Metabolic aspects of the ghrelin system

Carlotta Gauna

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Metabolic aspects of the ghrelin system

Metabole aspecten van het ghrelin systeem

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Chapter I

General introduction

THE GHRELIN SYSTEM

In the last decade the discovery of ghrelin, a gut peptide discovered in 1999 by Kojima and colleagues (1), has led to the identification of a complex system that introduced new perspectives in neuroendocrine and metabolic research.

Ghrelin is a peptide-hormone of 28 amino acids, predominantly produced by the stomach and detected in a lower amount in other central and peripheral tissues (1-11). The ghrelin peptide has a biological peculiarity, which is the esterification of a fatty (mostly n-octanoic) acid at its third serine residue (1). This modification is necessary for binding and activation of the growth hormone secretagogue receptor type 1a (GHS-R1a), the only cloned ghrelin receptor so far (1, 12, 13). Before the discovery of ghrelin, the GHS-R1a was an orphan G-protein coupled receptor specific for a family of synthetic molecules exerting a strong GH-releasing activity and therefore named Growth Hormone Secretagogues (GHS). The acyl-modified forms of ghrelin (AG), as well as some of the synthetic GHSs, have pleiotropic activities, including modulation of insulin secretion and glucose homeostasis.

Besides the acylated form of ghrelin (AG), an unacylated ghrelin molecule (unacylated ghrelin, UAG) is also present in circulation. The absence of the acyl modification makes UAG unable to bind or activate the GHS-R1a (1). Moreover, although a specific UAG receptor has not been isolated to date, its existence has been strongly suggested. UAG shares with AG a variety of biological actions, but it also exerts AG-independent activities (11). Recently, a third molecule has been identified as a ghrelin-associated peptide and named obestatin (14). Obestatin is encoded by the same ghrelin gene and is a 23-amino acid product of the pro-ghrelin peptide. However, it does not bind the GHS-R1a (14).

The growing body of literature over the last few years profiled the complex identities and interactions of these newly discovered molecules and their known and unknown receptor(s), which constitute the ghrelin system.

The line of research and the studies included in this thesis focus on the involvement of the ghrelin system in the regulation of glucose metabolism, with particular emphasis on AG, UAG and their receptor(s).

1. HISTORICAL BACKGROUND: SYNTHETIC GROWTH HORMONE SECRETAGOGUES (GHSs)

Ghrelin was identified as an endogenous ligand for the GHS-R, a previously orphan receptor specific for synthetic GHSs (1). Curiously, this receptor was identified and cloned 20 years later than the discovery of its synthetic ligands (15). This makes

the discovery of ghrelin an example of "reverse pharmacology", starting with the synthesis of synthetic analogs, leading to the isolation of a natural receptor and then to the discovery of an endogenous ligand for the natural receptor.

Synthetic GHSs are a family of synthetic, peptidyl and non-peptidyl, molecules designed by Bowers and Momany in the late 1970s (15). GHSs are potent stimulators of GH secretion (for review see (11, 16)). The first synthesized GHSs were short peptides, also called GH-releasing peptides (GHRPs), metenkefalin derivatives devoid of opioid activities (17). The main purpose of the GHS research was to find a candidate molecule for the treatment of GH deficiency in childhood and a possible "anti-aging" therapy in the frail elderly. Therefore, the requirements for such a compound were high oral bioavailability and pharmacokinetics suitable for once daily administration. GHRP-6 was the first peptide to actively and potently release GH after oral administration in vivo, more in humans than in animals, although it had a low bioavailability and a short half-life (16, 18-20). Further research led to non-peptidyl, orally active, GHSs. The most representative was the spiropiperidine L-163,191, introduced in the clinic as MK-0677, endowing a high bioavailability and able to enhance the 24-h GH secretion after a single oral administration (16). Appealing therapeutic perspectives gave new impulse to the research in the field, aimed to identify a receptor for GHSs and the regulatory mechanisms involved. MK-0677 was shown to bind with high affinity (K_D = 200 pM) to membranes isolated from pituitary and hypothalamic tissues (21, 22). Binding studies indicated that the specific bindig sites were G-protein coupled, because high-affinity binding was inhibited non-competitively by the guanine nucleotide analogue guanosine 5'-[γ-^{35}S]thiotriphosphate (GTP-g-S). Following ligand binding, the signal transduction mechanism was identified (22, 23) (see section 2). In 1996 Howard and colleagues exploited this signalling pathways to clone the receptor, using Xenopus oocytes for expression cloning (22, 24).

2. GHS RECEPTOR (GHS-R)

The GHS-R is encoded by a single gene at human chromosomal location 3q26.2, which is highly conserved across species (25, 26). This gene is composed of two exons separated by one intron. Alternate processing of a pre-mRNA generates two types of GHS-R mRNA, designated as GHS-R type 1a (GHS-R1a) and type 1b (GHS-R1b) (24, 27-30). The human full-length GHS-R1a, the functional receptor subtype, encodes for a predicted polypeptide of 366 aminoacids with seven transmembrane domains (TM 1-7) that confer high-affinity GHSs and ghrelin binding (24). By using molecular modeling and site-directed mutagenesis in combination with binding and activation data, the ligand-binding pocket of GHS-R1a was mapped. Synthetic

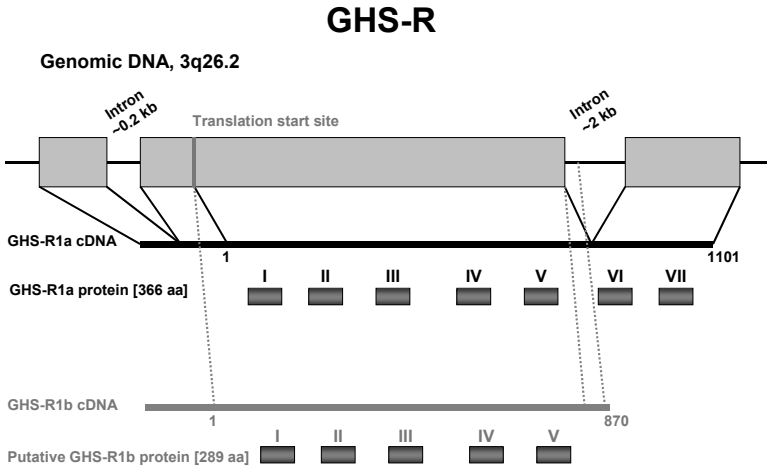

Figure 1. GHS-R gene and transcripts: GHS-R type 1a and type 1b. Adapted from (32).

GHSs and ghrelin share a common binding domain at TM3. Other contact points (at TM2, TM5, TM6 and extracellular loop 1) are specific for different, peptidyl and non-peptidyl, GHSs, suggesting that GHS-R can be activated by ligands binding at different pockets (31).

By contrast, the GHS-R1b is encoded by one exon, which results in a truncated polypeptide of 289 aminoacids with only 5 transmembrane regions (TM 1-5) fused to a short conserved reading frame of 24 amino acid at the carboxyl-terminal region. GHS-R1b fails to bind either synthetic GHSs or ghrelin (24, 26, 27, 30) and its functional activity has to be determined to date. Therefore, this GHS-R variant will not be further discussed in this thesis.

The GHS-R1a is the first of a subfamily of the rhodopsin-like family of G-protein coupled receptors, and a close relative of the neurotensin receptor-1 (NT-1) and the TRH receptor, with 59% and 56% similarity, respectively. The characterization of the GHS-R1a allowed the cloning of new GHS-R family members (FMs): i) GPR38 or FM1, later identified as motilin cognate receptor, having a 52% protein sequence identity to human GHS-R (25); ii) GPR39 or FM2 (25), with a 32% protein sequence identity to human GHS-R; iii) GPR66 or FM3, with a 33% protein identity to GHS-R, specific for neuromedin U (33, 34).

2.1 Alternative ligands for the GHS-R1a

Besides natural and synthetic GHSs, binding studies have identified other synthetic molecules able to bind the GHS-R1a.

Binding studies conducted to elucidate the yet unclear interactions between the GHSs system and somatostatin (SS) showed, unexpectedly, that some SS-peptidomimetics such as vapreotide, lanreotide and to a lesser extent octreotide, but not the native SS or SS-short fragments, caused a substantial displacement of the radiolabeled GHS [^{125}I]Tyr-Ala hexarelin, a synthetic GHS-R ligand, from pituitary binding sites, although at a concentration of the ligand in the micromolar range (35, 36). Cortistatin (CST) is a neuropeptide showing high structural homology with SS that binds to all five SS-receptors with an affinity similar to that of SS (37). CST competes with [^{125}I]Tyr-Ala hexarelin for (GHS-) receptor binding, exhibiting an affinity for binding similar to ghrelin (36). Moreover, CST displaced radiolabeled ghrelin from hypothalamic membranes with an affinity in the nanomolar range, similarly to ghrelin and hexarelin (38) (Table 1). These data suggest that CST might indeed be another endogenous ligand of the GHS-R1a, although the functional implications of this interaction have not been elucidated yet.

In the last few years several reports showed conflicting results regarding the role of adenosine as ligand of the GHS-R1a. Basing on in vitro observations, it was proposed that adenosine acts as a partial agonist of the GHS-R1a, via binding to a binding pocket distinct from that of GHSs (39) and stimulating a different signalling pathway, involving calcium mobilization and cAMP (see 2.3). However, more recently it has been elucidated that adenosine is not a direct agonist of GHS-R1a (40, 41) and that its action on intracellular pathways is mediated by the endogenous adenosine receptors types –2B and -3, which are able to partially use the intracellular signaling machinery of the GHS-R1a (41).

Table 1. Binding affinity of non-GHS molecules for binding sites specific for synthetic and natural GHSs in hypothalamus. IC_{50}: concentration of competitor required to inhibit radiotracer binding by 50%.

Compound **Ref.**	**[^{125}I]Ghrelin** **IC_{50} (mol/L)** **(38)**
GHSs	
Ghrelin	(24 ± 0.9) x 10^{-9}
Hexarelin	(26 ± 1.0) x 10^{-9}
SS peptidomimetics	
CST-14	(27 ± 2.0) x 10^{-9}
Vapreotide	(16 ± 7.0) x 10^{-9}

2.2. Alternative GHSs binding sites

The presence of binding sites for peptidyl GHSs (*i.e.* Tyr-Ala-hexarelin, GHRP-2, GHRP-6 and hexarelin) was shown in some peripheral tissues, with a density even higher than in the pituitary, including rat and human heart (42), lung, arteries, skeletal muscle, kidney, liver (20, 43-45). The fact that these binding sites have weaker capacity to bind MK-0677 or ghrelin than hexarelin (44, 45) (Table 2), along with the absence of detection of GHS-R1a mRNA in some tissues, suggests that they may differ from the known GHS-R1a (45, 46). Moreover, a growing body of literature has pinpointed the existence of binding sites recognized by AG as well as by UAG, in the presence of both AG- and UAG- dependent biological actions, regardless the presence (*i.e.* in pituitary) or the absence (*i.e.* in liver) of GHS-R1a expression (46-55). In some of these biological systems both AG and UAG have been shown to modulate proliferation upon activation of MAPK-dependent signaling cascades, including: i) MAPK and Extracellular signal-Regulated Kinase 1/2 (ERK1/2) (49, 50, 56-59); ii) tyrosine-kinase dependent MAPK p42/44, (30, 55, 60-62); iii) PI3 kinase/Akt and MAPK patways (30, 63-65).

A receptor able to bind GHSs has been identified in the cardiovascular system. In myocardium (43, 66) and different human cardiovascular tissues (ventricles, atria, aorta, coronaries, carotid, endocardium, and vena cava), studies using covalent binding of a photoactivatable benzoylphenylalanine (Bpa) radio-labelled derivative [^{125}I] Tyr-Bpa-Ala-hexarelin revealed a structure that is identical to CD36, also known as glycoprotein type B scavenger receptor, a multifunctional receptor of 84 kDa that is expressed in human myocardium and microvascular endothelium (43, 67-70). However, the functional role of CD36 in the cardiovascular actions of ghrelin and synthetic GHSs (see section 5) remains to be elucidated.

2.3. Tissue distribution of the GHS-R

Expression of GHS-R1a was found primarily in pituitary somatotrophs and hypothalamus (24). In the hypothalamus, in particular in neurons of the arcuate nucleus, ghrelin and synthetic GHSs induced the expression of markers of neuronal activity (*i.e.* c-fos and early growth response factor-1) (71, 72). The activated hypothalamic cells include GHRH-containing neurons, but also cells expressing the appetite-stimulating neuropeptide Y (NPY) and the agouti-related protein (AgRP), the latter being an agonist of the endogenous melanocortin receptor that prevents the intrinsic, ligand-independent, activity of the receptor (also referred as “inverse agonist” of receptor with “constitutive activity”) (73, 74). GHS-R1a distribution was initially detected using RT-PCR technique, which showed very low expression levels (24,

75). Localization of expression was determined by ribonuclease protection assays and *in situ* hybridization (24, 75), which showed the presence of GHS-R1a mRNA also in other extra-hypothalamic areas of CNS, such as hippocampus (dentate gyrus, CA2 and CA3 regions), substantia nigra, ventral tegmental area, dorsal and medial raphe nuclei, Edinger-Wesfal nucleus, pons and medulla oblongata (10, 75, 76). Localization studies of the GHS-R1a in peripheral tissues gave controversial results. This might be explained by the use of techniques with different sensitivity, along with the fact that, in the first studies using RT-PCR, the use of non-intron-spanning primers made GHS-R1a undistinguishable from GHS-R1b, which is widely expressed in tissues (4, 8, 63, 77). The more sensitive real-time PCR technique with intron-spanning primers detected GHS-R1a mRNA in a limited number of normal human tissues, including intestine, pancreas, adrenal, myocardium, spleen and testis (78), as well as in pituitary adenomas and endocrine neoplasms of lung, stomach and pancreas (5, 6, 45, 46, 79). This pattern of distribution of GHS-R1a has also been mapped with binding studies using radiolabelled synthetic GHS and ghrelin (45). However, binding sites for peptidyl GHSs may represent, at least in part, receptor subtypes other than GHS-R1a (see section 2.2.)

2.4. Signal transduction pathways coupled to GHS-R activation

Binding of ghrelin or synthetic GHSs (*i.e.* the peptidyl GHRP-6 and the non peptidyl MK-0677) to GHS-R1a activates the phospholipase C signaling pathway by coupling with $G_{\alpha 11}$, leading to inositol phosphate turnover and protein kinase C (PKC) activation, followed by the mobilization of intracellular calcium ($[Ca^{2+}]_i$) (23). Phospholipase C hydrolyzes phosphatidyl-inositol-4,5-biphosphate, stored in the plasma membrane, to give both diacylglycerol (DAG) and inositol-triphosphate (IP_3). IP_3 binds to the IP_3 receptor on the endoplasmic reticulum to release calcium from intracellular storage. In addition, DAG activates protein kinase C (PKC) that inhibits potassium channels via tyrosine phosphorilation, thus causing membrane depolarization, which is followed by influx of Ca^{2+} via voltage dependent L-type calcium channels (23).

A second possible signaling pathway for the GHS-R1a is cyclic AMP (cAMP) activation, although it has only been observed in the presence of GHRH receptor activation (32, 80) and therefore it may be expression of a synergism between GHRH- and GHSs- receptors, rather than an independent mechanism of action of the GHS-R1a itself. It has been hypothesized that this effect may be mediated by interactions between the $G_{\beta\gamma}$ subunits associated with the GHS-R and the $G_{\alpha s}$ of the GHRH receptor complex (23). However, the exact mechanisms have not been elucidated so far.

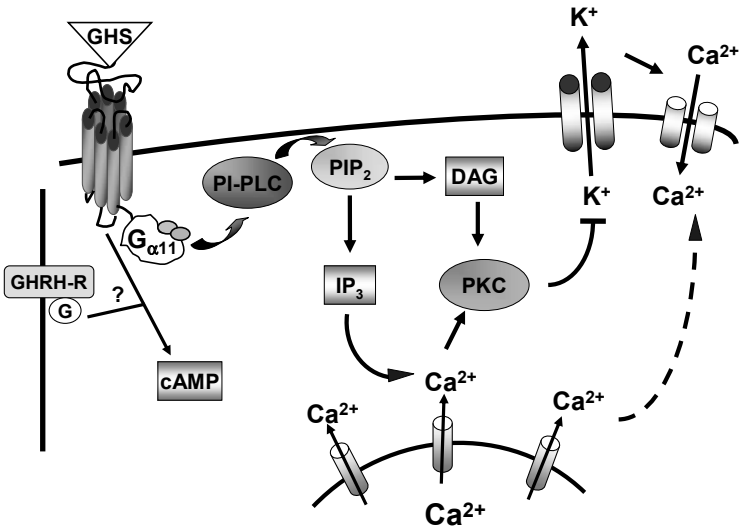

Figure 2. Signal transduction pathways of GHS-R (type 1a) in somatotroph cells. PLC: Phospholipase C; PIP_2: phosphatidyl-inositol-1,4-biphosphate; DAG: diacylglycerol; IP_3: inositol triphosphate; PKC: protein kinase C. Dotted line indicates an alternative calcium influx possibly due to calcium channels at the plasma membrane. Adapted from (30) and (32).

Besides being (further) activated upon ligand-receptor interaction, the GHS-R1a has been shown to have a strong intrinsic (ligand-independent) activity, also referred as "constitutive activity", in transfected COS-7 and HEK-293 cells, as detected by measuring inositol phosphate turnover and by using a reporter assay for transcriptional activity controlled by cAMP responsive element (81).

2.5. Regulation of GHS-R

The regulation of GHS-R1a responsiveness involves desensitization and receptor down regulation.

Exposure of the GHS-R1a to GHS results in rapid desensitization (occurring by 20 minutes), which is due to uncoupling of the receptor from heterotrimeric G proteins and to receptor internalization from the cell surface to intracellular compartments, which occurs via clathrin-coated pits (30, 82). Once the ligand-receptor complex is internalized into vescicles, the GHS-R1a is sorted into endosomes where the ligand-receptor complex is dissociated. The receptor is then recycled to the plasma membrane. Recycling of GHS-R1a requires approximately 1 hour. Surface binding slowly recovers and returns to baseline after 3-6 hours (30, 82).

The mRNA expression of GHS-R1a is down regulated by GH and GHSs. On the other hand, GH deficiency, GHRH agonists, glucocorticoids, estrogens and thyroid hormones have been reported to upregulate GHS-R1a expression (for review see (32)).

3. GHRELIN

Ghrelin was isolated in 1999 by Kojima and coworkers as an endogenous full agonist of GHS-R1a (1). The name ghrelin comes from the Proto-Indo-European word "ghre", which means grow, and "relin" that refers to its GH-releasing activities. Ghrelin is a 28-amino acid peptide produced predominantly by the acid-secreting part of the stomach fundus (1). Ghrelin is the cleavage product of a preproghrelin precursor of 117 aminoacids that in humans was found to be almost identical to the prepromotilin-related peptide, isolated by Tomasetto and coworkers (83). However, human ghrelin and motilin show only 36% homology (1, 83-86) and neither motilin activates GHS-R1a (29, 85, 86) nor ghrelin activates motilin receptors (87). The ghrelin peptide has as a post-translational modification the esterification of a fatty (n-octanoic) acid, which occurs on its third serine (Ser^3) residue (1). Alternative splicing of the ghrelin gene transcript can result in the translation of a second biologically active molecule, Des-Gln14-ghrelin, a 27-aminoacid peptide missing the glutamine in position 14, which undergoes the same process of acylation as ghrelin (88).

Biologically active analogues of ghrelin were described in much smaller amounts with acyl- chains of 10 or 11 C (84). These peptides, formed by 28 (full length) or 27 aminoacids (missing the last arginine residue in their C-terminus) showed a lower activity than octanoylated ghrelin in terms of calcium mobilization in GHS-R1a expressing cells and GH-release in rats (84).

Studies dealing with the minimal sequence needed to activate the GHS-R1a showed that not the entire ghrelin sequence is necessary for activity: short N-terminus tetra- or pentapeptides including the first Gly-Ser-Ser(n-octanoyl)-Phe aminoacids are the "active core", able to induce calcium mobilization in cells overexpressing the GHS-R1a (89, 90). Intriguingly, these short ghrelin analogues are similar to peptidyl GHSs or GHRPs (90). The n-octanoyl group of ghrelin (and its truncated derivatives) is one of the principal structural features determining its potency on GHS-R1a (89), which requires bulky, flexible, or rigid hydrophobic groups at the side chain of Ser^3. However, the ester group is not essential for binding and activity, since it can be replaced by an amide group (89). A ghrelin molecule that lacks this bulky (acyl) group on Ser^3 is also present in circulation: it is the unacylated (or des-octanoyl or des-acyl) ghrelin (UAG), which does not bind or activate the GHS-R1a (1), does not displace radiolabelled (acylated) ghrelin from hypothalamic or pituitary membranes (38) and is devoid of neuroendocrine activities (13). Nevertheless, there is emerging evidence that UAG is a biologically active molecule, which is likely to activate a putative, yet unknown, UAG-receptor (11).

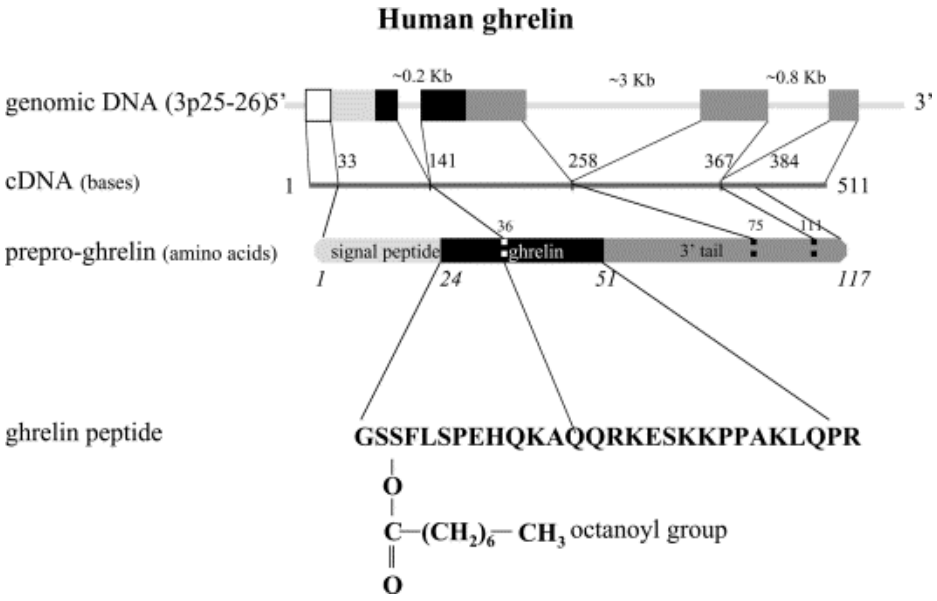

Figure 3. Gene and protein structure of human ghrelin. Reprinted, with permission, from (32)

3.1. Control of ghrelin secretion

Regulation of ghrelin levels and action involves several mechanisms that are, at least in part, independent. As suggested by van der Lely and coauthors (11), these mechanisms include: 1) regulation of transcription and translation of the ghrelin gene; 2) regulation of post-translational processes of the ghrelin molecule (*i.e.* acylation and deacylation) and regulation of levels and/or activity of the putative enzymes involved in the post-translational processing; 3) secretion rates of the bioactive ghrelin molecules; 4) possible existence of ghrelin binding proteins and their effects on hormone's bioactivity; 5) accessibility of target tissue (*i.e.* blood-brain barrier transport); 6) clearance or degradation of ghrelin by kidney or liver passage; 7) circulating concentration of additional endogenous ligands or other possibly cross-reacting hormones; 8) ghrelin receptor(s) levels of expression and activity in target tissues.

