**Abstract**

**Objective:** To clarify the metabolic effects of an overnight i.v. infusion of unacylated ghrelin (UAG) in humans. UAG exerts relevant metabolic actions, likely mediated by a still unknown ghrelin receptor subtype, including effects on β-cell viability and function, insulin secretion and sensitivity, and glucose and lipid metabolism.

**Design:** We studied the effects of a 16-h infusion (from 2100 to 1300 h) of UAG (1.0 μg/kg per h) or saline in eight normal subjects (age (mean ± S.E.M.), 29.6 ± 2.4 years; body mass index (BMI), 22.4 ± 1.7 kg/m²), who were served, at 2100 and 0800 h respectively, with isocaloric balanced dinner and breakfast. Glucose, insulin, and free fatty acid (FFA) levels were measured every 20 min.

**Results:** In comparison with saline, UAG induced significant (P < 0.05) changes in glucose, insulin, and FFA profiles. UAG infusion decreased glucose area under the curve (AUC) values by 10% (UAG0–960 min : 79.0 ± 1.7 mg/dl per min vs saline0–960 min : 87.5 ± 3.8 mg/dl per min) and the AUC at night by 14% (UAG 180–660 min : 28.4 ± 0.5 mg/dl per min vs saline 180–660 min : 33.2 ± 1.1 mg/dl per min). The overall insulin AUC was not significantly modified by UAG infusion; however, insulin AUC observed after meals was significantly increased under the exposure to UAG with respect to saline at either dinner or breakfast. The FFA AUC values were decreased by 52% under the exposure to UAG in comparison with saline (UAG0–960 min : 0.3 ± 0.02 mEq/l per min vs saline0–960 min : 0.6 ± 0.05 × 10⁻³ mEq/l per min).

**Conclusions:** Exposure to the i.v. administration of UAG improves glucose metabolism and inhibits lipolysis in healthy volunteers. Thus, in contrast to the diabetogenic action of AG, UAG displays hypoglycemic properties.
UAG is able to positively modulate glucose and lipid metabolism (19, 20). In fact, the acute administration of UAG has been shown to antagonize the effects of AG on insulin secretion and glucose levels in normal subjects (19). At the same time, the combined administration of AG and UAG significantly improved insulin sensitivity, compared with placebo, for at least 6 h in patients with adult-onset GH deficiency (20).

Moreover, UAG counteracted the effects of AG on glucose output from pig hepatocytes in vitro (21) and, in glucose-stimulated conditions in vivo, exerted a potent insulin secretagogue action that was completely blocked by the coadministration of AG (22).

In addition to the effect on glucose homeostasis, it has also been demonstrated that UAG, as well as its acylated form, has an inhibitory effect on isoproterenol-induced lipolysis from rat adipocytes (23).

In all, it is clear that UAG is an active peptide, also able to bind to receptors other than GHS-R1a receptor (4, 18). In this context, considering also the meal-related secretory pattern of circulating total ghrelin suggested by some authors (24), but not by others (25, 26), the negative association observed between body mass index (BMI) and ghrelin levels (4, 27), and their relationship with energy restriction and food intake (4, 8), insulin and glucose levels have been hypothesized to modulate peripheral ghrelin secretion that, in turn, is likely to influence insulin secretion and glucose metabolism (13, 28). Accordingly, total ghrelin levels have been shown to be reduced not only during an euglycemic hyperinsulinemic clamp and after insulin-induced hypoglycemia (8, 29, 30), but also after either an oral or i.v. glucose load (8, 29, 31, 32). Moreover, it has been hypothesized that UAG levels would be more remarkably reduced in obese patients and that this would significantly contribute to the reduction of insulin sensitivity (33).

The aim of this study was to clarify the metabolic effects of the prolonged i.v. infusion of UAG in humans. We studied the effects of a 16-h continuous infusion (from evening to the following early afternoon) of UAG or saline in normal young subjects, who were served a standard dinner and breakfast.

The infusion of UAG and placebo was double blind as it was performed by a third researcher. Subjects were admitted to the clinical facility 10 h before initiation of the testing session and were served an isocaloric lunch at 1300 h. The testing sessions began in the evening at 2100 h, 30 min after the indwelling of two catheters into two different antecubital veins of the forearm, kept patent by the slow infusion of isotonic saline. At 2100 h and 0900 h respectively, a standardized balanced isocaloric dinner (~900 total kcal: 50% carbohydrates, 20% proteins, and 30% lipids) and breakfast (~400 total kcal: 50% carbohydrates, 10% proteins, and 40% lipids) were served to the subjects who were instructed to eat everything.

