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Ghrelin Stimulates, Whereas Des-Octanoyl Ghrelin Inhibits, Glucose Output by Primary Hepatocytes

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Ghrelin exerts various metabolic activities, including regulation of glucose levels in humans. To verify whether the glucose response to ghrelin reflects a modulation of an insulin-independent hepatic phenomenon, we studied glucose output by primary porcine hepatocytes in suspension culture, after incubation with acylated ghrelin (AG), unacylated ghrelin (UAG), and hexarelin (HEX). AG induced glucose output dose dependently after 20 min of incubation ($P < 0.001$), whereas HEX, a GH secretagogue receptor type 1a (GHS-R1a) agonist, had no effect. UAG inhibited glucose release also dose dependently and after 20 min ($P < 0.001$). Moreover, UAG completely reversed AG-induced glucose output ($P < 0.01$). Using real-time PCR, GHS-R1a gene expression was undetectable in all the hepatocyte preparations studied. The lack of efficacy of HEX, the efficacy of UAG, and the absence of GHS-R1a expression indicate the involvement of a yet uncharacterized ghrelin receptor type.

In conclusion, glucose output by primary hepatocytes is time- and dose-dependently stimulated by AG and inhibited by UAG. Moreover, UAG counteracts the stimulatory effect of AG on glucose release. These actions might be mediated by a different receptor than GHS-R1a, and apparently, we must consider AG and UAG as separate hormones that can modify each other's actions on glucose handling, at least in the liver. (*J Clin Endocrinol Metab* 90: 1055–1060, 2005)

GHRELIN IS A 28-amino acid peptide with an n-octanoyl ester at its third serine residue (1) that is isolated from rat and human stomach and characterized as a natural ligand for the GH secretagogue (GHS) receptor (GHS-R) (1). Ghrelin is predominantly produced by the stomach but also detectable in many other tissues (1–4). The n-octanoyl group at serine 3 of the ghrelin molecule seems to be essential for the hormone's binding and bioactivity, at least in terms of endocrine actions (1, 5, 6), because the unacylated form of ghrelin, des-octanoyl ghrelin, does not bind the GHS-R type 1a (GHS-R1a) and is devoid of any endocrine activity (1, 6, 7). However, unacylated ghrelin (UAG) is not biologically inactive; it is able to share with ghrelin antiproliferative effects on human breast and prostate cancer lines (8, 9), has negative inotropic effects on papillary muscle (10), and can stimulate bone marrow adipogenesis (11), although the signal transduction mechanism(s) for these effects has not been determined. Acylated ghrelin (AG), as well as synthetic GHS, besides having a strong GH-releasing activity, has broader actions, including stimulation of lactotroph and corticotroph production, modulation of the activity of the pituitary-gonadal axis, stimulation of appetite, control of energy balance, influence on sleep and behavior, control of gastric motility and acid secretion, antiproliferative effects on thyroid and breast tumors, and influence on pancreatic function as well as on glucose metabolism (3, 12, 13). These actions are in agreement with the central and peripheral distribution of GHS-Rs, either GHS-R1a or still undefined subtypes (3, 9–11, 14).

Ghrelin seems to play a role in the neuroendocrine and metabolic response to food intake (3). Indeed, its circulating levels are increased in anorexia and cachexia but reduced in obesity (3, 15–17), and plasma ghrelin levels are negatively correlated with body mass index, body fat mass, and plasma leptin, insulin, and glucose levels (3, 18, 19). The hypothesis that ghrelin could exert a role in the modulation of glucose metabolism had been predicted by clinical studies in which synthetic GHS caused hyperglycemia (20–22). This effect was shown to be independent of their GH-releasing activity by the observation that the administration of GH-releasing peptide-6 to normal individuals significantly increased insulin and glucose levels, but only when given to subjects who were pretreated with the GH receptor antagonist pegvisomant (23). In humans, the acute administration of ghrelin elicited a prompt increase in glucose levels (24, 25), whereas no changes in insulin or glucose levels were recorded after iv administration of UAG and hexarelin (HEX), a synthetic GHS with a high affinity for the GHS-R1a (6, 24). Strikingly, the administration of UAG could totally block the hyperglycemic effects of AG bolus injection in normal subjects (26). This rapid increase in serum glucose was observed before a decrease in insulin levels was recorded, suggesting that ghrelin could directly affect hepatic glucose output. Although the influence of ghrelin on glucose output by the liver was

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Abbreviations: AG, Acylated ghrelin; Ct, cycle threshold for the sequence detector; GHS, GH secretagogue; GHS-R, GHS receptor; GHS-R1a, GHS receptor type 1a; GHS-R1b, GHS receptor type 1b; HEX, hexarelin; UAG, unacylated ghrelin.