3.1.1. Ghrelin mRNA and protein expression levels

Ghrelin was first isolated from rat and human stomach, where it is produced by the X/A-like cells of the oxyntic mucosa, in the acid secreting part of the fundus. Ghrelin production occurs, in a smaller amount (approximately 30%), also in the small intestine and the lower gastrointestinal tract (91), with caudally decreasing density of expression, in agreement with the fact that X/A-like cells are not strictly confined to oxyntic mucosa (91, 92). Ghrelin-containing cells mostly have no continuity with

the lumen, probably respond to physical and/or chemical stimuli from the basolateral side and are closely associated with the capillary network running through the lamina propria (91, 92). The concentration of ghrelin found in the circulation of rats decreases by 80% following surgical removal of the acid-producing part of the stomach suggesting that the oxyntic mucosa is the major source of ghrelin (92). A similar drop of plasma ghrelin levels was found in humans following gastric bypass (93). However, plasma levels of ghrelin after total gastrectomy have been reported to gradually increase again (84), suggesting that also other tissues can compensate for the loss of ghrelin production.

Ghrelin mRNA expression was shown in all normal human tissues (11, 32, 78), as well as in several tumors including pituitary adenomas, neuroendocrine tumors, thyroid and medullary thyroid carcinomas, and in endocrine tumors of the pancreas and lung (5-7, 46, 77, 79, 94-96).

Ghrelin peptide was observed in the pituitary (96), placenta (3), lung (7), immune system (8, 97), ovary with cyclical expression (98, 99), testis (100, 101) and kidney (9). In the pancreas, some studies demonstrated localization of ghrelin in the β-cells (6), the α-cells (77) and in the ε cells, a new islet cell type (102). Ghrelin peptide has also been shown to be expressed and secreted in the hypothalamus (5, 103), where immunohistochemical studies showed ghrelin expression in the internuclear space between the lateral hypothalamus, the arcuate nucleus (ARC), the ventromedial nucleus (VMN), the dorsomedial nucleus (DMN), the paraventricular nucleus (PVN). Moreover, ghrelin was localized in the axon terminals of a group of neurons in the ependymal layer of the third ventricle. These neurons send efferents onto NPY, AgRP, proopiomelanocortin (POMC), and CRH neurons, suggesting that local ghrelin would represent a novel regulatory circuit controlling energy homeostasis (103). Peripherally produced ghrelin has been shown to cross the blood-brain barrier. Banks and coworkers identified a saturable system transporting human ghrelin from brain-to-blood and from blood-to-brain, whereas mouse ghrelin, differing from human ghrelin by two amino acids, was transported predominantly from brain-to-blood, but only minimally from blood-to-brain (104). On the contrary, UAG entered the brain by nonsaturable transmembrane diffusion (104).

3.1.2. Circulating ghrelin levels

The measurement of ghrelin immunoreactivity involves technical difficulties, implying that quantification of ghrelin concentration by commercially available immunoassays should be interpreted with caution. Initially, measurements of circulating ghrelin have been performed using an antiserum targeting the C-terminal end of the molecule, which recognizes both acylated and unacylated ghrelin. A radioimmunoassay (RIA) targeting the octanoyl side chain of the molecule at its

N-terminus became available, allowing determination of acylated ghrelin only (105). The difference between total and acylated ghrelin levels as obtained by RIA methods was supposed to reflect unacylated ghrelin levels (105). More recently, an enzyme-linked immunosorbent assay (ELISA) has been introduced as the first assay that can specifically measure the unacylated and the acylated forms of ghrelin (106). Data from the comparison of these two methods (*i.e.* unacylated ghrelin levels as measured with the ELISA assay versus those derived from the difference between total and acylated ghrelin levels) suggest the presence of ghrelin peptide fragments in plasma (106). Furthermore, there are controversial data on the stability of ghrelin in plasma samples, the influence of storing time and thaw/freeze cycles, pH changes, or the necessity for enzyme-blocking additives to plasma samples before measuring ghrelin. Although absolute plasma ghrelin levels and ghrelin reference standards still have to be determined, it appears reasonable to investigate ghrelin regulation and physiology by measurement of relative changes in ghrelin (total, acylated or unacylated) levels, using available assays (11), after taking the necessary precautions to limit deacylation and proteolysis.

Spontaneous ghrelin secretion in rats is pulsatile and displays an ultradian rhythmicity, with number of peaks and the interval between peaks similar to those observed for GH (107). In humans, a diurnal and nocturnal rhythmicity of ghrelin levels has also been observed by some (108, 109), but not by other authors (110). Ghrelin secretion is reported to be sexually dimorphic in humans, with women in the late follicular stage having higher levels than men (110). Among the determinants of ghrelin secretion, the most important appear to be: i) nutritional state; ii) insulin; iii) glucose and diet, iv) parasympathetic nervous system (vagus nerve). However, GH, leptin, melatonin, thyroid hormones, glucagon also play a role in ghrelin metabolism.

i) Nutritional state

Endogenous ghrelin levels change according to acute as well as chronic nutritional status. Ghrelin levels are enhanced by food deprivation and decrease after food intake, with nadir post-prandial levels occurring by 60–120 min (91, 93, 108, 111-114). The postprandial ghrelin suppression has been reported to be proportional to the ingested calorie load (115, 116). After prolonged fasting (approximately 3 days) ghrelin levels did not change significantly compared to the baseline state, suggesting that the meal-related changes are rather decreases after food intake than increases due to fasting (117).

A preprandial increase of ghrelin levels has been observed, leading to the hypothesis that ghrelin might be an important factor in meal initiation (91, 108, 118). Fasting ghrelin levels are decreased in obesity and are restored after weight loss (11,

93, 111, 119, 120). On the contrary, in conditions of negative energy balance, such as anorexia nervosa, ghrelin levels are increased and can be diminished by weight gain (121).

ii) Insulin

Insulin has been shown to inhibit ghrelin levels both in animals and in humans (111, 113, 118, 122, 123), although some authors did not find any effect of insulin on ghrelin levels (124). Hyperinsulinemic clamp lowers ghrelin levels in the euglycemic state (122), but also in the presence of increased or decreased blood glucose levels (123, 125, 126), suggesting that insulin is the major regulator of ghrelin levels.

iii) Glucose and diet

Postprandial ghrelin suppression was initially reported in rodents and humans ingesting meals of mixed macronutrient content, and in rodents receiving intragastric glucose infusions. It appears that all three classes of macronutrients (carbohydrates, proteins, fat) can suppress plasma ghrelin, but with varying efficacy (127-130). In rodents, circulating ghrelin levels are substantially suppressed by isocaloric glucose, amino acid, or intralipid infusions into the gastrointestinal tract (131). Ghrelin levels are most effectively reduced by glucose, with similar effects after oral and intravenous administration (132), whereas the fat infusion induced a lesser suppression of ghrelin (130). In humans, inhibition of ghrelin levels following glucose load was reported by some authors (118), but not by others (125). Monitoring of plasma ghrelin after subjects consumed isocaloric, isovolemic beverages consisting of 80% carbohydrate, protein, or fat gave results similar to those obtained in rodents (130), with the carbohydrate beverage being most effective and the fat beverage being least effective (130), in agreement with other reports (129, 133, 134). However, in contrast with a consistent inhibitory effect of carbohydrates and, among these, glucose, the role of protein and lipids in the regulation of ghrelin levels is controversial (128, 135, 136). More recently, it has been reported that the administration, either oral or parenteral, of free fatty acids and amino acids does not affect ghrelin secretion, suggesting that the effects observed after mixed (protein-enriched and lipid-enriched) may be due to the even low carbohydrate content (137).

iv) Parasympathetic nervous system (vagus nerve)

Further insight into the mechanisms regulating ghrelin secretion is based on studies showing an increase of circulating ghrelin levels in rats after surgical interventions such as vagotomy and hypophysectomy.

Mundinger and coworkers have recently shown that the neural, but not the neurohumoral, branch of the sympathetic nervous system can directly stimulate ghrelin

secretion (138). Moreover, blockade of the gastric vagal efferent did not affect suppression of ghrelin by a nutrient load, whereas it prevented the fasting-induced increase in ghrelin levels (139), suggesting that high tonic vagal activity is responsible for the fasting-induced ghrelin rise. On the other hand, pharmacological or surgical vagotomy abolishes ghrelin-induced feeding, GH secretion, NPY-producing and GHRH-producing neurons activity, gastric motility and acid secretion (85, 140-142). The GHS-Rs are synthesized in vagal afferent neurons and transported to the afferent terminals (142).

v) Others

One month after hypophysectomy ghrelin levels are increased by three times in rats (100, 143). Among pituitary hormones, GH and LH appear to play a role in ghrelin regulation, although the physiological significance remains to be elucidated.

GH is suggested to inhibit ghrelin levels. In fact, acute injection of GH decreased circulating ghrelin levels in normal rats by 50% (143) and GH treatment decreased stomach ghrelin mRNA levels significantly (144). However, in human GH deficiency ghrelin levels do not differ from those in normal subjects, neither basally (145-149), nor in terms of ghrelin decrease after insulin-induced hypoglycemia (149) and not even after long-term GH replacement therapy (145). Similarly, plasma ghrelin levels in acromegaly do not seem to be significantly altered (147, 150). The fact that ghrelin levels and regulation are not altered in pathological conditions concerning GH-IGF-I axis suggests that GH is not a predominant modulator of the ghrelin system.

Ghrelin levels appear to be regulated also by LH agonists, at least when tissue (testicular) levels are concerned. In rats, testicular ghrelin mRNA and protein expression decreased to negligible levels after long-term hypophysectomy, whereas replacement with human chorionic gonadotropin (CG) (as superagonist of LH) partially restored ghrelin mRNA and peptide expression (100). A transient increase in testicular ghrelin (mRNA and protein) levels was also observed after acute CG administration to intact rats (100). In humans, hypogonadism is accompanied by decreased ghrelin levels, which are increased after 6-month of replacement testosterone therapy (151).

Also, hyperthyroidism is associated with decreased ghrelin levels (152-155), which normalize after anti-thyroid medical treatment (155), whereas in hypothyroid states ghrelin levels are increased (156).

Somatostatin (SS), its homologue CST and SS-peptidomimetics have been reported to suppress ghrelin in physiological (110, 125, 157, 158) as well as pathological conditions, such as acromegaly (125).

The adipose tissue-derived hormone leptin seems to play a role in regulating ghrelin levels. Leptin administration stimulated gastric ghrelin mRNA in the leptin deficient ob/ob mice that, as well as the leptin receptor deficient db/db mice, have

lower ghrelin levels (113, 119). Leptin transgene expression in the rat hypothalamus increased circulating ghrelin levels (159). However, leptin administration in physiological and pharmacological doses in humans did not regulate ghrelin over several hours up to a few days, suggesting that leptin does not regulate ghrelin levels independently of changes in adiposity (117).

3.1.3. Ghrelin "binding proteins"

Interestingly, studies of affinity chromatography showed that ghrelin binds to a species of high-density lipoprotein (HDL) in which apoA-I, the plasma esterase paraoxonase, and clusterin (apolipoprotein J) associate (160). In affinity chromatography columns both free ghrelin and paraoxon, a substrate for paraoxonase, can inhibit the binding of the HDL species with immobilized ghrelin. Some endogenous ghrelin is found to co-purify with HDL during density gradient centrifugation. This interaction links the orexigenic peptide hormone ghrelin to lipid transport and a plasma enzyme that breaks down oxidized lipids in low-density lipoprotein. Furthermore, the interaction of the esterified ghrelin with a HDL species containing an esterase (*i.e.* paraoxonase) suggests a possible mechanism for the conversion of ghrelin to des-acyl ghrelin (160).

4. BIOLOGICAL ACTIONS OF ACYLATED AND UNACYLATED GHRELIN (AG AND UAG)

Ghrelin is a pleiotropic hormone with a wide spectrum of biological actions. For reasons of clarity, this chapter revises the neuroendocrine, central and peripheral effects that represent a useful background for the studies presented in chapters 2-6. An overview of AG and UAG biological actions is summarized in Table 2.

4.1. Hypothalamo-pituitary actions

4.1.1. GH-releasing activity

The GH-releasing effect was the first recognized biological action of AG and synthetic GHSs (1, 23). This GH-releasing activity is strong, dose-dependent and GHS-R1a mediated (1, 209) and it is not exerted by UAG (1, 11). Higher in vivo than in vitro, GHSs-induced GH release is more marked in humans than in animals (1, 10, 11, 23, 210-214).

Natural and synthetic GHSs and GHRH have a synergistic effect both in vivo and in vitro indicating that they act, at least partially, via different mechanisms taking

Table 2. Overview of AG and UAG biological actions.

Biological actions		AG	UAG	References
Hypothalamo-pituitary	**GH**	↑	–(↓?)	Section 5.1.1
	ACTH	↑	–	Section 5.1.2
	PRL	↑	–	(11, 20, 161, 162)
	LH	↓	↓	(163-166)
Central	**Feeding**	↑	↓↑	Section 5.2.1
	Sleep	↑	**?**	(107, 167-172)
	Behavior	anxiety	**?**	(11, 173)
Peripheral	**Glucose metabolism**	↑ glucose levels ↓ circulating insulin	**?** **?**	Section 5.3.1
	Lipid metabolism	↑ adipogenesis ↓ lipolysis	↑ adipogenesis ↓ lipolysis	Section 5.3.2
	Stomach	↑ gastric acid secretion ↑ gastric emptying Disruption of gastric motility	↓ gastric emptying Disruption of gastric motility	(4, 11, 85, 140, 141, 174-181)
	Cardiovascular system	↓↑ Vascular resistance ↑ Inotropism ↑ Cardiac output ↓ Infart size and Protection on myocardial ischemia	↓ Vascular resistance ↑ Inotropism Protection on myocardial ischemia	(11, 56, 67-70, 182-203)
	Proliferation	↑↓	↑↓	(11, 46, 48-55, 57, 58, 60-62, 64, 204-208)

place at pituitary and hypothalamic level (20, 23, 71, 73). GHSs and AG need GHRH activity to fully express their GH-releasing effect and probably act by triggering GHRH-secreting neurons at the hypothalamic level (20, 23, 71, 73). In keeping with this, the GH response to GHSs is strongly blunted in humans by a GHRH receptor antagonist, as well as by hypothalamo-pituitary disconnection (215). Moreover, patients with GHRH-receptor deficiency show no GH response to GHS, whereas GHSs-induced stimulation on PRL, adenocorticotropin hormone (ACTH) and cortisol secretion is maintained (216). Although AG and GHSs do not inhibit somatostatin release, they probably act as functional somatostatin antagonists both at the pituitary and the hypothalamic level (11, 20, 23, 73, 217, 218). In humans, the GH response to GHSs is not modified by substances such as acetylcholine receptor agonists and arginine, which inhibit somatostatin and potentiate the GHRH-induced GH rise (20, 219). Moreover, the GH-releasing activity of GHSs is partially refractory to the inhibitory

effect of molecules acting via stimulation of hypothalamic somatostatin (such as acetylcholine receptor antagonists, β-adrenoceptor agonists, glucose), which almost abolish the somatotroph responsiveness to GHRH (20). GHSs are also partially refractory to the effects of inhibitors of pituitary somatotroph cells, such as free fatty acids and even to exogenous somatostatin (20, 218, 219), and to the negative GH autofeedback (20). However, GHSs show sensitivity to the negative Insulin-like Growth Factor I (IGF-I) feedback action (20).

The GH-releasing effect of AG and GHSs undergoes marked age-related variations: increasing at puberty, remaining constant during adulthood and decreasing with age (20, 161, 220). The mechanisms underlying the age-related variations in the GH-releasing activity of GHSs differ by age. At puberty, the enhanced GH-releasing effect of GHSs reflects positive influence of estrogens, which could trigger an increase in GHS-R expression (20, 221-223). However, the reduced GH response to GHSs in postmenopausal women is not due to estrogen insufficiency (20, 224, 225). During aging the most important mechanism accounting for reduced GH-releasing activity of GHSs is probably represented by age-related variations in the neural control of somatotroph function including GHRH hypoactivity and somatostatinergic hyperactivity (20, 226). However, the GH response to hexarelin in elderly subjects is increased, although not restored, by supramaximal doses (20), in agreement with the reduction in hypothalamic GHS-R in human aging brain (20, 42). It has been hypothesized that declining GH secretion during lifespan would reflect age-related decrease in AG levels and/or GHS-R activity/expression levels (42, 226) (12).

However, data from ghrelin knock out animals (ghrelin -/-) do not show a clear phenotype: their size, growth rate and IGF-I levels, food intake, body composition, reproduction, gross behavior, and tissue pathology are indistinguishable from wild-type littermates (227). Deletion of GHS-R1a gene (Ghsr -/-) in mice proved unambiguously that the stimulatory effect of AG and GHSs on GH release is mediated by the GHS-R, since in Ghsr -/- mice AG, as well as MK-0677, failed to stimulate GH release. However, similarly to ghrelin -/- animals, Ghsr -/- were not dwarf, and their phenotype was comparable to wild type animals (209).

Overall, considering this picture, it seems that AG or GHSs do not play a pivotal role in the physiological control of GH secretion. However, GHSs represent a useful diagnostic tool to assess GH/IGF-1 axis activity and they may have therapeutic implications (20) (11).

4.1.2. ACTH- releasing activity

Activity of both AG and synthetic GHSs at the pituitary level is not fully specific for GH, because it also includes stimulatory effects on both the lactotroph and

corticotroph system (11, 20, 211, 212, 214). However, some synthetic GHS that exclusively stimulate GH secretion have been reported (228).

The AG- and GHSs-induced activation of the hypothalamo-pituitary-adrenal axis in humans is remarkable and similar to that exerted by naloxone, a μ-opioid receptor competitive antagonist, by the hypothalamic hormone arginine vasopressin (AVP) and even by CRH. Interestingly, the effect of AG on ACTH secretion is even more pronounced than that elicited by synthetic GHSs (11, 20, 211, 214, 229-232). The ACTH-releasing effect of GHSs is acute, being attenuated during prolonged treatment, is independent of gender and shows age-related variations (12, 20, 233, 234). In fact, it rises at puberty, decreases in adulthood and shows a trend toward an increase in aging, when the GH-releasing activity of GHSs is clearly reduced (161).

Under physiological conditions, the ACTH-releasing activity of GHSs is mediated via CNS, involving hypothalamic release of CRH, AVP, NPY and γ-aminobutyric acid (GABA) (11, 20, 85, 229, 230, 234-237). The ACTH response to natural and synthetic GHSs is generally sensitive to the negative cortisol feedback mechanism (234, 238).

Nevertheless, it seems unlikely that AG plays a role in the regulation of corticotroph function in physiological conditions. In fact, two-fold increments of plasma ghrelin, which reflect physiological fluctuations in healthy subjects, do not elicit ACTH levels in humans, whereas they stimulate GH secretion (162). At least three-fold increase in circulating ghrelin is required to stimulate corticotroph function (162). Such a magnitude of variation has been observed in pathological conditions associated with severe malnutrition and weight loss, such as anorexia nervosa, liver cirrhosis, cancer, cardiac cachexia and end-stage renal failure (162).

4.2. Central actions of ghrelin and GHSs

4.2.1. Effects on feeding

In the late 1990's some studies in rodents, aimed to define the pharmacological properties of synthetic GHSs, reported positive effects on food intake. Such effects were observed after central as well as peripheral administration and independently of their GH-releasing activity (239-242). At the same time, a growing amount of data showed GHSs-induced neuronal activity in those hypothalamic areas that are currently considered the central units controlling food intake and energy balance (11, 237, 243, 244). These hypothalamic areas include the dorsomedial- (DMN), ventromedial- (VMN), paraventricular- (PVN) and arcuate- (ARC) nuclei (103, 245); the ARC being considered the most important for integration of signals from the periphery (246). A previously uncharacterized group of neurons adjacent to the third ventricle between DMN, VMN, PVN and ARC has been found to produce acylated- as well

as unacylated- ghrelin (AG ans UAG, respectively) (103, 245, 247). These neurons project on to hypothalamic circuits that produce NPY/agouti-related protein (AgRP) in the ARC, proopiomelanocortin (POMC), corticotrophin-releasing hormone (CRH) and orexin neurons in the lateral hypothalamus (LHA) (32, 103, 248). Upon fasting conditions the upregulation of hypothalamic AG, UAG, NPY and AgRP, coupled with a decrease in POMC, stimulates food intake (246, 247). A high density of expression and GHSs-induced activation of the GHS-R1a was observed in the ARC, but not in the LHA (11, 24-26, 249)

The surge of studies relating ghrelin effects to food intake started in 2000, when Tschoep and coworkers showed that two-week AG treatment caused increased weight gain in normal as well as GH-deficient rats (112). Other studies with AG and synthetic GHSs soon confirmed this finding (72, 250-253). The AG- and GHSs- induced weight gain is due to increased food intake and is characterized by accretion of fat mass and a decrease in lean (muscle) mass and in the absence of changes in longitudinal growth (251). However, changes in fat mass were also observed independently of feeding behaviour, thus reflecting a direct effect of AG on lipid metabolism (see section 5.3.2).

The increase in food intake after AG injection in rodents occurs rapidly (< 60 min) (250) and after both central and peripheral administration, in contrast with other orexigenic neuropeptides (*i.e.* NPY, AgRP, and MCH) that are only active when centrally administered (112). However, AG effects on feeding are more potent after central than after peripheral administration (112, 250).

AG administration into central sites of feeding regulation increased food intake as well as *c-fos* expression and immunoreactivity in ARC, PVN and DMN as well as the nucleus of the solitary tract (141, 254, 255) and the dorsal vagal nucleus (256) in the brainstem, which are the sensory and the visceromotor nuclei of the vagus. In keeping with the effects of AG on feeding, also peripheral injection of GHSs or AG increased *c-fos* expression in the hypothalamus, predominantly in the ARC and to a lesser extent in the PVN (257), although this effect was lower than after central administration (114, 243, 244). Peripherally administered AG can easily reach the ARC via the blood stream, as in this area the blood–brain barrier is semipermeable (246). The importance of the ARC in the central regulation of energy balance is supported by the fact that rats in which damage of ARC is induced (by treatment with monosodium glutamate) show diminished GH response and no increased food intake after AG administration (258).

