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Dose-Dependent Inhibition by Ghrelin of Insulin Secretion in the Mouse

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Ghrelin is produced by stomach oxyntic cells and thought to be involved in the regulation of body weight and food intake. We demonstrate here that the peptide inhibits insulin secretion from overnight-incubated mouse islets in the presence of 8.3, 11.1, and 22.2 mmol/liter glucose. Ghrelin was most efficient at 1 nmol/liter and its effect disappeared by raising the dose more than 25 nmol/liter. Also, insulin secretion in the presence of high K^+ concentrations (20 mmol/liter) was inhibited by ghrelin. Furthermore, when administered iv to mice together with glucose (1 g/kg), ghrelin (50 nmol/kg) inhibited both the rapid 1-min insulin response (364 ± 90 *vs.* 985 ± 114 pmol/liter in controls, $P < 0.001$) and the area under the 50 min curve of insulin concentration (12.6 ± 1.2 *vs.* 15.6 ± 1.2 nmol/liter × 50 min; $P = 0.046$) without affecting the glucose disposal rate, insulin sensitivity or glucose effectiveness, *i.e.* glucose disposal independent from any dynamic change in insulin. The insulinostatic effect of ghrelin was inversely related to insulin sensitivity. In contrast, ghrelin had no influence at the lower dose of 5 nmol/kg and only slightly inhibited insulin secretion at the higher dose of 150 nmol/kg. These findings therefore show that ghrelin inhibits glucose-stimulated insulin secretion in the mouse. The effect is dependent on the dose and elicited on distal signaling steps in islet cells. The results suggest that the islet β-cells are targets for ghrelin. (*Endocrinology* 144: 916–921, 2003)

GHRELIN IS A PEPTIDE that in 1999 was isolated from rat stomach and postulated to be the natural ligand for the GH secretagogue receptor (GHSR) (1). A corresponding peptide in the mouse stomach was discovered by Tomasetto *et al.* (2), when isolating an mRNA of a novel 117-amino-acid peptide. This corresponding peptide was named motilin-related peptide and turned out to be the mouse equivalent to preproghrelin (2, 3). Ghrelin is produced by the A-like endocrine cells of the stomach (4, 5). It consists of a 28-amino-acid sequence with a unique octanoyl-modification of the serine moiety in position 3 (1). The peptide sequence is strongly conserved between mammalian species: rat and mouse ghrelin are identical having 26 of the 28 amino acids identical with human ghrelin (1, 4, 6). The peptide potently releases GH through an action on GHSR in pituitary cells (1, 7, 8) and exhibits orexigenic properties upon intracerebroventricular administration in rats (9). Ghrelin markedly enhances food intake and induces obesity in rats, suggesting a role for ghrelin in the central regulation of feeding behavior (10, 11). This is supported by results that transgenic rats expressing an antisense GHSR mRNA in the hypothalamus display reduced food intake and disturbances in fat deposition (12). In addition, ghrelin mRNA levels in the murine stomach are up-regulated by fasting (13), and circulating ghrelin levels are increased by fasting (5, 13) and reduced in obesity (14).

GHRs are expressed also in the pancreatic islets (15). This suggests that ghrelin is involved in the regulation of islet function. Studies performed so far have, however, resulted in conflicting results, because ghrelin has been shown to inhibit insulin secretion in the perfused rat pancreas (16) and in humans (17) but to stimulate insulin secretion in isolated rat islets (15) and *in vivo* in rats (6). These discrepancies may be explained by species differences, by the use of different dose levels of the peptide or by the use of *in vitro vs. in vivo* models; ghrelin might affect insulin secretion through both direct islet actions and indirectly. In this study, we have investigated whether ghrelin, at several different dose levels, affects glucose-stimulated insulin secretion from isolated mouse islets and *in vivo* in mice. Finally, we have also studied the influence of ghrelin on insulin sensitivity and glucose disposal independent from any changes in dynamic insulin.

