

# Ghrelin differentially modulates glucose-induced insulin secretion according to feeding status in sheep

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## Abstract

The present study was conducted to investigate roles of ghrelin in glucose-induced insulin secretion in fasting- and meal-fed state in sheep. Castrated Suffolk rams were fed a maintenance diet of alfalfa hay cubes once a day. Hyperglycemic clamp (HGC) was carried out to examine glucose-induced insulin response from 48 to 53 h (fasting state) and from 3 to 8 h (meal-fed state) after feeding in Experiment 1 and 2 respectively. Total dose of 70 nmol/kg body weight of D-Lys3-GHRP6, a GH secretagogue receptor 1a (GHS-R1a) antagonist, was intravenously administered at 0, 60, and 120 min after the commencement of HGC. In the fasting

state, the ghrelin antagonist significantly ( $P < 0.01$ ) enhanced glucose-induced insulin secretion. In the meal-fed state, i.v. administration of synthetic ovine ghrelin (0.04 µg/kg body weight per min during HGC) significantly ( $P < 0.05$ ) enhanced glucose-induced insulin secretion. D-Lys3-GHRP6 treatment suppressed ghrelin-induced enhancement of the insulin secretion. In conclusion, ghrelin has an inhibitory and stimulatory role in glucose-induced insulin secretion via GHS-R1a in fasting- and meal-fed state respectively.

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## Introduction

Ghrelin is a novel peptide that acts on the growth hormone secretagogue receptor (GHS-R) in the pituitary and hypothalamus to stimulate GH secretion (Kojima *et al.* 1999, Takaya *et al.* 2000). In some species, there is evidence that ghrelin also stimulates food intake and reduces energy expenditure (Tschop *et al.* 2000, Nakazato *et al.* 2001, Wren *et al.* 2001).

Apart from these actions in the brain, ghrelin has been reported to have a dual role in the regulation of pancreatic insulin secretion. Some studies in rats show that ghrelin stimulates insulin secretion *in vivo* (Lee *et al.* 2002) and *in vitro* (Date *et al.* 2002, Adeghate & Ponery 2002). Others show that ghrelin inhibits insulin secretion from rat pancreatic islets in a dose- and glucose-dependent manner (Colombo *et al.* 2003) and from mouse islets in the presence of glucose (Reimer *et al.* 2003). GHS-R antagonist and immunoneutralization of endogenous ghrelin enhance glucose-induced insulin release from perfused rat pancreas (Dezaki *et al.* 2006). In humans, ghrelin has been shown to cause hyperglycemia by reducing insulin secretion (Broglia *et al.* 2001). These discrepancies among the effects of ghrelin on insulin secretion have not been examined.

On the other hand, blood ghrelin levels are affected by nutritional states. Plasma ghrelin levels are increased after fasting and reduced after feeding in humans (Ariyasu *et al.* 2001, Cummings *et al.* 2001, Shiya *et al.* 2002). Lee *et al.* (2002) showed that a high-fat diet decreases plasma ghrelin levels, whereas a low-protein diet increases plasma ghrelin levels in rats. Therefore, ghrelin secretion may be enhanced under negative energy balance but inhibited under positive energy balance.

Overall, it appears that ghrelin may play an important role in glucose metabolism, through modulation of insulin secretion, but this could be dependent on whether the organisms are in negative or positive energy balance. We observed that plasma levels of ghrelin were inversely related with those of insulin around feeding in sheep (unpublished data). Furthermore, we have demonstrated that ghrelin infusion stimulates glucose-induced insulin secretion in meal-fed sheep (Takahashi *et al.* 2006). These observations led us to hypothesize that ghrelin regulates insulin secretion dependent on energy balance. In the present study, we have explored this hypothesis by examining the involvement of GHS-R1a in glucose-induced insulin secretion in fasting- and meal-fed sheep.

## Materials and Methods

### Experimental animals and treatments

Twenty two-year-old neonatally castrated Suffolk rams of  $51.4 \pm 0.3$  kg were placed in metabolism cages and held at 20 °C ambient temperature under a 12 h light:12 h darkness cycle (0730–1930 h light:1930–0730 h darkness). The animals were fed a maintenance diet of alfalfa hay cubes at 0900 h each day for 10 days prior to the experimental period, with free access to water. Bilateral jugular venous cannulas were inserted one day prior to the experimentation and closed with two-way taps and filled with heparinized (40 U/ml) normal saline for infusion and blood sampling. The animals were divided into two groups ( $n=4$  per group) in Experiment 1 and into three groups ( $n=4$  per group) in Experiment 2.