Several hypothalamic pathways seem to mediate AG effects on feeding. They involve: i) NPY/AgRP neurons; ii) orexin; iii) vagus nerve.

i) NPY/AgRP neurons in the ARC are the major mediators of AG effects on feeding/ energy balance. NPY neurons contain both NPY and AgRP, a POMC-derived antagonist of melanocortin receptors with orexigenic effects (its POMC-derived counterpart with anorexigenic action is α-MSH). AG augments both AgRP and NPY expression after acute and chronic administration (74, 253) via interaction with the GHS-R1a, which is expressed in NPY/AgRP neurons in the ARC (249). This is clearly shown by the abolishment of AG- and GHSs- induced feeding by AG antibody pre-treatment, GHS-R1a antagonism, NPY and AgRP antibodies or NPY-Y1 receptor antagonists (72, 250, 252, 253, 259, 260). Furthermore, AG administration to GHS-R1a deficient mice failed in stimulating food intake (209, 260).

ii) Orexin is an orexigenic hormone in the lateral hypothalamus (LHA). Intracerebroventricular (icv) administration of AG induces *c-fos* expression in orexin immunopositive cells (254), which are also activated by AG in vitro (261). Morover, AG-induced feeding was attenuated by pretreatment with anti-orexin-A and -B antibodies and in orexin deficient mice (260). Interestingly, the majority of AG-responsive ARC neurons respond to orexin stimulation with an increase in intracellular calcium, suggesting that orexin cells stimulated by AG may activate NPY neurons in the ARC (32, 262). In accordance with this, the administration of a NPY receptor antagonist further attenuated AG-induced feeding in rats treated with anti-orexin antibody (260). This orexin pathway seems to be independent of GHS-R1a, which is not expressed in LHA (248).

iii) Abdominal vagal afferents terminate predominantly in the nucleus of the tractus solitarius of the dorsal brainstem. From here, information is disseminated to autonomic motor nuclei (*e.g.* the dorsal motor vagal nucleus) and "higher" regions of the brain including the hypothalamus. Indeed, GHS-R1a are synthesized in the vagal afferent neurons and transported to the afferent terminals. Blockade of the vagal afferent abolished peripheral AG-induced feeding, but also GH release and activation of NPY neurons. Electrophysiological studies showed that peripherally (intravenously) administered AG decreased the afferent activity of the gastric vagal nerve (85), in contrast to anorectic peptides (*i.e.* cholecystokinin, bombesin, leptin) that increase vagal afferent activity (11, 32, 85). Therefore, the effects of AG on vagal nerve activity and on feeding are opposite to those of feeding-inhibitory molecules, supporting vagal mediation of the orexigenic activity (142).

Some effects of UAG on feeding have also been described, although there are conflicting reports. Chen and coworkers showed that UAG significantly decreases food intake in food-deprived rats after central (intracisternal, ic) and peripheral

(intraperitoneal, ip) administration, whereas in the fed state ip administered UAG suppressed food intake only in the dark phase (175, 176). This effect seems to be mediated by different brain circuits than those activated by AG, since, differently from AG, peripherally injected UAG induced *c-fos* expression in PVN even more than in ARC, where most of *c-fos* neurons colocalized with CRF as detected by immunohistochemical studies (176). Moreover, UAG-induced suppression of food intake was not mediated by vagal afferent pathways, as indicated by the absence of effects of truncal vagotomy or treatment with capsaicin, a selective vagal afferent blockade (176).

On the other hand, more recently Toshinai and colleagues found a stimulatory effect of UAG on food intake, less impressive than that elicited by AG, after central (icv) but not after peripheral (ip and iv) administration (260). In this study *c-fos* immunoreactivity was found in the orexin neurons, but not in MCH neurons, of LHA (260), thus suggesting an involvement of the orexin pathway. In fact, treatment with anti-orexin completely abolished UAG-induced feeding, which was not modified by anti-NPY antibodies. In addition, UAG did not induce effects on feeding in orexin deficient mice (260). A mediation of UAG actions by the GHS-R1a was excluded by the observation that in GHS-R deficient mice UAG-induced food intake was even more pronounced than in wild type animals (260).

Recent data suggest a novel molecular mechanism for appetite regulation in hypothalamic cells. AMP-activated protein kinase (AMPK) acts as an intracellular energy sensor and maintains appropriate energy level in the cell. Leptin and adiponectin activate AMPK to switch to energy synthesis in muscle and liver cells (263, 264). Hypothalamic AMPK activity is stimulated by AG, leading to increased food intake (265), whereas leptin has opposite effects (266).

In summary, three different pathways have been proposed for the appetite-inducing effect of AG (see Figure 4):

1) Circulating AG reaches the hypothalamus trough the bloodstream and activates the orexigenic NPY/AgRP neuronal cell bodies and/or terminals in the ARC, which in turn inhibit anorexigenic POMC cells within the ARC.
2) Circulating AG or AG produced locally in the stomach acting via afferent vagal fibers innervating the nucleus tractus solitarius which then relays to the hypothalamic appetite-regulating nuclei.
3) AG is produced locally in the hypothalamus, connecting to and stimulating orexigenic NPY/AgRP neurons in the ARC, and orexin neurons in the lateral hypothalamic area.

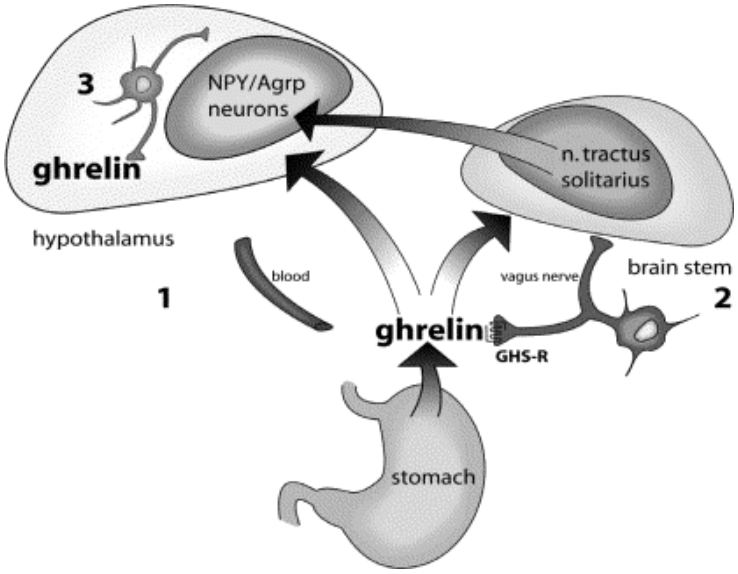

Figure 4. Schematic representation of the pathways mediating AG effects on feeding. Reprinted, with permission, from (32).

The effects of UAG on food intake need further elucidation. However, they seem to occur via circuits at least in part different from AG, involving orexin and CRF.

4.3. Peripheral actions of ghrelin and GHS

4.3.1. Effect on carbohydrate metabolism

The hypothesis that ghrelin could play a role in the regulation of glucose homeostasis and insulin secretion was based on the observation that, as shown in the previous sections, several biological activities of AG are mediated by the cholinergic system/vagus nerve, which also plays a pivotal role in the regulation of the endocrine pancreas. Moreover, ghrelin (including both AG and UAG) is expressed in pancreatic islets (6, 77) (267, 268), where it is present already during fetal development, whereas it decreases during adulthood (267, 268). The expression of GHS-R1a in the endocrine pancreas has been found by several groups (6, 75, 77, 78). Furthermore, previous reports in the literature described an effect of synthetic GHSs on insulin and glucose levels, although these metabolic actions were supposed to be mediated by the neuro-endocrine activity that GHSs exert at pituitary level. In fact, the increase in plasma glucose levels induced by sustained treatment with GHSs in obese rats was thought to be due to GHSs-induced activation of hypothalamo-pituitary adrenal axis (231). Similarly, chronic treatment with MK-0677, a non-peptidyl GHS, induced

hyperglycemia and insulin resistance in lean, but not in obese, elderly subjects and this phenomenon was supposed to reflect increased GH secretion (269, 270). However, a possible mediation by GH of the GHSs-induced modulation of glucose homeostasis was ruled out by the observation that GHRP-6, in fed conditions, induced a rise in glucose as well as in insulin and free fatty acid (FFA) levels in the presence of GH receptor antagonism by pegvisomant (271).

The first report showing an effect of ghrelin on glucose homeostasis was by Broglio *et al.*, who observed that AG administration to healthy subjects induced an acute and significant increase in glycemia that was followed by a transient decrease in circulating insulin levels (161, 272, 273). These metabolic effects were not induced by UAG (13) or by a synthetic GHS, although the latter potently stimulated GH release to the same extent as AG (272). This, along with the fact that glucose and insulin changes persisted over 2 hours after AG administration, in contrast with a more transient increase in GH levels, suggested that the metabolic actions of AG were GH-independent. The fact that insulin levels were suppressed despite the rise in blood glucose led to the hypothesis that AG could differentially modulate hepatic glucose metabolism (*i.e.* glycogenolysis) and insulin secretion (161, 272, 273). Supporting the hypothesis of a direct effect of AG on hepatic glucose handling, it was shown in vitro that AG hampered the inhibitory effects of insulin on gluconeogenesis in a hepatoma cell line (63). Whether this effect was exerted via the GHS-R1a was not elucidated, since GHS-R1a expression in the liver has not been clearly demostrated (45, 272).

Regarding the influence of AG on insulin secretion, different studies reported conflicting results. In fact, AG was able to stimulate insulin secretion from isolated rat pancreatic islets (77) and in vivo (144, 274), whereas it suppressed insulin secretion stimulated by glucose, arginine, and carbachol in isolated rat pancreas perfused *in situ* (275). In humans, AG induced a transient decrease of spontaneous insulin secretion and selectively blunted the insulin response to arginine, but not to an oral glucose load (276). The mechanisms by which AG selectively modulates the gluco-insulinemic response to arginine in humans remains unclear, although it was hypothesized that AG may act via depolarization of β-cells and/or via enhanced somatostatin release, which was shown both in animals and in humans (213, 217, 273, 276). However, in agreement with an inhibitory effect of AG on insulin secretion is the clear negative association between AG and insulin, reported in humans as well as in animals by the majority of authors (11, 91, 107, 108, 113, 122), although not by all (124).

Overall, these findings suggest that the gut hormone AG may exert a significant role in the regulation of insulin secretion and glucose metabolism. AG might integrate the

hormonal and metabolic response to fasting that, at least in humans, is accompanied by a clear-cut increase in GH secretion coupled with inhibition of insulin secretion and activation of mechanisms devoted to maintaining glucose levels (11, 277).

4.3.2. Effect on lipid metabolism

Ghrelin has been reported to increase body fat, also independently of changes in food intake (112). A specific effect of ghrelin on lipid metabolism was suggested by the observation that rodents treated with AG showed enhanced fat content independently of feeding behaviour, as assessed by magnetic resonance imaging (MRI), increased respiratory quotient (suggesting enhanced carbohydrates utilization and decreased fat utilization), dual energy x-ray absorptiometry (DEXA) and weight of omental and retroperitoneal fat pads (32, 112, 251, 278). In fat tissue AG and UAG promote adipogenesis and inhibit lipolysis, whereas they also modulate lean tissue fat distribution and metabolism. In fact, AG as well as UAG were shown to favour adipogenesis when infused to rodent bone marrow (47). The increase in respiratory quotient following both central and peripheral administration of AG is likely to reflect reduced whole body lipid oxidative utilization (112). In vitro studies demonstrated that AG stimulates differentiation of rat parametrial preadipocytes *in*

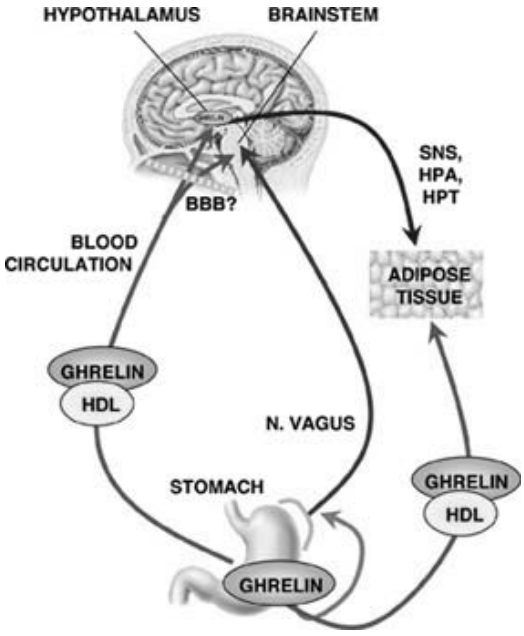

Figure 5. Ghrelin is involved in central and peripheral circuits regulating body fat. SNS = sympathetic nervous system; HPA = hypothalamo-pituitary-adrenal axis; HPT = hypothalamo-pituitary-thyroid axis. Reprinted, with permission, from (11).

vitro (279) and that both AG and UAG inhibited isoproterenol-induced lipolysis by primary adipocytes (280).

More recently, Barazzoni and colleagues (281) showed that sustained AG administration in rats (twice-daily for four days) modulates lipid metabolism also in non adipose tissues, including liver and skeletal muscle, increasing body weight, but not food intake. In the liver, AG induced lipogenic and glucogenic patterns of gene expression and triglyceride content, whereas the activity of the stimulator of FFA oxidation, AMP-activated kinase (AMPK), was reduced and mitochondrial oxidative enzyme activities were unchanged (264, 281). In muscle, AG reduced triglyceride content, increased mitochondrial oxidative enzyme activities and increased mRNA encoding uncoupling protein-2, independent of changes in expression of fat metabolism genes and phosphorylation of AMPK. Thus, AG favors triglyceride deposition in liver over skeletal muscle, suggesting that AG could be involved in adaptive changes of lipid distribution and metabolism in the presence of caloric restriction and loss of body fat.

AG treatment also significantly increased the mRNA levels of important regulators of tissue fat metabolism and content, such as peroxisome proliferator activated receptor (PPAR)-γ, in primary cultured rat differentiated adipocytes and in muscle (279, 281).

The actions of AG on lipid metabolism are unlikely to be mediated by GHS-R1a, since epididymal adipose tissue or isolated adipocytes did not express GHS-R1a mRNA, but showed a common high-affinity binding site recognized by AG and UAG and also synthetic, peptidyl and non-peptidyl GHSs (280). In keeping with this, bone marrow adipogenesis was stimulated also by UAG, but not by a potent GHS-R1a agonist (47).

In conclusion, both AG and UAG promote adipogenesis and inhibit lipolysis, probably acting via a yet unknown receptor, different from GHS-R1a.

5. AIM OF THE THESIS

As illustrated in the general overview, the ghrelin system owes its complexity to several factors, including:

i) The integrated neural, endocrine and metabolic regulation of ghrelin molecules and their receptor(s) expression and activity;
ii) The biological peculiarities that characterize the ghrelin molecules (AG and UAG);

iii) The pleiotropy of their biological actions, which require central and peripheral networks;
iv) The involvement of known and unknown receptor(s).

Until now, the majority of published literature has aimed to clarify the neuroendocrine, orexigenic, gastric and cardiovascular effects of ghrelin, the newly discovered endogenous ligand of the GHS-R1a. More recently, the finding that ghrelin exerted also metabolic actions opened a novel field in GHSs research. The first observations suggesting a direct role of ghrelin and (peptidyl) GHSs on glucose homeostasis came from our group and our collaborators (13, 271, 272). This new perspective seemed particularly intriguing, since it was the missing and direct link between central regulation of energy balance and peripheral fuel utilization in acute as well as in chronic conditions.

With the studies presented in this thesis the overall hypothesis that the ghrelin system is involved in glucose homeostasis and metabolism is pursued. Given the complexity of the ghrelin system itself and the limited amount of data in the literature, a multifaceted approach will be used in order to verify:

1) Whether unacylated ghrelin, as well as acylated ghrelin, modulates glucose control in physiological conditions. To this aim, clinical studies in humans will be carried out and a sensitive animal (rat) model will be used.
2) Whether unacylated and acylated ghrelin have an impact on hepatic glucose handling, independently of insulin, by using an *in vitro* system.
3) Whether the peripheral action of ghrelin molecules on glucose metabolism are mediated by the known GHS-R1a or by a yet unidentified receptor subtype(s). For this purpose, different *in vitro* models will be used.

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Chapter II

Administration of acylated ghrelin reduces insulin sensitivity, whereas the combination of acylated plus unacylated ghrelin strongly improves insulin sensitivity

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ABSTRACT

We investigated the metabolic actions of ghrelin in humans by examining the effects of acute administration of acylated ghrelin, unacylated ghrelin and the combination in eight adult onset growth hormone deficient patients. We followed glucose, insulin and free fatty acid concentrations before and after lunch, and with or without the presence of growth hormone in the circulation.

We found that acylated ghrelin, which is rapidly cleared from the circulation, induced a rapid rise in glucose and insulin levels. Unacylated ghrelin, however, prevented the acylated ghrelin-induced rise in insulin and glucose when it was co-administrated with acylated ghrelin. Surprisingly, the injection of acylated ghrelin induced an acute increase in unacylated ghrelin, and therefore total ghrelin levels. Finally, acylated ghrelin decreased insulin sensitivity up to the end of a period of 6 hours after administration. This decrease in insulin sensitivity was prevented by co-injection of unacylated ghrelin. This combined administration of acylated and unacylated ghrelin even significantly improved insulin sensitivity compared to placebo for at least 6 hours, which warrants studies to investigate the long-term efficacy of this combination in the treatment of disorders with disturbed insulin sensitivity.

INTRODUCTION

Ghrelin is predominantly produced by the stomach, but it is also detectable in many other tissues as well (1-12). Ghrelin can stimulate growth hormone (GH) secretion, which is mediated by the activation of the so-called GH secretagogue receptor type 1a (GHS-R1a). However, ghrelin exhibits additional activities including *e.g.* stimulation of prolactin and ACTH secretion, stimulation of a positive energy balance, gastric motility and acid secretion, but also modulation of pancreatic exocrine and endocrine function as well as effects on glucose levels (2, 9, 13-28). Ghrelin is the first natural hormone known in which the hydroxyl group of one of its serine residues is acylated by *n*-octanoic acid (1). This acylation is essential for binding to the GHS-R1a, for the GH-releasing capacity of ghrelin and likely also for its other endocrine actions (1, 29-31). Ghrelin is expressed by pancreatic endocrine α-cells, in rat and human tissue, according to some authors (32) and by pancreatic β- cells according to one group only (33). Moreover, ghrelin is not co-expressed with any known islet hormone, and the ghrelin cells may therefore constitute a new islet cell type (34). Ghrelin seems to exert a tonic inhibitory regulation on insulin secretion from pancreatic β-cells, and a clear negative association between ghrelin and insulin secretion has been found in humans as well as in animals by some (23, 35-39), although not by others (40). Also, ghrelin induces a significant increase in human plasma glucose levels, which are surprisingly followed by a reduction in insulin secretion (17). We, as well as others, already reported that acute, as well as chronic treatment with GHS, particularly non-peptidyl derivatives, induces hyperglycemia and insulin resistance in a considerable number of elderly subjects and obese patients (41-43). This suggests that ghrelin exerts a significant role in the fine-tuning of insulin secretion and glucose metabolism. Also, ghrelin secretion may be suppressed, at least in part, by an increased plasma glucose level as well as by insulin, as shown by hyperinsulinemic euglycemic clamp studies in healthy subjects (38, 44-45). However, it has also been suggested that ghrelin could have direct stimulatory effects on glycogenolysis (17).

To further investigate the effects of ghrelin on glucose and insulin handling in humans, we studied in adult onset GH deficient patients the effects of a single intravenous (i.v.) administration of placebo, acylated ghrelin (AG), unacylated human ghrelin (UAG) and a combination of AG and UAG on glucose and free fatty acid (FFA) metabolism before and after lunch, and with or without the presence of GH.

With this study design we wanted to address the acute effects of human ghrelin on parameters of glucose and lipid metabolism with or without the presence of GH and to determine whether or not UAG has any intrinsic metabolic effects as well as whether or not UAG can modify the effects of AG.

SUBJECTS AND METHODS

Subjects

Eight male subjects with a pituitary insufficiency but otherwise healthy, were asked to participate (range 21–69 yrs (age 55 ± 10; mean ± SEM)) and a body mass index of 29.4 ± 2.8 (mean ± SEM). All were treated by transsphenoidal surgery at least two years before enrolment for non-functioning pituitary tumors and all were on stable replacement therapy for their pituitary dependent thyroidal, adrenal and gonadal insufficiency, including GH therapy for at least more than 1 year and all had a serum total IGF-I concentration within the age and sex adjusted normal range. All subjects were admitted at the Clinical Research Unit. No alcoholic beverages were allowed from the day prior to admission until the end of the study. Also, all subjects were asked to skip the administration of their GH replacement every night prior to each of the 5 admission days. All subjects gave their written informed consent to participate in the study, which had been approved by the hospital's Ethical Committee.

All subjects underwent the following five testing sessions, each after an overnight fast, in random order and at least 1 week apart: 1) placebo (saline 3 ml, administered i.v.). 2) acylated ghrelin (Neosystem S.A.; Strasbourg, France; 1.0 µg/kg i.v., using a bacterial filter system). 3) unacylated ghrelin (Neosystem S.A.; Strasbourg, France; 1.0 µg/kg iv, using a bacterial filter system). 4) acylated ghrelin (1.0 µg/kg i.v.) but this time after the normal GH replacement dose was administered 15 minutes before. 5) acylated ghrelin in combination with unacylated ghrelin (both 1.0 µg/kg i.v., but via separate injection sites). All tests started in the morning at 09:30 h, 30 min after one or two indwelling catheters had been placed into an antecubital vein, kept patent by slow infusion of isotonic saline.

After the administration at 10:00 h of AG, or the combination of UAG and AG, blood samples were collected for two hours, after which a meal was given that consisted of two slices of bread with butter and preservative, along with a glass of milk. This meal was taken by all subjects on all test days.

Assessments

Insulin was assessed with a radioimmunoassay (Medgenix Diagnostics, Brussels, Belgium; intra- and interassay CV 13.7 and 8.0% respectively). Glucose was assessed with an automatic hexokinase method (Roche, Almere, The Netherlands). Free fatty acids were determined with an enzymatic colorimetric method (Wako Chemicals GmbH, Neuss, Germany; intra- and interassay CV 1.1 and 4.1% respectively).

Acylated and total ghrelin concentrations were measured, using a commercially available RIA (Linco research Inc., St. Charles, MO). This assay uses an antibody, which is specific for ghrelin with the n-octanoyl group on Serine-3. The Linco Ghrelin (active) assay utilizes ^{125}I-labeled Ghrelin and a ghrelin antiserum to determine the level of active ghrelin in serum, plasma or tissue culture media by the double antibody / PEG technique. The lowest level of ghrelin that can be detected by this assay is 10 pg/ml when using a 100 µl sample size. Within and between assay variations of the acylated ghrelin assay are respectively 7 and 13%. The Linco total ghrelin within and between assay variations are respectively 5 and 15 %.

Statistical analysis

Differences between the several study days were calculated, using a Newman-Keuls Multiple Comparison one-way ANOVA test (GraphPad Prism 4 for Windows; GraphPad Inc. USA). P-values <0.05 were considered significant. Areas under the curve were calculated using the trapezoid rule.

The results are expressed as mean ± SEM.

RESULTS

Ghrelin levels

The iv administration of 1 µg/kg of pure AG only induced a relatively small peak in AG levels in serum, which had already disappeared within 2 h. Apparently, most of the AG was almost immediately degraded or eliminated (Figure 1). However, as shown in Table 1, the total ghrelin concentration after administration of AG was significantly higher than when UAG was administered ($p<0.05$). Moreover, total ghrelin

Table 1. Total ghrelin concentrations the first 4 hours following an injection of 1 µg/kg acylated ghrelin (AG), 1 µg/kg unacylated ghrelin (UAG) and/or growth hormone (GH; normal replacement dose) in 6 GH deficient subjects after an overnight fast.

	AUC of total ghrelin levels (ng/L * 240min.)	p value
Placebo	$0.06*10^6$	
AG	$1.61*10^6$	P < 0.05 versus UAG
UAG	$1.12*10^6$	
AG + UAG	$2.22*10^6$	P < 0.05 versus UAG
AG + GH	$1.58*10^6$	NS versus AG

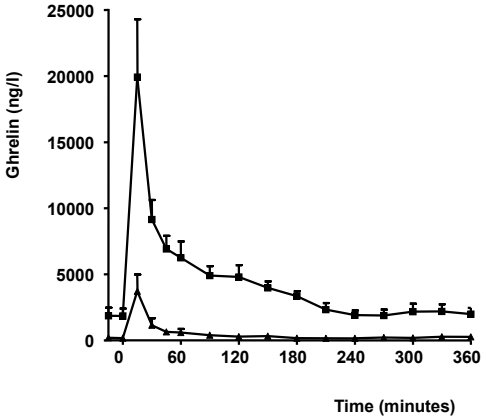

Figure 1. Total (■) and acylated (▲) ghrelin concentrations (ng/l) after an i.v. bolus injection of 1 µg/kg acylated ghrelin in 6 GH deficient subjects after an overnight fast.

levels after injection of UAG + AG were significantly higher than after the injection of UAG (Table 1 and Figure 2).