Methods and Materials

Animals

Female C57BL/6J mice, weighing 23.0 ± 0.3 g, obtained from the Taconic M&B A/S (Ry, Denmark), were used for this study. The mice were fed a normal laboratory chow diet (fat 11.4%, carbohydrate 62.8%, protein 25.8% as energy; Research Diets, New Brunswick, NJ) and tap water *ad libitum*. The mice were kept in an air-conditioned room with lights on between 0600 and 1800 h. All studies were performed in the morning hours after a 3-h fast of the mice. The study was approved by the Ethics Committee of Lund University.

In vitro study

Islets from mice were isolated by collagenase digestion, hand-picked under a stereomicroscope, and incubated overnight in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin G, 0.1 mg/ml streptomycin (all from Kebo Laboratory, Spånga, Sweden), and 2.5 μg/ml Amphotericin B (Life Technologies, Inc., Täby, Sweden). Islets were then incubated in groups of three in 96-well microtiter plates in a HEPES buffer containing (in mmol/liter) 125 NaCl, 5.9 KCl, 1.28 $CaCl_2$, 1.2 $MgCl_2$, 25 HEPES, and 0.1% BSA (pH 7.36), with 3.3, 5.5, 8.3, 11.1, or 22.2 mmol/liter D-glucose (all from Sigma, St. Louis, MO) containing different concentrations of synthetic rodent ghrelin (Bachem, Bubendorf, Switzerland). In one series of experiments, the KCl concen-

Abbreviations: $AUC_{insulin}$, Area under the 50-min curve of insulin concentration; GHSR, GH secretagogue receptor; K_G, tolerance index; S_G, glucose effectiveness; S_I insulin sensitivity index.

tration was 20 mmol/liter and, in another series of experiments, arginine at 5 mmol/liter was added (Sigma). After a 60-min incubation at 37 C, the supernatant was collected and stored at –20 C until analysis.

In vivo study

The *in vivo* studies were performed in anesthetized mice after a 3-h fast during the late morning hours. The animals were anesthetized with an ip injection of midazolam (Dormicum, Hoffman-La-Roche, Basel, Switzerland; 0.14 mg/mouse) and a combination of fluanison (0.9 mg/mouse) and fentanyl (0.02 mg/mouse; Hypnorm, Janssen Pharmaceuticals, Beerse, Belgium). After 30 min, a blood sample (75 μl) was taken from the retrobulbar, intraorbital, capillary plexus in a 100-μl pipette that had been prerinsed in heparin solution (100 U/ml in 0.9% NaCl; Lövens, Ballerud, Denmark). Thereafter, D-glucose (British Drug Houses, Poole, UK) was injected iv over 3 sec at the dose of 1 g/kg in a tail vein without flushing of the 27-gauge needle after injection, either alone (n = 20) or together with synthetic ghrelin (Bachem; 5, 50, or 150 nmol/kg body weight; n = 6–15). The volume load was 10 μl/g body weight. Additional blood samples (75 μl each) were taken either at 1, 5, 20, and 50 min or at these time points and also at 10 and 30 min. Plasma was immediately separated and stored at −20 C until analyses.

Analyses

Insulin concentration was determined by a double-antibody RIA using guinea pig antirat insulin antibodies, ^{125}I-labeled human insulin, and, as standard, rat insulin (Linco Research, Inc., St. Charles, MO). Ghrelin concentration was determined by a double-antibody RIA using rabbit antihuman ghrelin antibodies, ^{125}I-labeled human ghrelin, and, as standard, purified recombinant human ghrelin; the antibody showing cross reactivity with rodent ghrelin (Linco Research, Inc.). Glucose was measured by the glucose oxidase technique.

Calculations

Insulin and glucose data from the seven sample iv glucose tolerance test were analyzed with the minimal model technique as already reported in details (18). This analysis provides parameter S_I (insulin sensitivity index) that is defined as the ability of insulin to enhance net glucose disappearance and inhibit glucose production, and the parameter S_G, which is the glucose effectiveness, representing net glucose disappearance *per se* from plasma without any change in dynamic insulin. Total insulin secretion was assessed from the 50-min curve of insulin concentration ($AUC_{insulin}$) using the trapezoidal rule. Glucose disappearance rate was evaluated with the tolerance index (K_G), calculated as the slope for the interval 1–20 min after glucose injection after logarithmic transformation of the individual plasma glucose values.