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not investigated, the possibility that ghrelin had a direct peripheral action on liver was supported by an *in vitro* study, showing that in rat and human hepatoma cell lines, ghrelin was able to activate the intracellular signaling of the insulin receptor and to reverse the inhibitory effect of insulin on the expression of key gluconeogenic enzymes at the transcriptional level (27).

The aim of our study was to develop a model in which we could confirm the human *in vivo* findings with a hepatic mechanism that might contribute to the observed hyperglycemic effects of ghrelin. Because the clinical observations suggest a very rapid response to ghrelin, our approach was to examine whether AG and UAG can directly affect glucose release by porcine primary hepatocytes in short-term suspension cultures. We also investigated the effects of HEX, which shares with ghrelin some activities mediated by the GHS-R1a (*e.g.* GH secretion) but does not induce changes in glycemia *in vivo* (3, 24). The expression of GHS-R1a and GHS-R type 1b (GHS-R1b) by the primary hepatocytes was also evaluated.

Materials and Methods

Materials

Liver perfusion medium, liver digestion medium, and DMEM without glucose or pyruvate were purchased from GIBCO-Invitrogen (Paisley, Scotland, UK). Glucagon (Glucagen) was provided by Novo Nordisk (Bagsværd, Denmark). Glucose (TRINDER) was purchased from Sigma Diagnostics (Steinheim, Germany). The Protein Assay Kit was obtained from Bio-Rad (Munich, Germany). Human acyl-ghrelin was kindly provided by Neosystem (Strasbourg, France), human desoctanoyl ghrelin was provided by Theratechnologies Inc. (Montreal, Quebec, Canada), and HEX was provided by Europeptides (Argenteuil, France). The High-Pure RNA isolation kit was purchased from Roche Diagnostics (Mannheim, Germany). All other reagents were purchased from Sigma (Steinheim, Germany).

Hepatocyte isolation

All experiments were performed on porcine primary hepatocytes. In pigs as well as in humans, ghrelin is produced by endocrine cells of the stomach (28). In pigs, human ghrelin exerts GH-releasing activity on pituitary somatotropes, with conserved interaction with GHRH and somatostatin (29), and can activate the GHS-R1a (30).

Livers were obtained from 6-month-old female pigs (n = 11) after 12 h of fasting. The tissues were kindly provided by the Experimental Animal Center and Experimental Cardiology Department (Erasmus MC, Rotterdam) with approval of the local animal ethics committee.

Hepatocytes were isolated by a modification of the two-step *in situ* collagenase perfusion method based on the procedure described by Seglen (31). Within 15 min of the animal being killed, the right lobe of the liver was removed and then perfused with liver perfusion medium at 37 C for 15–20 min, followed by liver digestion medium for 20–30 min. Hepatocytes were isolated by gentle disruption of the digested liver in suspension medium [26.5 mM $NaHCO_3$, 8.99 mM Na-HEPES, 0.2% (wt/vol) BSA fraction V, 2.22 mM D-fructose, in DMEM with 5.5 mM glucose and 1 mM Na pyruvate] and filtered through a 200-μm mesh. The resulting cell suspension was then centrifuged at 500 rpm, the supernatant was discarded, and the cell pellet resuspended in prewarmed (37 C) suspension medium. Cell viability was assessed using the Trypan blue exclusion method (Life Technologies, Grand Island, NY) and was consistently higher than 85%. Cell counts were performed in triplicate, and the mean value was obtained.

Suspension cultures

Immediately after the isolation, hepatocytes were washed three times with serum-free DMEM without glucose or pyruvate and then resuspended in 600 μl of the same medium at a cell density of 30×10^6/ml in 50-ml conical tubes. The tubes were then incubated at 37 C for 10, 20, and 40 min with continuous shaking, alone or with increasing concentrations (1, 10, and 100 nM) of AG, UAG, or their combination. The glucose release by hepatocytes after 10 and 20 min of incubation with 100 nM HEX was also investigated. In each experiment, glucose output after stimulation with 100 nM glucagon was used as positive control.