In Experiment 1, hyperglycemic clamp (HGC; see below) was conducted in both groups from 48 to 53 h after the last feeding (fasting state), when plasma ghrelin levels reached plateau (Sugino *et al.* 2002). Ghrelin antagonist-treated group received a total dose of 70 nmol/kg body weight D-Lys3-GHRP-6 (Sigma) in normal saline via the right jugular cannula every 60 min from 0 to 120 min after the commencement of a glucose infusion via the contralateral cannula. The dose of D-Lys3-GHRP-6 was determined according to several reports (Fujino *et al.* 2003, Dezaki *et al.* 2004, Dong *et al.* 2006). The control group received saline vehicle alone. In order to determine physiological effects of ghrelin as far as possible, we avoided administering ghrelin to the fasting animals in which plasma ghrelin levels had reached plateau (2.0 ng/ml).

In Experiment 2, HGC was conducted in all groups from 3 to 8 h after feeding (meal-fed state) when plasma ghrelin levels were nadir (Sugino *et al.* 2002). Concomitantly, two ghrelin-treated groups received synthetic ovine ghrelin (Peptide Institute Inc., Osaka, Japan) in saline (0.9% NaCl, 0.1% sheep serum albumin) at a rate of 0.04 µg/kg body weight per min through the left jugular cannula. The control group received saline vehicle alone. The ghrelin plus antagonist-treated group received a total dose of 70 nmol/kg body weight D-Lys3-GHRP-6 in normal saline (or saline vehicle alone) via the right jugular cannula every 60 min from 0 to 120 min after the commencement of a glucose infusion via the contralateral cannula. In order to determine physiological effects of ghrelin as far as possible, we avoided administering the antagonist alone to the fed animals in which plasma ghrelin levels had reached nadir (0.5 ng/ml).

Blood samples were collected through the right cannula, immediately placed into a heparinized tube with aprotinin (1000 KIU/ml of blood) and centrifuged for 10 min at 4 °C. Harvested plasma was stored at –80 °C until assay.

### Hyperglycemic clamp

The HGC technique was used to determine insulin responsiveness to glucose. Glucose solution was prepared at

20% (wt/vol). Basal glucose concentrations were determined three times at 10-min interval before glucose infusion. In the HGC, 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 infusing the glucose solution via the right cannula with a peristaltic pump (Mode AC-2120, Atto Co. Ltd, Tokyo, Japan). Blood glucose levels were measured with a glucose analyzer (GLU-1, TOA Electronics Ltd, Tokyo, Japan) at 5-min intervals throughout the experiment, and glucose infusion rate was empirically determined.

### Time-resolved fluoro-immunoassay of plasma ghrelin, insulin, and GH

**Ghrelin** An assay for bioactive ghrelin was done as described previously (Sugino *et al.* 2002). The ghrelin concentration was measured by competitive solid-phase immunoassay using Europium (Eu)-labeled synthetic rat ghrelin and polystyrene microtiter strips (Nalge Nunc Int., Tokyo, Japan) coated with anti-rabbit γ-globulin. Intra- and inter-assay 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** Insulin assay was done as described previously (Takahashi *et al.* 2006). The insulin concentration was measured by competitive solid-phase immunoassay using Europium (Eu)-labeled synthetic bovine insulin and polystyrene microtiter strips (Nalge Nunc Int.) coated with anti-guinea pig γ-globulin. The anti-human insulin was kindly supplied by Dr. K. Wakabayashi (Biosignal Research Center, Institute for Molecular and Cellular Regulation, Gunma University). Intra- and inter-assay coefficients of variation were 3.2 and 3.1% respectively. Least detectable dose and IC50 in this assay system were 0.016 and 1.073 ng/ml respectively.