Statistics

Data and results are reported as means ± SEM. Statistical comparisons between two groups were performed with unpaired Student's *t* test. ANOVA with Bonferroni *post hoc* analysis was exploited for multiple comparisons. Pearson's product moment correlation coefficients were obtained to estimate linear correlations between variables.

Results

Effect of ghrelin on insulin secretion from isolated mouse islets

In the presence of 8.3, 11.1, or 22.2 mmol/liter glucose, ghrelin at doses between 0.01 and 1 nmol/liter inhibited insulin secretion from islets incubated for 60 min (Fig. 1). Ghrelin was most effective at 1 nmol/liter, whereas higher doses showed less efficient response. At more than 25 nmol/liter, the peptide was without effect. In contrast, in the presence of 3.3 or 5.5 mmol/liter glucose, ghrelin did not affect insulin secretion, showing that ghrelin requires a stimulatory level of glucose for inhibiting insulin secretion (*lower panel* in Fig. 1). Furthermore, ghrelin (10 nmol/liter) also inhibited

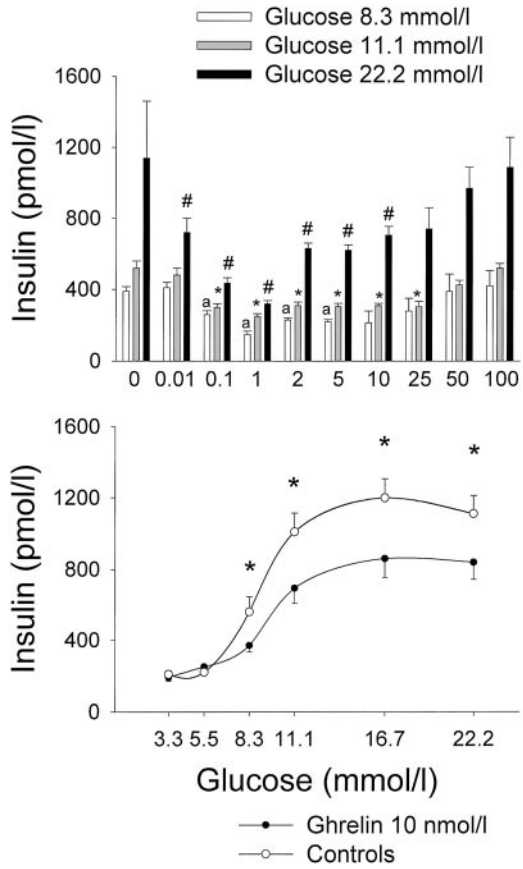

FIG. 1. *Upper panel*, Medium insulin concentrations after a 60-min incubation of isolated mouse islets (in groups of three) in the presence of 8.3, 11.1, or 22.2 mmol/liter glucose and different concentrations of ghrelin. Means ± SEM are shown (n = 20–24 in each column). *Asterisks* indicate probability level of random difference of $P < 0.05$ *vs.* respective controls incubated without ghrelin as assessed by ANOVA with Bonferroni *post hoc* test for each glucose concentration. [a], $P < 0.05$ against 8.3 mmol/liter glucose; *, $P < 0.05$ against 11.1 mmol/liter glucose; #, $P < 0.05$ against 22.2 mM glucose. *Lower panel*, Medium insulin concentrations after a 60-min incubation of isolated mouse islets (in groups of three) in the presence of glucose at 3.3, 5.5, 8.3, 11.1, 16.7, or 22.2 mmol/liter with or without ghrelin at 10 nmol/liter (n = 12–24 in each group). *Asterisks* indicate probability level of random difference of $P < 0.05$ *vs.* respective controls incubated without ghrelin as assessed by Student's *t* test.

the insulin response to high K^+ (20 mmol/liter), both at 3.3 and 11.1 mM glucose ($P < 0.001$; Fig. 2). To study whether ghrelin is released from isolated mouse islets, the islet were incubated for 60 min in the presence of 3.3 mmol/liter glucose (n = 8 incubations with three islets in each), of 16.7 mmol/liter glucose (n = 8) or of 3.3 mmol/liter glucose together with 5 mmol/liter arginine (n = 8), and medium ghrelin levels were determined by RIA. However, under all these conditions, ghrelin levels were below detection limit in the assay (100 pg/ml).

