Leptin downregulates ghrelin levels in streptozotocin-induced diabetic mice

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Submitted 4 November 2004; accepted in final form 9 August 2005

INSULIN-DEFICIENT DIABETES induced in rodents by the β-cell toxin streptozotocin (STZ) is characterized by marked hyperphagia (2). Researchers have examined the contribution of hypothalamic neuropeptides to the feeding response of diabetic animals (10) and hypothesize that two orexigenic peptides, hypothalamic neuropeptide Y (NPY) and agouti-related protein (AGRP), as well as anorexigenic corticotropin-releasing hormone are involved in diabetic hyperphagia (18, 20, 21). Changes in the hypothalamic neuropeptides and hyperphagia in diabetic animals were regulated by insulin or leptin supplementation (1, 7, 17).

Ghrelin, a novel gastrointestinal hormone identified in the stomach, is an endogenous ligand for the growth hormone secretagogue receptor (3, 5, 11, 12), and the peripheral or central administration of ghrelin increases food intake and body weight (14–16, 19, 22). Recent research has reported increased levels of stomach and plasma ghrelin in STZ-DM rats (6, 9).

Hypoleptinemia is another important factor that induces behavioral responses and changes in the hypothalamic neuropeptides of STZ-DM animals (1, 7, 17). Insulin supplementation in STZ-DM rats is accompanied by a simultaneous restoration of the endogenous leptin level, and we have previously shown that the central administration of leptin regulated the elevation of hypothalamic AGRP in STZ-DM rats (8). Therefore, the pathogenesis of diabetic hyperphagia with regard to the involvement of leptin and feeding-related peptides, such as ghrelin, remains uncertain. This study sought to clarify the regulation of plasma ghrelin and ghrelin mRNA levels by leptin in STZ diabetic mice.

MATERIALS AND METHODS

Animals. We housed 12-wk-old male C57Bl/6N mice (Seac Yoshitomi, Fukuoka, Japan) in a room at 21 ± 1°C and 55 ± 5% humidity with daily illumination from 0700 to 1900 (12:12-h light-dark cycle). The mice were allowed access to standard powdered mouse food (CLEA Japan, Tokyo, Japan) and tap water ad libitum. We measured daily food consumption and body weights of the mice. Protocols were approved by the Committee of Laboratory Science of Oita University. The animals were treated in accordance with the Oita University Guidelines for the Care and Use of Laboratory Animals.

Reagents. Murine leptin (Sigma, St. Louis, MO) was dissolved in PBS at a concentration of 22 μM, and the pH was adjusted to 6.8–7.4. STZ (Sigma) was also dissolved in PBS. Each solution was freshly prepared on the day of use.

Leptin treatment procedures. Mice were divided into four groups and sorted by body weight and food intake during the 2-wk acclimation period. Each group contained 4–8 mice, and we administered STZ for 7 days before the beginning of the feeding and leptin treatment studies as follows: 1) control; 2) STZ (70 mg/kg ip one-shot bolus); 3) STZ-subcutaneous leptin (STZ-LEP); and 4) STZ pair-fed groups. The food intake of the latter group matched that of the STZ-LEP group to exclude the secondary effect of leptin-induced feeding suppression. After the food consumption by the STZ-LEP group was analyzed, the same amount of food was administered to the pair-fed group. For the pair-feeding regimen, we calculated the daily food intake for the leptin-treated group. On the basis of a preliminary study of food consumption during day and night, one-fourth of the total amount was provided in the morning (0700), while the remaining three-fourths was provided before dark (1900). We did not observe any differences in food consumption between leptin-treated and pair-fed groups, which were injected with PBS instead of leptin. Mice were injected at 1900 once daily for 7 days with either PBS or leptin at a dose ranging from 0–27 nmol/kg sc. The cumulative food intake was measured once daily for 7 days, and each group was measured for changes in body weights, plasma ghrelin, and ghrelin mRNA expression.

Measurement of food consumption and body weight. The cumulative 24-h daily food intake of each mouse (all males, 4–8 group) and its body weight were measured. Each mouse was housed alone during the monitoring period under the ambient conditions described in animals.

Procedures of measuring blood samples. The stomach of each mouse was dissected, frozen in liquid nitrogen, and stored at −80°C until RNA extraction. During days 0–7 of the leptin treatment, blood samples were taken through the jugular veins at 1800, which were then separated into plasma and immediately frozen at −20°C until assayed. We placed blood samples in Vacutainer tubes containing EDTA, and immediately after blood collection, rocked the tubes

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several times to prevent coagulation. Next, the blood was transferred from the Vacutainer tubes into centrifuge tubes containing aprotinin and gently rocked several times to inhibit proteinase activity. The samples for plasma glucose, insulin, and murine leptin were assayed using commercial kits (Eiken Chemical, Tokyo, Japan), and the samples for plasma full ghrelin were also measured with commercial kits (Phoenix Pharmaceuticals, St. Joseph, MO).