Glucose levels

During fasting, directly after administration of study drug

Fasting glucose concentrations at baseline were 100.9 ± 2.9 mg/dl (5.6 ± 0.16 mmol/L; 1 mg/dl = 0.05551 mmol/l). Figure 3 shows the serum glucose levels after administration of placebo, AG and UAG, alone or together with AG the first two hours after administration, but before lunch, so when these GH deficient subjects were still fasting. The administration of AG, and to a lesser extent UAG, induced significant hyperglycemia. Interestingly, when GH was administered 15 min prior to the administration of AG, this hyperglycemia did not occur, which was also true when AG and UAG were given simultaneously.

After lunch, 2 – 6 hrs after administration of study drug

Figure 4 shows that the administration of AG and UAG still changes serum glucose levels after lunch. Both AG and UAG increased serum glucose levels significantly in these GH deficient subjects ($p<0.001$). However, when AG was given after the administration of the normal replacement dose of GH in the morning, these changes in serum glucose levels after lunch could no longer be observed. Interestingly, the combination of AG + UAG seemed to lower serum glucose levels significantly, when compared to the changes in glucose seen after the single administration of AG or UAG. Finally, UAG increased serum glucose levels significantly more than AG ($p<0.01$).

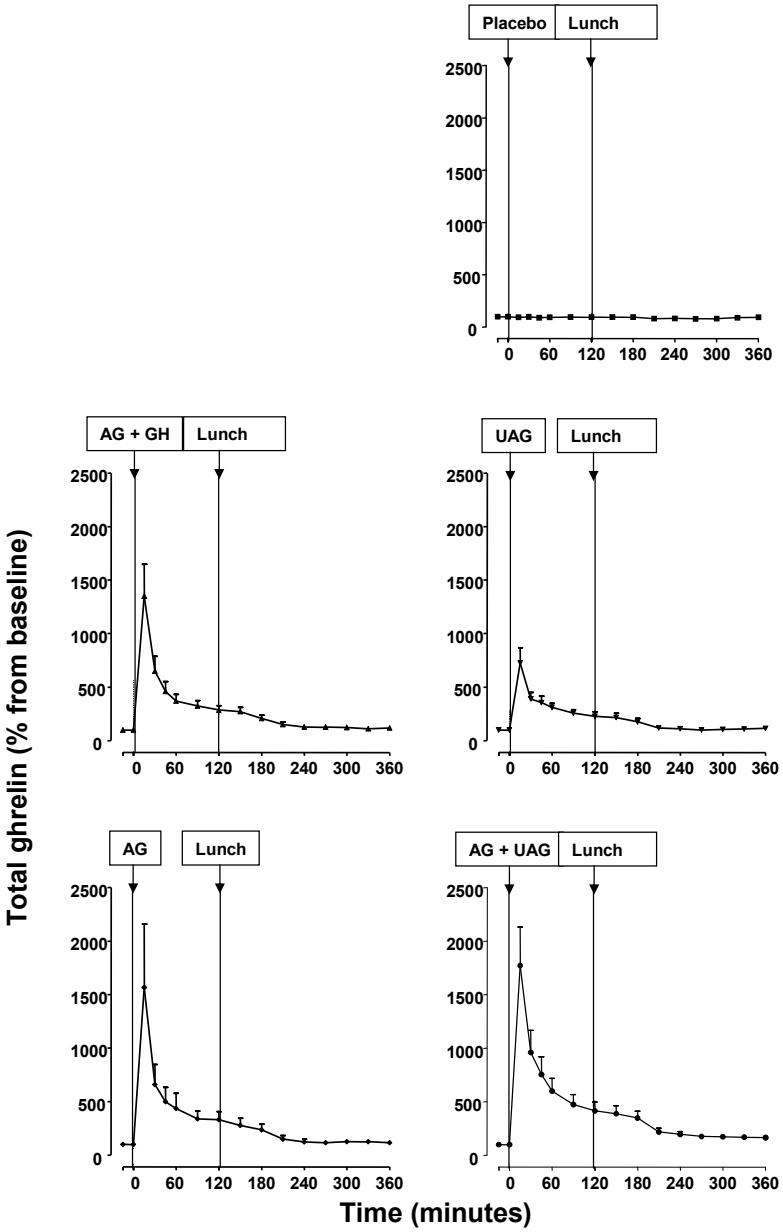

Figure 2. Changes in serum total ghrelin concentrations as % of baseline in 6 GH deficient subjects after administration of placebo, AG (with or without GH), UAG and AG + UAG. AG = acylated ghrelin (1 µg/kg i.v.); UAG = unacylated ghrelin (1 µg/kg i.v.); GH = growth hormone (normal daily replacement dose).

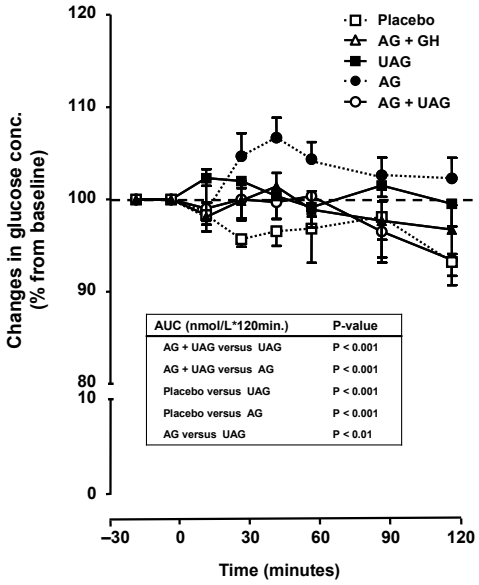

Figure 3. Acute changes in serum glucose concentrations as % of baseline in 6 GH deficient subjects after administration of placebo, AG (with or without GH), UAG and AG + UAG. AG = acylated ghrelin (1 µg/kg i.v.); UAG = unacylated ghrelin (1 µg/kg i.v.). Only the data from the first two hours after administration when subjects were still fasting are shown. The inserted table indicates which parameters significantly differ in area under the curve.

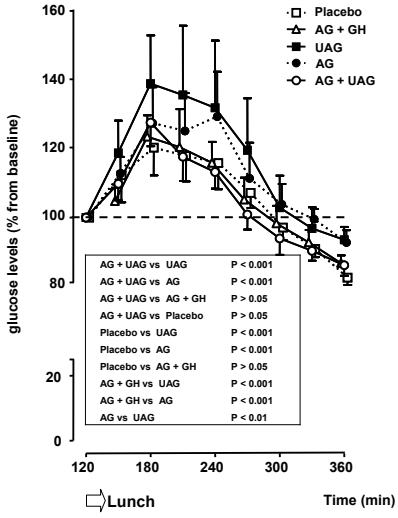

Figure 4. Sub-acute changes in serum glucose concentrations as % of baseline (t = 120 min) in 6 GH deficient subjects after administration of placebo, AG (with or without GH), UAG and AG + UAG. AG = acylated ghrelin (1 µg/kg i.v.); UAG = unacylated ghrelin (1 µg/kg i.v.); GH = growth hormone (normal daily replacement dose). Only the data starting two hours after administration and after lunch are shown. The inserted table indicates which parameters significantly differ in area under the curve.

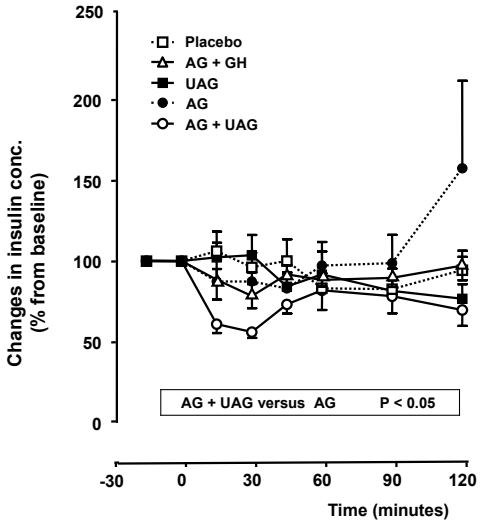

Figure 5. Acute changes in serum insulin concentrations as % of baseline in 6 GH deficient subjects after administration of placebo, AG (with or without GH), UAG and AG + UAG. AG = acylated ghrelin (1 µg/kg i.v.); UAG = unacylated ghrelin (1 µg/kg i.v.). Only the data from the first two hours after administration are shown. The inserted table indicates which parameters significantly differ in area under the curve.

Insulin levels

During fasting, directly after administration of study drug

Fasting insulin concentrations at baseline were 32.7 ± 6.2 µU/ml (196 ± 37 pmol/L; 1 µU/ml = 6.0 pmol/l). Figure 5 shows the serum insulin levels after administration of placebo, AG in the absence or the presence of GH, and UAG, alone or together with AG, during the first two hours after administration, but before lunch, when these GH deficient subjects were still fasting. The administration of AG with UAG induced a significant reduction in serum insulin levels ($p<0.05$). All other interventions did not significantly change serum insulin levels during the first 2 h after administration.

After lunch, 2 – 6 hrs after administration of study drug

Figure 6 shows that the coadministration of AG and UAG impressively reduced serum insulin levels after lunch ($p<0.001$), as the serum insulin levels after lunch when AG was given together with UAG were significantly lower than observed after the administration of placebo, and after AG (with or without GH) or UAG alone.

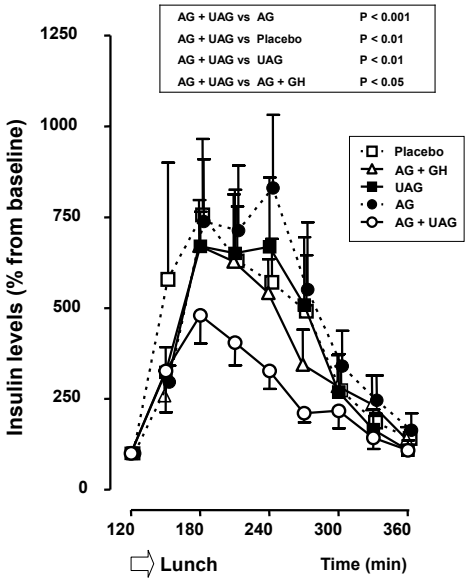

Figure 6. Sub-acute changes in serum insulin concentrations as % of baseline (t = 120 min) in 6 GH deficient subjects after administration of placebo, AG (with or without GH), UAG and AG + UAG. AG = acylated ghrelin (1 µg/kg i.v.); UAG = unacylated ghrelin (1 µg/kg i.v.); GH = growth hormone (normal daily replacement dose). Only the data starting two hours after administration and after lunch are shown. The inserted table indicates which parameters significantly differ in area under the curve.

Free fatty acid levels

During fasting, directly after administration of study drug

Fasting concentrations of free fatty acids at baseline were 0.94 ± 0.09 mmol/L. There were no significant differences in the effects of the various compounds on FFA levels during the first two hours, when subjects were still fasting. Interestingly, the administration of whatever compound, including placebo, induced an increase in FFA concentrations (data not shown).

After lunch, 2 – 6 hrs after administration of study drug

Figure 7 shows that the administration of AG and UAG impressively reduced serum FFA levels, compared to placebo, AG (with or without GH) and UAG, after lunch ($p<0.001$ for all comparisons). AG (with or without GH), administered 2–6 hrs before, significantly increased FFA levels after lunch, compared to placebo; something that could not be observed when UAG was administered.

When put together, Figure 8 shows the glucose, insulin and FFA levels, in the first two hours following the administration of AG (with or without GH), UAG and the

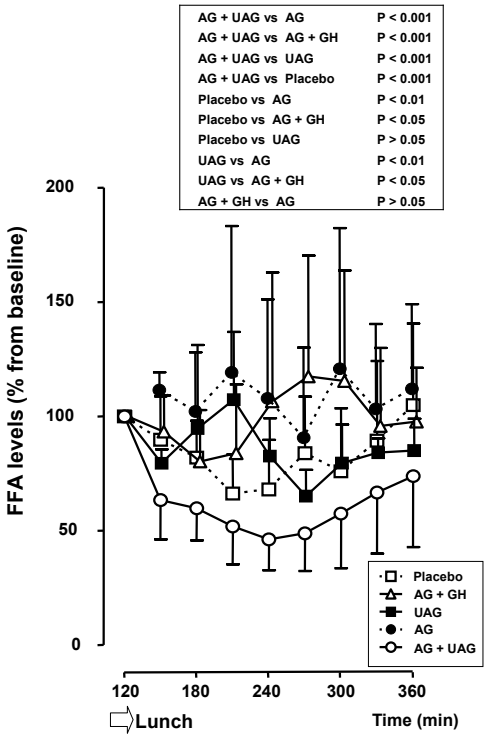

Figure 7. Sub-acute changes in serum free fatty acids (FFA) concentrations as % of baseline in 6 GH deficient subjects after administration of placebo, AG (with or without GH), UAG and AG + UAG. AG = acylated ghrelin (1 μg/kg i.v.); UAG = unacylated ghrelin (1 μg/kg i.v.); GH = growth hormone (normal daily replacement dose). Only the data starting two hours after administration and after lunch are shown. The inserted table indicates which parameters significantly differ in area under the curve.

combination of AG + UAG, expressed as areas under the curve (AUC). Apparently, the combination of AG + UAG improved insulin sensitivity. Figure 9 shows the same parameters as AUC, but for the period following lunch. This fig. 9 clearly demonstrates that AG decreased insulin sensitivity, compared to placebo, but it also shows again that the combination of AG + UAG impressively improved insulin sensitivity, which was also translated into the lower FFA levels in this situation.

DISCUSSION

The results of all AG and UAG levels in this study, measured with the Linco assay, were within the same range per assay as those that we observed in normal individuals. This was also true for the levels of total ghrelin when we used another assay [ghrelin assay for total ghrelin (Phoenix Pharmaceuticals, Belmont, CA), although

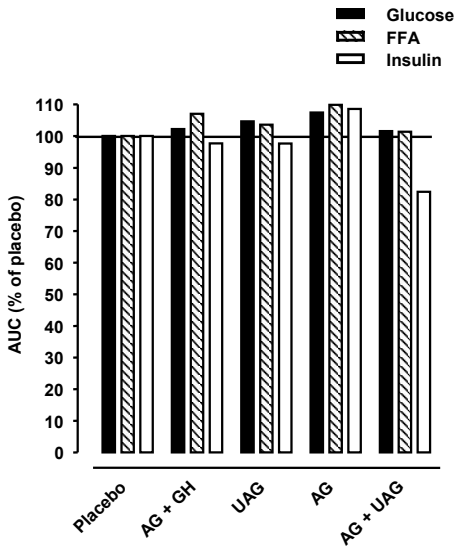

Figure 8. Changes in areas under the curve of serum glucose, insulin and free fatty acid concentrations as % of baseline in 6 GH deficient subjects during first 2 hrs after the intravenous administration of placebo, AG (with or without GH), UAG and AG + UAG. AG = acylated ghrelin (1 µg/kg i.v.); UAG = unacylated ghrelin (1 µg/kg i.v.); GH = growth hormone (normal daily replacement dose).

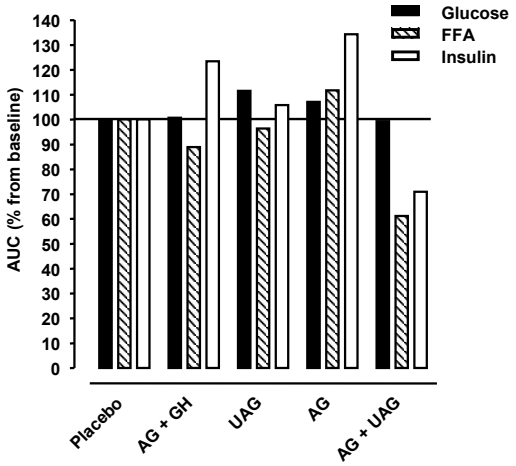

Figure 9. Changes in areas under the curve of serum glucose, insulin and free fatty acid concentrations as % of baseline in 6 GH deficient subjects during the first 4 hrs after lunch at 12:00 am following the intravenous administration at 10:00 am of placebo, AG (with or without GH), UAG and AG + UAG. AG = acylated ghrelin (1 µg/kg i.v.); UAG = unacylated ghrelin (1 µg/kg i.v.); GH = growth hormone (normal daily replacement dose).

this assay showed consistent lower results in total ghrelin than the Linco assay] (46), although we must emphasize that not much is known and/or reported of AG levels

in any condition. We also have to emphasize that our patients were GH deficient, which means that baseline observations in them can only be extrapolated to normal healthy subjects with caution. Our first conclusion is that a single intravenous injection of 1 µg/kg pure acylated ghrelin is very rapidly cleared from circulation, because hardly any of it can be found in serum in the following hours after injection (Fig. 1). Surprisingly, we also observed that the injection of AG induces an instant and significant increase in total ghrelin levels, *i.e.* in UAG levels (Fig. 2 and Table 1). We think this might be caused by an active release of unacylated ghrelin from an unknown source. However, an acute change in the capacity of the body to clear ghrelin might of course be another plausible explanation. Whatever the cause of it might be, apparently the body responds to an acute increase of AG by increasing UAG levels instantly. One might speculate on the function of this, but it seems that UAG could be used to blunt the effects of AG. Whatever the reason is of our observation that the administration of AG induced a higher level of total ghrelin than the administration of the same amount of UAG, we could not attribute this to an assay problem. We tried whatever combination in test tubes, but could not find an interaction between AG and UAG; *i.e.* both AG and UAG induced the same changes in concentration when added alone or in combination to fresh serum.

In concordance with this is the observation that a single intravenous (i.v.) injection of 1 µg/kg of AG early in the morning after an overnight fast induces a direct increase in glucose (Figs. 3 and 4) and insulin concentrations (Fig. 6) in GH deficient subjects. Interestingly, pretreatment of the subjects with their normal replacement dose of growth hormone, however, prevented these hyperglycemic changes. The changes in glucose were less after a single i.v. injection of 1 µg/kg UAG (Fig 3). Moreover, the co-administration of 1 µg/kg UAG together with 1 µg/kg of AG (at two separate injection sites) prevented the changes that were seen after the injection of AG alone, as it blunted the hyperglycemic and hyperinsulinemic effects, as was also observed by us in another study in normal individuals without GH deficiency (47). These data indicate that not only AG, but also UAG has metabolic effects, as they both can induce hyperglycemia and change insulin levels. This indicates a GHS receptor 1a (GHS-R1a) independent effect, because UAG can not bind to the GHS-R1a (1,48-49). Maybe even more important is the observation that the combination of AG and UAG can blunt the effects of AG, which therefore might give us a clue why the administration of AG in our study immediately increased the levels of UAG in an attempt of the body to temper these metabolic effects of AG.

Recently, another study demonstrated a clear metabolic role for UAG, as Thompson *et al.* reported not only that AG promotes bone marrow adipogenesis *in vivo* by a direct peripheral action, but that this effect was also observed with UAG. Moreover, this effect of UAG could not be antagonized by administration of a potent

synthetic GHS-R1a agonist. They concluded that the ratio of AG and UAG production might help regulate the balance between adipogenesis and lipolysis in response to nutritional status (50).

Another important observation we made is that the administration early in the morning of AG, but not of UAG, was still able to induce a state of insulin resistance in the period after lunch (so at least 6 hours after a single administration). This indicates that growth hormone secretagogues can influence insulin sensitivity for many hours, as we already observed in earlier work, using GH-releasing peptide-6 (GHRP-6) as a ghrelin receptor agonist (43). In that study, we found that GHRP-6, given in the morning, was able to induce insulin resistance in the afternoon in normal subjects, provided that GH action was knocked out using pegvisomant as a GH receptor antagonist, so again when GH action was low. This effect might be of pathophysiological relevance as we think that these changes in insulin sensitivity, induced by AG, are most prominent in those subjects with low intrinsic GH levels, *e.g.* GH deficient patients and subjects with syndrome-X, as well as during physiological ageing (15, 35-37, 51-55). In other words, when GH action is reduced, GHSs and AG can apparently induce a state of insulin resistance that might explain, at least in part, why in these situations people become more obese. Our data also indicate that these undesired changes in metabolism and phenotype could be counteracted by an increase in GH levels again, or by increasing the UAG over AG ratio, e.g. by the administration of GH.

However, there might be another way to counteract the undesired effects of AG on insulin sensitivity, especially when GH action is low, as we observed that the postprandial effects after lunch of a bolus injection of the combination of both AG and UAG in the morning not only resulted in a significant improvement of insulin sensitivity compared to the injection of AG alone, but also compared to the injection of placebo. This improvement of insulin sensitivity resulted in significant decreases in serum insulin and FFA levels. These results might indicate that in humans the administration of UAG, alone or in combination with AG, might improve insulin sensitivity. This might at least be true for subjects with a relative or absolute GH deficiency, but maybe also for subjects with normal GH levels, as our results indicate that even in the presence of GH, we could improve insulin sensitivity by the co-administration of AG + UAG.

At least we think that AG and UAG should be considered as separate hormones, and that UAG is more than just an inactive form of ghrelin. Future studies will have to address issues such as *e.g.* tachyphylaxis, and which receptor system and control systems are involved etc. We already observed, however, that AG can induce a glucagon-independent increase in hepatic glucose output in an isolated porcine hepatocyte model, which might be one of the mechanisms by which AG can increase

serum glucose levels. Again in this model, UAG was able to counteract these effects of AG (Gauna C et al., submitted for publication).

In conclusion, we found the following:

- An intravenous bolus injection of acylated ghrelin is almost immediately cleared from the circulation.
- Both AG and UAG immediately increase glucose and insulin levels.
- When AG and UAG are injected together, this combination can prevent the acute hyperglycemic and hyperinsulinemic effects of AG and UAG when injected alone. Moreover, this combination of AG and UAG can improve insulin sensitivity for many hours, even compared to the worsening of insulin sensitivity of AG administration and even compared to placebo administration.
- AG induces an acute increase in UAG (and therefore total ghrelin) levels, via either a decrease in the clearance of ghrelin or an active release of UAG or a combination of both.
- AG can induce a decrease in insulin sensitivity up to at least 6 hours after administration, which again can be prevented or even actively improved by co-injection of UAG.

These data clearly demonstrate that the ghrelin system, using both the acylated and unacylated molecules, is actively involved in the acute and long-term control of glucose metabolism and insulin sensitivity in humans, which might enable new treatment modalities for the many disorders in which insulin sensitivity is disturbed.

ACKNOWLEDGMENTS

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Chapter III

Ghrelin stimulates, whereas des-octanoyl ghrelin inhibits, glucose output by primary hepatocytes

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J Clin Endocrinol Metab 2005; 90:1055-1060

ABSTRACT

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, following incubation with acylated ghrelin (AG), unacylated ghrelin (UAG) and hexarelin (HEX). AG induced glucose output dose-dependently after 20 min incubation ($p<0.001$), while HEX, a GHS 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 GHSR-1a expression indicate the involvement of a yet uncharacterized ghrelin receptor type.

