Unacylated ghrelin acts as a potent insulin secretagogue in glucose-stimulated conditions

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Unacylated ghrelin acts as a potent insulin secretagogue in glucose-stimulated conditions. Am J Physiol Endocrinol Metab 293: E697–E704, 2007. First published June 19, 2007; doi:10.1152/ajpendo.00219.2007.—Acylated and unacylated ghrelin (AG and UAG) are gut hormones that exert pleiotropic actions, including regulation of insulin secretion and glucose metabolism. In this study, we investigated whether AG and UAG differentially regulate portal and systemic insulin levels after a glucose load. We studied the effects of the administration of AG (30 nmol/kg), UAG (3 and 30 nmol/kg), the ghrelin receptor antagonist [d-Lys3]GHRP-6 (1 μmol/kg), or various combinations of these compounds on portal and systemic levels of glucose and insulin after an intravenous glucose tolerance test (IVGTT, d-glucose 1 g/kg) in anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced in anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced anesthetized fasted Wistar rats. UAG administration po...
regulates the portal and systemic insulin response to glucose and/or modulates hepatic insulin clearance.

We therefore studied in rats the effects of the administration of AG, UAG, the ghrelin receptor antagonist [d-Lys3]GHRP-6, or their combinations on portal and peripheral glucose and insulin levels during an intravenous glucose tolerance test (IVGTT).

MATERIALS AND METHODS

Materials

Plasma glucose levels were measured using a glucose oxidase method (Instruchemie, Delfzijl, The Netherlands). Rat insulin was measured using a rat insulin ELISA kit (Merckodia, Uppsala, Sweden). Total and acylated ghrelin were measured using radioimmunoassays (RIAs) from Linco Research (St. Charles, MO). Rat acylated and unacylated (des-octanoyl) ghrelin, as well as [d-Lys3]GHRP-6, were obtained from NeoMPS (Strasbourg, France). Pentobarbital sodium (250 mg/5 ml) was prepared and provided by the hospital pharmacy (Erasmus MC, Rotterdam, The Netherlands). EDTA-containing tubes were obtained by Greiner Bio-One (Alphen aan den Rijn, The Netherlands). Silicone catheters (3-French size) were provided by UNO Roestvaststaal (Zevenaar, The Netherlands); suture needles (Dafilon 8/0) were by B. Braun Melsungen (Melsungen, Germany).

Animals

Male Wistar rats (age 10–12 wk, weight 350–400 g; Harlan Netherlands, Horst, The Netherlands) were housed in groups in a temperature-controlled room under a 12:12-h light-dark cycle and maintained on pelleted chow with free access to water. The animals were housed for at least 1 wk before the start of the experiments to allow for acclimatization. Animal protocols were in compliance with the principles of laboratory animal care and Dutch regulations on animal welfare and were approved by the institutional Animal Welfare Committee.

Surgery and Experimental Design

All studies were performed after a fasting period of 18 h (overnight). Studies were performed under anesthesia, and the rats were euthanized at the end of the experiment.

Animals were anesthetized using an intraperitoneal (ip) injection of pentobarbital sodium (60 mg/kg induction, 20 mg/kg maintenance administered at the end of the surgical procedure, before the start of the experimental session). Deep anesthesia was confirmed by the absence of reflexes. Animals were kept on a warming mat to maintain core body temperature and were connected to a breathing apparatus (O2, 1 l/min), to improve oxygenation, for the entire duration of the experiment (including surgical procedure).

The surgical procedure was performed under aseptic conditions, as follows.

**Cannulation of the jugular vein.** An incision was made just above the right clavicle, the connective and adipose tissues were pushed aside, and the jugular vein was exposed. After the jugular vein was mobilized, a catheter previously connected to a syringe and filled with saline solution was pushed inside the vessel until it reached the right atrium. Patency of the catheter was checked by aspirating blood and flushing the catheter with saline solution. The free end of the catheter was used for saline injection, treatment administration, and sampling.

**Cannulation of the portal vein.** A midline incision was made from the level of the symphysis pubis to the xiphoid cartilage. The intestines were lifted out and laid next to the animal on gauze moistened with warm saline solution to minimize dehydration. A purse-string (diameter ~1 mm) was made in the wall of the portal vein, opposite the gastroduodenal vein. The center of the purse-string was cut, and the catheter was inserted into the portal vein and pushed in for a few millimeters with the tip secured ~1 mm caudal to the liver. The patency of the catheter was checked by aspirating blood and injecting saline. The free end of the cannula was used for sampling procedure during the experiment.

