Effects of acute administration of acylated and unacylated ghrelin on glucose and insulin concentrations in morbidly obese subjects without overt diabetes

Short Title: Effects of AG and UAG in morbid obesity

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Abstract

Objective
To investigate the effects of unacylated ghrelin (UAG) and co-administration of acylated ghrelin (AG) and UAG in morbid obesity, a condition characterized by insulin resistance and low growth hormone (GH) levels.

Design and Method
Eight morbidly obese non-diabetic subjects were treated with either UAG 200µg, UAG 100µg+AG 100µg (Comb), or placebo in 3 episodes of 4 consecutive days in a double blind randomized cross-over design. Study medication was administered as daily single i.v. bolus injections at 09.00h after an overnight fast. At 10.00h a standardized meal was served. Glucose, insulin, GH, free fatty acids (FFA) and ghrelin were measured up to 4 hours after administration.

Results
Insulin concentrations decreased significantly after acute administration of Comb only, reaching a minimum at 20 minutes: 58.2±3.9% of baseline, vs. 88.7±7.2% and 92.7±2.6% after administration of placebo and UAG, respectively (P<0.01). After 1 hour, insulin concentration had returned to baseline. Glucose concentrations did not change after Comb. UAG administration alone, however, did not change glucose, insulin, FFA or GH levels.

Conclusion
Co-administration of AG and UAG as a single i.v. bolus injection causes a significant decrease in insulin concentration in non-diabetic subjects suffering from morbid obesity. Since glucose concentration did not change in the first hour after Comb administration, our data suggests a strong improvement in insulin sensitivity. These findings warrant studies in which UAG with or without AG
is administered for a longer period of time. Administration of a single bolus injection of UAG did not influence glucose and insulin metabolism.
Introduction

Ghrelin, a 28-amino acid peptide produced mainly by the stomach, was originally discovered as the natural ligand of the Growth Hormone Secretagogue Receptor type 1a (GHS-R1a). Its unique molecular structure is characterized by n-octanoylation of serine at position 3 (acylated ghrelin, AG), which is essential for binding to the GHS-R1a. However, in vivo, most circulating ghrelin is unacylated (UAG), which was consequently thought to be devoid of any endocrine action. Indeed, UAG does not share with AG its potent growth hormone (GH) stimulating effect, but more recent studies have shown that UAG does have biological effects.

Despite being primarily identified as a potent GH stimulating factor, ghrelin has been demonstrated to have a wide spectrum of biological activities, such as stimulation of prolactin and ACTH secretion, promotion of gastric motility and acid secretion, and modulation of cardiovascular function. One of its most intriguing functions is the long-term and short-term regulation of energy balance. Continuous administration of ghrelin to rodents induces increased food intake resulting in weight gain, whereas in humans 24-h plasma profiles show marked preprandial increases and postprandial decreases in circulating ghrelin concentrations, which suggests an orexigenic effect. Since insulin displays an exactly opposite meal-related pattern, the interaction between insulin and ghrelin has been extensively studied. In general, it is assumed that insulin has a negative effect on ghrelin concentrations, whereas administration of AG results in insulin resistance. On the other hand, the effect of UAG on insulin metabolism is still a matter of debate.

Since the main biological difference between AG and UAG is its ability to bind to the GHS-R1a, the question arises whether this receptor and consequently GH release is involved in ghrelin effects on glucose and insulin metabolism. To answer this question, our group has previously studied the effects of administration of AG, UAG and a combination of AG and UAG in adult-onset GH deficient subjects. Surprisingly, the combination of AG and UAG strongly improved insulin sensitivity in these individuals, whereas AG as well as UAG alone was shown to increase glucose concentration at constant insulin levels.
Since decreased insulin sensitivity plays a key role in the pathophysiology of type 2 diabetes, ways to improve insulin sensitivity could be beneficial to individuals prone to develop this disease. Obesity is typically associated with insulin resistance and, in a later phase, with type 2 diabetes.\textsuperscript{24} Additionally, obesity is characterized by low GH levels, comparable to GH-deficient subjects.\textsuperscript{25}

In the present study we therefore evaluated the effects of UAG and co-administration of AG and UAG on glucose and insulin metabolism in individuals suffering from morbid obesity, a condition characterized by insulin resistance and low growth hormone levels. As we were only interested in potential ways to improve insulin sensitivity, we did not study the effects of AG administration only, as this substance is known to worsen insulin sensitivity in all animal and human models studied so far.
Materials and Methods