We studied hepatocytes isolated from 11 different livers, each condition being run on at least five different preparations, with six replicates each.

After the incubation period, glucose released into the medium was determined with the glucose-oxidase method using a Trinder assay kit (Sigma), and the results of all replicates have been normalized for protein content, which was determined using the Bio-Rad protein assay kit.

RNA extraction and RT-PCR for GHS-R1a and GHS-R1b

The expression of GHS-R1a and GHS-R1b mRNA was also investigated in 10 preparations of hepatocytes isolated by different livers using RT-PCR. Total RNA was isolated from primary hepatocytes using a High-Pure RNA isolation kit (Roche). The quality and quantity of RNA was assessed using both an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) and amplification of β-actin mRNA. RNA samples (100 ng) underwent conventional reverse transcription followed by one-step TaqMan real-time PCR (ABI PRISM 7700 sequence Detection System; Perkin-Elmer, Wellesley, MA) for the GHS-R1a and GHS-R1b genes. Porcine-specific primers and probes were designed using a similar strategy of detection as Korbonits *et al.* (32) and conditions similar to those described by these authors. Intron-spanning primers were used for the GHS-R1a (forward: 5′-CggTgggCTCCTCgC-3′; reverse: 5′-gTATgAAAgCAAACACCACTACAgC-3′; probe: 5′-FAM-CAgggAC-CAgAACCACAAACAAACCg-TAMRA-3′), whereas a special reverse primer was designed for the intronic sequence of GHS-R1b (forward: 5′-CggTgggCTCCTCgC-3′; reverse: 5′-gTATgAAAgCAAACACCAC-TACAgC-3′; probe: 5′-FAM-CAgggACCAgAACCACAAACAAACCg-TAMRA-3′). Each sample was assayed in duplicates in at least two different reactions.

Statistical analysis

The results are expressed as percentage of control values from untreated controls. Statistical analysis was carried out with StatSoft, version 6.0 (StatSoft Inc., Tulsa, OK). Data were revised, and outliers and extremes were eliminated according to a coefficient of outlier of 2.0. Results were then tested for statistical significance using ANOVA, followed by least significant difference *post hoc* test. $P < 0.05$ was considered significant.

Functional assay for AG and UAG in CHO cells expressing GHS-R1a

Because there is very little published data on the inability of UAG to activate the GHS-R1a or antagonize activation of this receptor by ghrelin, we tested the activity of the UAG in a functional bioassay. CHO-K1 cells stably expressing both mitochondrially targeted apoaequorin and GHS-R1a (GHS-R-A5 cells, kindly provided by Euroscreen, Gosselies, Belgium) were resuspended in BSA assay buffer (DMEM/HAM's F12, with HEPES, 0.1% BSA, amphotericin, penicillin, and streptomycin) at 5×10^6 cells/ml, and then coelenterazine h (Sigma, St Louis, MO) was added to a final concentration of 2.5 μM. Cells were incubated at room temperature for 4 h and kept in suspension by gentle rotation. Cells were then diluted with BSA assay buffer to 5×10^5 cells/ml, and 100 μl was injected into wells of a 96-well plate containing 100 μl of various concentrations and combinations of UAG and ghrelin. Luminescence was measured for 20 sec using a Victor2 1420 multilabel counter (Perkin-Elmer). After the collection of data (response to agonist: x), 100 μl of 1% Triton X-100 (vol/vol in water) was injected into each well, and luminescence was measured (response to Triton: y). Data were calculated as the fractional response to agonist relative to the total response of the cells to agonist and Triton [$FR = x \times (x + y)^{-1}$].

^{125}I-ghrelin binding assay

Competition binding assay was performed on membranes of the GHS-R-A5 cell line.