Effect of ghrelin on insulin secretion and glucose disposal in mice

The influence of ghrelin on insulin secretion *in vivo* was studied by administering the peptide together with glucose (1 g/kg) in an iv glucose tolerance test. It was found that the

1-min insulin response was inhibited by the peptide at 50 and 150 nmol/kg, whereas ghrelin at 5 nmol/kg had no effect (Table 1, Fig. 3). The action of ghrelin (50 nmol/kg) on glucose disposal was studied using the minimal model analyses of insulin and glucose data derived from the seven sample iv glucose tolerance test (Fig. 3). It was found that whereas ghrelin inhibited insulin secretion both when calculated as the 1-min insulin response and the $AUC_{insulin}$ (Table 1), the glucose elimination rate, K_G, was not different between the groups (1.79 ± 0.24%/min in mice given glucose and ghrelin *vs.* 1.86 ± 0.19%/min in controls; $P > 0.2$). The minimal model analysis of glucose disappearance showed that ghrelin did not affect S_I (1.12 ± 0.10 *vs.* 1.02 ± 0.09 10^{-4} min^{-1}/(pmol/liter) of controls) or S_G (0.055 ± 0.006 *vs.* 0.060 ± 0.009 min^{-1}). However, analyses of the effect of ghrelin in relation to the insulin sensitivity of each mouse revealed that the insulinostatic action of ghrelin was dependent on the ambient S_I. This was demonstrated by two approaches. First, the effects of ghrelin on insulin secretion in mice with low *vs.* with high insulin sensitivity were compared. This was achieved by arbitrarily dividing the mice in two equally sized groups with high [>1.08 10^{-4} min^{-1}/(pmol/liter); n = 14] or low [≤1.08 10^{-4} min^{-1}/(pmol/liter); n = 14] S_I. When comparing the influence of ghrelin in these two groups, $AUC_{insulin}$ was not reduced by ghrelin in mice with low S_I (15.8 ± 1.9 *vs.* 16.8 ± 1.6 nmol/liter × 50 min; $P = 0.464$), whereas ghrelin inhibited insulin secretion in mice with high S_I (10.8 ± 0.6 *vs.* 13.5 ± 0.7 nmol/liter × 50 min; $P = 0.030$). Second, S_I was related to $AUC_{insulin}$ across all animals. S_I and $AUC_{insulin}$ were thereby related to each other in a hyperbolic manner ($r = -0.72$, $P < 0.001$), resulting in a linear regression between the logarithmically transformed data for S_I and $AUC_{insulin}$. Significant correlations were found also in the single groups ($r = -0.58$, $P = 0.025$ in controls and $r = -0.53$, $P = 0.039$ in mice given glucose and ghrelin). The slopes of the regression lines were, however, different between the groups, being −0.50 ± 0.15 in the group given glucose and ghrelin *vs.* −0.36 ± 0.14 in the controls ($P = 0.042$; Fig. 4).