Study population

Eight morbidly obese female Caucasian subjects (age 45.4 ± 10.3 (mean ± SD), range 28-62 years, mean Body Mass Index 42.4 ± 4.8 kg/m$^2$) were recruited from an affiliated clinic for bariatric surgery. All were on a waiting list to undergo gastric banding or gastric bypass (criteria: Body Mass Index (BMI) > 40 kg/m$^2$ or BMI > 35 kg/m$^2$ in combination with relevant comorbidity). Exclusion criteria for the present study were: overt diabetes mellitus, liver enzyme test abnormalities, pregnancy and previous bariatric surgery. All subjects gave their written informed consent to participate in the study, which had been approved by the ethical committee of our hospital. Two participants were suffering from hypertension, for which they were treated with antihypertensive drugs. Six were healthy, not suffering from any relevant comorbidities.

Study design

The present double blind randomized cross-over study design consisted of 3 study episodes in which 3 treatment regimens were administered: 1) UAG 200 µg (UAG), 2) UAG 100 µg in combination with AG 100 µg (Comb), 3) placebo (placebo). Every patient underwent all treatment regimens, which were separated by a wash out period of at least 2 weeks. Every study episode consisted of 4 consecutive days. Study medication was administered as a single daily intravenous bolus injection.

After an overnight fast, an indwelling catheter was placed in the forearm and kept patent by a slowly running saline infusion. At 9.00h study medication was administered as an acute bolus injection. Blood samples were taken before administration of study medication and at regular intervals up to 240 minutes: at 10, 20, 30, 45, 60, 75, 90, 120, 180, 240 minutes. Subjects were kept fasted during the first hour after administration of study medication. At 10.00h they received a standard breakfast containing 595 kcal (23 gr protein, 27 gr fat, 65 gr carbohydrate) and at 13.00h they received a standard lunch, comparable to breakfast. After lunch up to midnight, patients were free to choose their food intake.
Study medication

Both AG and UAG were obtained from Bachem AG, Bubendorf, Switzerland. To prevent degradation of ghrelin vials were stored at -80°C up to 90 minutes before administration. To prevent interaction of AG and UAG in vitro two separate samples were administered to the patients, followed by 5 ml of saline after each infusion. Samples were blinded and randomized.

Assessments

Blood samples for total ghrelin and acylated ghrelin measurements were collected in EDTA tubes. Samples were stored on ice until centrifuging. After centrifuging serum samples were stored at -20°C until processing. Acylated and total ghrelin levels were determined using a commercially available RadioImmunoAssay (Linco Research, St. Charles, Missouri, USA). Intra- and interassay variation of the AG assay are 7% and 13% respectively, and of the total ghrelin assay 6% and 16% respectively.

Both insulin and growth hormone were measured using a chemiluminescent immunometric assay (Immule 2000, Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA). Intra- and interassay variation of the insulin assay are 4% and 5% respectively, while intra- and interassay variation of the growth hormone assay are 4% and 6% respectively. Glucose was measured on a Hitachi 917 (Roche Diagnostics, Mannheim, Germany) by a glucose-oxidase method. FFA concentrations in the presence of tetrahydrolipstatin (final concentration 1 mg/L, prepared from Xenical® capsules) were measured in EDTA plasma on a Hitachi 912 using the Wako Chemicals kit.27

Statistical analysis

Results are presented as mean ± SEM unless otherwise specified. P < 0.05 was considered significant. Differences between the three study periods were calculated using the Friedman test, the nonparametric equivalent of a one-sample repeated measures design. To determine correlations between various parameters a two-tailed Spearman’s rank test was used. Areas under the curve were calculated using the trapezoid rule.
Statistic calculations were performed using Statistical Package for the Social Sciences (SPSS release 14.0; SPSS Inc, Chicago).