**Treatment Administration and Sampling**

Rats (fasted overnight) were assigned to one of the following treatment groups: 1) Saline (1 ml), n = 12; 2) IVGTT, n = 12. IVGTT was performed by injecting d-glucose at a dose of 1 g/kg (50%, 1 ml maximal volume) through the jugular catheter. The dose of 1 g/kg was chosen taking into account the reduction of insulin sensitivity caused by abdominal surgery (23) and the possible interference due to anesthesia (1, 19). Pentobarbital sodium was used, since compared with other anesthetics it has been shown to interfere less with insulin secretion and glucose metabolism in both the fed and the fasted conditions (1, 19), in accord with our previous observations (unpublished data). The other groups were 3) IVGTT + rat AG (30 nmol/kg), n = 7; 4) IVGTT + rat UAG (3 nmol/kg), n = 6; 5) IVGTT + UAG (30 nmol/kg), n = 10; 6) IVGTT + [d-Lys3]GHRP-6 (1 nmol/kg), n = 6; 7) IVGTT + [d-Lys3]GHRP-6 (1 µmol/kg) + AG (30 nmol/kg), n = 6; 8) IVGTT + [d-Lys3]GHRP-6 (1 µmol/kg) + UAG (30 nmol/kg), n = 7; and 9) IVGTT + AG (30 nmol/kg) + UAG (30 nmol/kg), n = 7.

After baseline samples had been taken from both catheters, treatments were administered through the jugular cannula at time 0, and samples were taken from both catheters at 1, 5, 10, 20, 30, and 50 min after treatment administration to measure glucose and insulin levels. At baseline, total and acylated ghrelin levels were also measured in 24 rats (before they were assigned to different treatment groups). At every time point, the blood volume withdrawn from each catheter (350 µl) was replaced by an equal volume of saline solution.

Blood samples were collected using ice-cold EDTA-containing tubes, to which aprotinin (Trasylol, 5000 KIE, 40 µl/ml) was added. Samples were immediately centrifuged, and plasma aliquots for AG measurements were acidified with 1 N HCl (1:10 vol/vol). All aliquots were kept at 4°C until the end of the experiment and then stored at –20°C. Multiple freeze-thaw cycles were avoided, and aliquots were thawed only for the ghrelin assay. This procedure has been indicated by Hosoda et al. (17) and by Groschl et al. (15) as a standard procedure for collection of blood samples to determine ghrelin concentrations.

At the end of each experiment the animals were killed by exsanguination under deep anesthesia.

Serum total ghrelin and AG levels (pg/ml) were measured using RIA kits that utilize 125I-labeled ghrelin as a tracer. The specificity for rat ghrelin (total and AG, respectively) is 100%. Total ghrelin is recognized by polyclonal rabbit antibodies raised against full-length ghrelin. This antibody recognizes intact and des-octanoyl ghrelin and ghrelin residues 14–28. The sensitivity of the assay is 93 pg/ml; the intra-assay coefficient of variation (CV) averages 6.4%, the interassay CV 16.3%. AG is recognized by a guinea pig anti-ghrelin specific for the ghrelin molecule octanoylated at its Ser3 residue. This antibody recognizes intact and des-octanoyl ghrelin and ghrelin residues 14–28. The sensitivity of the assay is 7.8 pg/ml; the intra-assay CV is 7.4% and the interassay CV is 13.5%.

Insulin was measured using a rat insulin ELISA kit according to the manufacturer’s instructions. The sensitivity of the assay is 0.07 µg/L.

Calculations

**UAG.** UAG levels were calculated by subtracting AG from total ghrelin concentrations at every time point either in the portal or in the peripheral (i.e., right atrium) vein samples.