**Real-time quantitative RT-PCR.** Ghrelin mRNA was amplified using PCR and measured by real-time quantitative PCR as follows. Total cellular RNA was prepared from selected mouse tissues using Trizol (Lifetech, Tokyo, Japan), according to the manufacturer’s protocol. Total RNA (20 μg) was electrophoresed on 1.2% formaldehyde-agarose gels. RNA quality and quantity were assessed using EtBr-agarose gel electrophoresis and by measuring the absorbance at 260 nm relative to that at 280 nm; cDNA was synthesized from 150 ng total RNA in a 20 μl-volume using a ReverTra-Dash reverse transcriptase kit (Toyobo, Tokyo, Japan) with random hexamer priming.

**Ghrelin mRNA was amplified using PCR and measured by real-time quantitative PCR as follows.** Total cellular RNA was prepared from selected mouse tissues using Trizol (Lifetech, Tokyo, Japan), according to the manufacturer’s protocol. Total RNA (20 μg) was electrophoresed on 1.2% formaldehyde-agarose gels. RNA quality and quantity were assessed using EtBr-agarose gel electrophoresis and by measuring the absorbance at 260 nm relative to that at 280 nm; cDNA was synthesized from 150 ng total RNA in a 20 μl-volume using a ReverTra-Dash reverse transcriptase kit (Toyobo, Tokyo, Japan) with random hexamer primers. Reactions were diluted to 50 μl with sterile distilled water and stored at −20°C. Primers for ghrelin were designed, synthesized, optimized, and provided as a preoptimized kit (Applied Biosystems, Foster City, CA): ghrelin (cat no. Mm00445450m1). The GenBank ghrelin accession number was NM 021488. Primers for ribosomal RNA used as internal controls were also provided as a preoptimized kit (cat no. Hs99999901). Using an ABI PRISM 7000 sequence detector, PCR amplification was performed in 50-μl volumes containing a 100-ng cDNA template in PCR Master Mix (Roche Laboratories, Nutley, NJ) according to the following regimen: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. Samples were analyzed in duplicate using sequence detection software (Applied Biosystems), and the level of ghrelin mRNA expression was normalized to that of ribosomal RNA, as outlined in Perkin-Elmer’s User Bulletin No. 2.

**Statistical analysis.** All data are expressed as means ± SE. We used a repeated two-way ANOVA to analyze differences for multiple comparisons (StatView 4.0); when appropriate, we used the unpaired t-test or Mann-Whitney U-test. Pearson’s correlation coefficient was used to examine the relationship between levels of plasma glucose and levels of plasma ghrelin and dose-dependent changes. The time-course and dose-dependent changes were also examined by the Pearson’s correlation coefficient.

**RESULTS**

**Effects of treatment with leptin on food intake and body weight.** We analyzed the changes in food intake and body weight over time. Figure 1A shows the changes in the daily food intake of STZ mice, which was greater than in the control group (P < 0.01). There was also a decrease in the STZ-LEP group’s food intake compared with that of the STZ group (P < 0.05). There was also a decrease in the STZ group’s body weights compared with the control group (P < 0.01; Fig. 1, B and C). The STZ-LEP group had greater body weights compared with the STZ and STZ PF groups (P < 0.05 for each group; Fig. 1, B and C). In this study, the leptin treatment had a significant effect on food intake and body weight at a dose range of 0–27 nmol/kg sc on the 7th day (food intake g: 0 nmol, 6.4 ± 0.3; 0.9 nmol, 6.1 ± 0.2; 2.7 nmol, 5.8 ± 0.2; 9 nmol, 4.7 ± 0.3; 27 nmol, 4.6 ± 0.4; body weight g: 0 nmol, −2.0 ± 0.1; 0.9 nmol, −1.8 ± 0.1; 2.7 nmol, −1.8 ± 0.1; 9 nmol, −0.8 ± 0.1; 27 nmol, −0.8 ± 0.1) (P < 0.01 for each group), but did not correct either to control levels.

**Effects of treatment with leptin on plasma glucose.** We analyzed the changes in plasma glucose over time; Fig. 1D shows the changes in STZ mice. The STZ group had higher plasma glucose levels than the control group (P < 0.01), and treatment with leptin decreased plasma glucose compared with levels in the STZ and STZ PF groups (P < 0.05 for each; Fig. 1D).