Unacylated ghrelin concentrations were determined calculating the difference between total ghrelin and acylated ghrelin. Glucose to insulin ratio was used as an estimate of insulin sensitivity.
Results

Concentrations of AG and UAG
After acute administration of AG 100 µg i.v. (in combination with UAG 100 µg) baseline AG concentration of 64 pg/ml increased to a peak of 2325 pg/ml after 10 minutes. The half-life was short: AG concentrations returned to baseline 100 minutes after administration (Fig.1A). Baseline concentrations of UAG were 844 pg/ml, increasing to 10499 pg/ml and to 11205 pg/ml 10 minutes after administration of UAG 200 µg i.v. alone and 100 µg i.v. in combination with AG 100 µg respectively. At termination of the measurements, 4 hours after administration, UAG concentrations had not completely returned to baseline (Fig.1B).

Effects of administration of UAG
During fasting, first hour after administration
Acute administration of UAG 200 µg did not induce any change in GH concentration.

Fasting baseline insulin concentrations were 166.8 ± 32.6 pmol/L and 145.8 ± 30.4 pmol/L, on day 1 and day 4 respectively. No changes in insulin concentrations were observed in the first hour after administration of UAG. Additionally, insulin concentrations after UAG administration were not different from placebo (Fig. 2A).

Figure 3A demonstrates corresponding results in glucose. Fasting baseline glucose concentrations were 4.4 ± 0.47 mmol/L and 4.8 ± 0.4 mmol/L on day 1 and 4, respectively. Glucose concentrations did not change during the first hour after UAG administration and were not different from placebo.

UAG did not have any acute effects on FFA levels (data not shown).

After breakfast, 1 – 4 h after administration
As shown in figure 2C and 3B, UAG did not have any effects on glucose and insulin concentrations in fed conditions, starting 1 hour after administration. Additionally, no effects on FFA metabolism were observed (data not shown).
Effects of administration of UAG in combination with AG (Comb)

During fasting, first hour after administration

Administration of Comb induced a rapid and significant peak in GH levels. Maximum concentration of GH was reached at 20 minutes after administration: 20.9 ± 3.37 µg/L and 13.1 ± 2.70 µg/L on day 1 and 4 respectively, vs. placebo 0.6 ± 0.12 µg/L and 0.6 ± 0.21 µg/L respectively, and UAG 0.6 ± 0.21 µg/L and 0.3 ± 0.08 µg/L, respectively (P < 0.001, data not shown).

Insulin concentrations decreased strongly after acute administration of Comb, reaching a minimum at 20 minutes (Fig. 2A). Insulin concentrations at T20 were 58.3 ± 5.4% and 58.2 ± 6.3% of baseline on day 1 and 4 respectively, whereas after administration of placebo and UAG on day 1 and day 4 insulin concentrations were 92.0 ± 11.6%, 84.5 ± 7.4%, 93.7 ± 4.8% and 91.8 ± 3.0%, respectively (P < 0.01) Fig. 2B shows AUC/h, which demonstrates that insulin concentration is significantly lower throughout the first hour after administration of Comb, compared to both placebo and UAG (P < 0.05).

Comb administration did not have any effect on glucose concentration (Fig. 3A). Therefore, calculating glucose over insulin ratio resulted in a strong improvement in insulin sensitivity after Comb administration: at T20 insulin sensitivity is 184.3 ± 19.7% and 169.3 ± 16.7% of baseline on day 1 and 4, respectively.

Comb administration did not have any effect on FFA levels (data not shown).

After breakfast, 1 – 4 h after administration

After breakfast, the suppressing effect of Comb on insulin concentration could not be observed anymore. Insulin concentration after Comb administration was not significantly different from either placebo or UAG (Fig. 2C). However, no rebound effect was observed as well. Again, no effects on glucose (Fig. 3B) and FFA metabolism were observed (data not shown).

Tachyphylaxis
We did not observe any change in effects after repeated administration of study medication, especially no reduction of improvement in insulin sensitivity after Comb administration. Results on day 1 were not different from day 4, in the UAG period as well as in the Comb period.

**Correlations with change in insulin sensitivity**

None of the subjects studied was suffering from diabetes mellitus, but nevertheless both baseline insulin concentration as well as 2-hour post-prandial insulin concentration had a high interindividual variability. Baseline insulin concentration in the placebo period varied from 72.9 pmol/L to 365.8 pmol/L, whereas 2-hour post-prandial insulin concentration varied from 222.5 pmol/L to 1513.8 pmol/L. Additionally, GH responses to Comb administration varied strongly as well, with a GH peak range 20 minutes after administration of 9.3 – 31.2 µg/L. To evaluate which individuals would benefit the most of the positive effect of Comb on insulin sensitivity a correlation study was performed. Neither baseline and postprandial insulin concentrations nor GH response showed any correlation with change in insulin sensitivity after Comb administration.