**Hepatic clearance.** To estimate whether the liver might play a role in the clearance of ghrelin produced by the gut, we calculated the percentage of hepatic clearance by using a method originally proposed
by Kaden et al. (20). The percentage of hepatic extraction of any given hormone is calculated as (hormone presented to the liver − hormone leaving the liver) × 100/(hormone presented to the liver). The ratio of the relative contribution of a "hormone presented to the liver" by the portal vein vs. the hepatic artery (concentration × flow) is assumed to be 3:1 (2). The percentage of portal hormone extraction is calculated as (hormone concentration in the portal vein − hormone concentration in the hepatic vein) × 100/(hormone concentration in the portal vein). Since the contribution to posthepatic insulin levels due to tissues that do not drain in the portal vein is negligible, we assumed that the insulin gradient between portal vein and right atrium is a valid proxy of hepatic clearance, although in the right atrium insulin concentration may be affected by a greater dilution (due to the ancillary venous return) than in the hepatic vein.

Results are expressed as absolute Δ changes vs. baseline (means ± SE) and as Δareas under the curve (ΔAUCs) (means ± SE).

Statistical Analysis

Statistical analysis was performed using SPSS for Windows 10.0 (Chicago, IL). The one-way analysis of variance (ANOVA) was used to compare the several treatment groups for baseline levels and ΔAUCs of each parameter. The one-way repeated-measures ANOVA was used to verify whether, for each group and each parameter, there was an overall difference over the 50-min time course. An independent t-test was performed to compare two groups, whereas a paired t-test was also run to compare changes vs. baseline and jugular vs. portal values within each group. A difference was considered significant when P < 0.05.

RESULTS

AG and UAG Baseline Levels

The AG concentration in the portal vein was 1.7-fold higher than in the systemic circulation (108 ± 13 vs. 63 ± 5 pg/ml, respectively, P < 0.001), whereas the portal-peripheral gradient of UAG was 1.1 (1,449 ± 92 vs. 1,286 ± 71 pg/ml). The AG/UAG ratio was already very low in the portal vein, and it decreased further in the systemic circulation (0.075 ± 0.006 vs. 0.049 ± 0.003, respectively, P < 0.01).

Effects of IVGTT, Alone or Combined With Different Treatments, on Glucose and Insulin Levels

Baseline glucose and insulin levels were not significantly different among all groups both in the portal and in the systemic circulation (Table 1).

Table 1. Baseline absolute levels of glucose and insulin in portal and in systemic circulation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose, mmol/l</th>
<th>Insulin, μg/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Portal</td>
<td>Systemic</td>
</tr>
<tr>
<td>Saline (n = 12)</td>
<td>7.6±1.1</td>
<td>9.2±1.2</td>
</tr>
<tr>
<td>IVGTT controls (n = 12)</td>
<td>7.9±0.8</td>
<td>10.1±0.7</td>
</tr>
<tr>
<td>IVGTT + AG, 30 nmol/kg (n = 7)</td>
<td>10.2±1.0</td>
<td>10.1±0.8</td>
</tr>
<tr>
<td>IVGTT + UAG, 3 nmol/kg (n = 6)</td>
<td>9.1±1.2</td>
<td>9.5±1.4</td>
</tr>
<tr>
<td>IVGTT + UAG, 30 nmol/kg (n = 10)</td>
<td>6.2±0.8</td>
<td>7.7±1.1</td>
</tr>
<tr>
<td>IVGTT + AG + UAG (n = 7)</td>
<td>6.9±0.9</td>
<td>7.3±1.2</td>
</tr>
<tr>
<td>IVGTT + [d-Lys3]GHRP-6 (n = 6)</td>
<td>9.8±1.0</td>
<td>9.8±1.0</td>
</tr>
<tr>
<td>IVGTT + [d-Lys3]GHRP-6 + AG (n = 6)</td>
<td>9.5±1.9</td>
<td>10.5±1.2</td>
</tr>
<tr>
<td>IVGTT + [d-Lys3]GHRP-6 + UAG (n = 7)</td>
<td>8.2±0.9</td>
<td>10.1±2.2</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of animals. IVGTT, iv glucose tolerance test; AG, acylated ghrelin; UAG, unacylated ghrelin. Baseline absolute levels of glucose and insulin were not significantly different among the treatment groups in either the portal or the systemic circulation.

After saline injection (1 ml), insulin levels showed a small and transient decrease in both the portal and the peripheral circulation (ΔAUC, P < 0.01 and P < 0.05 vs. baseline, respectively; Fig. 1, A and C), whereas glucose levels did not show significant variations at any time point (Fig. 2, A and C). The AG concentration in the portal vein was 1.7-fold higher than in the systemic circulation (Table 1).