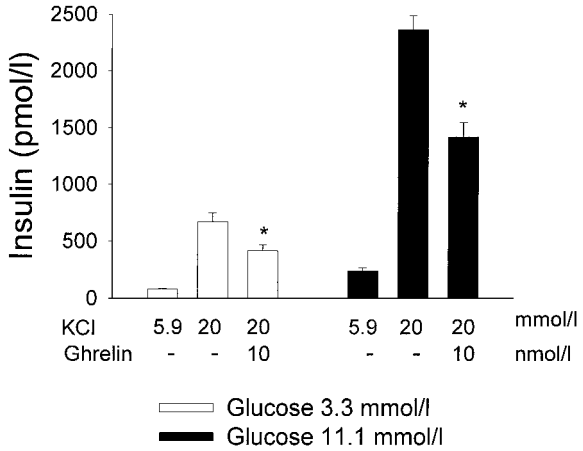

FIG. 2. Medium insulin concentrations after 60-min incubation of isolated mouse islets (in groups of three) in the presence of 3.3 or 11.1 mmol/liter glucose with or without 20 mmol/liter KCl and/or 10 nmol/liter ghrelin. Means ± SEM (n = 22–24 in each column). *Asterisks* indicate probability level of $P < 0.001$ of random difference in respective groups incubated at high KCl with *vs.* without ghrelin as assessed by Student's *t* test.

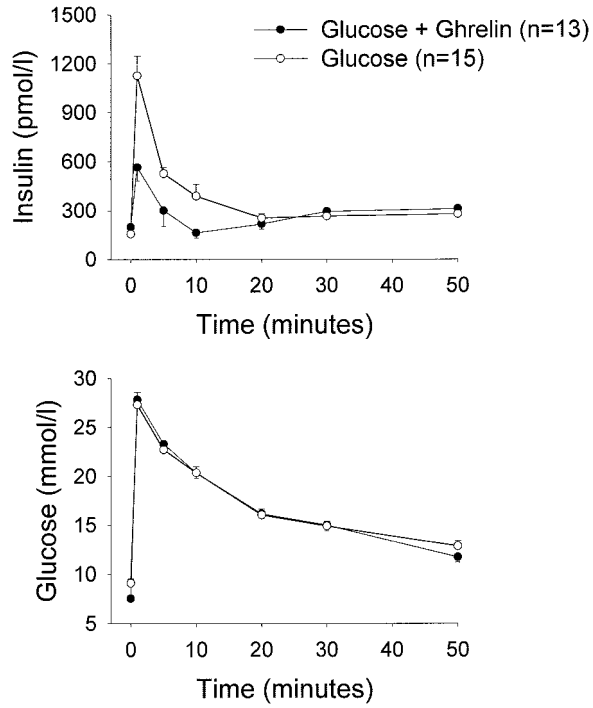

FIG. 3. Glucose and insulin levels during the iv glucose test (1 g/kg) with or without addition of ghrelin at 50 nmol/kg. Means ± SEM are shown.

TABLE 1. The insulin response to iv glucose (1 g/kg) with or without concomitant administration of ghrelin (5, 50, or 150 nmol/kg) in anesthetized mice

Experiment	1-min insulin response (pmol/liter)		$AUC_{insulin}$ (nmol/liter × 50 min)	
	Ghrelin	Controls	Ghrelin	Controls
Ghrelin 5 nmol/kg (n = 12)	818 ± 106	1036 ± 96 ($P = 0.018$)	14.1 ± 2.3	16.4 ± 2.0
Ghrelin 50 nmol/kg (n = 13–15)	364 ± 90	985 ± 114 ($P < 0.001$)	12.6 ± 1.2	15.2 ± 1.2 ($P = 0.046$)
Ghrelin 150 nmol/kg (n = 6)	493 ± 86	878 ± 103 ($P = 0.026$)	13.9 ± 2.1	15.3 ± 1.9

Samples were taken before and at 1, 5, 20, and 50 min after injection in the experiments with ghrelin at 5 and 150 nmol/kg and additionally at 10 and 30 min in the experiments with ghrelin at 50 nmol/kg. Controls injected with glucose alone were run in all individual experiments. The 1-min insulin response (difference in insulin levels between 1 min postinjection and preinjection samples) and the $AUC_{insulin}$ over 50 min are shown (means ± SEM). *Numbers in parentheses* indicate number of animals in each experimental group, and *P* values indicate probability level of random difference between respective experimental groups.