**Side effects**

Three patients experienced a short episode of flushing and dizziness shortly after administration of Comb. They all developed this mild and self-limiting side effect on one day, randomly in four days during the Comb study period.
Discussion

This study demonstrates that co-administration of acylated and unacylated ghrelin induces a strong decrease in insulin concentration in morbidly obese subjects without overt diabetes. A single injection of AG + UAG resulted in almost 50% reduction of insulin concentration with unaffected glucose levels, suggesting a strong improvement in insulin sensitivity. During repeated administration, no tachyphylaxis was observed. Broglio et al. previously demonstrated that in healthy young men UAG was able to counteract the insulin resistance induced by AG alone. Additionally, co-administration of AG and UAG was shown to improve insulin sensitivity in GH-deficient patients. Nevertheless, the present study population is the first that could actually benefit from a treatment able to improve insulin sensitivity. Since obesity induces insulin resistance and consequently causes diabetes mellitus, the present findings could lead towards a new approach in treating diabetes.

The observed decrease in insulin concentration after acute injection of AG + UAG with unaffected glucose levels suggests an improvement in insulin sensitivity, as stated above. However, glucose/insulin ratio is only partially correlated with the variation in insulin action and insulin sensitivity, since insulin levels also depend on secretion, distribution and degradation of insulin. Nevertheless, in the present study we at least replicated the effect of AG + UAG on insulin concentration as previously observed in our study in GH-deficient subjects. Therefore, future studies evaluating the effect of AG + UAG on insulin sensitivity using an euglycaemic insulin clamp are warranted and indicated.

Co-administration of AG and UAG affected insulin concentration in the first hour after administration only. The most likely explanation of this short-lived effect is the observed short half-life of AG and, to a smaller extent, UAG. Additionally, plasma concentrations of UAG were comparable 10 minutes after administration of UAG 200 µg and UAG 100 µg + AG 100 µg respectively. Therefore, the AG plasma peak concentration must have been significantly earlier than 10 minutes, followed by a rapid degradation of AG to UAG. Since subjects were fasted during the first hour of the study protocol and insulin concentrations had returned to baseline at breakfast, no
conclusions can be drawn about the acute effect of AG + UAG on insulin sensitivity in fed conditions. Nevertheless, at least no rebound effect was observed after breakfast.

In considering co-administration of AG and UAG as a treatment of insulin resistance, it is important to be aware of the risk of tachyphylaxis. Up till now, no data are available on the long-term effects of AG and UAG administration. In the present study, AG + UAG were administered on four consecutive days while no decrease in effect was observed. We found the sustained effects of the combination of AG and UAG after 4 days of q.d. administration reassuring suggesting the absence of acute tachyphylaxis.

Shortly after the discovery of the orexigenic effect of ghrelin, it was hypothesized that obese subjects would have elevated ghrelin concentrations that could contribute to the pathogenesis of obesity. On the contrary, total ghrelin concentrations were found to be decreased in obesity. More recent studies, however, assessed both AG and UAG levels and AG/UAG ratios. UAG, but not AG, is decreased in obesity, while insulin resistant obese subjects display a higher AG/UAG ratio than equally obese insulin sensitive subjects. These data suggest that relatively high AG levels combined with lower UAG levels might contribute to insulin resistance in obesity. In the present study, however, we administered UAG and AG in a 1:1 ratio, which is much higher than in vivo where UAG/AG is about 9:1. Since this 1:1 ratio was previously observed to improve insulin sensitivity, we decided to continue using these concentrations. Nevertheless, future studies are needed to evaluate the effect on insulin resistance of co-administration of AG and UAG in different proportions.