As expected, IVGTT induced a prompt increase in insulin levels in both the portal and in the jugular samples. The insulin peak occurred at 1 min of our time course and was larger in the portal vein than in the systemic circulation (Fig. 1, A and C). ΔInsulin levels were higher in the IVGTT than in the saline group during the whole time course (ΔAUC, P < 0.0005; Fig. 1, A and C). Of course, IVGTT promptly increased glucose levels, which were higher in the systemic than in the portal circulation and were reduced by the elevated circulating insulin, although they had not normalized yet after 50 min (P < 0.0005 vs. baseline and vs. saline; Fig. 2 and Table 2).

The administration of exogenous AG (30 nmol/kg) did not change the insulin response to IVGTT significantly, although a small and transient decrease was recorded in portal, but not in systemic, insulin levels (Fig. 1, A–D). Moreover, the administration of AG did not modify glucose levels (excursion curves and ΔAUCs) after IVGTT either in the portal or in the systemic samples (Fig. 2 and Table 2).

Administration of UAG dose-dependently increased the second-phase insulin response to IVGTT in the portal vein. In fact, after peaking at 1 min, insulin decreased and started gradually to rise again at 10 min and reached the highest level at 50 min (ΔAUC, IVGTT + UAG 3 nmol/kg vs. IVGTT, P < 0.004; IVGTT + UAG 30 nmol/kg vs. IVGTT, P < 0.0005; Fig. 1A). The insulin response to IVGTT during the whole time course (ΔAUC) was clearly and dose-dependently increased by UAG, although statistical significance was reached only at 30 nmol/kg (P < 0.001 vs. IVGTT; Fig. 1B). In the systemic circulation, the stimulatory effect of UAG at 30 nmol/kg was still detectable, although much less than in the portal vein (ΔAUC, P < 0.05; Fig. 1, C and D). However, portal and systemic glucose levels after IVGTT were not modified significantly by UAG (Fig. 2 and Table 2).

The GHS-R1a antagonist [d-Lys3]GHRP-6 (1 μmol/kg), like UAG, enhanced the second-phase insulin response to glucose in the portal vein. Portal insulin levels gradually
significant for \[ \text{P} \]\[D-Lys3\]GHRP-6 rats treated with \[ \text{D-Lys3} \]\[D-Lys3\]GHRP-6/H9004

Portal insulin (AG) did not modify insulin levels. This insulin-secretagogue effect of UAG was much larger in the portal vein (Fig. 1). Unacylated ghrelin (UAG) dose-dependently stimulated the second-phase insulin response to an iv glucose load (IVGTT, 1 g/kg), whereas exogenous acylated ghrelin (AG) did not modify insulin levels. This insulin-secretagogue effect of UAG was much larger in the portal vein (Fig. 1).

Unacylated ghrelin (UAG) dose-dependently stimulated the second-phase insulin response to an iv glucose load (IVGTT, 1 g/kg), whereas exogenous acylated ghrelin (AG) did not modify insulin levels. This insulin-secretagogue effect of UAG was much larger in the portal vein (Fig. 1).

### Table 2. Glucose and insulin (levels \( \Delta \text{AUC} \)) in portal and systemic circulation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Glucose ( \Delta \text{AUC}, \text{mmol}^{-1} \text{min}^{-1} )</th>
<th>Insulin ( \Delta \text{AUC}, \mu g^{-1} \text{min}^{-1} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Portal</td>
<td>Systemic</td>
</tr>
<tr>
<td>Saline IVGTT</td>
<td>( -4 \pm 2.7 )</td>
<td>( 14 \pm 1.6 )</td>
</tr>
<tr>
<td>IVGTT controls (n = 12)</td>
<td>( 711 \pm 65 )</td>
<td>( 778 \pm 68 )</td>
</tr>
<tr>
<td>IVGTT + AG, 30 nmol/kg (n = 7)</td>
<td>( 604 \pm 55 )</td>
<td>( 730 \pm 44 )</td>
</tr>
<tr>
<td>IVGTT + UAG, 3 nmol/kg (n = 6)</td>
<td>( 693 \pm 61 )</td>
<td>( 818 \pm 60 )</td>
</tr>
<tr>
<td>IVGTT + UAG, 30 nmol/kg (n = 10)</td>
<td>( 716 \pm 51 )</td>
<td>( 819 \pm 46 )</td>
</tr>
<tr>
<td>IVGTT + AG + UAG (n = 7)</td>
<td>( 666 \pm 50 )</td>
<td>( 856 \pm 59 )</td>
</tr>
<tr>
<td>IVGTT + [n-Lys3]GHRP-6 (n = 6)</td>
<td>( 815 \pm 65 )</td>
<td>( 997 \pm 107 )</td>
</tr>
<tr>
<td>IVGTT + [n-Lys3]GHRP-6 + AG (n = 6)</td>
<td>( 785 \pm 66 )</td>
<td>( 734 \pm 74 )</td>
</tr>
<tr>
<td>IVGTT + [n-Lys3]GHRP-6 + UAG (n = 7)</td>
<td>( 652 \pm 35 )</td>
<td>( 703 \pm 67 )</td>
</tr>
</tbody>
</table>

