Ghrelin enhances glucose-induced insulin secretion in scheduled meal-fed sheep

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Abstract

The purpose of this study was to investigate the effects of physiologic levels of ghrelin on insulin secretion and insulin sensitivity (glucose disposal) in scheduled fed-sheep, using the hyperglycemic clamp and hyperinsulinemic euglycemic clamp respectively. Twelve castrated Suffolk rams (69.8 ± 0.6 kg) were conditioned to be fed alfalfa hay cubes (2% of body weight) once a day. Three hours after the feeding, synthetic ovine ghrelin was intravenously administered to the animals at a rate of 0.025 and 0.05 $\mu\text{g/kg}$ body weight (BW) per min for 3 h. Concomitantly, the hyperglycemic clamp or the hyperinsulinemic euglycemic clamp was carried out. In the hyperglycemic clamp, a target glucose concentration was clamped at 100 mg/100 ml above the initial level. In the hyperinsulinemic euglycemic clamp, insulin was intravenously administered to the animals for 3 h at a rate of

2 mU/kg BW per min. Basal glucose concentrations (44 ± 1 mg/dl) were maintained by variably infusing 100 mg/dl glucose solution. In both clamps, plasma ghrelin concentrations were dose-dependently elevated and maintained at a constant level within the physiologic range. Ghrelin infusions induced a significant (ANOVA; $P < 0.01$) increase in plasma GH concentrations. In the hyperglycemic clamp, plasma insulin levels were increased by glucose infusion and were significantly ($P < 0.05$) greater in ghrelin-infused animals. In the hyperinsulinemic euglycemic clamp, glucose infusion rate, an index of insulin sensitivity, was not affected by ghrelin infusion. In conclusion, the present study has demonstrated for the first time that ghrelin enhances glucose-induced insulin secretion in the ruminant animal.

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Introduction

Ghrelin, a recently discovered gastric hormone, has been implicated in the control of food intake and energy homeostasis. Changes in blood ghrelin levels are associated with feeding behavior in rats (Date *et al.* 2002a) and goat (Hayashida *et al.* 2001). A large preprandial rise and a postprandial fall in plasma ghrelin levels were observed in man (Cummings *et al.* 2001) and ruminants (Sugino *et al.* 2002). In addition, ghrelin expression and plasma ghrelin levels are increased by fasting (Kim *et al.* 2003). In contrast, plasma ghrelin levels are decreased by oral or intravenous administration of glucose (Shiia *et al.* 2002). In rodents, ghrelin stimulates food intake, promoting weight gain and adiposity (Tschop *et al.* 2000, Kamegai *et al.* 2001, Wren *et al.* 2001, Beck *et al.* 2002, Lawrence *et al.* 2002). Thus, ghrelin may be closely related to energy metabolism in association with feeding status.

Insulin is a critical regulator of energy metabolism, and evidence suggests a close relationship between circulating

ghrelin levels and insulin secretion. Blood ghrelin and insulin concentration fluctuate reciprocally before and after feeding (Cummings *et al.* 2001). Growth hormone (GH) secretagogue receptor is detected in pancreatic B cells containing insulin (Kageyama *et al.* 2005), suggesting that ghrelin plays a role in regulating insulin secretion in the pancreas. Inconsistent results have been reported in the effects of ghrelin administration on insulin secretion. Intravenous administration of ghrelin stimulates insulin secretion in free-feeding rats (Lee *et al.* 2002). *In vitro* studies have also shown that ghrelin increases insulin secretion from isolated rat pancreatic islets (Adegate & Ponery 2002, Date *et al.* 2002b). In contrast, peripheral ghrelin administration suppresses insulin secretion in man (Broglia *et al.* 2001, Arosio *et al.* 2003) and mice (Reimer *et al.* 2003). Moreover, ghrelin inhibits insulin secretion in isolated mouse islets (Reimer *et al.* 2003). However, it has not been determined whether ghrelin modulates insulin secretion in ruminants.

