/A-like endocrine cells in the oxyntic glands of the gastric fundus [3,4]. The biological activities of ghrelin require octanoylation of the peptide on Ser3, an unusual posttranslational modification that is catalyzed by the enzyme ghrelin *O*-acyltransferase (GOAT) [5,6].

Increased ghrelin signaling has been shown to contribute to the pathogenesis of diabetic hyperphagia [7,8] using streptozotocin-induced diabetic rat models with an observation period of 14 days or less, presumably due to the difficulty of maintaining the animals in the absence of insulin treatment. As central and peripheral administration of insulin in rats is known to suppress food intake and decrease neuropeptide Y messenger RNA (mRNA) [9], a model using treatment with insulin injection is not appropriate for investigating the appetite and eating disorders in the diabetic state.

However, a new inbred strain, the spontaneously diabetic Torii (SDT) rat, survives for extended periods of time characterized by hyperglycemia and hypoinsulinemia in the absence of ketonuria without insulin treatment [10] and thus can serve as a model of nonobese type 2 diabetes mellitus. As no exploratory study has been performed to date for the long-term involvement of ghrelin in eating behavior, we investigated this mechanism using SDT rats in the present study.

2. Methods

2.1. Animals and protocols

Male SDT rats (Japan SLC, Tokyo, Japan) and age-matched male Sprague-Dawley (SD) rats, used as controls, were housed in a controlled room under light conditions (lights on from 7:00 AM to 7:00 PM) with ad libitum access to standard laboratory chow and water from 8 to 35 weeks of age. The body weight and daily food intake of the rats were measured once a week throughout study period. The SDT and SD (n = 8, respectively) rats were euthanized at 8, 25, and 38 weeks of age; and blood and whole stomach samples were obtained. All experiments were conducted in accordance with the Regulation for Animal Experimentation in Kurume University.

2.2. Blood sampling and biochemical analysis

Blood samples were obtained via heart puncture into fluoride tubes for blood glucose, EDTA-2Na tubes for insulin and leptin measurement, and EDTA-2Na tubes with aprotinin for ghrelin analysis. After centrifugation (3500 rpm for 15 minutes at 4°C), plasma glucose was measured using a standard method; and the plasma insulin and leptin concentrations were measured using an enzyme-linked immunosorbent assay with commercially available kits (Morinaga Biochemical Research Laboratory, Yokohama, Japan).

2.3. Radioimmunoassay for ghrelin

Plasma samples for ghrelin were prepared by adding HCl (final concentration of 0.1 N) followed by extraction using a Sep-Pak C18 cartridge (Waters, Milford, MA) [4,11]. The eluate was lyophilized and stored at –80°C until the assay was performed. Stomach samples were extracted following homogenization. The supernatants were centrifuged for 15 minutes at 15 000 rpm, lyophilized, and stored at –80°C until the assay was performed.

Lyophilized samples were dissolved in radioimmunoassay (RIA) buffer on the day of assay. Ghrelin RIAs were performed as previously described [12,13] using 2 rabbit antisera against the N-terminal (Gly1-Lys11 with *O*-n-octanoylation at Ser3) or C-terminal (Gln13-Arg28) fragments of rat ghrelin to determine either the active form or total amount of peptide.

2.4. Immunohistochemistry of ghrelin-immunopositive cells in the stomach

The stomach samples were prepared for immunohistochemical analysis as described previously [14] using a rabbit anti-*O*-ghrelin antiserum (the same antiserum used for the ghrelin RIA as described above). The number of ghrelin-immunopositive cells (ip-ghrelin cells) per unit area of mucosal area (square millimeter) was counted using the Meta Morph software system (Molecular Devices, Tokyo, Japan).