In conclusion, glucose output by primary hepatocytes is stimulated by AG and inhibited by UAG, time- and dose-dependently. Moreover, UAG counteracts the stimulatory effect of AG on glucose release. These actions might be mediated by a different receptor than GHS-R1a, while apparently we must consider AG and UAG as separate hormones that can modify each other actions on glucose handling, at least in the liver.

INTRODUCTION

Ghrelin is a 28 amino acid peptide with an *n*-octanoyl ester at its third serine residue (1), 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), since the unacylated form of ghrelin, des-octanoyl ghrelin, does not bind the GHS-R1a and is devoid of any endocrine activity (1, 6, 7). However, unacylated ghrelin (UAG) is not biologically inactive, being able to share with ghrelin antiproliferative effects on human breast and prostate cancer lines (8, 9), negative inotropic effect on papillary muscle (10) and stimulation of bone marrow adipogenesis (11), although the signal transduction mechanism(s) for these effects has not been determined. Acylated ghrelin (AG), as well as synthetic GHSs, 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, 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 receptors, 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 GHRP-6 to normal individuals significantly increased insulin and glucose levels, but only when given to subjects pretreated with the GH receptor antagonist pegvisomant (23). In humans, the acute administration of AG elicited a prompt increase in glucose levels (24, 25), while no changes of 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 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. Since 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 hexarelin, that 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 1b by the primary hepatocytes was also evaluated.

MATERIALS AND METHODS

Materials

Liver perfusion medium, Liver digestion medium and Dulbecco's modified Eagle's medium (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 Neosystems (Strasbourg, France), human des-octanoyl ghrelin by Theratechnologies Inc. (Montreal, Quebec, Canada) and hexarelin 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 somatotrophes, with conserved interaction with GHRH and somatostatin (29), and can activate the GHS-R 1a (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, The Netherlands) 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 1mM 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 pre-warmed (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 obtained.

Suspension cultures

Immediately after the isolation, hepatocytes were washed 3 times with serum free DMEM without glucose or pyruvate and then resuspended in 600 µl of the same medium at a cell density of $30x10^6$/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 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 at least on 5 different preparations, with 6 replicates each.

Following 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, determined using the Bio-Rad protein assay kit.

RNA extraction and RT-PCR for GHS-R type 1a and 1b

The expression of GHSR type 1a and 1b mRNA were 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 and

amplification of β-actin mRNA. RNA samples (100 ng) underwent conventional RT followed by one-step TaqMan real-time PCR (ABI PRISM 7700 sequence Detection System, Perkin Elmer) for the GHS-R type 1a and 1b genes. Porcine specific primers and probes were designed employing 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-R type 1a (forward: 5'-CggTgggCTCCTCgC-3'; reverse: 5'-gTATgAAAgCAAACACCACTACAgC-3'; probe: 5'-FAM-CAgggACCAgAACCACAAACAAACCg–TAMRA-3'), while a special reverse primer was designed for the intronic sequence of GHS-R 1b (forward: 5'-CggTgggCTCCTCgC-3', reverse: 5'-gTATgAAAgCAAACACCACTACAgC-3', probe: 5'-FAM-CAgggACCAgAACCACAAACAAACCg-TAMRA-3'). Each sample was assayed in duplicates in at least 2 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, USA). Data were revised and outliers and extremes eliminated according to a coefficient of outlier of 2.0. Results were then tested for statistical significance using ANOVA, followed by least significant difference (LSD) post-hoc test. P values < 0.05 were considered significant.

Functional assay for acylated and unacylated ghrelin in CHO cells expressing GHS-R1a

Since 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% bovine serum albumin, amphotericin, penicillin and streptomycin) at $5x10^6$ cells/mL, 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 $5x10^5$ 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 seconds using a Victor2 1420 multilabel counter (Perkin-Elmer Wallac). Following the collection of data (response to agonist: x), 100µL of 1% Triton X-100 (v/v in water) was injected into each well and luminescence 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 ($FR = x\ (x + y)^{-1}$).

^{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^{++}- and Mg^{++}-free Phosphate Buffer Saline (PBS). The cells were then centrifuged for 3 min at 1500 *x g* and the pellet resuspended in Buffer A (15 mM Tris-HCl pH 7.5; 2 mM $MgCl_2$; 0.3 mM EDTA; 1 mM EGTA) and homogenized in a glass homogenizer. The crude membrane fraction was collected by two consecutive centrifugation steps at 40.000 *x 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; 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 $CaCl_2$; 5 mM $MgCl_2$; GHS-R-A5 membrane extracts (20 µg protein/tube) and fixed concentrations of the radioligand ^{125}I-ghrelin (NEN, NEX388) with increasing concentration 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 gamma counter.

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

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 resulted to be lower than that exerted by equimolar glucagon (158% of controls, $p<0.001$ 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 (Figure 1). The stimulating effect of AG on glucose release by hepatocytes was dose-dependent, being significant at 100 nM AG (Figure 2).

Hexarelin (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

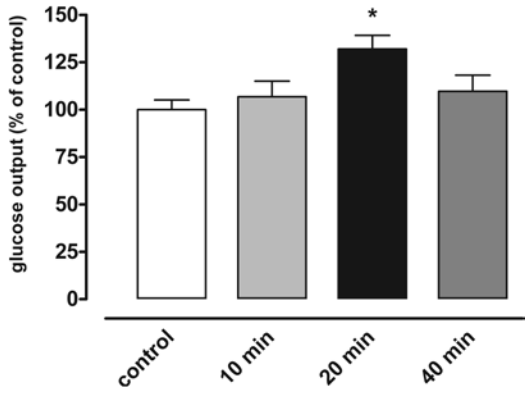

Figure 1. Time course of the glucose release by primary hepatocytes in suspension culture incubated for 10, 20 and 40 min in DMEM without glucose in presence of 100 nM ghrelin. At each time-point experiment, glucose output is expressed as percentage of control values. Bars represent standard error of the mean (*, p≤0.001).

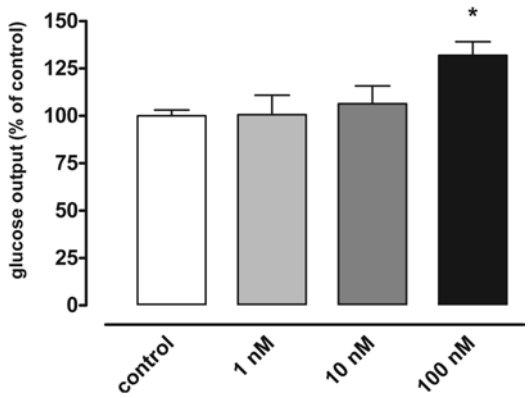

Figure 2. Glucose output by primary hepatocytes in suspension culture after 20 min incubation with DMEM without glucose in absence or presence of 1, 10 and 100 nM ghrelin. Bars represent standard error of the mean (*, p≤0.001).

shown that acute treatment with HEX does not induce changes in glucose levels (24). This has led to speculate 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 the hepatocytes after 10 or 20 min incubation with 100 nM HEX was unaffected relative to control values (99% of control, p=0.9).

UAG has been shown to exert peripheral actions (11), although it has been demonstrated not to activate the GHS-R1a (1). Surprisingly, we found that UAG was able to inhibit glucose release. This effect was also dose-dependent, significant at 100 nM (76% of controls, p<0.001) and detectable after 20 min incubation (Figure 3).

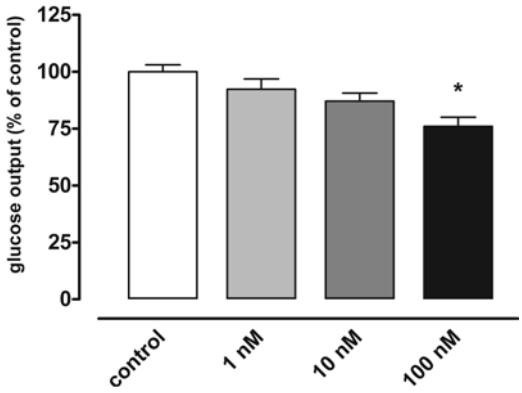

Figure 3. Glucose output by primary hepatocytes in suspension culture after 20 min incubation with DMEM without glucose in absence or presence of 1, 10 and 100 nM des-octanoyl ghrelin. Bars represent standard error of the mean (*, p≤0.001).

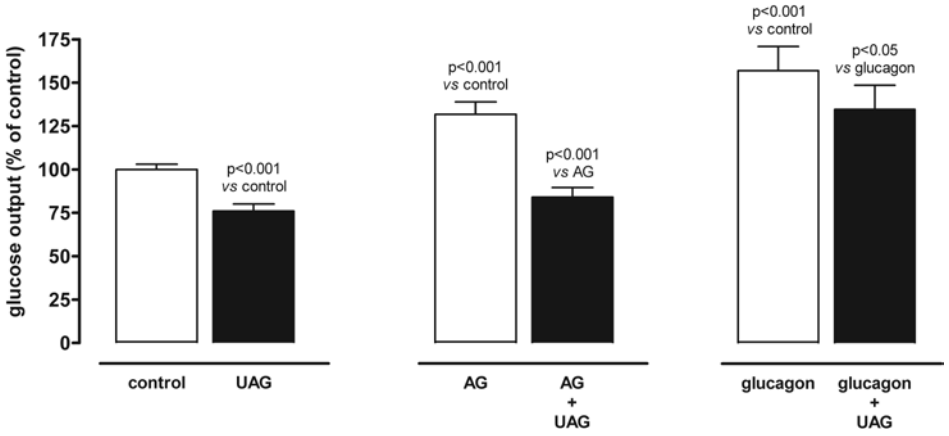

Figure 4. Effects of des-octanoyl ghrelin (unacylated ghrelin, UAG) on ghrelin (AG)- or glucagon-stimulated glucose output by primary hepatocytes. The glucose output, expressed as percentage of control values, was evaluated after 20 min incubation with 100 nM ghrelin or glucagon, alone or in combination with equimolar des-octanoyl ghrelin. Bars represent standard error of the mean.

Moreover, equimolar UAG completely reversed the 100 nM AG-induced glucose output to control levels (132% down to 84%, $p<0.01$) (Figure 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-R type 1a and 1b

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 to pancreas (Ct 17-20), which we used as a positive control. 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 AG on hepatocytes (at least with glucose output as an endpoint). A possible explanation was that UAG was able to modulate the interaction of AG with GHS-R1a. However, UAG does not activate this receptor (1), making such a mechanism of action unlikely. To examine this experimentally, we utilized a CHO cell-line that co-expressed 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 AG 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 AG with an IC_{50} of approximately 2 nM in the same system (data not shown). These results demonstrate that UAG is incapable of modulating AG interaction/activity at the GHS-R1a. Since 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 an intravenous bolus 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*, as the results of the present study demonstrate for the first time that AG, 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 20 minutes, 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 the liver as the primary suspect responsible for this acute hyperglycemic effect of AG. 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 AG on glucose release by liver cells has not been investigated to date.

So, in order 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, while in normal subjects *in vivo* UAG administered alone was inert as far as glucose output was concerned. In fact, it was inert in any 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 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. 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 signalling 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 already learned 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 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, since 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 signalling 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 from GHS-R1a. In this context, we cannot exclude that UAG modulates glucagon receptor activity, since 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 even 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 the 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, while 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 demonstrate that UAG suppresses glucose release by hepatocytes. Furthermore, we report that UAG 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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Chapter IV

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

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ABSTRACT

Acylated and unacylated ghrelin (AG and UAG, respectively) 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-Lys^3]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 1g/kg) in anesthetized fasted Wistar rats.

UAG administration potently and dose-dependently enhanced the rise of insulin concentration induced by IVGTT in the portal and, to a lesser extent, in the systemic circulation. This UAG-induced effect was completely blocked by the co-administration of exogenous AG at equimolar concentrations. Similarly to UAG, the ghrelin receptor antagonist [D-Lys^3]GHRP-6, alone or in combination with AG and UAG, strongly enhanced the portal insulin response to IVGTT, whereas exogenous AG alone did not exert any further effect.

Our data demonstrate that in glucose-stimulated conditions exogenous UAG acts as a potent insulin-secretagogue, whereas endogenous AG exerts a maximal tonic inhibition on glucose-induced insulin release.

INTRODUCTION

Ghrelin is a gut hormone predominantly produced in the stomach and, to a lesser extent, in other regions of the gastrointestinal tract (1-3). Ghrelin circulates in the bloodstream in two different forms: acylated (or n-octanoylated) and unacylated (or des-octanoylated or des-acylated) (1). Acylated ghrelin (AG) has a unique feature: a post-translational esterification of a fatty (n-octanoic or, to a lesser extent, n-decanoic) acid on serine residue at position 3 (1). This acylation is considered necessary for AG's actions via the growth hormone secretagogue receptor type 1a (GHS-R1a), also called ghrelin receptor (GRLN-R) (1, 4). However, normally AG accounts for less than 10% of the total ghrelin in circulation. The majority of circulating ghrelin is unacylated (UAG), which binds with high affinity to a receptor, different from GHS-R1a and yet unknown (1, 5).

Both AG and UAG have pleiotropic activities, including regulation of insulin secretion and glucose metabolism. It has been shown that endogenous AG and UAG are also produced in the endocrine pancreas, which also expresses the GHS-R1a (6-10). It has been found that endogenous AG in the pancreas inhibits the glucose-induced insulin release via the GHS-R1a (10), as demonstrated by the marked increase of insulin response to glucose after blockade of endogenous AG (*i.e.* via receptor antagonism, anti-AG antiserum, deletion of the ghrelin gene) (3, 10, 11). Moreover, ablation of the ghrelin gene improved glucose tolerance, insulin secretion and insulin sensitivity in genetically, leptin-deficient (ob/ob), obese mice (11). Administration of exogenous AG suppressed further insulin secretion both in fasting and in glucose-stimulated conditions, and it worsened insulin sensitivity and glucose tolerance after a meal or a glucose load (3, 11-13). UAG administration neither had effects on glucose-induced insulin release in a perfused pancreas model (3), nor did it induce significant changes in systemic fasting levels of insulin and glucose in vivo (3, 10, 13, 14). However, UAG increased insulin release in vitro by insulinoma cell lines exposed to high glucose concentrations (15, 16) and over-expression of (endogenous) UAG in pancreatic islets improved the insulin sensitivity to an intraperitoneal glucose load in mice (17). Moreover, when co-administered with AG, UAG completely prevented the AG-induced increase in circulating glucose levels and worsening of insulin sensitivity (13, 18, 19).

Together, these data elucidate the role of AG in the negative regulation of insulin secretion, insulin sensitivity and glucose metabolism. On the other hand, they show that an excess of endogenous UAG improves insulin sensitivity and suggest that UAG, or more likely the ratio of AG/UAG, might be implicated in the modulation of insulin release. However, at present the metabolic role of UAG remains to be defined. The reported effects of AG and UAG on glucose and insulin levels in vivo

are based on measurements of systemic blood samples, while both AG and UAG are secreted into the portal circulation before they reach the systemic circulation. Moreover, these peptides also have hepatic effects. Therefore we hypothesized that, concerning insulin secretion, assessment of insulin concentration in the portal vein might be more informative than that in the systemic circulation.

The aim of this study was to investigate whether the blockade of endogenous AG action (*i.e.* blockade of the GHS-R1a) or administration of exogenous AG and UAG differentially regulate the portal and systemic insulin response to glucose and/or modulate hepatic insulin clearance.

We therefore studied in rats the effects of the administration of AG, UAG, the ghrelin receptor antagonist [D-Lys^3]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 (Mercodia, Uppsala, Sweden). Total and acylated ghrelin were measured using radioimmunoassays (RIAs) from Linco Research Inc. (St. Charles, Missouri, USA). Rat acylated and unacylated (des-octanoyl) ghrelin, as well as [D-Lys^3]GHRP-6 were obtained from NeoMPS SA (Strasbourg, France). Sodium pentobarbital (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 BV (Alphen aan den Rijn, The Netherlands). Silicone catheters (3-french size) were provided by UNO Roestvaststaal BV (Zevenaar, The Netherlands); suture needles (Dafilon 8/0) by B. Braun Melsungen AG (Melsungen, Germany).

Animals

Male Wistar rats (age: 10-12 weeks; weight: 350-400 g, Harlan Netherlands BV, Horst, The Netherlands) were housed in groups in a temperature-controlled room under a 12-hour light/12-hour dark cycle, and maintained on pelleted chow with free access to water. The animals were housed for at least one week before starting the experiments, in order to allow 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 hours (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 sodium pentobarbital (60 mg/kg induction, 20 mg/kg maintenance administered at the end of the surgical procedure, before starting 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 (O_2, 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:

i) 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 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.

ii) 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 approximately 1 mm) was made in the wall of the portal vein, opposite to the gastroduodenal vein. The center of the purse-string was cut, the catheter inserted into the portal vein and pushed in for a few millimetres, with the tip secured about 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. Intravenous Glucose Tolerance Test (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 in account the reduction of insulin sensitivity caused by abdominal surgery (20) and the possible interference due to anesthesia (21, 22). Sodium pentobarbital was used, since, compared to other anesthetics, it has been shown to interfere less with insulin secretion

and glucose metabolism both in the fed and the fasted conditions (21, 22), in accordance with our previous observations (unpublished data).

3. IVGTT + rat Acylated ghrelin (AG) (30 nmol/kg), *n = 7*
4. IVGTT + rat Unacylated ghrelin (UAG) (3 nmol/kg), *n = 6*
5. IVGTT + UAG (30 nmol/kg), *n = 10*
6. IVGTT + [D-Lys^3]GHRP-6 (1 μmol/kg), *n = 6*
7. IVGTT + [D-Lys^3]GHRP-6 (1 μmol/kg) + AG (30 nmol/kg), *n = 6*
8. IVGTT + [D-Lys^3]GHRP-6 (1 μmol/kg) + UAG (30 nmol/kg), *n = 7*
9. IVGTT + AG (30 nmol/kg) + UAG (30 nmol/kg), *n = 7*

After baseline samples were 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 minutes after treatment administration to measure glucose and insulin levels. At baseline total and acylated ghrelin levels were also measured in 24 rats (before assigning them 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 the aprotinin (Trasylol®, 500.000 KIE, 40 μl/ml) was added. Samples were immediately centrifuged and plasma aliquots for AG measurements were acidified with 1N 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.* (23) and by Groschl *et al.* (24) 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 utilizes [^{125}I]-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 variation: (average) 6.4% CV, inter-assay variation: 16.3 %CV. AG is recognized by a Guinea Pig anti-Ghrelin specific for the ghrelin molecule octanoylated at its Ser^3 residue. This antibody recognizes octanoyl ghrelin, intact and (residues 1-10). Cross-reactivity with unacylated ghrelin is less than 0.1% and with ghrelin fragments (residues 14-28) is zero. The sensitivity of the assay is 7.8 pg/ml; the intra-assay variation: 7.4% CV, inter-assay is 13.5% CV.

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

Calculations

Unacylated ghrelin

Unacylated ghrelin (UAG) levels were calculated by subtracting acylated ghrelin (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

In order to estimate whether the liver may play a role in the clearance of ghrelin produced by the gut, we calculated the percentage of hepatic clearance using a method originally proposed by Kaden *et al*. (25, 26). The percentage hepatic extraction of any given hormone is calculated as: [(hormone presented to the liver – hormone leaving the liver) x 100 / (hormone presented to the liver)]. The ratio of the relative contribution of a "hormone presented to the liver" by the portal vein versus the hepatic artery (concentration x flow) is assumed to be 3:1 (26). The percentage of portal hormone extraction is calculated as: [(hormone concentration in the portal vein – hormone concentration in hepatic vein) x 100 / (hormone concentration in the portal vein)]. Since the contribution to post-hepatic 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 delta (Δ) changes versus baseline (mean ± SEM) and as Δ areas under the curve (ΔAUCs) (mean ± SEM).

Statistical analysis

Statistical analysis was performed using SPSS for Windows 10.0 (Chicago, IL, USA). The one–way analysis of variance (ANOVA) was used to compare the several treatment-groups for baseline levels and ΔAUC 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. Independent t-test was performed to compare two groups, whereas paired t-test was also run to compare

Δ changes versus baseline and jugular versus 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 pg/ml vs 63 ± 5 pg/ml, respectively, $p<0.001$), whereas the portal-peripheral gradient of UAG was 1.1 (1449 ± 92 pg/ml *vs* 1286 ± 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 intravenous glucose administration (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).

After saline injection (1 ml) insulin levels showed a small and transient decrease both in the portal and the peripheral circulation ($\Delta_{5\text{-}0}$, $p<0.01$ and $p<0.05$ *vs* baseline, respectively) (Figure 1, A and C), whereas glucose levels did not show significant variations at any time point (Figure 2, A and C represent Δ variations during the time course; ΔAUCs are reported in Table 2).

Table 1. Baseline absolute levels (mean ± SEM) of glucose and insulin were not significantly different among the treatment groups, either in the portal or the systemic circulation.

Baseline levels					
Groups (number of animals)		**Glucose** (mmol/l)		**Insulin** (μg/l)	
		Portal	*Systemic*	*Portal*	*Systemic*
Saline *(n = 12)*		7.6±1.1	9.9±1.2	4.2±0.9	1.7±0.4
IVGTT	**IVGTT controls** *(n = 12)*	7.9±0.8	10.1±0.7	5.3±0.8	1.8±0.3
	AG (30 nmol/kg) *(n = 7)*	10.2±1.0	10.1±0.8	4.6±0.1	1.6±0.3
	UAG (3 nmol/kg) *(n = 6)*	9.1±1.2	9.5±1.4	3.6±0.4	1.7±0.4
	UAG (30 nmol/kg) *(n = 10)*	6.2±0.8	7.7±1.1	4.3±0.8	1.4±0.2
	AG + UAG *(n=7)*	6.9±0.9	7.5±1.2	3.5±0.9	1.0±0.1
	[D-Lys^3]GHRP-6 *(n = 6)*	9.8±1.0	9.8±1.0	3.1±0.6	1.3±0.4
	[D-Lys^3]GHRP-6+AG *(n = 6)*	9.5±1.9	10.5±1.2	2.9±0.7	1.1±0.2
	[D-Lys^3]GHRP-6+UAG *(n = 7)*	8.2±0.9	10.1±2.2	3.5±0.5	1.8±0.4

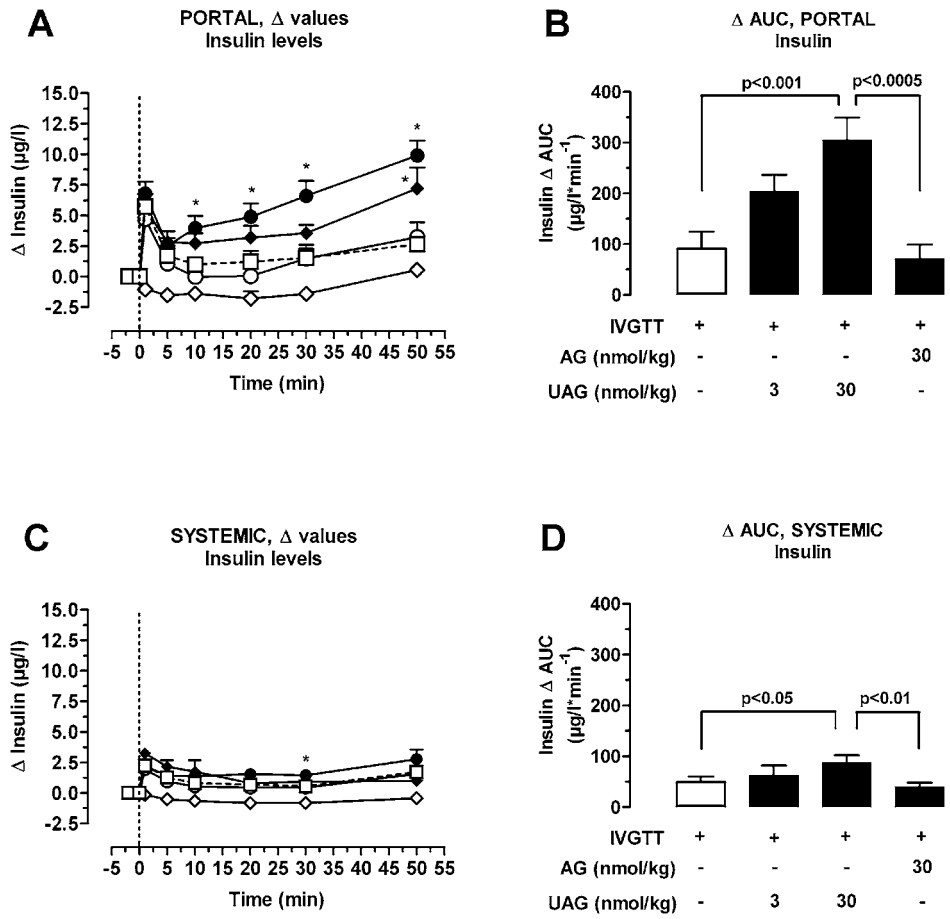

Figure 1. Unacylated ghrelin (UAG) dose-dependently stimulated the second-phase insulin response to an intravenous glucose load (IVGTT, 1g/kg), whereas exogenous AG did not modify insulin levels. This insulin-secretagogue effect of UAG was much larger in the portal vein (A, B) than in the systemic circulation (C, D). Left panels represent the values during the time course relative to the baseline value which was set as 0 (Δ). Right panels represent ΔAUCs 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.