In the preliminary study, we found that plasma ghrelin and insulin levels were reciprocally changed by feeding in sheep. Thus, we expected that ghrelin might participate in glucose metabolism modulating insulin secretion. However, the effect of ghrelin on insulin secretion has not been reported in ruminants. In scheduled meal-fed sheep, therefore, we examined the effects of ghrelin administration on insulin secretory response to glucose load and on insulin sensitivity in the postprandial period, when insulin secretory response is high and blood ghrelin level is low, using the hyperglycemic and hyperinsulinemic euglycemic clamps respectively.

Materials and Methods

Experimental animals and treatments

Twelve castrated Suffolk rams, aged 2 years, weighing 69.8 ± 0.6 kg (mean $\pm$ S.E.M.) were kept in a metabolic crate in a chamber kept at 20 °C under a 12-h light-dark cycle (0730–1930 h, light; 1930–0730 h, dark). The animals were fed alfalfa hay cubes at 1200 h at the level of 2% of body weight (BW) to meet 120% of their daily metabolizable energy (ME) requirements on the basis of metabolic body size during a 10-day pre-experimental period. Drinking water was freely available during the whole experiment. The animals were fitted with a catheter on both sides of the jugular vein at least 1 day before the experimental day. The catheters were filled with 40 U/ml heparinized saline. The hyperglycemic clamp was established at 3–6 h after feeding, when blood ghrelin levels should be very low. The rams were equally divided into three groups. During the glucose clamp, synthetic ovine ghrelin (Peptide Institute, Osaka, Japan) dissolved in saline (0.9% NaCl, 0.1% SSA) was continuously infused at a rate of 0.025 and 0.05 μ g/kg BW per min for ghrelin-treated animals, and saline (0.9% NaCl, 0.1% SSA) for control animals. All solutions were infused through the left catheter at a rate of 1 ml/min with a peristaltic pump. Blood samples were collected through the right catheter, immediately placed into a heparinized tube with aprotinin (1000 KIU/ml blood) and centrifuged for 10 min at 4 °C. Harvested plasma was stored at –80 °C until assay.

The hyperglycemic clamp technique

The hyperglycemic clamp technique was used to determine insulin responsiveness to glucose. Glucose solution was prepared at 20% (w/v). Basal glucose concentrations were determined three times at 10-min intervals before glucose infusion. In the hyperglycemic clamp, blood glucose levels were raised to the desired hyperglycemia (100 mg/100 ml higher than the basal blood glucose) and were maintained at that plateau by variably infusing the glucose solution through the left catheter with a peristaltic pump (Mode AC-2120; Atto Co, Tokyo, Japan).

Blood glucose levels were measured with a glucose analyzer (GLU-1; TOA Electronics, Tokyo, Japan) at 5-min intervals throughout the experiment, and the glucose infusion rate was empirically determined.

The hyperinsulinemic euglycemic clamp technique

The hyperinsulinemic euglycemic clamp experiment was carried out to determine insulin sensitivity (glucose disposal). The insulin solution (100 U/ml) (Actrapid mono-component porcine insulin; Novo Industry, Bagsvaerd, Denmark) was infused through the left jugular catheter at a constant rate of 2 mU/kg BW per min for 3 h. Blood glucose concentrations were measured with a glucose analyzer (GLU-1; TOA Electronics Ltd) every 5 min, and the glucose solution (20% (w/v)) was variably infused into the left jugular catheter to maintain the preinfusion blood glucose concentrations.