**Effects of treatment with leptin on plasma levels of insulin and leptin.** Next, we analyzed the dose-dependent changes of insulin and leptin on the 3rd (Fig. 2, A and C) and 7th days (Fig. 2, B and D). Pearson’s correlation coefficient test analyzed the dose-dependent effects of leptin on plasma leptin.
Effects of treatment with leptin on plasma and ghrelin mRNA. Plasma ghrelin levels increased in the STZ group compared with levels in the control group, and in the STZ-LEP group, leptin treatment decreased plasma ghrelin levels compared with levels in the STZ group. We also examined dose-dependent changes in ghrelin levels on the 3rd (Fig. 3A) and 7th days (Fig. 3B); there was a dose-dependent change in plasma ghrelin levels in the STZ-LEP group on both the 3rd ($P < 0.05$) and 7th days ($P < 0.01$; Fig. 3A and B). The leptin treatment had a significant effect on the plasma parameters at a dose range of 0–27 nmol/kg sc on the 7th day (plasma ghrelin pg/ml: 0 nmol, 4,093 ± 342; 0.9 nmol, 3,442 ± 364; 2.7 nmol, 1,948 ± 214; 9 nmol, 1,771 ± 152; 27 nmol, 1,833 ± 194) ($P < 0.01$), but it did not correct food intake or body weight to control levels. In addition, Pearson’s correlation coefficient test analyzed relationships between plasma glucose/ghrelin and leptin/ghrelin. Negative relationships were observed between glucose/ghrelin and leptin/ghrelin (glucose/ghrelin, $P < 0.01$; leptin/ghrelin, $P < 0.05$). Furthermore, ghrelin mRNA levels were also higher in the STZ group than in the control group ($P < 0.01$). The leptin treatment decreased the ghrelin mRNA/ rRNA levels relative to those in the STZ group ($P < 0.05$).

DISCUSSION

The STZ-DM mouse is a widely used animal model characterized by insulin deficiency and several other pathological features, including marked hyperphagia (2, 7, 13, 17). Plasma insulin levels are reduced in STZ-DM mice, and insulin deficiency is the main cause of STZ-DM hyperphagia because insulin in the central nervous system plays a suppressive role in food intake. In addition, previous studies have demonstrated that decreased plasma leptin levels and leptin secretion are related to hyperphagia in STZ-DM mice (8, 17). In the STZ model of diabetes, reduced leptin levels induce NPY expression and may act in concert with a reduction in levels of corticotropin-releasing hormones to cause hyperphagia.

This study demonstrated that leptin improved weight loss in STZ-induced diabetic mice, even in a pair-fed condition. These results may be due to changes in hyperglycemia and/or insulin action on body weight caused by the leptin treatment. Recent
research has reported that plasma ghrelin concentrations in untreated diabetic rats are significantly higher than levels in control rats, that elevated plasma ghrelin levels can contribute to diabetic hyperphagia by increasing hypothalamic NPY, and that plasma ghrelin levels can be normalized using insulin treatment (9). The present study supported previous research indicating that plasma ghrelin concentration is significantly higher in STZ-DM mice than in control mice; however, the mechanism by which plasma ghrelin is elevated in STZ-induced diabetes is largely unknown. Therefore, the goal of our study was to clarify leptin regulation of plasma ghrelin and ghrelin mRNA levels in STZ-DM mice.

This study demonstrated that peripheral leptin action improves blood glucose and food intake values, supporting previous research (4). There was a dose-dependent difference in blood glucose levels between STZ-LEP and STZ groups; however, at a 0–27 nmol/kg dose range, the leptin action was incomplete, and thus blood glucose levels were higher in the STZ-LEP group than in the control group. In this study, the STZ group had elevated plasma ghrelin levels compared with levels in the control group. Peripheral leptin action reduced food intake as well as blood ghrelin and ghrelin mRNA levels. STZ-LEP mice exhibited decreased plasma ghrelin concentrations and ghrelin mRNA levels compared with levels in STZ-LEP pair-fed mice, suggesting that leptin regulation of plasma and ghrelin mRNA levels is independent of food intake. Peripheral leptin action elevated blood leptin levels but not blood insulin levels, indicating that leptin reverses elevated plasma and ghrelin mRNA levels independent of the insulin concentration. However, we cannot rule out the involvement of glucose or insulin in the regulation of ghrelin in STZ mice because several studies have demonstrated that glucose and/or insulin can regulate ghrelin levels (6, 9). Further studies are needed to examine active ghrelin as well as full ghrelin.

In conclusion, we demonstrated that leptin regulates the suppression of food consumption in STZ-DM mice. This study also resulted in a major new finding: leptin can reverse elevated plasma ghrelin levels independent of insulin concentration and food intake.

GRANTS

This work was supported by Grant-in-Aid 136770067 from the Japanese Ministry of Education, Science and Culture, and by Research Grants for Intractable Diseases from the Japanese Ministry of Health and Welfare, 2001–2002. This work was also supported in part by a grant from the Smoking Research Foundation, Venture Business Laboratory at Oita University, and Health and Labor Sciences Research and Grant 16922171 from Japan Society for the Promotion of Science.

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