2.5. Reverse transcriptase polymerase chain reaction for mRNAs of ghrelin and GOAT

The expression levels of mRNA for ghrelin and GOAT in stomachs of rats were examined using semiquantitative reverse transcriptase polymerase chain reaction (PCR) as described previously [15]. The PCR was performed using a commercially available PCR kit (Go-Taq Master Mix; Promega, Madison, WI, USA) with each primer set necessary to amplify the transcripts for ghrelin (accession no. NM_021669, 32 cycles) and GOAT (accession no. NM_001107317, 36 cycles). The National Institutes of Health Image (http://rsb.info.nih.gov/nih-image">http://rsb.info.nih.gov/nih-image">http://rsb.info.nih.gov/nih-image) program was used to determine the relative amount of each PCR product after gel electrophoresis, and the amount was normalized using the simultaneously amplified β-actin.

2.6. Statistical analysis

All tests were performed using SAS version 9.2 (SAS Institute, Cary, NC). Data are presented as the mean ± SD. Two-way analysis of variance followed with a post hoc test (Dunnett test) was used for the comparisons of the data obtained from 3

groups of rats with different ages. For the comparisons of the data between SD and SDT rats of the same age, Student unpaired *t* test was used for parametric data; or Mann-Whitney *U* test was used for nonparametric data. A *P* value < .05 was considered to be statistically significant.

3. Results

3.1. Body weight, daily food intake

The body weight of the SDT rats was significantly lower than that of the SD rats, despite a significantly larger amount of food intake, at both 25 and 38 weeks of age. Furthermore, in SDT rats, the daily food consumption at 38 weeks of age exceeded that at 25 weeks of age (Table 1).

3.2. Plasma concentrations of glucose, insulin, and leptin

Nonfasting plasma glucose concentrations of SDT rats at both 25 and 38 weeks of age were significantly higher than those of SD rats of the same age, respectively. At both 25 and 38 weeks of age, the plasma insulin and leptin concentrations of SDT rats were significantly lower than at 8 weeks of age and significantly lower than those of SD rats of the same age, respectively (Table 1).

3.3. Plasma and stomach levels of active ghrelin, total ghrelin, and the ratio of active ghrelin to total ghrelin

The plasma active ghrelin levels of SDT rats were significantly higher than those of SD rats at 38 weeks of age, whereas plasma total ghrelin levels in SDT rats were significantly lower compared with those of SD rats at 25 weeks of age. Consequently, at 38 weeks of age, the plasma ratios of active ghrelin to total ghrelin levels (A/T ratios) in SDT rats were significantly higher than those of SD rats.

Both active and total ghrelin contents in the stomach of SDT rats were significantly lower than those of SD rats at both 25 and 38 weeks of age. However, the stomach A/T ratios of SDT rats were significantly higher compared with those of SD rats at 25 and 38 weeks of age (Fig. 1).

3.4. Distribution of ghrelin-producing cells in the stomach

Ghrelin-immunopositive cells in the stomachs of both SD and SDT rats at 8 and 38 weeks of age were sparsely distributed in the lower part of the gastric mucosal layer, where they were moderately abundant. At 38 weeks of age, the number of ip-ghrelin cells per unit area of the stomach mucosa in SDT rats was significantly lower than that in SD rats (Fig. 1).

3.5. Stomach expression levels of ghrelin and GOAT mRNA

The relative mRNA levels of ghrelin in the stomach, corrected to β-actin levels, in SDT rats were significantly ($P < .05$) higher at both 25 and 38 weeks of age (1.17 ± 0.12 and 1.81 ± 0.31, respectively) compared with those in SD rats at the same ages (1.02 ± 0.04 and 1.38 ± 0.20, respectively). Concurrently, the GOAT mRNA level was significantly ($P < .05$) higher in SDT rats at 38 weeks of age (1.96 ± 0.23) than in SD rats (1.50 ± 0.26). The expression levels of these 2 transcripts were not significantly different between SD and SDT rats at 8 weeks of age.