Time-resolved fluoroimmunoassay (TR-FIA) of plasma ghrelin, insulin and GH

Ghrelin The ghrelin assay was done as described previously (Sugino *et al.* 2002). The ghrelin concentration was measured by competitive, solid-phase immunoassay with Eu-labeled synthetic rat ghrelin and polystyrene microtiter strips (Nalge Nunc, Tokyo, Japan) coated with antirabbit gamma globulin. The ghrelin was extracted by the following procedure. A volume of 1 ml of 1 mol/l acetic acid (pH 2) was added to 1 ml ovine plasma, and plasma protein was precipitated by addition of 4 ml acetone. After centrifugation, the supernatant was evaporated and resuspended in assay buffer (50 mM Tris–HCL, 140 mM NaCl, 0.5% gamma globulin, 0.00078% DTPA, 0.05% sodium azide, and 0.01% Tween 40 (pH 7.8)) with 10 KIU/ml aprotinin. The mean recovery of ghrelin from ovine plasma was 97.6%. Ghrelin (3 μ g/100 μ l 10 mM bicarbonate saline (pH 8.5)) was labeled with europium according to the manufacturer's instructions (Wallac Oy, Turku, Finland). Diluted antighrelin rabbit serum (1:2 000 000) was incubated in each well overnight. After washing the wells, serial diluted ghrelin standards (0.01–10 ng/ml) and extracted ghrelin in plasma, dissolved in assay buffer (100 μ g/well), were incubated in wells overnight. After washing the wells, Eu-labeled ghrelin (about 50 pg/100 μ l) was distributed in all wells, and incubated for 3 h. After washing, 100 μ l enhancement solution were added to each well, and fluorescence was measured by time-resolved fluorometer (Multilabel Counter, 1420 ALVO; Wallac Oy). Intra- and interassay coefficients of variation were 6.9 and 5.5% respectively. Least detectable dose and IC₅₀ in this assay system were 0.025 and 0.831 ng/ml respectively.

Insulin The insulin concentration was measured by competitive, solid-phase immunoassay with Eu-labeled

Figure 1 Dose-response lines of ovine insulin ● and ovine plasma ○ in the TR-FIA system. Plasma samples were adjusted to 0.1, 0.32, 1.0, 3.12, 10.0, 36.2 and 100 μl. The curve was linearized by the method of least squares on logarithmic amounts of percent sample bound vs maximum binding (B/B_0). Each point is the mean for triplicate determinations.

synthetic bovine insulin and polystyrene microtiter strips (Nalge Nunc) coated with anti-guinea pig gamma globulin. A diluted antibody to human insulin (NIDDK-anti-h insulin, 1:500 000) was distributed in all wells coated with anti-guinea pig gamma globulin antiserum, and incubated overnight. After washing off the insulin antibody, serial diluted GH standards (0.1–100 ng/ml), dissolved in assay buffer and plasma, were added to the wells (100 μl/well) and incubated overnight. After incubation, Eu-labeled insulin (NIDDK-b insulin-I-5, about 1250 pg/50 μl) was distributed in all wells, and incubated at 6 °C for 2 h. After washing, 100 μl enhancement solution were added to each well, and fluorescence in each well was measured by time-resolved fluorometer. Intra- and interassay of coefficients of variation were 3.2% and 3.1% respectively. Least detectable dose and IC_{50} in this assay system were 0.016 and 1.073 ng/ml respectively (Fig. 1).

GH assay GH assay was done as described previously (Sugino *et al.* 2002). The GH concentration was measured by competitive, solid-phase immunoassay with Eu-labeled synthetic ovine GH and polystyrene microtiter strips (Nalge Nunc) coated with antirabbit gamma globulin. A diluted antibody to ovine GH (NIDDK-anti-o GH, 1:100 000) was distributed in all wells coated with anti-rabbit gamma globulin antiserum, and incubated overnight. After washing off the GH antibody, serial diluted GH standards (0.1–100 ng/ml), dissolved in assay buffer and plasma, were added to the wells (100 μl/well) and incubated in wells overnight. After washing, Eu-labeled GH (NIDDK-oGH-I-5, about 250 pg/100 μl), was

distributed in all wells, and incubated for 3 h. After washing, 100 μl enhancement solution were added to each well, and fluorescence in each well was measured by time-resolved fluorometer. Intra- and interassay coefficients of variation were 4.1 and 9.3% respectively. Least detectable dose and IC_{50} in this assay system were 0.158 and 8.738 ng/ml respectively.