**GH** GH assay was done as described previously (Sugino *et al.* 2002). The GH concentration was measured by competitive solid-phase immunoassay using Europium (Eu)-labeled synthetic ovine GH and polystyrene microtiter strips (Nalge Nunc Int.) coated with anti-rabbit γ-globulin. Intra- and inter-assay coefficients of variation were 4.1 and 9.3% respectively. Least detectable dose and IC50 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 means ± S.E.M. Repeated measures of two-way ANOVA was used to evaluate statistical significance of treatment effects on each parameter over time. Statistical comparisons for glucose, ghrelin, GH and insulin among the treatments at each time point was evaluated using the *post hoc* Fisher's test.

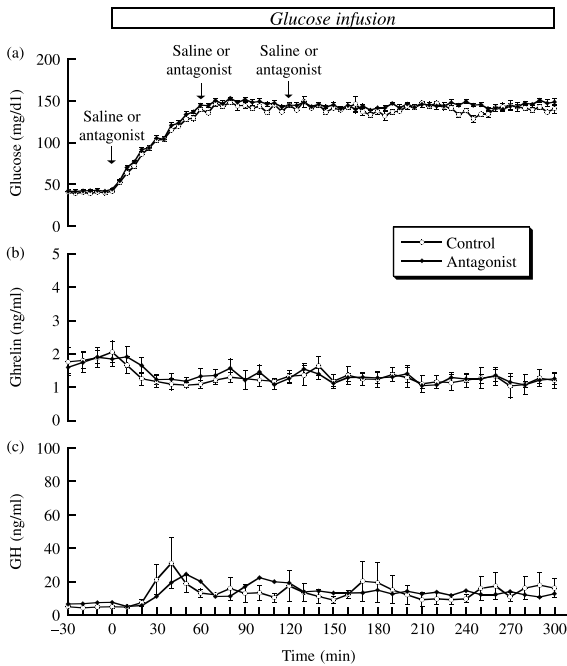
**Results**

In the HGC (Experiments 1 and 2), plasma glucose concentrations were clamped at 100 mg/100 ml above the initial level between 60 and 300 min after the start of glucose infusion (Figs 1a and 3a). There was no difference in basal plasma glucose concentrations before glucose infusion between the fasting (Fig. 1a) and meal-fed state (Fig. 3a).

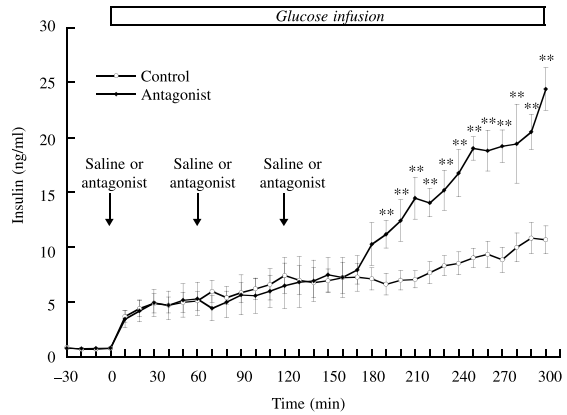
In the fasting state (Experiment 1), average plasma ghrelin level was 1.8 ng/ml before glucose infusion (Fig. 1b). Plasma ghrelin levels were significantly ( $P < 0.01$ ) decreased after the commencement of glucose infusion in both groups (Fig. 1b). Plasma GH levels were significantly ( $P < 0.05$ ) increased after glucose infusion in both groups (Fig. 1c). Plasma ghrelin and GH levels were not affected by the ghrelin antagonist (Fig. 1b and c).

Changes in plasma insulin levels in the fasting state are presented in Fig. 2. Plasma insulin levels were significantly ( $P < 0.01$ ) increased by glucose infusion in both groups. There was a greater incremental increase ( $P < 0.01$ ) in plasma insulin concentrations in the D-Lys3-GHRP6 group when compared with the control beginning about 60 min after the third administration of the antagonist.