As expected, IVGTT induced a prompt increase in insulin levels both in 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). Δ insulin 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

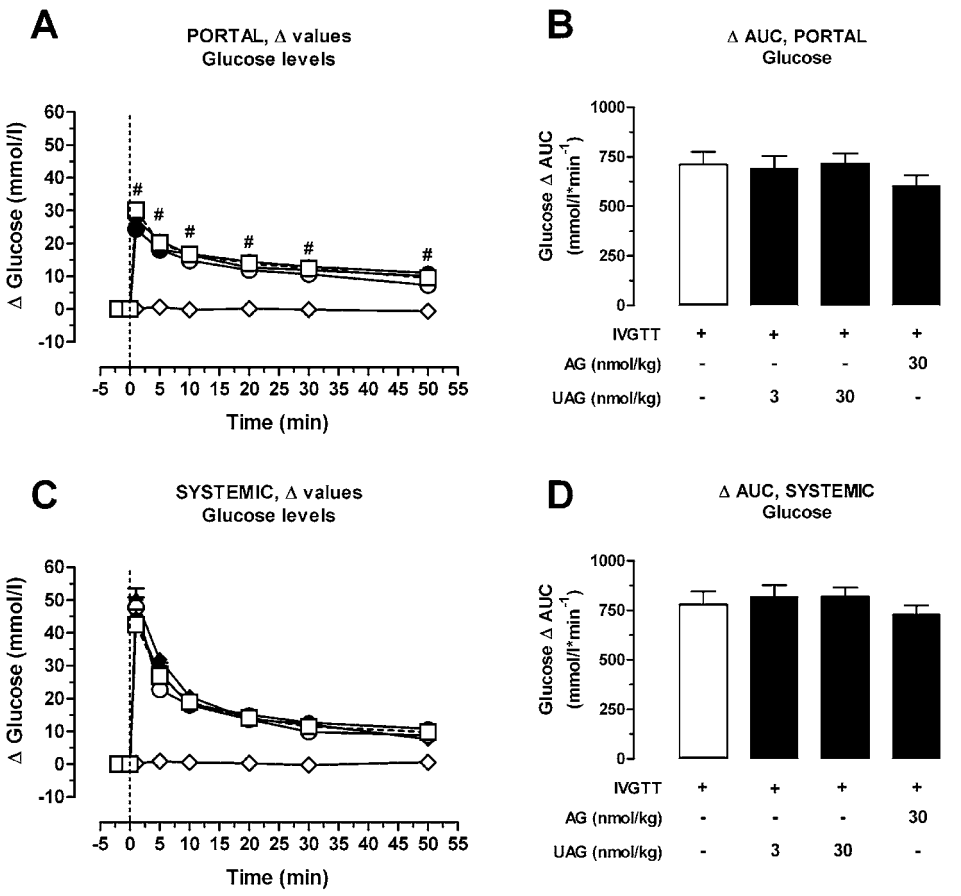

Figure 2. Administration of exogenous AG (30 nmol/kg) or UAG (3 and 30 nmol/kg) did not modify glucose levels either in the portal vein (A, B) or in the peripheral circulation (C, D).
Left panels represent the values during the time course relative to the baseline value which was set as 0 (Δ). Right panels represent ΔAUCs 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.

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 Tab. 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 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 Tab. 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

Table 2. Glucose and insulin levels (ΔAUC of the whole 50-min time course) in both portal and systemic circulation are reported for all treatment groups. The effects of acylated ghrelin (AG), unacylated ghrelin (UAG) and the ghrelin receptor antagonist [D-Lys^3]GHRP-6, alone or in combination, were studied during an intravenous glucose tolerance test (IVGTT, D-glucose 50%, 1 g/kg). In all groups treatments were administered at t=0 min. Values are reported as mean ± SEM. P values are reported for significant differences ($p<0.05$) *vs* the IVGTT group.

		Glucose		**Insulin**	
		Δ AUC (mmol/l*min^{-1})		**Δ AUC** (μg/l*min^{-1})	
Groups		*Portal*	*Systemic*	*Portal*	*Systemic*
Saline		-4±27	14±16	-53±20	-31±11
IVGTT	**IVGTT controls** *(n=12)*	711±65	778±68	91±33	50±11
	AG (30 nmol/kg) *(n=7)*	604±55	730±44	72±27	40±9
	UAG (3 nmol/kg) *(n=6)*	693±61	818±60	204±33	63±19
	UAG (30 nmol/kg) *(n=10)*	716±51	819±46	305±44$^{p<0.001}$	88±15 $^{p<0.05}$
	AG + UAG *(n=7)*	666±50	855±59	73±35	39±12
	[D-Lys^3]GHRP-6 *(n=6)*	815±65	997±107	280±68$^{p<0.01}$	68±18
	[D-Lys^3]GHRP-6+AG *(n=6)*	785±66	734±74	234±54$^{p<0.03}$	69±13
	[D-Lys^3]GHRP-6+UAG *(n=7)*	652±35	703±67	257±81$^{p=0.05}$	60±26

and started gradually to rise again at 10 min and reached the highest level at 50 min ($\Delta_{50\text{-}0}$, 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 Tab. 2).

The GHS-R1a antagonist [D-Lys^3]GHRP-6 (1 μmol/kg), like UAG, enhanced the second-phase insulin response to glucose in the portal vein. Portal insulin levels gradually increased from 20 min ($p<0.05$) to 50 min ($\Delta_{50\text{-}0}$, [D-Lys^3]GHRP-6+IVGTT *vs* IVGTT: $p<0.03$) (Figure 3A). Portal insulin ΔAUC was significantly higher ($p<0.01$) in rats treated with [D-Lys^3]GHRP-6+IVGTT than in those that received IVGTT alone (Figure 3B). In the systemic circulation, the stimulatory effect on insulin release induced by the GHS-R1a antagonist was lost and the ΔAUC of the whole time course was similar to that in the IVGTT group (Figure 3, C and D). Moreover, the effect exerted by [D-Lys^3]GHRP-6+IVGTT on glucose-induced insulin secretion was not modified by the simultaneous administration of AG or UAG. Figures 3A and 3B clearly show that [D-Lys^3]GHRP-6, alone or co-administered with AG or UAG, stimulated the second-phase portal insulin response to IVGTT and that this effect was again similar

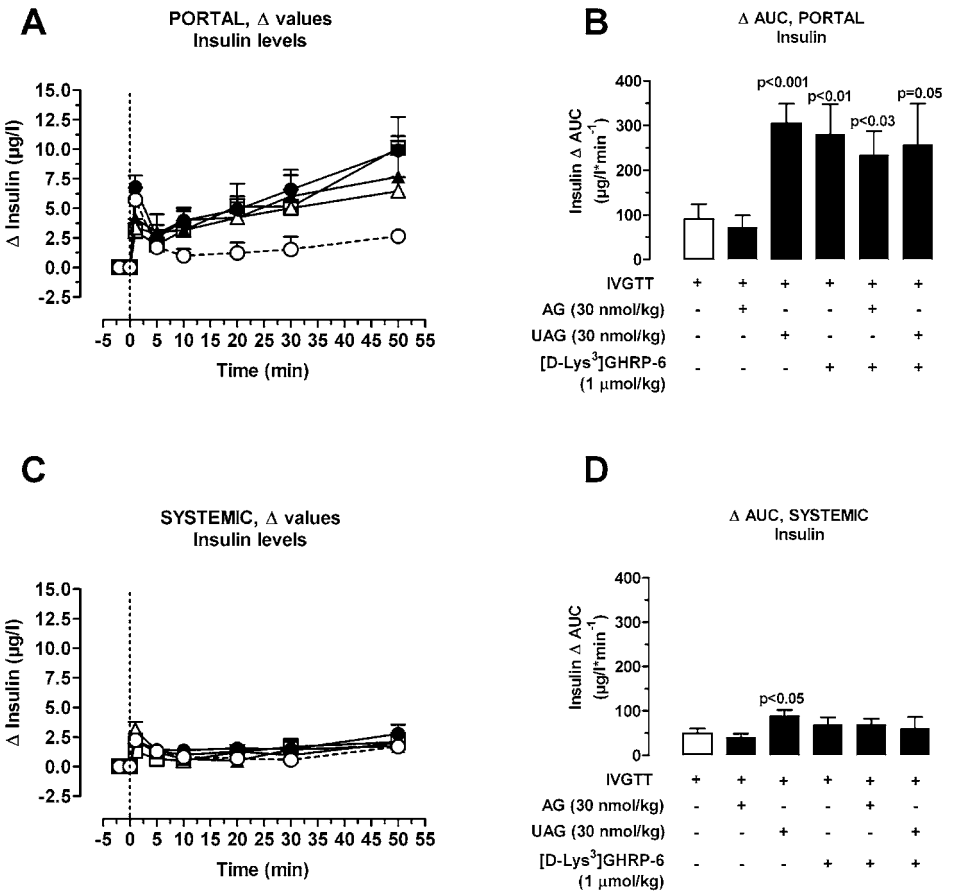

Figure 3. The insulin-secretagogue effect of UAG in glucose-stimulated conditions was similar to that of [D-Lys^3]GHRP-6, alone or in combination with AG or UAG (A, B), whereas in the peripheral circulation, only a slight stimulatory effect of UAG was still detectable (C, D). Left panels represent the values during the time course relative to the baseline value which was set as 0 (Δ). Right panels represent ΔAUCs of all parameters after treatment administration. Vertical dotted line: treatment administration at t=0. ○: IVGTT, ●: IVGTT+UAG (30 nmol/kg) (n = 10), □: IVGTT+[D-Lys^3]GHRP-6 (1 µmol/kg) (n = 6), △: IVGTT+[D-Lys^3]GHRP-6+AG (30 nmol/kg) (n = 6), ▲: IVGTT+[D-Lys^3] GHRP-6+UAG (30 nmol/kg) (n = 7). P values are reported in the figure, differences were considered significant for p<0.05.

in extent, pattern and timing to that observed after UAG (30 nmol/kg) alone. ΔAUC of portal insulin concentrations in the group treated with [D-Lys^3]GHRP-6, alone or combined with AG and UAG were 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 (ΔAUC) in all the groups treated with [D-Lys^3]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-Lys^3]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 ΔAUC (Table 2) and curve profile (data not shown).

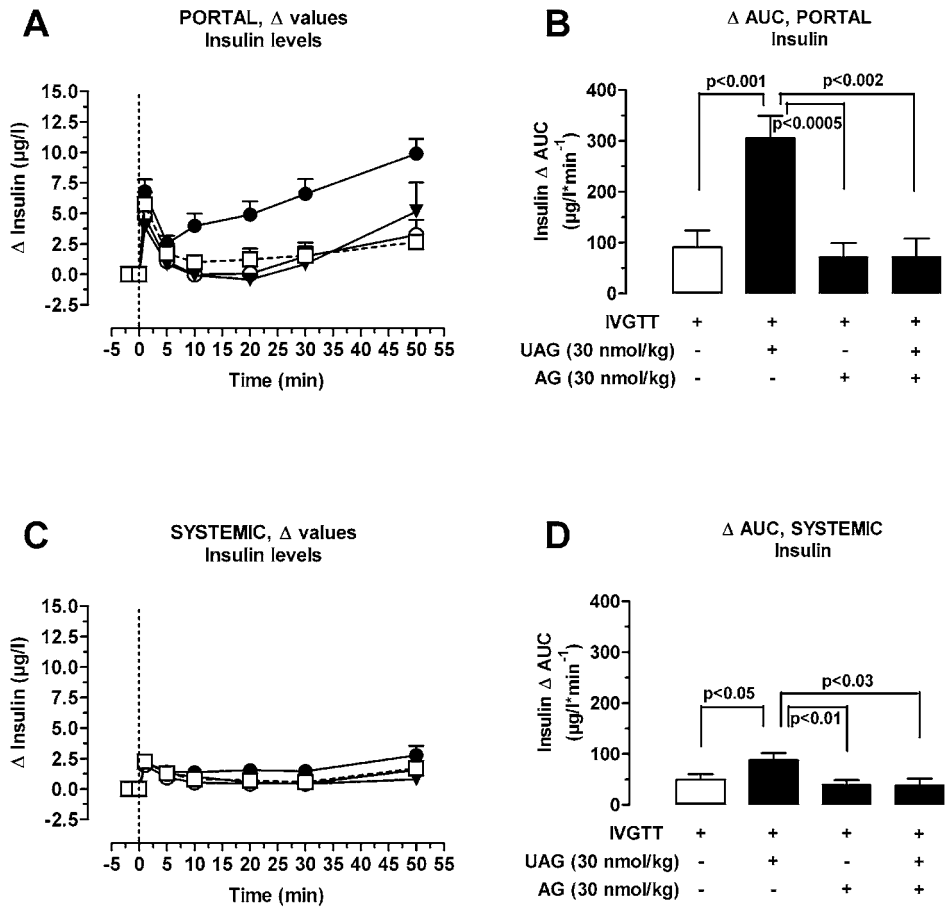

Figure 4. The co-administration of AG (30 nmol/kg) and UAG (30 nmol/kg) abolished completely the UAG-induced enhancement of insulin response to glucose both in the portal vein (A, B) and in the peripheral circulation (C, D). Panels A and C represent the values during the time course relative to the baseline value, which was set as 0 (Δ). Panels B and D represent ΔAUCs after treatment administration. Vertical dotted line: treatment administration at t=0. □: IVGTT, ○: IVGTT+AG (30 nmol/kg) (n = 7), ●: IVGTT+UAG (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.

Interestingly, the co-administration 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 (ΔAUC: $p<0.002$) and in peripheral ($p<0.03$) circulation (Figure 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 affect not only 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* IVGTT: 59 ± 4 % and *vs* IVGTT+AG: 57 ± 5 %). Like UAG, [D-Lys^3]GHRP-6, alone or combined with AG and UAG, slightly increased hepatic insulin clearance when compared to rats treated with IVGTT alone or with AG. However, the statistical significance was reached only by the group that received IVGTT+AG+[D-Lys^3]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 co-administration of AG and was similar to that exerted by blockade of the GHS-R1a using the specific antagonist [D-Lys^3]GHRP-6. Moreover, UAG as well as [D-Lys^3]GHRP-6 slightly increased hepatic insulin clearance. This may partly explain why we observed a marked increase in insulin levels in the portal circulation, but not in the peripheral blood.

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, completely prevented the AG-induced and partially blocked the glucagon-dependent glucose release (27). However, it was also found that UAG alone does not improve hepatic insulin sensitivity in a euglycemic hyperinsulinemic clamp model in mice (19). 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 stimulates 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-Lys^3]GHRP-6, a GHS-R1a antagonist. The effect of [D-Lys^3]GHRP-6 likely reflects the blockade of the inhibitory action of endogenous AG on beta cells. This is in accordance 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 (3, 10, 11). 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 (20) and/or possible effects of the anesthesia (21, 22).

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 reconfirmed in our model, exerts already 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 co-administration of the GHS-R1a antagonist together with exogenous AG elicited the insulin response to glucose load to the same extent as [D-Lys^3]GHRP-6 alone, *i.e.* removing the inhibitory tone of endogenous AG on insulin secretion. Our findings differ from previous reports by Dezaki and colleagues (3), 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 co-administered together 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 co-administration of UAG with AG was able to prevent the AG-induced decrease in circulating insulin and worsening of insulin sensitivity (12, 13, 18, 19).

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-Lys^3]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, differently from [D-Lys^3]GHRP-6, does not block the GHS-R1a (1), 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: i) either UAG or [D-Lys^3]GHRP-6 exert a maximal antagonistic activity on endogenous AG; ii) [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 (27), suggests that UAG action is mainly targeted 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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Chapter V

Intravenous glucose administration in fasting rats has differential effects on acylated and unacylated ghrelin in the portal and the systemic circulation (A comparison between portal and peripheral concentrations in anesthetized rats)

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ABSTRACT

Ghrelin is produced by the gastrointestinal tract and its systemic concentrations are mainly regulated by nutritional factors.

Our aim was to investigate: i) endogenous portal and systemic acylated- and unacylated- ghrelin levels (AG and UAG, respectively); ii) whether an intravenous glucose tolerance test (IVGTT) modifies AG and UAG; iii) whether the liver passage plays a role in regulating systemic AG and UAG. To elucidate this, we evaluated the effects of IVGTT or saline injection on endogenous portal and systemic concentrations of glucose, insulin, AG and UAG in anesthetized fasting rats. Hepatic extraction of insulin, AG and UAG, and the ratio of AG/UAG were also measured.

IVGTT suppressed both portal ($p<0.03$) and peripheral ($p<0.05$) UAG, whereas it only blunted pre-hepatic, but not peripheral, AG. During fasting, hepatic clearance of UAG was 11% and it was decreased to 8% by IVGTT. AG was cleared by the liver by 38%, unaffected by glucose. The AG/UAG ratio was higher in the portal than in the systemic circulation, both in the saline ($p<0.004$) and in the IVGTT ($p<0.0005$) rats.

In conclusion, this study shows that: i) the ratio of AG/UAG is very low in the portal vein and decreases further in the systemic circulation; ii) IVGTT in anesthetized fasting rats inhibits UAG, whereas it only blunts pre-hepatic, but not systemic, AG; iii) hepatic clearance of AG is much higher than that of UAG. Thus, our results suggest that peripheral AG metabolic regulation and action are mainly confined within the gastrointestinal tract.

INTRODUCTION

Ghrelin is a gut hormone predominantly produced by the stomach and, to a lesser extent, by other regions of the gastrointestinal tract (1-3). Ghrelin circulates in the bloodstream in two different forms: acylated (or *n*-octanoylated) and unacylated (or des-octanoylated or des-acylated) (1). The acylated ghrelin (AG) bears a unique post-translational modification due to the esterification of a fatty (*n*-octanoic or, to a lesser extent, *n*-decanoic) acid on its third serine residue (1). This acylation is necessary for ghrelin action via the growth hormone secretagogue receptor type 1a (GHS-R1a), also designated as ghrelin receptor (GRLN-R) (1, 4). AG accounts for a small amount (approximately 10%) of total ghrelin. The majority of circulating ghrelin is unacylated (UAG) and has been suggested to interact with a different receptor than the GHS-R1a (1).

Ghrelin secretion is regulated by the nutritional state. Thus, systemic ghrelin levels are reduced in obesity and elevated in conditions of negative energy balance (5-7) and acute changes in energy disposal determine circadian ghrelin secretion, which is increased by short-term fasting (*i.e.* before meals) and suppressed immediately after feeding (8). The extent of ghrelin suppression after acute energy intake has been shown to be dependent on the type of macronutrient, carbohydrates and, among them, glucose, being the most potent inhibitors of peripheral ghrelin levels (9-11). Moreover, the magnitude of ghrelin suppression by glucose load was similar after oral and intravenous administration (10), and three hours of glucose infusion halved ghrelin levels (9). The majority of reports in the literature about the nutritional regulation of ghrelin refer to peripheral total ghrelin levels, which may differ from the amount released by the gut source into the local circulation. In fact, since the main source of ghrelin is the gastrointestinal tract, ghrelin is secreted into the portal vein and has to pass the liver before it reaches the peripheral circulation. It is known that the liver passage is crucial for clearance/extraction of other hormones secreted into the hepatic portal vein (such as insulin and glucagon) and this process is subjected to acute nutritional regulation (12-15). Moreover, it cannot be excluded that the liver also regulates the ratio between AG and UAG and thereby affects the physiological role of these hormones. To our knowledge, portal concentrations of total ghrelin, but not AG or UAG, have been reported by Mundinger and colleagues (16). However, these authors did not compare portal with peripheral levels (16).

Therefore, our aims were: i) to investigate whether peripheral ghrelin levels differ from those secreted into the portal vein; ii) if acute nutritional changes can modify ghrelin secretion and/or metabolism; ii) to clarify whether the first pass effect by the liver on ghrelin secreted by the gut plays a role in regulating ghrelin metabolism (*i.e.* the ratio between AG and UAG). To address these questions, we used a fasting rat

model with a catheterization of the portal and the jugular veins, under anesthesia. We compared AG and UAG variations in fasted rats under basal conditions and after an intravenous glucose load.

This study shows that: i) the ratio of AG/UAG is very low in the portal vein and decreases further in the systemic circulation; ii) IVGTT in anesthetized fasting rats inhibits portal and systemic UAG, whereas it only blunts pre-hepatic, but not systemic, AG; iii) hepatic clearance of UAG is small, whereas hepatic clearance of AG is about 40%. Thus, our results suggest that peripheral AG metabolic regulation and action are mainly confined within the gastrointestinal tract.

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 (Mercodia, Uppsala, Sweden). Total and acylated ghrelin were measured using radioimmunoassays (RIAs) from Linco Research Inc. (St. Charles, Missouri, USA). Sodium pentobarbital (250 mg/5 ml) was prepared and provided by the ErasmusMC pharmacy. EDTA containing tubes were obtained by Greiner Bio-One BV (Alphen aan den Rijn, The Netherlands). Silicone catheters (3-french size) were provided by UNO Roestvaststaal BV (Zevenaar, The Netherlands); suture needles (Dafilon 8/0) by B. Braun Melsungen AG (Melsungen, Germany).

Animals

Male Wistar rats (age: 10-12 weeks; weight: 350-400 g, Harlan Netherlands BV, Horst, The Netherlands) were housed in groups in a temperature-controlled room under a 12-hour light/12-hour dark cycle, and maintained on pelleted chow with free access to water. The animals were housed for at least one week before starting the experiments, in order to allow acclimatization. Animal protocols were in compliance with the Dutch regulations on animal welfare and approved by the institutional Animal Welfare Committee.