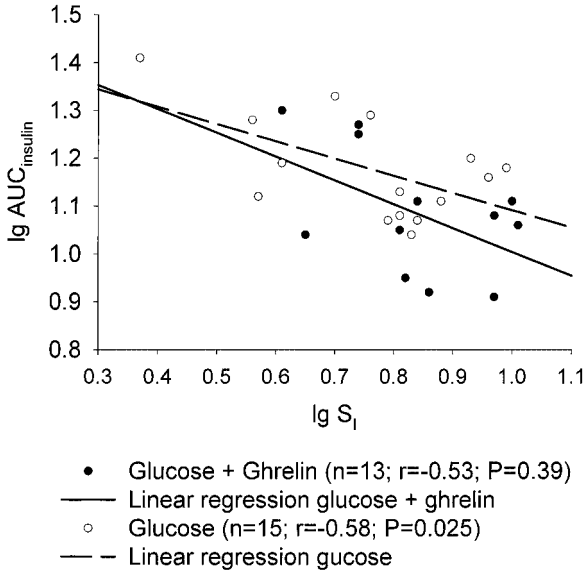

FIG. 4. Logarithmic plot of S_I *vs.* $AUC_{insulin}$ in mice given iv glucose (1 g/kg) alone (n = 15, *open circles*, *dotted line*) or glucose with addition of ghrelin (50 nmol/kg; n = 13; *closed circles*, *continuous line*). Statistics of the regression lines are reported in the text.

Thus, ghrelin augmented the slope between lg $AUC_{insulin}$ and lg S_I, indicating higher inhibition of insulin secretion at elevated S_I.

Discussion

We show that ghrelin *in vivo* inhibits glucose-stimulated insulin secretion at 50 and 150 nmol/kg in the mouse. We also show that this effect is executed by a direct islet action of the peptide because at dose levels between 10 pmol/liter and 10 nmol/liter, ghrelin inhibited the insulin response to glucose from isolated mouse islets. A stimulatory level of glucose was required to detect the inhibitory effect of ghrelin; hence, the peptide does not seem to affect baseline insulin secretion. These results confirm previous observation in the perfused rat pancreas, where Egido *et al.* (16) showed that ghrelin at 10 nmol/liter inhibits glucose-stimulated insulin secretion. Similarly, iv administration of ghrelin at 1 μg/kg reduced circulating insulin in humans in spite of increasing circulating glucose (17). In contrast, ghrelin at 1 pmol/liter stimulated insulin secretion at 8.3 mmol/liter glucose from isolated rat islets (15) and iv administration of ghrelin (25 nmol/rat) increased circulating insulin in rats (6). Because we found an inhibitory influence of ghrelin both *in vitro* and *in vivo* and no hint of a stimulatory action, the discrepancy in the literature is difficult to explain. One possibility is species differences but also other possibilities exist. One such is long-term *vs.* short-term actions of the peptide. Thus, the stimulatory action of ghrelin on insulin levels in rats was observed at 15 min but not at 5 min after ghrelin administration (6), whereas we found the inhibition when administering ghrelin together with glucose.

We found that the inhibitory action of ghrelin on insulin secretion *in vitro* was dose and glucose dependent. We also found that ghrelin inhibited insulin secretion induced by high K^+-concentrations. Both glucose and K^+ cause depolarization of the β-cells allowing inflow of Ca^{2+}, which activates the exocytosis machinery (19). The glucose effect requires, however, intracellular metabolism for the generation of ATP, which closes ATP-regulated K^+ channels causing the depolarization, whereas K^+ directly depolarizes the cell membrane, thereby bypassing the metabolism of glucose (19). The finding that ghrelin inhibits K^+-induced insulin secretion indicates, therefore, that the effect is executed at a distal step beyond the metabolism of glucose and depolarization by ATP-regulated K^+-channels of the plasma membrane in the islet β-cells. This supports the study by Egido *et al.* (16) in the perfused rat pancreas, where ghrelin inhibited insulin secretion stimulated by glucose, by arginine and by the cholinergic agonist carbachol, which all use different proximal stimulus-secretion coupling mechanisms for eliciting insulin secretion. Whether the effect is due to a direct inhibitory action on the exocytosis machinery or to an inhibition of the action of intracellular Ca^{2+} remains to be established.