Cell membrane extracts were obtained as indicated. Briefly, GHS-R-A5 cells were scraped from the culture flasks in Ca^{2+}- and Mg^{2+}-free PBS. The cells were then centrifuged for 3 min at 1500 × *g*, and the pellet was resuspended in buffer A (15 mM Tris-HCl, pH 7.5; 2 mM $MgCl_2$; 0.3 mM EDTA; and 1 mM EGTA) and homogenized in a glass homogenizer. The crude membrane fraction was collected by two consecutive centrifugation steps at 40,000 × *g* for 25 min separated by a washing step in buffer A. The final pellet was resuspended in buffer B (75 mM Tris-HCl, pH 7.5; 12.5 mM $MgCl_2$; 1 mM EGTA; and 250 mM sucrose) and flash frozen in liquid nitrogen. Protein content was determined using the Bio-Rad protein assay kit.

Competition binding assays were performed in tubes containing binding buffer (25 mM HEPES, pH 7.4; 1 mM $CaCl_2$; and 5 mM $MgCl_2$), GHS-R-A5 membrane extracts (20 μg protein/tube), and fixed concentrations of the radioligand ^{125}I-ghrelin (NEX388; NEN Life Science Products, Boston, MA) with increasing concentrations of either AG or UAG. The samples were incubated in a final volume of 100 μl for 1 h at room temperature and then washed twice with ice-cold binding buffer. The washing steps were followed by centrifugation at 14,000 rpm at 4 C for 3 min. The pellet was then counted in a γ-counter.

The IC_{50} was calculated using PRISM (GraphPad Software Inc, San Diego, CA).

Results

Effect of ghrelin on glucose output

After 20 min, 100 nM AG induced an increase of glucose output by primary hepatocytes reaching a maximum of 132% of control values ($P < 0.001$), which was lower than that exerted by equimolar glucagon (158% of control values; $P < 0.001$, glucagon *vs.* control), although the difference did not reach a statistical significance. The inductive effect of AG on glucose output was already lost by 40 min (Fig. 1). The stimulating effect of AG on glucose release by hepatocytes was dose dependent and was significant at 100 nM AG (Fig. 2).

HEX is a synthetic GHS that shares with ghrelin a potent GH-releasing activity, which is mediated by GHS-R1a (3). However, recent *in vivo* studies have shown that acute treatment with HEX does not induce changes in glucose levels (24). This has led to speculation that the hyperglycemic effect of ghrelin may not involve the GHS-R1a. To confirm the clinical data and demonstrate a potential differential effect of AG and HEX in the liver, we examined whether HEX could alter glucose release by primary hepatocytes. In contrast to the effect of AG, and in agreement with the clinical data, glucose release by hepatocytes after 10 or 20 min of incubation with 100 nM HEX was unaffected relative to control values (99% of control, $P = 0.9$).

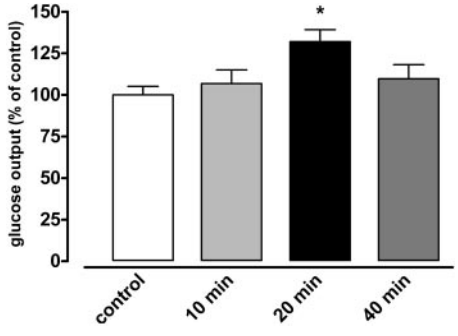

FIG. 1. Time course of the glucose release by primary hepatocytes in suspension culture incubated for 10, 20, and 40 min in DMEM without glucose in the presence of 100 nM ghrelin. At each time-point experiment, glucose output is expressed as percentage of control values. *Bars* represent SEM (*, $P \leq 0.001$).

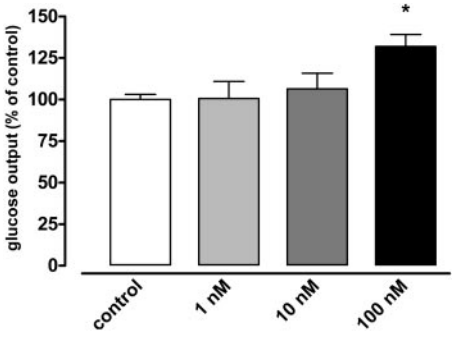

FIG. 2. Glucose output by primary hepatocytes in suspension culture after 20 min of incubation with DMEM without glucose in the absence or the presence of 1, 10, and 100 nM ghrelin. *Bars* represent SEM (*, $P \leq 0.001$).