Statistical analysis

The values of plasma ghrelin, insulin and GH concentrations, and glucose infusion rates were expressed as mean $\pm$ S.E.M. Repeated-measures ANOVA was performed to evaluate the statistical significance of treatment effects on each parameter over time. Statistical comparisons for ghrelin, insulin, GH and glucose concentrations among the three treatments at each time point were performed with the *post-hoc* Fisher test.

Results

In the hyperglycemic study, plasma glucose concentrations were clamped at 100 mg/100 ml above the initial level at 60–180 min after the start of glucose infusion (Fig. 2A). Plasma ghrelin levels dose-dependently increased and reached a plateau within 10 min after the commencement of ghrelin infusion (Fig. 2B).

Plasma GH levels showed a dose-dependent and significant increase ($P < 0.01$), peaking at 30 min after the commencement of ghrelin infusion (Fig. 3A). Changes in plasma insulin concentration are presented in Fig. 3B. Plasma insulin levels were increased by glucose infusion in all treatments. Furthermore, an elevation in plasma insulin levels was significantly ($P < 0.01$) greater in ghrelin-infused groups at several time points.

In the hyperinsulinemic euglycemic clamp, plasma insulin concentrations reached a plateau within 60 min after the commencement of insulin infusion (Fig. 4A). There was no difference in insulin levels among the three treatments. Plasma glucose concentrations were successfully clamped at preinfusion levels during insulin infusion in all treatments (Fig. 4B). Plasma ghrelin levels were dose-dependently elevated to a plateau within 30 min after the commencement of ghrelin infusion and successfully maintained within the physiologic range (Fig. 4C). A significant ($P < 0.01$) increase was observed in plasma GH levels during ghrelin infusion (Fig. 5).

The glucose infusion rate (GIR) of every 10 min was significantly ($P < 0.05$) less in the low- ghrelin-infused group (G1) in the time periods of 60–70, 120–150, and 170–180 min (Fig. 6A). There was no difference in GIR between control and the high-ghrelin-infused group (G2). There was no significant difference in mean GIR among the three treatments (Fig. 6B).

Figure 2 Glucose and ghrelin infusion. Average glucose (A) and ghrelin (B) plasma concentrations in four sheep continuously receiving saline (control: ●), low level of ghrelin (G1, 0.025 $\mu\text{g/kg BW}$ per min: ■) and high level of ghrelin (G2, 0.05 $\mu\text{g/kg BW}$ per min: ◇) during the hyperglycemic clamp. Values are means $\pm$ S.E. ** $P < 0.01$ vs control; † $P < 0.05$ vs G1; †† $P < 0.01$ vs G1.

Figure 3 Average GH (A) and insulin (B) plasma concentrations in four sheep continuously receiving saline (control: ●), low level of ghrelin (G1, 0.025 µg/kg BW per min: ■) and high level of ghrelin (G2, 0.05 µg/kg BW per min: ◇) during the hyperglycemic clamp. Values are means ± s.e. * $P < 0.05$ vs control; ** $P < 0.01$ vs control; † $P < 0.05$ vs G1; †† $P < 0.01$ vs G1.

Figure 4 Insulin, glucose and ghrelin infusion. Average insulin (A), glucose (B) and ghrelin (C) plasma concentrations in four sheep continuously receiving saline (control: ●), low level of ghrelin (G1, 0.025 μ g/kg BW per min: ■) and high level of ghrelin (G2, 0.05 μ g/kg BW per min: ◇) during the hyperinsulinemic euglycemic clamp. Values are means $\pm$ s.e. * P <0.05 vs control; ** P <0.01 vs control; † P <0.05 vs G1; †† P <0.01 vs G1.