We found a U-shaped dose-response relationship between the dose of ghrelin and inhibition of insulin secretion in which concentrations around 1 nmol/liter exerted most potent action, whereas the effect disappeared at levels above 25 nmol/liter. This is similar to the action of leptin on isolated rodent islets, where the inhibitory action of leptin on glucose-stimulated insulin secretion disappeared by raising the dose of the peptide (20). The dose-response relationship for leptin on insulin secretion has been ascribed to desensitization of oligomerization of the Ob receptor by a high dose (20). However, it is unlikely that ghrelin works through a similar signaling, because the GHSR, which transmits the effects of ghrelin and is expressed in the pancreas (15, 21), is a G protein-coupled seven-transmembrane receptor (22), whereas the Ob-receptor is a cytokine-like receptor (23). The pharmacological properties of ghrelin in our experiments might instead suggest that more than one receptor form of GHSR with different sensitivities to the ligand are responsible for the islet effects of ghrelin. However, also this explanation is unlikely because although several different receptor forms of GHSR exist, ghrelin seems to be a ligand for only GHSR-1a (24, 25). It is therefore more likely that the biphasic effect of ghrelin is explained by ligand-receptor characteristics, the nature of which however needs to be explored in more detail.

Because ghrelin is mainly a hormone produced by the stomach, it is possible that the inhibitory effect of ghrelin on insulin secretion reflects a hormonal function of the peptide. This is consistent with the inhibitory effect of ghrelin in the subnanomolar concentration range because the circulating level of the peptide has been shown to be in the range of 100–150 pmol/liter (14, 26). Plasma ghrelin is increased by fasting (5, 13) and therefore it may be suggested that the peptide contributes to the inhibition of insulin secretion under fasting conditions to maintain glucose levels. Ghrelin might therefore not only function as a hunger signal to the brain, but also to the periphery. However, recent studies have localized ghrelin also inside the pancreatic islets: a study in rat islets showed expression in the peripherally located glucagon-containing cells (15), whereas a study in human islets showed expression in the centrally located in-

sulin-containing cells (27). Islet expression of ghrelin would suggest that also a local islet action of the peptide may underlie its insulinostatic property. This is, however, still an open question because we were unable to detect any release of ghrelin from incubated islets under conditions when the release insulin (high glucose) or glucagon (high arginine) is stimulated.

The use of the iv glucose tolerance test in the present study allowed conclusions of any potential influence of ghrelin on glucose disposal. This process consists of a combination of insulin-dependent mechanisms (insulin sensitivity) and glucose effectiveness, *i.e.* the glucose disappearance independent from any dynamic change of insulin (18). The minimal model analysis of glucose tolerance test data allowed calculation of these parameters whose result was unchanged with ghrelin. This explains why ghrelin did not affect glucose tolerance in spite of its marked insulinostatic action; in fact, it has been demonstrated that K_G in mice is more dependent on glucose effectiveness than on insulin secretion and sensitivity (18).

It is known that insulin sensitivity and insulin secretion display an inverse curvilinear relation, *i.e.* mice with high insulin sensitivity have a low insulin response to glucose and *vice versa* (18). In both mice and humans, the relation between insulin sensitivity and insulin secretion is mathematically described by a hyperbolic function (18, 28), which is equivalent to a linear relation between the logarithmically transformed data, as illustrated in Fig. 4. We found that ghrelin significantly altered this relation, and this was due to a failure of ghrelin to inhibit insulin secretion in mice with low insulin sensitivity, whereas ghrelin exerted a marked inulinostatic action in mice with high insulin sensitivity. This suggests that the control by ghrelin of insulin secretion is modulated by insulin sensitivity: when more insulin is required to counteract high insulin resistance, the inhibitory effect of ghrelin on insulin release is relaxed. However, the nature of the biochemical mechanisms involved in this putative process is yet not known.

In conclusion, our study has shown that ghrelin at specific doses inhibits glucose-stimulated insulin secretion in mice by a direct action on the islets and, furthermore, that the effect most likely is executed by a distal action on the β-cell signaling machinery. This result therefore suggests that the β-cell is a target of action for ghrelin.

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