Values are means ± SE. Glucose and insulin levels (\( \Delta \text{AUC} \)) of the whole 50-min time course (ivglucose 50%, 1 g/kg) in all groups. Treatments were administered at \( t = \) 0 min. \( P \) values are reported for significant differences (\( P < 0.05 \)) vs. IVGTT group.

increased from 20 min (\( P < 0.05 \)) to 50 min (\( \Delta_{50-0} \)). [n-Lys3]GHRP-6 + IVGTT vs. IVGTT, \( P < 0.03 \); Fig. 3A). Portal insulin \( \Delta \text{AUC} \) was significantly higher (\( P < 0.01 \)) in rats treated with [n-Lys3]GHRP-6 + IVGTT than in those that received IVGTT alone (Fig. 3B). In the systemic circulation, the stimulatory effect on insulin release induced by the GHS-R1a antagonist was lost, and the \( \Delta \text{AUC} \) of the whole time course was similar to that in the IVGTT group (Fig. 3, C and D). Moreover, the effect exerted by [n-Lys3]GHRP-6 + IVGTT on glucose-induced insulin secretion was not modified.

**Fig. 1.** Unacylated ghrelin (UAG) dose-dependently stimulated the second-phase insulin response to an iv glucose load (IVGTT, 1 g/kg), whereas exogenous acylated ghrelin (AG) did not modify insulin levels. This insulin-secretagogue effect of UAG was much larger in the portal vein (A and B) than in the systemic circulation (C and D). Left: values during the time course relative to the baseline value, which was set as 0 (\( \Delta \)). Right: \( \Delta \text{AUCs} \) of all parameters after treatment administration. Vertical dotted line, treatment administration at \( t = 0 \). –, saline (n = 12); □, IVGTT (n = 12); ○, IVGTT + AG (30 nmol/kg; n = 7); ●, IVGTT + UAG (3 nmol/kg; n = 6); ●, IVGTT + UAG (30 nmol/kg; n = 10). *\( P < 0.01 \) vs. IVGTT. Other \( P \) values are reported in the figure; differences were considered significant for \( P < 0.05 \).
by the simultaneous administration of AG or UAG. Figure 3, A and B, clearly shows that [D-Lys3]GHRP-6, alone or coadministered with AG or UAG, stimulated the second-phase portal insulin response to IVGTT and that this effect was again similar in extent, pattern, and timing to that observed after UAG (30 nmol/kg) alone. △AUC of portal insulin concentrations in the group treated with [D-Lys3]GHRP-6, alone or combined with AG and UAG, was similar and higher than in the control (IVGTT) animals (P < 0.01, P = 0.05, and P < 0.03, respectively). Furthermore, glucose-stimulated portal insulin levels (△AUC) in all the groups treated with [D-Lys3]GHRP-6, alone or in combination with AG and UAG, were higher (P < 0.005, P < 0.01, and P < 0.04, respectively) than in animals that received exogenous AG alone (Fig. 3, B and D).

No effects were observed on peripheral insulin levels in rats treated with the GHS-R1a antagonist, alone or in combination with AG or UAG, compared with the IVGTT or the IVGTT + AG group (Fig. 3, C and D).

Despite the observed increase of insulin levels, after administration of the GHS-R1a antagonist [D-Lys3]GHRP-6, alone or in combination with AG or UAG, this was not accompanied by any significant changes in portal or peripheral glucose levels in terms of △AUC (Table 2) and curve profile (data not shown).