UAG has been shown to exert peripheral actions (11), although it has been demonstrated not to activate GHS-R1a (1). Surprisingly, we found that UAG was able to inhibit glucose release. This effect was also dose dependent and was significant at 100 nM (76% of controls, $P < 0.001$) and detectable after 20 min of incubation (Fig. 3). Moreover, equimolar UAG completely reversed the 100 nM AG-induced glucose output to control levels (132% down to 84%, $P < 0.01$; Fig. 4). Equimolar UAG also significantly suppressed the effect of 100 nM glucagon (158% down to 135%, $P < 0.05$; Fig. 4).

Gene expression of GHS-R1a and GHS-R1b

The consistent effect of ghrelin on glucose release suggested that this was potentially a classical GHS-R1a-mediated response. Therefore, we examined the gene expression of this receptor as well as its splice variant, GHS-R1b. Porcine hepatocyte preparations from 10 different livers were assessed for gene expression of GHS-R1a and GHS-R1b using 100 ng of reverse-transcribed total RNA.

Because of the low level of expression, the results are reported as Ct (cycle threshold for the sequence detector) values. In all the samples, the level of GHS-R1a mRNA was either low (Ct 35) or undetectable (Ct 40) when compared with pancreas (Ct 17–20), which we used as a positive con-

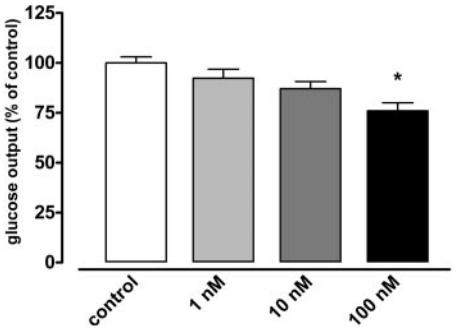

FIG. 3. Glucose output by primary hepatocytes in suspension culture after 20 min of incubation with DMEM without glucose in the absence or the presence of 1, 10, and 100 nM des-octanoyl ghrelin. *Bars* represent SEM (*, $P \leq 0.001$).

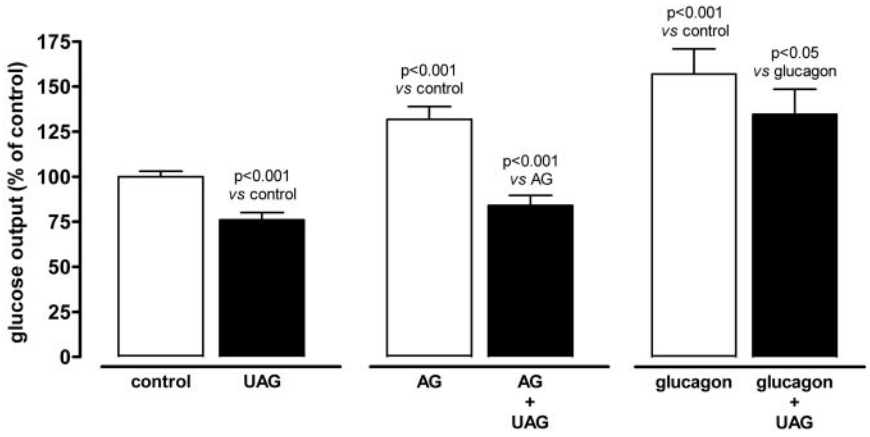

FIG. 4. Effects of des-octanoyl ghrelin on ghrelin- or glucagon-stimulated glucose output by primary hepatocytes. The glucose output, expressed as percentage of control values, was evaluated after 20 min of incubation with 100 nM ghrelin or glucagon, alone or in combination with equimolar des-octanoyl ghrelin. *Bars* represent SEM.

trol. GHS-R1b mRNA was also low to barely detectable (Ct 35–36) in all the samples studied.