Figure 5 Average GH plasma concentrations in four sheep continuously receiving saline (control: ●), low level of ghrelin (G1, 0.025 µg/kg BW per min: ■) and high level of ghrelin (G2, 0.05 µg/kg BW per min: ◇) during the hyperinsulinemic euglycemic clamp. Values are means ± S.E. * $P < 0.05$ vs control; † $P < 0.05$ vs G1; †† $P < 0.01$ vs G1.

Discussion

In the present study, glucose clamping and ghrelin infusion were started 3 h after feeding, when insulin secretory response to glucose is high and circulating ghrelin levels are low, to determine the effects of ghrelin administration on insulin secretion and sensitivity. Thus, we have clearly demonstrated that ghrelin is involved in energy metabolism by enhancing glucose-induced insulin secretion.

Ghrelin inhibits insulin secretion in human (Broglio *et al.* 2001) and mouse islets (Reimer *et al.* 2003). Furthermore, blood ghrelin levels are inversely related to postprandial insulin levels (Cummings *et al.* 2001). Therefore, we had expected that ghrelin would inhibit insulin secretion. In the present study, however, ghrelin administration distinctly enhanced insulin secretory response to glucose load in fed sheep. Our preliminary study showed that circulating ghrelin was elevated by food deprivation and then rapidly declined after feeding in sheep. These results suggest that circulating ghrelin decreases with enhancing insulin secretory response to glucose in the postprandial period when circulating insulin inversely increases. This is consistent with an *in vitro* study showing that glucose-stimulated insulin release is increased by ghrelin in isolated rat islets (Date *et al.* 2002b). However,

we should consider that in ruminants glucose is constantly synthesized from volatile fatty acids (VFAs), the main energy source, in the liver, and the change in circulating glucose is small. This suggests that ghrelin participates in glucose-dependent insulin secretion without change in circulating glucose in ruminants. Furthermore, VFAs stimulate insulin secretion in ruminants. Future studies should determine whether ghrelin modulates VFA-induced insulin secretion.

Fasting and nonfasting blood ghrelin levels are positively correlated with insulin resistance (McCowen *et al.* 2002, Gauna *et al.* 2004). In addition, ghrelin receptors are widely distributed in the whole body (Gnanapavan *et al.* 2002). Therefore, ghrelin may cause insulin resistance by directly acting on peripheral tissues. Furthermore, acute and chronic elevations of blood GH levels cause insulin resistance (Rizza *et al.* 1982, Moller *et al.* 1989, Hettiarachchi *et al.* 1996, Kim *et al.* 1999). Therefore, ghrelin administration might cause insulin resistance indirectly through stimulating GH secretion, because ghrelin administration induces a transient but significant increase in plasma GH concentrations. Postprandial insulin sensitivity, as evaluated by mean GIR, was not affected by ghrelin administration. But ghrelin-infused groups showed low GIR at several time points, suggesting that

Figure 6 (A) Every 10 min of glucose infusion rate (GIR) was significantly ($P<0.05$) less in the low-ghrelin-infused group (G1, $0.025 \mu\text{g/kg BW}$ per min) than in control at 60–70, 120–150 and 170–180 min during the hyperinsulinemic euglycemic clamp. * $P<0.05$; † $P<0.05$ vs G1; ‡ $P<0.01$ vs G1. (B) Mean GIR of G1 was less ($P<0.08$) than that of control during the hyperinsulinemic euglycemic clamp.

ghrelin might induce insulin resistance. The possibility that insulin secretion is complementally enhanced against GH-induced insulin resistance in the ghrelin-treated animals cannot be excluded.

In conclusion, the present study has demonstrated for the first time that ghrelin enhances glucose-induced insulin secretion in ruminants, but the mechanism underlying the facilitating effect of ghrelin on insulin secretion in ruminants must be determined in further studies.

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