Interestingly, the coadministration of AG (30 nmol/kg) with UAG (30 nmol/kg) completely abolished the UAG-mediated increase in the second-phase insulin release both in portal (△AUC; P < 0.002) and in peripheral (P < 0.03) circulation (Fig. 4, A–D), but this did not modify portal and peripheral glucose levels after IVGTT (Table 2).

**Hepatic Insulin Clearance**

Since modulation of insulin levels observed in the portal vein by various treatments were (severely) blunted in the systemic circulation, we hypothesized that the administered compounds might not only affect insulin secretion in the portal vein but also modify insulin cleared by the liver and thereby increase the portal-peripheral gradient of insulin.

Insulin clearance after saline injection (%AUC) was 63 ± 3%, and it did not change significantly after glucose load. UAG at 30 nmol/kg, but not at 3 nmol/kg, slightly increased hepatic insulin clearance, which was higher (P < 0.05) than in the IVGTT or IVGTT + AG groups (IVGTT + UAG: 69 ± 2% vs.

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**Fig. 2.** Administration of exogenous AG (30 nmol/kg) or UAG (3 and 30 nmol/kg) did not modify glucose levels either in the portal vein (A and B) or in the peripheral circulation (C and D). Left: values during the time course relative to the baseline value which was set as 0 (△). Right: △AUCs of all parameters after treatment administration. Vertical dotted line, treatment administration at t = 0. ◊, saline (n = 12); ◊, IVGTT (n = 12); ◊, IVGTT + AG (30 nmol/kg; n = 7); ●, IVGTT + UAG (3 nmol/kg; n = 6), ●, IVGTT + UAG (30 nmol/kg; n = 10). △P < 0.001 vs. IVGTT. Differences were considered significant for P < 0.05.
IVGTT: 59 ± 4% and vs. IVGTT + AG: 57 ± 5%). Like UAG, [d-Lys3]GHRP-6, alone or combined with AG and UAG, slightly increased hepatic insulin clearance compared with rats treated with IVGTT alone or with AG. However, statistical significance was reached only by the group that received IVGTT + AG + [d-Lys3]GHRP-6 (70 ± 3%, \( P < 0.05 \) vs. IVGTT, \( P < 0.02 \) vs. IVGTT + AG; data not shown).

**DISCUSSION**

The results of the present study show that UAG acts as a secretagogue of insulin in the portal vein in anesthetized rats. This UAG-induced increase in insulin levels was abolished by the coadministration of AG and UAG, slightly increased hepatic insulin clearance compared with rats treated with IVGTT alone or with AG. However, statistical significance was reached only by the group that received IVGTT + AG + [d-Lys3]GHRP-6 (70 ± 3%, \( P < 0.05 \) vs. IVGTT, \( P < 0.02 \) vs. IVGTT + AG; data not shown).

Our data demonstrate for the first time that UAG potently and dose-dependently enhances the insulin response to an intravenous glucose load in vivo. This insulin secretagogue effect of UAG was marked in the portal vein, whereas it was barely detectable in the systemic circulation, supporting the hypothesis that UAG plays an important role in glucose metabolism in the liver. In line with this, previous observations using primary hepatocyte cultures showed that UAG dose-dependently decreased glucose output and completely prevented the AG-induced and partially blocked the glucagon-dependent glucose release (10). However, it was also found that UAG alone does not improve hepatic insulin sensitivity in a euglycemic hyperinsulinemic clamp model in mice (16). In the present study, we estimated that UAG also slightly increased the fraction of insulin cleared by the liver, thus contributing to the augmentation of the portal-peripheral gradient of insulin. Although we did not perform real insulin clearance studies, we speculate that UAG might also influence hepatic insulin metabolism. Therefore, we suggest that UAG enhances insulin secretion by pancreatic islets and perhaps also improves insulin action on target tissues (e.g., the liver). Interestingly, the UAG-enhanced insulin response to glucose was similar in extent, timing, and pattern to that exerted by [d-Lys3]GHRP-6, a GHS-R1a antagonist. The effect of [d-Lys3]GHRP-6 likely reflects the blockade of the inhibitory action of endogenous AG on \( \beta \)-cells. This is in accord with the
evidence that endogenous AG tonically restricts glucose-induced insulin release and that pharmacological, immunological, and genetic blockade of AG action in pancreatic islets enhanced glucose-induced insulin release (8, 9, 24). Nevertheless, by using this model, we could not detect significant effects on glucose levels in any of the treatment groups, making difficult any interpretation of these data as variations in insulin sensitivity. This may be explained by the high glucose load that we administered during the experiments, the presence of an increased counterregulatory hormonal response in the studied rats due to abdominal surgery (23), and/or possible effects of the anesthesia (1, 19).