During the testing sessions, subjects were not allowed to drink alcohol, coffee, tea, or sweetened beverages or eat anything other than what they were provided with by the medical staff. All the subjects slept during the light-off period, although sleep was not instrumentally monitored. Lights were turned off at 2330 h and on at 0730 h.

Blood samples were collected every 20 min from 2100 to 1300 h during the following day. Glucose, insulin, and free fatty acid (FFA) levels were assayed at each time point for both sessions. Vials containing 100 mg lyophilized human UAG were purchased from Neosystem and administered using a bacterial filter system.

Plasma glucose levels (mg/dl; 1 mg/dl, 0.05551 mmol/l) were measured by the glucose oxidase colorimetric method (GLUCOFTX: Menarini Diagnostici, Florence, Italy).

Serum insulin levels (µU/ml) were measured in duplicate by immunoradiometric assay (INSIK-5; SORIN Biomedica, Saluggia, Italy). The sensitivity of the insulin assay was 2.5±0.3 µU/ml. The inter- and intra-assay coefficients of variation (CV) were 6.2–10.8 and 5.5–10.6% respectively.

Serum FFA levels (mEq/l) were measured by enzymatic colorimetric method (Wako Chemicals GmbH, Neuss, Germany). The inter- and intra-assay CV were 1.1 and 4.1% respectively. All the samples from an individual subject were analyzed in a single run of each assay.

The results are expressed as absolute mean ± S.E.M. The areas under the curves (AUC) of all variables were measured by the trapezoidal integration method for the whole infusion (960 min), the night time period (480 min), or the 60 min postprandial period. The ΔAUC values corrected for premeal levels were also calculated (ΔAUC). The differences observed between saline and UAG treatments were evaluated using the nonparametric Wilcoxon test. Statistical significance was assumed at P<0.05.

All statistical analyses were carried out using the SPSS 11.0 Software (SPSS, Chicago, IL, USA).

**Results**

The study subjects did not have any weight change between the two testing sessions (data not shown).
As presented in Figs 1 and 2, during the saline infusion, overall absolute glucose AUC values were lower on average by 10% with respect to saline (saline0–960 min: 87.5 $\pm$ 3.8 $\times$ 10³ mg/dl per min vs UAG0–960 min: 79.0 $\pm$ 1.7 $\times$ 10³ mg/dl per min; $P<0.01$), and this difference was even more pronounced during the nighttime period (0000–0800 h), when the absolute glucose AUC value was lower than saline by 14% (saline180–660 min: 33.2 $\pm$ 1.1 $\times$ 10³ mg/dl per min vs UAG180–660 min: 28.4 $\pm$ 0.5 $\times$ 10³ mg/dl per min; $P<0.01$). Interestingly, this glucose-lowering effect was detected for the overall treatment at night but not during the 180 min postprandial periods following dinner and breakfast.

**Saline treatment**

As presented in Figs 1 and 2, during the saline infusion, glucose and insulin levels showed the expected increase after both dinner (absolute glucose peak: 110 $\pm$ 7.9 mg/dl, $P<0.01$; glucose $\Delta\text{AUC}_{0–60\text{ min}}$: 317.5 $\pm$ 145.0 mg/dl per min, $P<0.01$; absolute insulin peak: 57.8 $\pm$ 14.7 $\mu$U/ml, $P<0.01$; insulin $\Delta\text{AUC}_{0–60\text{ min}}$: 232.0 $\pm$ 116.2 $\mu$U/ml per min, $P<0.01$) and breakfast (absolute glucose peak: 115.5 $\pm$ 6.8 mg/dl, $P<0.05$; glucose $\Delta\text{AUC}_{2–780\text{ min}}$: 505.0 $\pm$ 124.3 mg/dl per min, $P<0.05$; absolute insulin peak: 46.3 $\pm$ 9.5 $\mu$U/ml, $P<0.05$; insulin $\Delta\text{AUC}_{2–780\text{ min}}$: 1078.4 $\pm$ 124.3 $\mu$U/ml per min, $P<0.05$). In addition, as presented in Fig. 3, FFA levels decreased significantly ($P<0.05$) after both dinner (absolute nadir, 0.6 $\pm$ 0.1 mEq/l; $\Delta\text{AUC}_{0–60\text{ min}}$: 1.1 $\pm$ 3.9 mEq/l per min) and breakfast (absolute nadir, 0.5 $\pm$ 0.1 mEq/l; $\Delta\text{AUC}_{720–780\text{ min}}$: $-5.3 \pm 2.3$ mEq/l per min).