Since the present study did not evaluate the effects of AG in morbidly obese subjects, it could be discussed that the observed decrease in insulin concentration is the result of AG alone more than of the coadministration of AG and UAG. Three studies have evaluated the effect of ghrelin administration in obesity. One study did not show any change in glucose and insulin concentrations, while two studies reported an increase in glucose concentration with a slight decrease in insulin levels. These results are not in accordance with the present findings, that show a highly significant decrease in insulin concentrations without a reciprocal increase in glucose concentrations. This
difference suggests that the present findings do result from the coadministration of AG and UAG more than of AG alone, which is supported by the study in GH deficient subjects as well. 23

In the present study, UAG administration had no effect on glucose and insulin levels despite the presence of pharmacological concentrations. It is still unclear whether acute changes in UAG levels do have intrinsic effects on glucose and insulin concentrations. Some reports on acute effects of UAG described an increase in glucose levels 23, while other studies, like the present, did not observe any effect. 3 However, continuous administration of UAG, on the contrary, seems to improve insulin sensitivity. 34 Therefore, possible explanations for the observed effects of co-administration of AG + UAG remain speculative. Since UAG is not able to bind to the GHS-R1a, it is not likely that antagonism on this receptor plays a role. Additionally, GHS-R1a was previously shown not to be involved in mediating ghrelin’s effects on hepatic glucose output by primary porcine hepatocytes. 6 Whether a yet unidentified receptor to which AG and UAG are both able to bind mediates these effects needs to be studied.

Our study clearly opens new perspectives in the approach of insulin resistance in obesity. As mentioned before euglycaemic insulin clamp studies are needed to evaluate whether the present changes in glucose and insulin concentrations are mainly the result of improvement in insulin sensitivity, as currently expected. Further research is needed to evaluate whether the present findings can be extrapolated to fed conditions. However, attention must be paid to the possible adverse effects of continuous administration of AG, such as its impact on adipogenesis and food intake. 17 Finally, the effects of co-administration of AG and UAG in subjects suffering from diabetes should be studied.

In conclusion, the present study demonstrates, that co-administration of AG and UAG in a 1:1 molar ratio in fasted morbidly obese subjects, but without overt diabetes, strongly decreases insulin concentrations at unchanged glucose levels, suggesting an improvement in insulin sensitivity. Further studies are needed to provide information on the effects in fed conditions and in diabetic subjects.
The authors have no conflicts of interest to declare.

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Fig. 1 Changes in plasma concentrations of acylated and unacylated ghrelin after administration of study medication.
A: AG plasma concentration.
B: UAG plasma concentration
T₀ = administration of treatment: placebo (—○—), UAG 200 µg day 1 (- -▲- -), UAG 200 µg day 4 (- -▼- -), UAG 100 µg + AG 100 µg day 1 (⋯♦⋯), UAG 100 µg + AG 100 µg day 4 (⋯●⋯)

Fig. 2 Serum insulin concentration
A: First hour after administration of study medication. Concentration presented as % from baseline = before administration. T₀ = administration of treatment: placebo day 1 (—○—), placebo day 4 (—□—), UAG 200 µg day 1 (- -▲- -), UAG 200 µg day 4 (- -▼- -), UAG 100 µg + AG 100 µg day 1 (⋯♦⋯), UAG 100 µg + AG 100 µg day 4 (⋯●⋯). * P < 0.05 Comb day 1 and 4 vs. placebo day 1 and 4, UAG day 1 and 4.
B: Area Under the Curve/hour of insulin concentration, presented as % from baseline, in the first hour after administration of study medication. Treatment: placebo day 1 (P1), placebo day 4 (P4), UAG 200 µg day 1 (U1), UAG 200 µg day 4 (U4), UAG 100 µg + AG 100 µg day 1 (C1), UAG 100 µg + AG 100 µg day 4 (C4). * P < 0.05 C1 and C4 vs P1, P4, U1 and U4.
C: After breakfast. Concentration presented as % from baseline = before administration. T₀ = administration of treatment. T₆₀ = breakfast.

Fig. 3 Serum glucose concentration
A: First hour after administration of study medication. Concentration presented as % from baseline = before administration. T₀ = administration of treatment: placebo day 1 (—○—), placebo day 4 (—□—), UAG 200 µg day 1 (- -▲- -), UAG 200 µg day 4 (- -▼- -), UAG 100 µg + AG 100 µg day 1 (⋯♦⋯), UAG 100 µg + AG 100 µg day 4 (⋯●⋯)
B: After breakfast. Concentration presented as % from baseline = before administration. T₀ = administration of treatment. T₆₀ = breakfast
Fig. 1A
Fig. 2B

AUC/h Insulin (% from baseline)

P1  P4  U1  U4  C1  C4

*  *
Fig. 2C

[Graph showing insulin levels over time after breakfast]
Fig. 3A

Time (min) vs Glucose (% from baseline)