4. Discussion

The major findings of the present study are described below. First, after the onset of diabetes, SDT rats exhibited a significant weight loss despite exaggerated food consumption. Second, SDT rats showed overt hyperglycemia at 25 and 38 weeks of age with a concomitant hypoinsulinemia and hypoleptinemia. Third, the plasma active ghrelin levels and ghrelin A/T ratio of SDT rats increased after the induction of diabetes, which was correlated with an increase of stomach ghrelin A/T ratio and ghrelin and GOAT mRNA expression levels.

Interestingly, stomach ghrelin contents and ip-ghrelin cell numbers were decreased in SDT rats compared with control SD rats, suggesting that active ghrelin was produced de novo in the stomach. Nevertheless, the increased A/T ratio of stomach ghrelin content in SDT rats was indicative of an active ghrelin

Table 1 – Comparisons of parameters between SD and SDT rats at each age

	Age (wk)	Body weight (g)	Food intake (g/d)	Glucose (mmol/L)	Insulin (pmol/L)	Leptin (ng/mL)
SD	8	240.0 ± 7.1 (231.2-248.8)	27.6 ± 2.1 (25.0-30.2)	9.65 ± 0.56 (9.06-10.22)	359.1 ± 123.6 (167.8-720.6)	1.75 ± 0.45 (1.16-2.29)
	25	642.5 ± 26.6 * (620.3-664.7)	29.5 ± 2.2 (26.9-32.2)	9.32 ± 0.91 (8.56-10.07)	991.6 ± 182.4 * (469.5-1226.4)	5.81 ± 1.25 * (4.50-7.13)
	38	619.0 ± 66.4 * (549.0-689.0)	28.4 ± 1.5 (26.6-30.2)	9.61 ± 0.31 (9.28-9.93)	879.8 ± 166.2 * (671.8-1083.6)	5.29 ± 1.65 * (3.58-7.60)
SDT	8	302.1 ± 39.8 (265.4-338.9)	29.9 ± 1.2 (28.7-31.1)	9.04 ± 0.53 (8.57-9.69)	340.1 ± 78.8 (68.1-610.7)	2.20 ± 0.51 (1.67-2.73)
	25	454.3 ± 41.6 †,‖ (415.8-492.7)	45.5 ± 5.8 †,‖ (38.4-52.7)	28.45 ± 2.07 †,‖ (26.51-30.33)	73.1 ± 19.3 ‡,‖ (52.8-93.1)	0.94 ± 0.26 ‡,‖ (0.67-1.22)
	38	414.2 ± 10.0 †,‖ (403.8-424.5)	55.8 ± 5.9 †,§,‖ (48.4-63.1)	30.02 ± 1.26 †,‖ (28.68-31.32)	34.1 ± 1.4 ‡,‖ (31.9-36.3)	0.37 ± 0.08 ‡,‖ (0.28-0.46)

Values are presented as means ± SD. Ninety-five percent confidence intervals are shown in parentheses.
* $P < .001$ vs 8-week-old SD rats.
† $P < .001$ vs 8-week-old SDT rats.
‡ $P < .05$ vs 8-week-old SDT rats.
§ $P < .05$ vs 25-week-old SDT rats.
‖ $P < .001$ vs SD rats at the same age.