**UAG treatment**

With respect to saline, the UAG infusion induced significant variations of all metabolic variables studied.

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**Figure 1** Glucose levels (mean ± S.E.M.) expressed as absolute values and overall AUC (AUC_{0–960 min}) in eight young normal subjects during saline (gray) and UAG (black) i.v. infusion. The two vertical arrows correspond to the time of dinner and breakfast respectively. **$P<0.01$ vs saline.

**Figure 2** Insulin levels (mean ± S.E.M.) expressed as absolute values and after meals AUC (AUC_{0–60 min}) in eight young normal subjects during saline (gray) and UAG (black) i.v. infusion. The two vertical arrows correspond to the time of dinner and breakfast respectively. *$P<0.05$ vs saline; **$P<0.01$ vs saline.
Figure 3 FFA levels (mean ± s.e.m.) expressed as absolute values and overall AUC (AUC_{0–960 min}) in eight young normal subjects during saline (gray) and UAG (black) i.v. infusion. The two vertical arrows correspond to the time of dinner and breakfast respectively. **P < 0.01 vs saline.

dinner (ΔAUC_{saline0–180 min}: 3.7 ± 1.0 × 10^3 mg/dl per min vs ΔAUC_{UAG0–180 min}: 3.8 ± 0.3 × 10^3 mg/dl per min; P =NS) or breakfast (ΔAUC_{saline720–900 min}: 1.5 ± 0.7 × 10^3 mg/dl per min vs ΔAUC_{UAG720–900 min}: 1.5 ± 0.4 × 10^3 mg/dl per min; P = NS).

Figure 2 indicates that, although the UAG infusion did not have significant impact on overall insulin concentrations during the 16-h period (saline AUC_{0–960 min}: 27.1 ± 3.6 × 10^3 μU/ml per min vs UAG AUC_{0–960 min}: 26.5 ± 1.8 × 10^3 μU/ml per min; P = NS), there was a clear enhancing effect on insulin secretion immediately after both dinner (ΔAUC_{saline0–60 min}: 0.2 ± 0.1 × 10^3 μU/ml per min vs ΔAUC_{UAG0–60 min}: 1.3 ± 1.8 × 10^3 μU/ml per min; +472%; P < 0.01) and breakfast (ΔAUC_{saline720–780 min}: 1.1 ± 0.3 × 10^3 μU/ml per min vs ΔAUC_{UAG720–780 min}: 1.7 ± 1.1 × 10^3 μU/ml per min; +64%; P < 0.05).

As presented in Fig. 3, FFA AUC values decreased by 52% during the 16-h UAG infusion (UAG_{0–960 min}: 0.3 ± 0.02 × 10^3 mEq/l per min vs saline_{0–960 min}: 0.6 ± 0.05 × 10^3 mEq/l per min).

Side effects

No side effect was associated with saline infusion as expected. Similarly, UAG infusion was not associated with any significant adverse event. At the end of UAG infusion, without any evidence of cause-related effect, minimal facial edema was apparent in two subjects but disappeared within the following 2 h. Blood pressure and pulse rate were monitored during the awaking period of saline and UAG infusion and their results were similar in the two testing sessions.

Discussion

Although AG, the only active transcript of the ghrelin gene, was considered for long time, some biological actions of UAG were observed both in vitro and in vivo, in animal as well as in human models (4, 13, 14, 18, 28). Our study aimed to evaluate the effect of continuous i.v. administration of UAG on metabolic variables in healthy humans. We observed that in young healthy volunteers, the i.v. infusion of UAG significantly decreased glycemic profiles, particularly during nighttime. Also, UAG infusion was associated with an enhancement of the meal-induced early insulin response as well as with an impressive reduction in FFA levels. These metabolic variations occurred in the absence of any modifications of GH and cortisol levels (data not shown), in line with previous observations showing no neuroendocrine effect of UAG after acute administration (12, 18, 19).