In the meal-fed state (Experiment 2), average plasma ghrelin level was 0.5 ng/ml before glucose infusion (Fig. 3b). Plasma ghrelin levels significantly ( $P < 0.01$ ) increased and reached a plateau within 10 min after the commencement of ghrelin

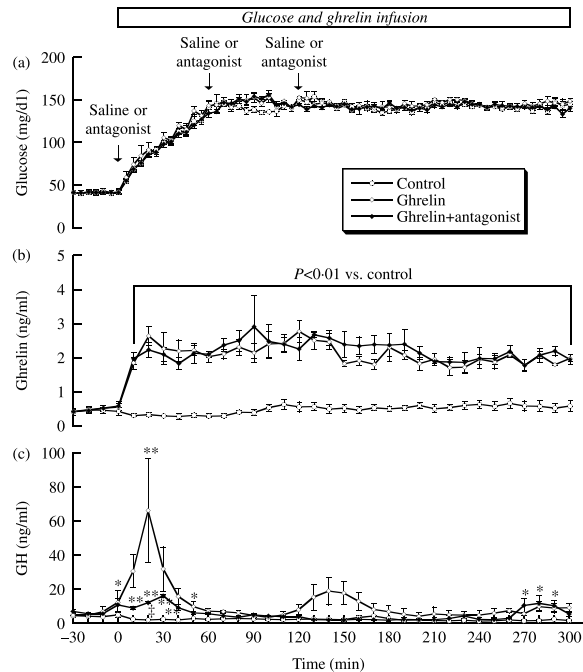


**Figure 1** (a) Average glucose, (b) ghrelin, and (c) GH plasma concentrations in fasted sheep receiving saline (control) or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) every 60 min during the first half of hyperglycemic clamp. Values are means  $\pm$  S.E.M. ( $n = 4$ ).

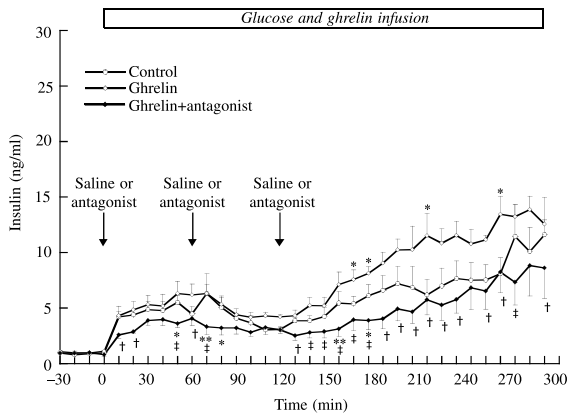


**Figure 2** Average insulin plasma concentrations in fasted sheep receiving saline (control) or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) every 60 min during the first half of hyperglycemic clamp. Values are means  $\pm$  S.E.M. ( $n = 4$ ). \*\* $P < 0.01$  versus control.

infusion (Fig. 3b). There were temporal increases ( $P < 0.05$ ) in plasma GH levels during ghrelin infusion (Fig. 3c). Increase in plasma GH levels was significantly ( $P < 0.01$ ) depressed by the ghrelin antagonist between the first- and second administration of the ghrelin antagonist (Fig. 3c).



**Figure 3** (a) Average glucose, (b) ghrelin, and (c) GH plasma concentrations in meal-fed sheep continuously receiving saline (control) or ghrelin (ghrelin, 0.04  $\mu$ g/kg body weight per min) during hyperglycemic clamp. Saline vehicle or D-lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) was administered every 60 min during the first half of hyperglycemic clamp. Values are means  $\pm$  S.E.M. ( $n = 4$ ). \* $P < 0.05$  versus control, \*\* $P < 0.01$  versus control, <sup>#</sup> $P < 0.01$  versus ghrelin.



**Figure 4** Average insulin plasma concentrations in meal-fed sheep continuously receiving saline (control) or ghrelin (ghrelin, 0.04 µg/kg body weight per min) during hyperglycemic clamp. Saline vehicle or D-Lys3-GHRP-6 (antagonist, total dose of 70 nmol/kg body weight) was administered every 60 min during the first half of hyperglycemic clamp. Values are means  $\pm$  S.E.M. ( $n=4$ ). \* $P<0.05$  versus control, \*\* $P<0.01$  versus control, † $P<0.05$  versus ghrelin, \* $P<0.01$  versus ghrelin.

Changes in plasma insulin levels in the meal-fed state are presented in Fig. 4. Plasma insulin levels were significantly ( $P<0.01$ ) increased by glucose infusion in all groups. There was a biphasic insulin increment in control and ghrelin-infused group. Ghrelin significantly ( $P<0.05$ ) enhanced only the second phase of insulin secretion. The ghrelin antagonist significantly ( $P<0.05$ ) depressed both the first- and second phase of insulin secretion.