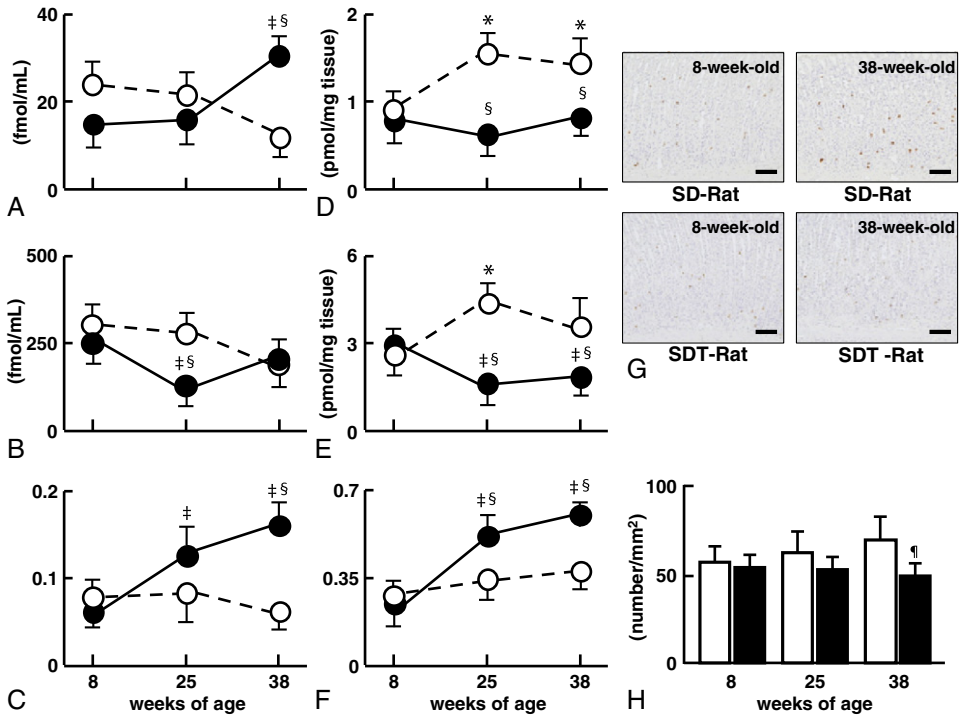

Fig. 1 – Plasma and stomach ghrelin contents and the localization of ip-ghrelin cells in the stomach mucosa of SD or SDT rats at different ages. A to C, Plasma ghrelin concentrations. D to F, Stomach ghrelin contents. A and B,: Active ghrelin. C and D, Total ghrelin. E and F, Ratio of active ghrelin to total ghrelin. G, Immunohistochemistry of ip-ghrelin cells (scale bar = 100 μm). H, The number of ip-ghrelin cells (the means of random 20 sections from each rat). White circles with dotted lines and white bars indicate SD rats, and black circles with solid lines and black bars indicate SDT rats. Data are presented as the mean ± SD. *$P < .01$ vs values from 8-week-old SD rats. †$P < .05$ vs values from 8-week-old SDT rats. ‡$P < .01$ vs 8-week-old SDT rats. §$P < .01$ vs SD rats at the same age. ¶$P < .001$ vs SD rats at the same age.

production to meet a demand, which was further confirmed by higher levels of ghrelin and GOAT mRNA in stomach of SDT rats after the induction of diabetes. The increased ghrelin production in this diabetic model may be a compensatory mechanism to keep weight stable under conditions of diabetic malnourishment. Future experiments involving ghrelin antagonists may be helpful to explore this hypothesis.

The hyperphagia observed in SDT rats appears to arise, at least in part, from deficient hypothalamic signaling by insulin and leptin, which in turn leads to the activation of neuropeptide Y/AgRP (agouti-related protein) neurons [9,16] separate from the participation of the ghrelin production. Furthermore, reciprocal relationships exist between ghrelin and insulin and between ghrelin and leptin in plasma concentration [17]. Notably, the peripheral administration of insulin to diabetic women suppressed plasma ghrelin concentrations [18]. Whereas the mechanisms involved in ghrelin production in SDT rats are largely unknown, suppressed insulin or leptin levels could be one of the possible mechanisms.

In conclusion, SDT rats with uncontrolled hyperglycemia may serve as a model of hyperphagia often observed in nonobese patients with type 2 diabetes mellitus. Active ghrelin production is concerned with diabetic hyperphagia solely or in concert with the suppression of insulin or leptin. This report elucidated the relevance of ghrelin to diabetic hyperphagia in a diabetic state over an extended period.