The reduction in glucose levels during UAG infusion in humans fits well with the previous studies indicating the glucose-lowering effect of the nonacylated form of ghrelin. In fact, it had been demonstrated in vitro that UAG reduces glucose output from pig hepatocytes (21); in this experimental model, UAG was also able to abolish the stimulatory effect on glucose output induced by AG and to partially counteract the stimulatory effect exerted by glucagon (21). Again, although unable to exert an acute hypoglycemic effect in fasted healthy volunteers following single i.v. bolus administration, it was demonstrated that UAG can abolish the hyperglycemic effect of AG in humans (19) and that the combined administration of AG and UAG significantly improved insulin sensitivity in patients with adult-onset GH deficiency (20).

The lowering of glucose profiles during exposure to UAG was associated with a more marked first-phase insulin response to meals and this might in part explain the observed favorable effect of UAG on glucose metabolism. AG can exert inhibitory effects on β-cell secretion and this has been reported by several groups from observations in both in vitro and in vivo studies in animal and human models (13, 14, 28). In particular, AG seems to be able to decrease insulin secretion and its response to glucose and arginine in humans as well as in vitro in perfused pancreas (4, 13, 14). Conversely,
it has been shown that UAG, dose dependently, can stimulate insulin secretion from rat β-cells in vitro either in basal condition or in response to exposure to glucose (14), while it is able to counteract the inhibitory effect of AG on insulin levels in humans (19, 20).

It has to be emphasized that the endocrine pancreas is a natural source of ghrelin that is synthesized and secreted by a new pancreatic islet population defined as ‘epsilon’ (ε) and that during fetal life the endocrine pancreas and not the stomach is the most important contributor of circulating ghrelin levels (14, 34). Within the endocrine pancreas, ghrelin, either acylated or not, likely plays a para/autocrine action on the regulation of insulin secretion (14). In fact, at the level of pancreatic endocrine islets, the presence of GHS-R1a as well as of the still uncharacterized ghrelin receptor subtypes has been demonstrated (14).

Thus, the ghrelin system is well represented in the endocrine pancreas, suggesting that it plays a relevant role in the modulation of insulin secretion. However, ghrelin might also play a role in the regulation of insulin sensitivity at the peripheral level (4, 8, 13). In this context, it is noteworthy that the prevalence of ghrelin in its acylated form is associated with a worsening in insulin sensitivity in humans (4, 8, 13). In fact, the ghrelin system has been suggested to be active in muscle and adipose tissue (4, 5). An example of these ghrelin effects on the adipose tissue is the remarkable inhibition of FFA levels that we observed during the infusion with UAG; this FFA decrease could partly explain the observed improvement in insulin sensitivity (35, 36). Actually, the observation of decreased FFA levels in our study does not necessarily imply an inhibition of lipolysis but could also suggest an increase in their tissutal uptake or inhibition of gastrointestinal fatty acid uptake and effects on hepatic fat metabolism. Nevertheless, our present findings fit well with the previous report that UAG inhibits isoproterenol-induced lipolysis (23) and stimulates lipid accumulation in human visceral adipocytes (37), as well as in murine preadipocyte cells (38). Interestingly, the inhibitory action on lipolysis seems to be the only common action for nonacylated ghrelin and AG (18, 23).

In summary, although a direct measurement of insulin sensitivity has not been performed, the results of this study, showing a concomitant reduction in insulin and glucose responses to meals, might support the hypothesis that UAG could play a metabolic role that improves glucose metabolism and insulin secretion and sensitivity and inhibits lipolysis in humans. All together, these data are consistent with transgenic animal models overexpressing UAG (39) as well as with a model of specific ghrelin-O-acyltransferase (MBOAT4) inhibition (40). In fact, on one hand, mice overexpressing UAG, under the control of the rat insulin II promoter in pancreatic islets, showed reduced blood glucose levels and increased insulin sensitivity, although in these models glucose-stimulated insulin secretion was reduced (39). On the other hand, normal mice treated with specific GOAT inhibitor, which are lacking AG but show normal or even enhanced levels of UAG, display improvement in glucose metabolism and insulin sensitivity (40).

In conclusion, we demonstrated that an overnight continuous i.v. administration of UAG improves glucose metabolism and inhibits lipolysis in healthy volunteers. This points toward a potential therapeutic role for UAG and its analogs in clinical conditions characterized by the presence of insulin resistance and metabolic derangement.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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