Surgery and experimental design

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

Animals were anesthetized using an intraperitoneal (ip) injection of sodium pentobarbital (60 mg/kg induction, 20 mg/kg maintenance administered at the end of the surgical procedure, before starting 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 (O_2, 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:

i) 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 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 catheters 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.

ii) 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 approximately 1 mm) was made in the wall of the portal vein, opposite to the gastroduodenal vein, then the center of the purse-string was cut and the cannula inserted into the portal vein and pushed in for a few millimetres, with the tip secured about 1 mm caudal to the liver. The patency of the cannula 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:

- Saline (1 ml), n = 12
- Intravenous Glucose Tolerance Test (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 in account the reduction of insulin sensitivity caused by abdominal surgery (17) and the possible interference due to anesthesia (18, 19). Sodium pentobarbital was used, since, compared to other anesthetics, it has been shown to interfere less with insulin secretion and glucose metabolism both in the fed and the fasted conditions (18, 19).

After baseline samples were 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 and 30 minutes after treatment administration to measure glucose,

insulin, total and acylated ghrelin. 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 the aprotinin (Trasylol®, 500.000 KIE, 40 µl/ml) was added. Samples were immediately centrifuged and aliquots for total and acylated ghrelin assays were made. Aliquots for AG measurement were promptly acidified by adding 1N HCl (1:10, vol/vol), whereas aliquots for total ghrelin measurement were not. All aliquots were kept at 4°C until the end of the experiment and then stored at -20°C until assay. Multiple freeze/thaw cycles were avoided and aliquots were thawed only for the ghrelin assay. This procedure has been indicated by Hosoda *et al.* (20) and by Groschl *et al.* (21) as a standard procedure for collection of blood samples to determine ghrelin concentrations.

Serum total ghrelin and AG levels (pg/ml) were measured using RIA kits that utilizes [^{125}I]-ghrelin as a tracer. The specificity for rat ghrelin (total and AG, respectively) is 100%. Total ghrelin is detected by polyclonal rabbit antibodies that recognizes residues $_{(14\text{-}28)}$, thereby including AG, UAG and ghrelin fragments $_{(14\text{-}28)}$ (Table 1). The sensitivity of the assay is 93 pg/ml; the intra-assay variation: (average) 6.4% CV, inter-assay variation: 16.3% CV. AG is recognized by a Guinea Pig anti-Ghrelin specific for the ghrelin molecule octanoylated at its Ser^3 residue. This antibody recognizes octanoyl ghrelin, residues $_{(1\text{-}10)}$. Cross-reactivity with unacylated ghrelin is <0.1% and with ghrelin fragments $_{(14\text{-}28)}$ is zero (Table 1). The sensitivity of the assay is 7.8 pg/ml; the intra-assay variation: 7.4% CV, inter-assay is 13.5% CV.

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

Table 1. Specificity of total and acylated ghrelin Linco radioimmunoassays (RIAs). Total ghrelin is recognized by a polyclonal rabbit antibodies that recognizes residues $_{(14\text{-}28)}$ of both acylated and unacylated ghrelin (AG and UAG, respectively). AG is recognized by a Guinea Pig anti-Ghrelin specific for the ghrelin molecule octanoylated at its Ser^3 residue (N-terminus, residues $_{(1\text{-}10)}$). n.d. = not detectable. "?" indicates that the antibody used in the assay is able to recognize peptide fragments containing residues $_{(14\text{-}28)}$ and, perhaps, shorter fragments at the C-terminus.

Total and acylated ghrelin radioimmunoassay		
Peptide regions recognized by the antibody used in the assay:	**Total ghrelin**	**Acylated ghrelin**
AG $_{(1\text{-}10)}$	n.d.	100%
AG $_{(14\text{-}28)}$	100%	n.d.
UAG $_{(1\text{-}10)}$	n.d.	<0.1%
UAG $_{(14\text{-}28)}$	100%	n.d.
AG or UAG C-terminus fragments	?	n.d.

Ghrelin spiking experiments in rat plasma

In order to confirm that ghrelin was not degraded under the sampling and storing conditions used, rat blood was spiked with a known concentration of rat (acylated) ghrelin and recovery of total and acylated ghrelin was measured in the presence and in the absence of sample acidification. Rat blood was withdrawn by cardiac puncture from anesthetized animals. Blood was collected in ice-cold vials containing EDTA and aprotinin (Trasylol®, 500.000 KIE, 40 µl/ml) and kept on ice. Before centrifugation, rat acylated ghrelin (AG) was added to different tubes at a concentration of 1000 pg/ml (in duplicate). Plasma samples without any addition of acylated ghrelin were kept as control samples (blank). After centrifugation, two aliquots for total ghrelin and two aliquots for acylated ghrelin measurement were made from each tube. One aliquot was acidified by adding 1N HCl (1:10, vol/vol), whereas the other aliquot was not. All aliquots were kept at 4°C until the end of the experiment and then stored at -20°C until assay.

Total and acylated ghrelin levels were then measured in duplicate both in acidified and in non-acidified samples (blank or non-spiked and 1000 pg/ml)."

Calculations

Unacylated ghrelin

Unacylated ghrelin (UAG) levels were calculated by subtracting acylated ghrelin (AG) from total ghrelin concentrations at every time point, either in the portal or in the jugular vein samples.

Hepatic clearance

In order to estimate whether the liver may play a role in the clearance of ghrelin produced by the gut, we calculated the percentage of hepatic extraction using a method originally proposed by Kaden *et al*. (22, 23). The percentage hepatic extraction of any given hormone is calculated as: [(hormone presented to the liver – hormone leaving the liver) x 100 / (hormone presented to the liver)]. The ratio of the relative contribution of a "hormone presented to the liver" by the portal vein versus the hepatic artery (concentration x flow) is assumed to be 3:1 (23). The percentage of "portal" hormone extraction is calculated as: [(hormone concentration in the portal vein – hormone concentration in hepatic vein) x 100 / (hormone concentration in the portal vein)]. We adapted this calculation to: [(hormone concentration in the portal vein – hormone concentration in jugular vein) x 100 / (hormone concentration in the portal vein)], being aware that jugular hormone concentrations may be affected by a greater dilution than the hepatic vein (due to the ancillary venous return) and/

or by other sources of hormone production other than those tissues that drain into the portal vein.

Basal absolute levels of glucose, insulin, total ghrelin, UAG and AG in the systemic (jugular) and in the portal circulation are reported in Table 2.

Table 3 reports absolute AUCs of all parameters in the portal and the systemic circulation, for each group.

Results are expressed as absolute delta (Δ) changes versus baseline (mean ± SEM) and as Δ Areas Under the Curve (ΔAUCs) (mean ± SEM).

Absolute baseline levels and AUC of total ghrelin and UAG are expressed in ng/ml, Δ variations and ΔAUC are expressed in pg/ml.

Statistical analysis

Statistical analysis was performed using SPSS for Windows 10.0 (Chicago, IL, USA). Row data were checked for the presence of outliers that could bias the analysis. Since we could not detect any outlier value either in the saline or the IVGTT groups, all cases have been included in the statistical analysis. Independent t-test was run to compare different groups, whereas paired t-test was performed to compare results within the same group (Δ changes versus baseline and jugular versus portal values). A difference was considered significant when $p < 0.05$.

RESULTS

Baseline levels of glucose, insulin, total, unacylated and acylated ghrelin in the systemic (jugular) and in the portal circulation were not significantly different between the saline and IVGTT treated group. (Table 2).

In both treatment groups, mean concentrations (AUCs) of insulin, total ghrelin and AG were higher in portal than in peripheral samples, whereas absolute concentrations of glucose in the portal vein were lower than in the jugular vein. UAG levels (absolute AUC) were lower in jugular than in portal plasma in the saline group, whereas in the glucose-injected group portal and peripheral concentrations were similar (Table 3).

Table 2. Baseline absolute levels of glucose, insulin, total-, unacylated- and acylated- ghrelin were similar in the saline-and the IVGTT- treated rats.

Baseline levels				
	Saline (mean ± SEM)		IVGTT (mean ± SEM)	
Parameters	*Portal*	*Systemic*	*Portal*	*Systemic*
Glucose (mmol/l)	7.6±1.1	9.9±1.2	7.9±0.8	10.1±0.7
Insulin (µg/l)	4.2±0.9	1.7±0.4	5.29±0.8	1.8±0.3
Total ghrelin (ng/ml)	1.8±0.1	1. 6±0.1	1.6±0.1	1.4±0.07
Unacylated ghrelin (ng/ml)	1.7±0.1	1.5±0.1	1.4±0.09	1.3±0.07
Acylated ghrelin (pg/ml)	118±14	87±10	108±13	63±5

Table 3. Absolute values of areas under the curve (AUCs) of glucose, insulin, total-, unacylated- and acylated- ghrelin in the saline-and the IVGTT- treated rats. P values represent significant difference between portal and jugular levels within each group.

AUC				
	Saline (mean ± SEM)		IVGTT (mean ± SEM)	
Parameters	*Portal*	*Systemic*	*Portal*	*Systemic*
Glucose (mmol/l*min^{-1})	231.7±26.0	306.3±31.2 $^{p<0.0005}$	729.4±29.2	858.5±41.3 $^{p<0.0005}$
Insulin (µg/l*min^{-1})	80.5±17.3	31.8±5.1 $^{p<0.03}$	207.8±16.3	81.5±8.1 $^{p<0.0005}$
Total ghrelin (ng/ml*min^{-1})	51.4±3.1	45.0±3.6 $^{p<0.001}$	37.8±3.0	33.4±1.7 $^{p<0.04}$
Unacylated ghrelin (ng/ml*min^{-1})	47.9±3.1	42.8±3.4 $^{p<0.004}$	35.3±2.8	31.9±1.6 $^{p=0.06}$
Acylated ghrelin (pg/ml*min^{-1})	3535±354	2157±328 $^{p<0.001}$	2533±316	1493±144 $^{p<0.001}$

Recovery of ghrelin spiked in rat plasma

Acylated ghrelin

In blank (non-spiked) samples, acylated ghrelin levels in non-acidified aliquots were 8% of those in acidified plasma.

When a concentration of 1000 pg/ml of acylated ghrelin was added, the recovery of acylated ghrelin in acidified plasma was 94%. Conversely, in non-acidified plasma acylated ghrelin concentration was not different from non-spiked controls, indicating that the exogenous acylated ghrelin was almost entirely degraded.

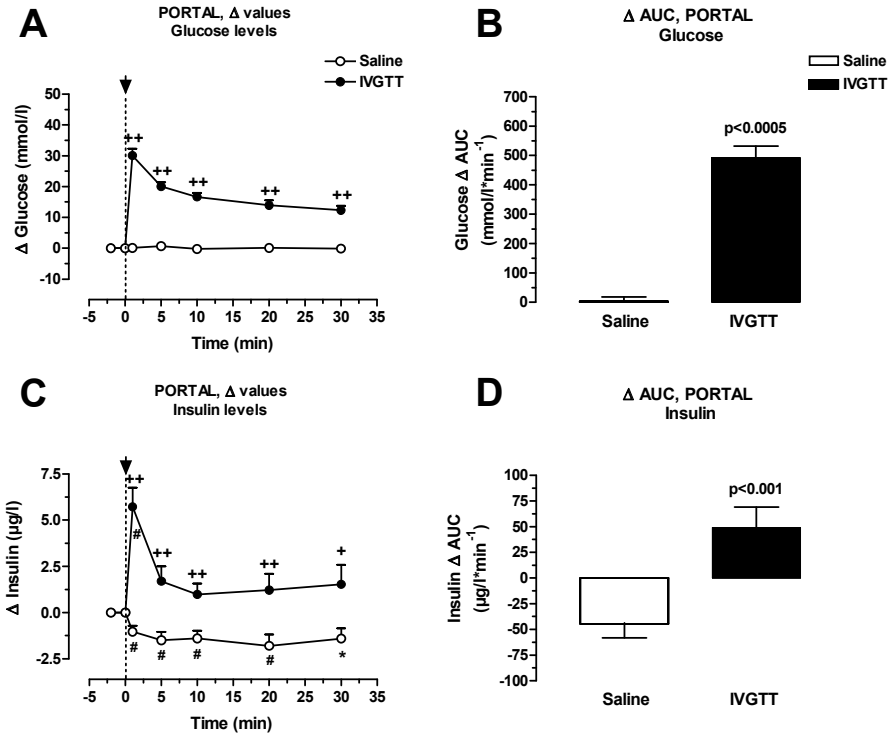

Figure 1. Effects of intravenous glucose tolerance test (IVGTT) on glucose, insulin, acylated and unacylated ghrelin (AG and UAG, respectively) concentrations in the portal vein, compared to the saline-injected group. Left panels represent the values during the time course relative to the baseline value which was set as 0 (Δ). Treatment injection: vertical dotted line at 0 min; saline group: open circles; IVGTT group: closed circles. Right panels represent ΔAUCs of all parameters after treatment administration. Saline group: open bars; IVGTT group: closed bars.

IVGTT induced an increase in portal concentrations of glucose (A, B) and insulin (C, D). Glucose administration suppressed UAG (E, F) as well as AG levels (G, H), although the effect on AG did not reach statistical significance when compared to saline.

* = p<0.05 *vs* baseline; # = p<0.01 *vs* baseline; + = p< 0.05 IVGTT *vs* saline; ++ = p<0.001 IVGTT *vs* saline.

Total ghrelin

In blank, non-spiked samples, total ghrelin levels were similar in acidified and non-acidified plasma samples.

When a concentration of 1000 pg/ml of acylated ghrelin was added, the recovery of total ghrelin in acidified plasma was 99% and this was similar to that found in non-acidified plasma.

Overall, the experiments show that recovery of acylated and total ghrelin after spiking is > 90% under the conditions that we have used and that almost complete de-acylation of AG occurs in non-acidified plasma.

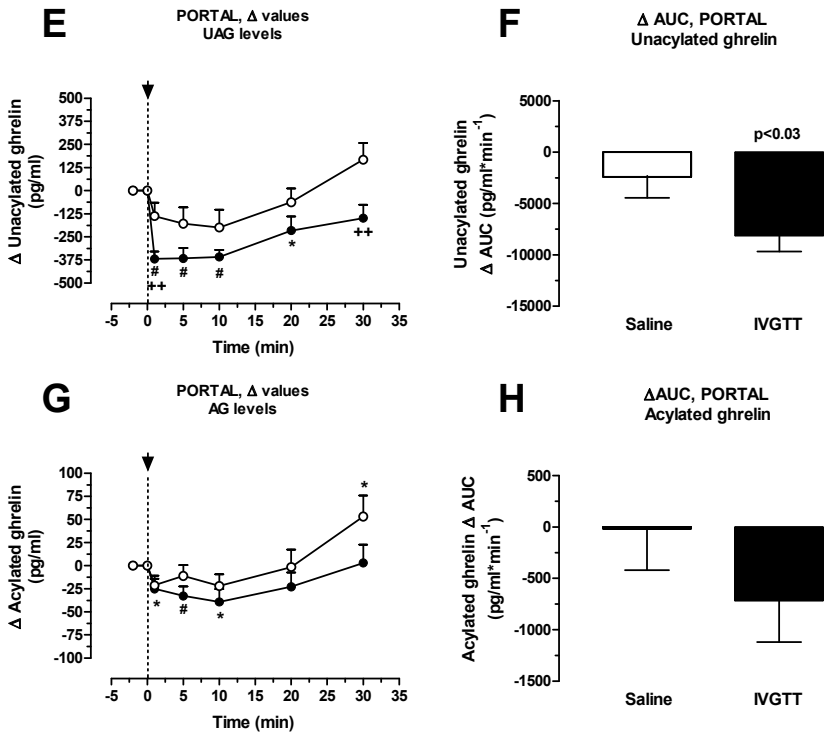

Figure 1.

Glucose

Saline injection (1 ml) did not modify glucose levels at any time point either in the portal or in the peripheral samples (Figures 1A and 1B; 2A and 2B, respectively).

As expected, IVGTT induced a prompt increase in glucose levels, which peaked at 1 min and decreased thereafter, although at 30 min they had not normalized yet ($p<0.0005$). In each group glucose levels were higher in jugular than in portal vein (peak, jugular $\Delta_{1\text{-}0}$: 42.6 ± 2.0 mmol/l, portal $\Delta_{1\text{-}0}$: 30.1 ± 2.3 mmol/l, $p<0.01$).

ΔAUCs were higher in the IVGTT than in the saline control group ($p<0.0005$), both in the portal and in the jugular samples. (Figures 1B and 2B).

Insulin

After saline injection, a slight but significant significant decrease of insulin levels was observed in the portal vein at 1 min ($\Delta_{1\text{-}0}$: -1.0 ± 0.3 µg/l, $p<0.01$) and persisted up to 30 min, without significant changes during the time course (Figure 1C). In the systemic samples insulin levels were reduced by saline at 5 min ($\Delta_{5\text{-}0}$: -0.5 ± 0.2 µg/l; $p<0.05$) and up to 30 min (Figure 2C).

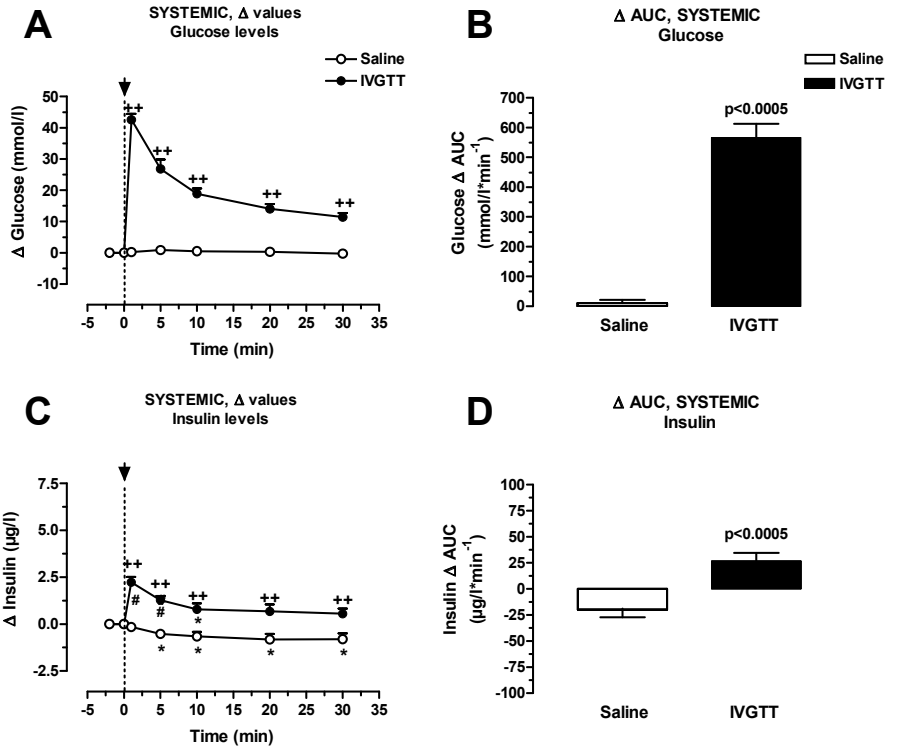

Figure 2. Effects of intravenous glucose tolerance test (IVGTT) on glucose, insulin, acylated and unacylated ghrelin (AG and UAG, respectively) concentrations in the jugular vein, compared to the saline-injected group. Left panels represent the values during the time course relative to the baseline value, which was set as 0 (Δ). Treatment injection: vertical dotted line at 0 min; saline group: open circles; IVGTT group: closed circles. Right panels represent ΔAUCs of all parameters after treatment administration. Saline group: open bars; IVGTT group: closed bars.

IVGTT in the systemic circulation induced an increase in glucose (A, B) and insulin (C, D) concentrations. After glucose administration UAG levels were inhibited (E, F), whereas the concentration of AG was unchanged (G, H).

* = p<0.05 *vs* baseline; # = p<0.01 *vs* baseline; + = p< 0.05 IVGTT *vs* saline; ++ = p<0.001 IVGTT vs saline.

As expected, IVGTT induced a prompt response in insulin levels both in the portal and in the systemic samples. In the portal vein, insulin peaked at 1 min ($\Delta_{1\text{-}0}$: 5.7 ± 1.0 µg/l, p<0.01) and at 5 min was back to baseline levels. However, Δ insulin levels were higher in the IVGTT than in the saline group during the whole time course (Fig. 1C), as well as the ΔAUC (49.2 ± 19.9 µg/l*min^{-1} *vs* -44.61 ± 13.6 µg/l*min^{-1}; p<0.0005) (Figure 1D).

In the peripheral samples, insulin responded to glucose administration following a pattern similar to that observed in the portal vein, although the Δ changes were less impressive (peak: $\Delta_{1\text{-}0}$: 2.2 ± 2.3 µg/l, p<0.01; ΔAUC IVGTT *vs* saline: 26.9 ±

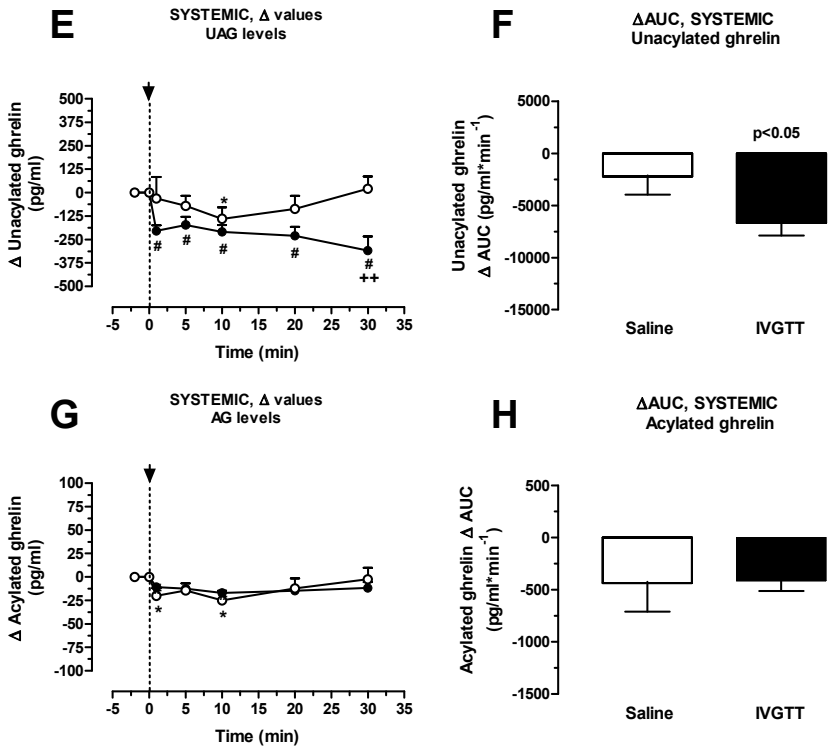

Figure 2.

7.9 µg/l*min^{-1} *vs* -31.9 ± 11.0 µg/l*min^{-1}, respectively, p<0.0005) (Fig. 2C and Figure 2D).

Unacylated ghrelin

UAG concentrations in the portal vein were not significantly modified by saline injection, although a slight, but not significant, transient decrease was observed at 10 min (nadir $\Delta_{10\text{-}0}$: -198 ± 95 pg/ml) (Figure 1E). In the peripheral samples, UAG levels were significantly blunted only at 10 min ($\Delta_{10\text{-}0}$: -140 ± 62 pg/ml, p<0.05), after which they returned to baseline. (Figure 2E).

IVGTT suppressed UAG levels in the portal circulation during the whole study period (nadir $\Delta_{1\text{-}0}$: -369 ± 40 pg/ml, p<0.0005) (Fig. 1E). ΔAUC of UAG was lower in the IVGTT than in the saline group (-8151 ± 1522 pg/ml*min^{-1} *vs* –2421±2021 pg/ml*min^{-1}, p<0.03) (Figure 1F).