UAG does not antagonize activation or binding of ghrelin to GHS-R1a

An intriguing finding that we had not anticipated was the ability of UAG to antagonize the effects of ghrelin on hepatocytes (at least with glucose output as an end point). A possible explanation was that UAG was able to modulate the interaction of ghrelin with GHS-R1a. However, UAG does not activate this receptor (1), making such a mechanism of action unlikely. To examine this experimentally, we used a CHO cell line that coexpressed GHS-R1a and the $[Ca^{2+}]$ reporter aequorin (GHS-R-A5 cell line, Euroscreen). This model allowed us to examine specifically whether UAG could antagonize ghrelin activation of the GHS-R1a. Initially, UAG was incapable of activating the GHS-R1a in this system (data not shown), despite characteristic stimulation of $[Ca^{2+}]_i$ by ghrelin with an EC_{50} of approximately 2 nM. We then examined whether UAG could antagonize ghrelin in this system. Cells were treated with 5 nM ghrelin combined with varying concentrations of UAG. No significant antagonistic effect of UAG was observed up to 10^{-6} M (data not shown). Finally, we examined the possibility that UAG could prevent ghrelin from binding to its receptor. In competitive binding experiments, we found that UAG at concentrations up to 10^{-7} M was incapable of displacing ^{125}I-ghrelin from GHS-R1a (membrane preparations prepared from cultures of GHS-R-A5 cells), despite complete displacement of ligand by unlabeled ghrelin with an IC_{50} of approximately 2 nM in the same system (data not shown). These results demonstrate that UAG is incapable of modulating ghrelin interaction/activity at the GHS-R1a. Because in hepatocytes ghrelin activity (measured by glucose release) can be antagonized by UAG, these data provide indirect evidence that the ghrelin response we observed in hepatocytes is not mediated by GHS-R1a.

Discussion

Although the gut hormone ghrelin was discovered as a factor that increased GH via central effects, recent observations have clearly indicated that ghrelin can exert significant direct actions on peripheral tissues that are essential in metabolic control (9, 11, 24, 25, 33). We have reported already that ghrelin has an acute hyperglycemic effect when injected as a bolus iv in normal human subjects (24, 26). Most interestingly, this hyperglycemic effect was only seen when AG, but not UAG, was administered (26). Moreover, and this was the most stunning observation, UAG could even block the hyperglycemic effect (26). To address the mechanism behind these phenomena, we studied the effects of AG and UAG on primary hepatocytes. Using this model, we could confirm these effects of ghrelin *in vitro* because the results of the present study demonstrate, for the first time, that ghrelin, but not HEX, induces a rapid increase of glucose output by primary hepatocytes, which supports the hypothesis that AG modulates glucose homeostasis by at least acting directly on the liver. We also found that the UAG itself exerts an inhibitory effect on glucose output and, as was seen in normal subjects *in vivo*, it is able to counteract the inductive effect of AG on glucose release.

Strikingly, we also observed that the maximal stimulation of glucose output by hepatocytes was by 20 min, which fits well with the observation that, in humans, ghrelin administration was rapidly followed by an increase in plasma glucose levels (24, 25), again within 20 min of the time of injection. After the administration of AG *in vivo*, a transient suppression of circulating insulin was observed but only after a rise in glucose levels. Because of the relative timing of the events, we postulated that the suppression of insulin could not be the cause of the hyperglycemic effect. This conclusion implied that ghrelin should be considered as a direct regulator of glucose metabolism, independent of insulin secretion. Obviously, we considered by then that the liver was the primary suspect responsible for this acute hyperglycemic effect of ghrelin. This possibility was supported by another observation that ghrelin was able to suppress Akt kinase activity and to partially reverse the inhibitory effect of insulin on phosphoenolpyruvate carboxykinase (PEPCK) mRNA expression in rat and human hepatoma cell lines (27). However, the influence of ghrelin on glucose release by liver cells has not been investigated to date.

So, to address the hyperglycemic effect of AG on the liver, we chose an *in vitro* model that enabled us to study the effects of the presence of AG and/or UAG in the absence of hormones that also regulate glucose output (*e.g.* GH, insulin, and