Funding

The current work was supported in part by Japanese Grant-in-Aid for Scientific Research (c) (nos. 19591089, 19591089, 22500361, and 22591145), a grant from the Kurume University Millennium Box Foundation for the Promotion of Science, and a grant from Morinaga Foundation for Health and Nutrition.

Acknowledgment

The authors thank Dr Kentaro Setoyama, Dr Junko Yoh, Mr Kazuyuki Ohba, and Mr Kazutoyo Ohkubo for their technical assistance.

Conflict of Interest

The authors declare that there is no conflict of interest associated with this manuscript.

REFERENCES

[1] Mannucci E, Tesi F, Ricca V, et al. Eating behavior in obese patients with and without type 2 diabetes mellitus. Int J Obes Relat Metab Disord 2002;26:848-53.

[2] Kenardy J, Mensch M, Bowen K, et al. A comparison of eating behaviors in newly diagnosed NIDDM patients and case-matched control subjects. Diabetes Care 1994;17:1197-9.
[3] Date Y, Kojima M, Hosoda H, et al. Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 2000;141:4255-61.
[4] Kojima M, Hosoda H, Date Y, et al. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. Nature 1999;402:656-60.
[5] Gutierrez JA, Solenberg PJ, Perkins DR, et al. Ghrelin octanoylation mediated by an orphan lipid transferase. Proc Natl Acad Sci U S A 2008;105:6320-5.
[6] Yang J, Brown MS, Liang G, et al. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. Cell 2008;132:387-96.
[7] Gelling RW, Overduin J, Morrison CD, et al. Effect of uncontrolled diabetes on plasma ghrelin concentrations and ghrelin-induced feeding. Endocrinology 2004;145:4575-82.
[8] Ishii S, Kamegai J, Tamura H, et al. Role of ghrelin in streptozotocin-induced diabetic hyperphagia. Endocrinology 2002;143:4934-7.
[9] Sipols AJ, Baskin DG, Schwartz MW. Effect of intracerebroventricular insulin infusion on diabetic hyperphagia and hypothalamic neuropeptide gene expression. Diabetes 1995;44:147-51.
[10] Shinohara M, Masuyama T, Shoda T, et al. A new spontaneously diabetic non-obese Torii rat strain with severe ocular complications. Int J Exp Diabetes Res 2000;1:89-100.
[11] Hosoda H, Kojima M, Matsuo H, et al. Ghrelin and des-acyl ghrelin: two major forms of rat ghrelin peptide in gastrointestinal tissue. Biochem Biophys Res Commun 2000;279:909-13.
[12] Nishi Y, Hiejima H, Mifune H, et al. Developmental changes in the pattern of ghrelin's acyl modification and the levels of acyl-modified ghrelins in murine stomach. Endocrinology 2005;146:2709-15.
[13] Nishi Y, Hiejima H, Hosoda H, et al. Ingested medium-chain fatty acids are directly utilized for the acyl modification of ghrelin. Endocrinology 2005;146:2255-64.
[14] Yabuki A, Ojima T, Kojima M, et al. Characterization and species differences in gastric ghrelin cells from mice, rats and hamsters. J Anat 2004;205:239-46.
[15] Nonoshita A, Nishi Y, Takushima S, et al. Dynamics of placental ghrelin production and its receptor expression in a Dahl salt-sensitive rat model of intrauterine growth restriction. Placenta 2010;31:358-64.
[16] Sindelar DK, Mystkowski P, Marsh DJ, et al. Attenuation of diabetic hyperphagia in neuropeptide Y–deficient mice. Diabetes 2002;51:778-83.
[17] Sun Y, Asnicar M, Smith RG. Central and peripheral roles of ghrelin on glucose homeostasis. Neuroendocrinology 2007;86:215-28.
[18] Gibson W, Liu J, Gaylinn B, et al. Effects of glucose and insulin on acyl ghrelin and desacyl ghrelin, leptin, and adiponectin in pregnant women with diabetes. Metabolism 2010;59:841-7.