## Discussion

Effects of ghrelin on insulin secretion are bi-directional, since ghrelin exerts both stimulatory and inhibitory effects on insulin secretion. Ghrelin inhibits insulin secretion in fasted humans (Broglia *et al.* 2001) and mice (Reimer *et al.* 2003). By contrast, i.v. injection of ghrelin accelerates insulin secretion in free-feeding rats (Lee *et al.* 2002) and fed sheep (Takahashi *et al.* 2006). Ghrelin stimulates insulin secretion from isolated pancreatic islets of freely fed rats (Date *et al.* 2002). Taken together, these reports suggest that ghrelin differentially modulates insulin secretion, dependent on feeding states. The present study has clearly demonstrated that ghrelin inhibits and stimulates glucose-induced insulin secretion in fasting- and meal-fed state respectively.

Baseline of plasma ghrelin fluctuates between 0.5 and 2.0 ng/ml depending on the feeding states in sheep. The fluctuation of plasma ghrelin makes it difficult for us to determine the effects of ghrelin, since it is uncertain which level of plasma ghrelin is most effective on insulin secretion in different feeding states. In order to determine physiological effects of ghrelin as far as possible, we avoided administering ghrelin to the fasting animals in which plasma ghrelin levels

had reached plateau (2.0 ng/ml), while we did not administer the ghrelin antagonist alone to the fed animals in which plasma ghrelin levels had reached nadir (0.5 ng/ml). Our previous study showed that ghrelin significantly enhanced glucose-stimulated insulin secretion at 1.0 ng/ml of plasma level in the meal-fed state (Takahashi *et al.* 2006). Therefore, the effect of the antagonist on insulin response at plasma ghrelin levels between 1.0 and 1.5 ng/ml during the HGC in the fasting states would be comparable with the effect of ghrelin administration in the meal-fed state.

In the meal-fed state, exogenous ghrelin enhanced glucose-induced insulin secretion as shown previously (Takahashi *et al.* 2006). The enhancement by ghrelin of glucose-stimulated insulin secretion was delayed in the present study when compared with the previous study. This may be related to weak insulin response to glucose in the animals newly applied to the HGC. Repeated doses of a ghrelin antagonist, D-Lys3-GHRP-6, counteracted the stimulatory effect of exogenous ghrelin on insulin secretion, suggesting that ghrelin stimulates insulin secretion via GHS-R1a in the meal-fed state. In addition, even lower insulin secretion by ghrelin plus antagonist compared with control suggests the possibility that basal ghrelin in the meal-fed state may also enhance insulin secretion.

In the fasting state, repeated doses of D-Lys3-GHRP-6 significantly enhanced glucose-induced insulin release, suggesting that endogenous ghrelin suppresses insulin secretion in the fasting state. The present result is supported by the finding that GHS-R antagonist enhances glucose-induced insulin release from perfused rat pancreas and increases plasma insulin levels in rats (Dezaki *et al.* 2006). In the present study, the insulin secretory response to the ghrelin antagonist emerged 60 min after the last dose of the drug. The hypoglycemic effect of the ghrelin antagonist emerges 2 days after the commencement of daily injections in diabetic mice where plasma ghrelin levels are high (Dong *et al.* 2006). In the fasting state, therefore, high levels of circulating ghrelin might compete with the ghrelin antagonist for GHS receptors, thus delaying the action of the drug.

Ghrelin stimulates GH secretion in sheep (Iqbal *et al.* 2006, Takahashi *et al.* 2006). In the present study, therefore, plasma GH concentrations were measured as an indicator for ghrelin receptor blockade. In the fed-state, D-Lys3-GHRP-6 attenuated ghrelin-induced GH secretion, indicating that the antagonist certainly blocked the ghrelin receptors. In the fasting state, however, the antagonist did not affect GH secretion, probably because GHS-R1a might not be involved in GH elevation after glucose infusion. Furthermore, ghrelin receptor mRNA expression is upregulated by fasting in the hypothalamus and pituitary (Kim *et al.* 2003). This may be related to the ineffectiveness of the ghrelin antagonist in GH suppression in the fasting state.

In conclusion, ghrelin inhibits and stimulates glucose-induced insulin secretion via GHS-R1a in fasting- and meal-fed state respectively. Further studies are required to identify

underlying mechanisms for alteration in the effects of ghrelin on insulin secretion in different feeding states.

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