glucagon). Using this model, we not only confirmed the *in vivo* data in humans by our *in vitro* studies, but we also showed that the effects of AG were dose dependent (Fig. 2). Surprisingly, we also found that UAG inhibited glucose release by primary hepatocytes, whereas, in normal subjects *in vivo*, UAG administered alone was inert as far as glucose output was concerned. In fact, it was inert in all of the metabolic parameters studied. As with AG, the suppressive effect of UAG on glucose release *in vitro* was dose dependent and most prominent after 20 min of incubation. *In vitro*, comparable to the *in vivo* data, UAG was again able to completely antagonize the AG-induced glucose release by hepatocytes, and it significantly suppressed glucagon-stimulated glucose output as well. One of the most important questions now is which receptor (system) might be involved in the modification of glucose metabolism by ghrelin. Most likely, the receptor that mediates UAG effects is different than the classical ghrelin receptor, GHS-R1a, because this receptor is not able to bind UAG as a ligand. We show that UAG was incapable of activating calcium mobilization in GHS-R1a-expressing cells, and it was also unable to antagonize the ghrelin-induced calcium release. One could hypothesize that UAG might bind to the GHS-R1a, activating a different intracellular signaling than calcium release. However, in competitive binding experiments, we found that UAG did not displace ^{125}I-ghrelin from GHS-R1a in membranes obtained from GHS-R-A5 cells. From other studies reported so far, we have learned already that UAG is not biologically inactive, being able to share with ghrelin antiproliferative effects on human breast and prostate cancer lines (8, 9), negative inotropic effects on papillary muscle (10), and stimulation of bone marrow adipogenesis (11). In these reported studies, the authors also came to the conclusion that these effects are mediated by other ghrelin receptors, although neither these authors nor any other research group so far have been able to characterize these non-GHS-R1a ghrelin receptors.

Our data again suggest the existence of non-GHS-R1a receptors that mediate the hepatic actions of at least UAG. Moreover, we cannot exclude that AG in this case could also act via a non-GHS-R1a because the GHS-R1a was not detectable in the hepatocytes studied. Human AG and UAG, when administered alone, exert an opposite regulation on glucose output by liver cells, making it possible that different receptors or signaling mechanisms are involved. We observed that UAG antagonized AG-induced glucose output. We speculate that AG and UAG can exert their hepatic biological effect activating separate receptors and/or compete for the same receptor subtype that is different than GHS-R1a. In this context, we cannot exclude that UAG modulates glucagon receptor activity because we observed that it was able to inhibit the glucagon effect on glucose output. Completely in line with this is our observation that the classical GHS-R1a agonist HEX (3, 14), a synthetic peptidyl GHS, does not modify glucose release by hepatocytes, an observation that is in agreement with human studies (24). Therefore, we come to the conclusion that AG and UAG are factors, produced by the gut, that directly influence glucose handling by the liver by as yet unidentified type(s) of ghrelin receptors. Moreover, AG and UAG seem to control each other's actions on glucose handling, which makes it even more important to obtain information on the levels of both these forms of active ghrelin in any report on these hormones. In fact, we have shown here that both AG and UAG should be considered as separate hormones. Further support for this postulation is provided by a recent report on the existence of an unidentified GHS-R other than the GHS-R1a that is involved in the peripheral actions of AG as well as UAG on adipocytes, prostate cancer cell lines, or papillary muscle (9–11). Another indirect way of showing the presence of new receptor(s) is to show the absence of the known GHS-R1a in those circumstances in which efficacy of AG is still detectable, along with UAG activity. Indeed, mRNA expression of GHS-R1a in liver has not been clearly demonstrated. To date, GHS-R1a gene expression has been detected in a human liver cDNA library and HepG2 cells (27) but not in whole human liver (4). Only the GHS-R1b, a splice variant of GHS-R1a, which neither binds nor is activated by AG or UAG (3, 34), is widespread in human tissues, including the liver (4). Using real-time PCR, we investigated the mRNA expression of the GHS-R subtypes in our hepatocyte preparations. We found that the gene expression of GHS-R1a was undetectable, whereas the expression of GHS-R1b was low to undetectable, thus indirectly proving that receptor subtype(s) other than GHS-R1a mediate the peripheral direct action of AG and UAG on liver glucose output.

In conclusion, these data show that AG, but not the classical GHS-R1a agonist HEX, elicits glucose output by primary hepatocytes, providing evidence that ghrelin modulates glucose metabolism by acting directly on the liver. We also demonstrated that des-octanoyl ghrelin suppresses glucose release by hepatocytes. Furthermore, we report that des-octanoyl ghrelin is able to antagonize AG-induced glucose output. These actions could be mediated by receptor(s) different from the GHS-R1a. Notwithstanding the fact that the metabolic pathways mediating these actions need to be clarified and the ghrelin receptor (sub)type(s) involved must be characterized, we postulate that both octanoyl- and des-octanoyl ghrelin should be considered as separate hormones able to modify hepatic glucose homeostasis.

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