We show that the administration of (exogenous) AG did not suppress insulin release any further, suggesting that after a glucose load endogenous AG at low concentrations, which we confirmed in our model, already exerts a maximal inhibitory effect on insulin secretion, at least under these experimental conditions. Another possible reason is that this maximal suppressive activity is due to autocrine and paracrine effects of AG produced in the pancreas. This would also explain why the coadministration of the GHS-R1a antagonist together with exogenous AG elicited the insulin response to glucose load to the same extent as [d-Lys³]GHRP-6 alone, i.e., removing the inhibitory tone of endogenous AG on insulin secretion. Our findings differ from previous reports by Dezaki et al. (9), who observed a suppressive effect of exogenous AG on glucose-induced insulin release, which was not modified by UAG in a perfused pancreas model. However, this discrepancy may be due to the fact that, differently from Dezaki et al., we used an in vivo model.

Intriguingly, when exogenous AG was coadministered with UAG, it completely blocked the insulin secretagogue effect of UAG. This finding once again reinforces the hypothesis that AG and UAG, at least at equimolar concentrations, interact with each other and have effects on glucose homeostasis. This is in agreement with previous reports in humans and in rodents, showing that the coadministration of UAG with AG was able to prevent the AG-induced decrease in circulating insulin and worsening of insulin sensitivity (3, 5, 12, 16).

Although our data do not provide evidence regarding the possible mechanism of action of UAG, we found a striking similarity between the insulin secretagogue effect of UAG and [d-Lys³]GHRP-6. This observation, coupled with the finding that exogenous AG could block the UAG-induced stimulation on insulin, led us to speculate that UAG may act as an antagonist of endogenous AG (i.e., removing the suppressive tone of AG on insulin release). However, since UAG, differ-

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**Fig. 4.** Coadministration of AG (30 nmol/kg) and UAG (30 nmol/kg) abolished completely the UAG-induced enhancement of insulin response to glucose both in the portal vein (A and B) and in the peripheral circulation (C and D). A and C: values during the time course relative to the baseline value, which was set as 0 (Δ). B and D: ΔAUCs after treatment administration. Vertical dotted line, treatment administration at time 0. ○, IVGTT; ●, IVGTT + AG (30 nmol/kg; n = 10); ●, IVGTT + AG (30 nmol/kg) + UAG (30 nmol/kg; n = 7). *P < 0.01 vs. IVGTT. P values for ΔAUCs are reported in the figure; differences were considered significant for P < 0.05.
ently from [D-Lys^3]GHRP-6, does not block the GHS-R1a (21), we suggest the existence of a putative UAG receptor (different from GHS-R1a) that mediates the stimulating effect of UAG on insulin. The fact that the actions of UAG and [D-Lys^3]GHRP-6 on glucose-stimulated insulin secretion were neither additive nor synergistic might be explained by two mechanisms: 1) either UAG or [D-Lys^3]GHRP-6 exerts a maximal antagonistic activity on endogenous AG; 2) [D-Lys^3]GHRP-6 is not only an (ant)agonist of the GHS-R1a but also an agonist of the putative UAG receptor. Indeed, the mechanisms of (inter)action of UAG, [D-Lys^3]GHRP-6, and AG on insulin release and glucose metabolism, as well as their physiological relevance, need to be further elucidated and may disclose a ghrelin system far more complex than it is currently known.

In conclusion, our data demonstrate that UAG at pharmacological concentrations is a potent insulin secretagogue. This, together with our previous observation that UAG blunts glucose output by primary hepatocytes (10), suggests that UAG action is targeted mainly at the liver. These effects of UAG in the regulation of glucose metabolism might be of therapeutic interest for those pathological conditions characterized by insulin resistance and impaired insulin release.

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