In the peripheral circulation, UAG was also inhibited during the whole study period (for all time points, Δ changes *vs* baseline: p<0.01) (Fig. 2E). The inhibition

of UAG was greater after IVGTT that after saline administration (ΔAUC: -6697 ± 1195 pg/ml*min^{-1} *vs* –2207 ± 1756 pg/ml*min^{-1}, respectively, p<0.05) (Figure 2F).

Acylated ghrelin

Portal AG levels were transiently and slightly, though not significantly, reduced by saline administration and rose above baseline levels at 30 min (p<0.05)(Figure 1G). Δ variations in the peripheral samples were also slightly inhibited, being statistically significant only at 1 and 10 min (p<0.01) (Figure 2G).

IVGTT induced a suppression of portal AG levels up to 10 min, with a gradual return to baseline at later time points (Fig.1G). Portal ΔAUC was lower during IVGTT than during saline (-716 ± 404 pg/ml*min^{-1} *vs* -16 ± 406 pg/ml*min^{-1}), although the difference was not statistically significant (Figure 1H). Systemic AG levels were decreased by IVGTT injection during the all time course (p<0.01) (Fig. 2G). However, the inhibition induced by IVGTT was not different than that observed after saline. (Figure 2H).

Hepatic clearance

Insulin

In the saline-treated group (*i.e.* in fasting conditions) the percentage of insulin clearance by the liver was 56.6 ± 3.7 % at baseline and did not vary during the time course. (Figure 3A).

IVGTT did not modify the percentage of insulin clearance, which was stable at all time points and did not differ from saline (% AUC: 58.8 ± 4.3 % and 51.0 ± 5.9 %, respectively) (Figure 3B).

Unacylated ghrelin

In the saline group hepatic clearance of portal UAG was 11.4 ± 3.8 % at baseline and it slightly increased (not significantly) during the time course, reaching a maximum at 30 min (19.8 ± 3.7 %, p = ns vs baseline) (Fig.3A).

IVGTT transiently but significantly reduced the percentage of UAG cleared by the liver (from 10.2 ± 2.8% at baseline down to a nadir of –3.6 ± 3.4 % at 5 min, p<0.004), which gradually increased to rise above baseline at 30 min (23.3 ± 3.6 %, p<0.01) (Fig. 3B). However, the percentage of UAG cleared by the liver during IVGTT was not different from the clearance in the saline treated animals (%AUC: 7.8 ± 3.3 % *vs* 11.3 ± 3.0 %, respectively)

Acylated ghrelin

In the saline-treated rats the hepatic removal of endogenous AG was 34.0±3.5% at baseline, without significant changes over the 30 min time course after saline injection (Fig. 3A).

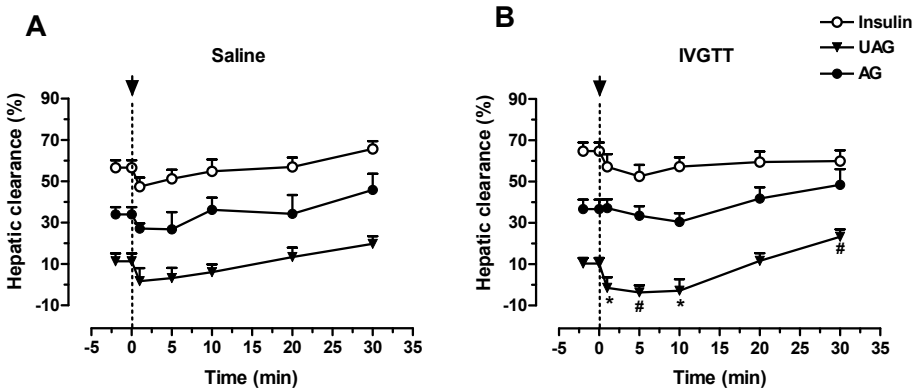

Figure 3. First liver passage after saline administration was 11% (%AUC) for unacylated ghrelin (UAG), 38% for acylated ghrelin (AG) and 57% for insulin, with no significant changes over the 30 min time course (A). Glucose administration did not modify hepatic clearance of AG and insulin, whereas it transiently abolished the uptake of unacylated ghrelin (B). * = $p<0.05$ *vs* baseline; # = $p<0.01$ *vs* baseline.

After IVGTT, AG extraction did not change over the time course and remained similar to that observed after saline (%AUCs: 37.8 ± 4.9 % *vs* 37.9 ± 6.0 %, respectively) (Fig. 3B).

Ratio of AG/UAG

Portal AG at baseline was approximately 7% of total ghrelin (saline 6.8 ± 0.8 %, IVGTT: 6.9 ± 0.6 %) and it slightly increased at 30 min in all animals (Figure 4A). In the systemic circulation, the percentage of AG was also similar in the saline and in the IVGTT groups (baseline: 5.8 ± 0.8 % and 4.7 ± 0.3 %, respectively), without significant variations over the 30 min observation (Figure 4B).

Mean portal and jugular AG/UAG were similar in the saline and in the IVGTT groups. In the saline as well as in the IVGTT treated animals, the ratio of AG/UAG was slightly higher in the portal than in the jugular samples (AUC ratio, saline: 0.08 ± 0.01 *vs* 0.05 ± 0.01, $p<0.004$; IVGTT: 0.07 ± 0.01 *vs* 0.05 ± 0.00, $p<0.0005$). (Figures 4C and 4D).

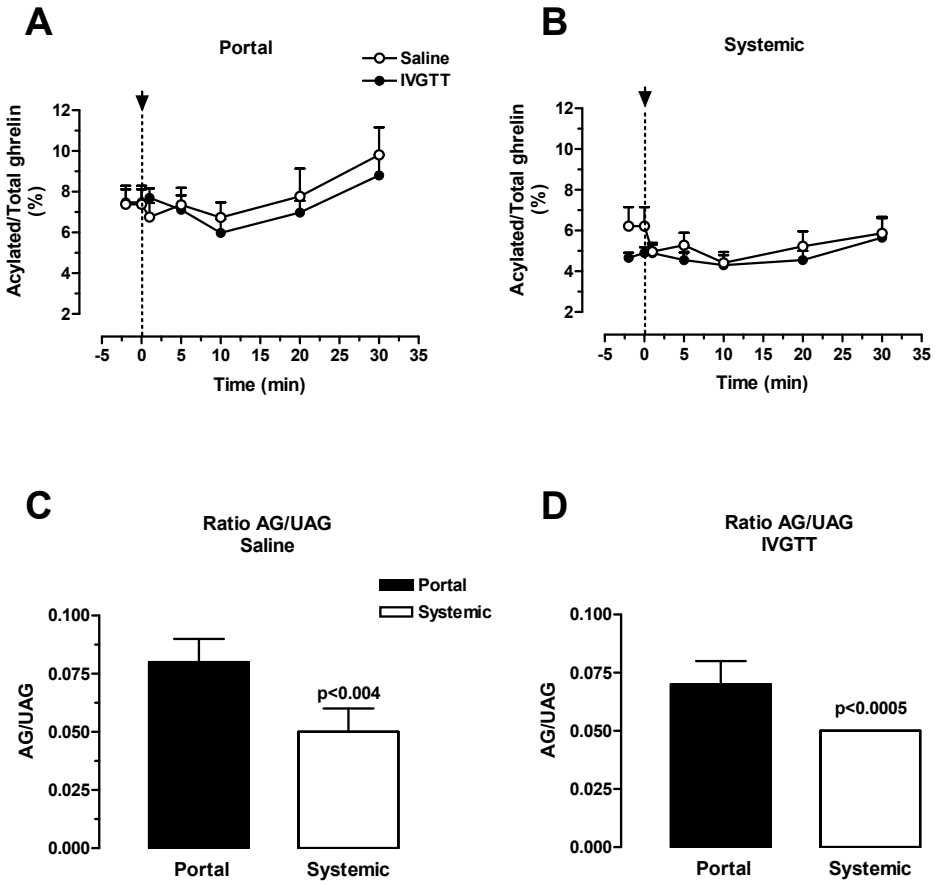

Figure 4. Acylated ghrelin accounted for approximately 7% in the portal vein and this fraction was blunted to approximately 4.5% in the systemic circulation. Both in the portal and the systemic circulation there was no difference between the saline and the IVGTT treated groups (A, B). Either in the saline or in the IVGTT groups the portal AG/UAG ratio was significantly higher than in the jugular vein (C, D). Open circles: saline group; closed circles: IVGTT group. Closed bars: portal AG/UAG; open bars: jugular AG/UAG.

DISCUSSION

The results of the present study show that intravenous glucose administration suppresses UAG levels in the portal and systemic circulations, whereas it blunts pre-hepatic, but not peripheral, AG in anesthetized fasted rats. This indicates that the regulation of peripheral total ghrelin by parenteral glucose administration is mainly due to changes in the concentration of the unacylated form.

Furthermore, we found that that the liver has a preference for the clearance of AG over UAG, as shown by the observation that the pre-hepatic AG/UAG ratio is higher than in the peripheral circulation. Both in fasting and in (intravenously)

glucose-stimulated conditions, approximately 38% of the AG was taken up by the liver. Conversely, the fraction of UAG removed after hepatic passage was relatively small (11%) and could be further reduced (down to 8%) by parenteral glucose.

Our data show for the first time that, in rats, an intravenous glucose load (slightly) blunts pre-hepatic, but not systemic AG levels. In fact, in our model glucose administration induced a decrease from baseline of portal and, to a much lesser extent and transiently, systemic AG levels. However, when compared to values obtained in the control (saline-injected) group, only a slight decrease in portal AG concentrations was recorded, whereas peripheral levels were similar to those observed in the controls. Our data are, at least in part, controversial with previous reports (24, 25). Gordon *et al.* (24) found that peripheral AG levels decreased from baseline values after glucose infusion in horses, although in this study the possible effects of a saline infusion were not evaluated. Hotta and coworkers (25) observed a suppression of AG levels after a 2-h glucose infusion in humans. The discrepancy between our data and those presented by Hotta *et al.* may be due to methodological aspects, and to the ghrelin assay used. In fact, in this study parameters were measured at 1 h and 2 h during a continuous glucose infusion, whereas we evaluated acute effects (up to 30 min) of a bolus administered at baseline. Secondly, in the paper by Hotta and colleagues glucose infusion inhibited AG (as measured by RIA and ELISA) and UAG (as measured by ELISA), whereas total ghrelin levels as measured by RIA showed a non-significant tendency to decrease (25) Conversely, previous studies in rodents reported an inhibitory effect of parenteral glucose administration on peripheral ghrelin levels using assays that recognized both acylated ghrelin and unacylated ghrelin (9, 10). In line with this, we observed a suppressive effect of glucose on total and UAG levels, which was more marked in the portal than in the systemic circulation. The changes in UAG and (pre-hepatic) AG levels were opposite to those observed in glucose and insulin concentrations, suggesting that, in accordance with the majority of data in the literature, either insulin, glucose, or both, suppressed ghrelin levels (5, 8, 9, 26-29).

Although we cannot fully exclude that some degradation and/or des-octanoylation occurred, we took all possible precautions to protect AG stability in our samples (20, 21). Therefore, the relative changes in UAG and AG levels probably reflect a physiological regulation in these conditions.

Moreover, a disadvantage of rat model used in these studies is that we observed a slight reduction of AG, UAG and insulin levels also in the saline-injected animals, which may reflect neuronal, neuro-hormonal and hemodynamic factors as a consequence of abdominal surgery and/or anesthesia. However, by comparing glucose-treated with control animals we think that our model is valid.

To our knowledge, this is also the first report in the literature showing that in basal and in glucose stimulated conditions portal ghrelin levels (either AG or UAG) are higher than the systemic levels. This is in agreement with a very recent publication by Dezaki and coworkers (3), who reported higher concentrations of AG and UAG (of pancreatic origin) in the pancreatic vein than in the pancreatic artery. However, in our rat model the higher levels in the portal vein do not only reflect an increased release of AG and UAG by the pancreas, but also by the stomach and/or the intestine. Interestingly, the gradient between portal and peripheral levels of UAG (1.1-fold), although statistically significant in fasted rats (*i.e.* saline group), was not as striking as the difference between portal and peripheral concentrations of AG (1.6-fold) or insulin (2.5-fold). Therefore, we hypothesize that the clearance of UAG and AG by the liver differs and is responsible for the observed different concentrations in the systemic circulation. In this respect, the metabolism of ghrelin may be comparable to that of insulin in the liver. It is known that the amount of insulin released in the periphery is regulated by hepatic extraction, which varies depending on acute nutritional changes (23, 30). We used insulin hepatic clearance as a reference to be able to compare to what extent ghrelin is taken up by the liver. We found that, in fasting conditions, the liver clears only a small fraction (approximately 11%) of UAG, which was further, but transiently, decreased by glucose, indicating that almost all of the UAG that reaches the liver is delivered to the systemic circulation. Conversely, AG was cleared by the liver by 38% and was not altered after intravenous glucose load. In accordance with Kaden *et al.* (22), insulin extraction after single liver passage was found to be 51% and, like AG, it was not significantly modified by the 'intravenous meal'.

It has been shown that the liver adapts rapidly to fluctuations in portal insulin concentrations, in order to quench the delivery of large oscillations of insulin from the portal vein to the systemic circulation (14, 31). In our study, despite the (2.5-fold) increase in portal insulin levels after a glucose load, the percentage of insulin extraction was not significantly changed, which suggests that the absolute amount of insulin cleared by the liver was largely increased in these circumstances. The fact that the decrease in portal AG levels after glucose was small, while the hepatic clearance of AG did not change, may account for the lack of an effect of glucose on systemic AG concentrations. Conversely, portal UAG levels were inhibited by glucose, while hepatic clearance of UAG was almost zero during glucose and therefore almost all UAG was delivered to the systemic circulation. Moreover, and in agreement with this, we found that the ratio AG accounts only for 7% of total ghrelin in the portal vein and that the ratio of AG/UAG further decreases in the systemic circulation. Although these data provide no evidence as to the fate of the AG fraction removed by the liver,

it is possible that the liver is responsible, at least in part, of AG de-acylation and/or degradation in inactive fragments. In this respect, De Vriese *et al.*(32) showed that a two-hour incubation of (acylated) ghrelin with liver homogenates leads to the formation predominantly of UAG and, to a much lesser extent, of (inactive) fragments derived from cleavage of the AG molecule at its N-terminus. In this study, a precise quantification N-terminus fragments of AG is difficult, because the assay used detects acylated peptide regions including residues $_{(1\text{-}10)}$, whose activity is not known. However, enzyme activity *in vivo* is likely to differ from that in tissue homogenates. Therefore, the hepatic mechanisms of ghrelin deacylation remain to be clarified.

Overall, these observations suggest that an acute glucose administration regulates mainly pre-hepatic concentrations of AG, thereby AG may play a role in glucose metabolism and/or insulin sensitivity in the liver. This is in accordance with previous studies showing a direct effect of AG on glucose output by primary porcine hepatocytes (33) and an AG-dependent modulation of insulin action in a hepatoma cell line (34). We speculate that the relative increase of UAG fraction in the peripheral circulation reflects the buffering of AG metabolic actions, perhaps in order to improve peripheral insulin sensitivity. Moreover, the hepatic clearance and/or de-acylation of AG might be an additional physiological mechanism modulating the central effects of AG (*i.e.* on energy homeostasis and feeding behaviour) in response to the nutritional state.

Our results show that acute nutritional changes may differentially regulate UAG and AG concentrations, at least after an acute parenteral administration. Moreover, this study suggests that liver clearance plays an important role in the regulation of the amount of AG released to the systemic circulation. Indeed, the relevance and the regulation of hepatic clearance of ghrelin need to be further elucidated. However, the assumption that systemic total ghrelin levels reflect acylated ghrelin secretion should be made with caution.

In conclusion, the present data show that:

- The ratio of AG/UAG is already very low in the portal vein and decreases further in the systemic circulation.
- An acute intravenous glucose load in anesthetized fasting rats inhibits UAG in the portal and the systemic circulation, whereas it blunts only pre-hepatic but not systemic AG concentrations.
- UAG is cleared by the liver in a small amount in fasting conditions. After glucose administration, all the UAG secreted into the portal vein was delivered to the systemic circulation. Conversely, the hepatic clearance of AG was not influenced by acute changes in glucose and/or insulin levels.

Overall, our results may suggest that AG acute metabolic regulation and action are mainly confined within the gastrointestinal tract.

ACKNOWLEDGEMENTS

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Chapter VI

AG and UAG receptor(s)

Chapter VI.1.

Unacylated ghrelin is active on the INS-1E rat insulinoma cell line independently of the growth hormone secretagogue receptor type 1a and the corticotropin releasing factor 2 receptor

Gauna C, Delhanty PJD, van Aken MO, Janssen JAMJL, Themmen APN, Hofland LJ, Culler M, Broglio F, Ghigo E and van der Lely AJ

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ABSTRACT

Both unacylated ghrelin (UAG) and acylated ghrelin (AG) exert metabolic effects. To investigate the interactions between AG and UAG on ghrelin receptors we evaluated the effects of AG and UAG on INS-1E rat insulinoma cells, using insulin secretion after 30 min static incubation as a read-out. A possible involvement of the growth hormone secretagogue receptor type 1a (GHS-R1a) or the corticotropin-releasing factor 2 (CRF2) receptor (CRF2R), as a putative receptor for UAG, was also studied determining their mRNA expression and the functional effects of receptor antagonists on insulin release. Both UAG and AG stimulated insulin release dose-dependently in the nanomolar range. The AG-induced insulin output was antagonized by two GHS-R1a antagonists ([D-Lys^3]GHRP-6 and BIM28163), which did not block UAG actions. These effects occurred in the presence of low levels of GHS-R1a mRNA. Neither CRF2R expression nor effects of the CRF2R antagonist (astressin$_2$B) on insulin output were observed. In conclusion, we provide a sensitive and reproducible assay for specific effects of UAG, which in this study is responsible for insulin release by INS-1E cells. Our data support the existence of a specific receptor for UAG, other than the CRF2R and GHS-R1a. The stimulatory effect on insulin secretion by AG in this cell line is mediated by the GHS-R1a.

INTRODUCTION

Ghrelin, a natural ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a) (1), is predominantly produced by the stomach. The ghrelin peptide has a unique feature, which is the esterification of a fatty (n-octanoic) acid at its third serine residue (1). Besides acylated ghrelin (AG), an unacylated form of the ghrelin molecule (unacylated ghrelin, UAG) is also present in circulation in far greater amount than AG. The acylation is necessary for binding and activation of the GHS-R1a that appears to be essential only for some, but not all, of its biological activities (1-3). The GHS-R 1a mediates the GH releasing and the orexigenic activity of acylated ghrelin (AG), as shown in GHS-R-knock-out mice (4, 5). These animals are unable to respond to the presence of AG, despite the fact that their phenotype is undistinguishable from the wild type (4, 5). However, since its discovery and characterization as a GH-releasing and orexigenic factor, AG has been demonstrated to be a pleiotropic molecule, displaying central, neuroendocrine, non-endocrine and metabolic effects (6), which do not necessarily require the presence of the GHS-R1a. An increasing number of reports on the presence of specific binding sites recognized by both AG and UAG (7, 8) suggests that the GHS-R1a is not "the" ghrelin receptor, but most likely "one" of the ghrelin receptor subtypes. The GHS-R1a is expressed in many central and peripheral tissues, but predominantly in the hypothalamo-pituitary unit (9), and this distribution perfectly matched the first reports of the ghrelin-induced regulation of energy balance and GH release (1, 6, 10, 11). Also, the *in vivo* studies showing an action of AG on glucose metabolism were in line with the GHS-R distribution (9, 12, 13). However, more recently it became apparent that also UAG exerts metabolic actions both *in vivo* and *in vitro* (7, 8, 14-17). Since it elicits neither GH secretion nor other neuroendocrine actions (3), most likely UAG uses a receptor different from the GHS-R1a to exert its activities.

Recently, Chen and coworkers suggested that central CRF2 receptors (CRF2R) could mediate at least some of UAG effects, such as the decrease in food intake and the regulation of motor activity of the antrum (17). Overexpression of UAG in mice has also been shown to slightly increase insulin levels, although not significantly (18). Interestingly, in humans the coadministration of UAG with AG prevented the increase in glycemia, the worsening of insulin sensitivity and the increase in FFA that were recorded after the administration of AG alone (12, 19). This was observed in both healthy subjects and in GH deficient individuals, ruling out a GH-dependency of these metabolic effects (19). We previously showed that UAG could counteract AG-dependent action *in vitro* as well, since it prevented AG-induced glucose output by primary porcine hepatocytes (20). Effects of UAG have also been reported in adipose tissue, as it has been shown that UAG as well as AG inhibited the isoproterenol-

induced lypolysis in the absence of GHS-R1a (21). Moreover, animal studies showed that both AG and UAG were able to stimulate bone marrow adipogenesis (22).

It has also been reported that AG can modulate insulin secretion, at least in rodents (23-29) and that it induces the expression of IA-2β in MIN6 mouse insulinoma cells (30). We studied whether AG and UAG can modify insulin secretion in a cloned rat insulinoma cell line (INS-1E) that has been thoroughly characterized. INS-1E cells have been documented to be responsive to glucose in terms of insulin secretion (31). We also studied the effects of two GHS-R1a antagonists ([D-Lys^3]-GHRP-6 and BIM28163) on the effects of AG and UAG on insulin release.

MATERIALS AND METHODS

Materials

INS-1E cells were kindly provided by the Dr Pierre Maechler (University of Geneva, Switzerland). Rat AG and UAG, as well as the GHS-R1a antagonist [D-Lys^3]-GHRP-6 were obtained from NeoMPS SA (Strasbourg, France); human Dap-octanoylated ghrelin (an AG-analog, resistant to de-acylation) was obtained from Phoenix (Phoenix Europe GmbH, Karlsruhe, Germany). Human UAG was kindly provided by Theratechnologies Inc. (Montreal, Quebec, Canada) and the GHS-R1a antagonist BIM28163 by Ipsen (IPSEN Group, Milford, MA, USA). Peptides were dissolved in water or acetic acid, according to manufacturer's instructions. Insulin released into the medium was assayed using a rat/mouse insulin ELISA kit (Linco Research Inc., St, Charles, Missouri, USA). The High-Pure RNA isolation kit was obtained from Roche Diagnostics (Mannheim, Germany). For immunocytochemical studies the primary antibody (rabbit anti-[cys^0]GHS-R1a (330-366) human antiserum) and the human peptide [Cys^0]-growth hormone secretagogue receptor type 1a (330-366), used for preabsorption experiments, were from Phoenix Pharmaceuticals, Inc. (Phoenix Europe GmbH, Karlsruhe, Germany). Culture media RPMI 1640 with and without glucose were from GIBCO (Invitrogen, Breda, The Netherlands). All other reagents were obtained from Sigma (Steinheim, Germany).

Cell culture

The clonal β-cell line INS-1E, derived from parental INS-1 cells was selected for its insulin content and proliferation (31). INS-1E cells were cultured in a humidified atmosphere containing 5% CO_2 in "Complete" medium, composed of RPMI 1640 (containing 11 mM glucose) supplemented with 5% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES,

100 U/ml penicillin and 100 µg/ml streptomycin. The maintenance culture was passaged once a week by trypsinization and cells were seeded at a density of $1.5x10^6$ cells in 75 cm^2 Falcon flasks with 20 ml complete medium. Media were regularly assayed for mycoplasma and the results were constantly negative.

Insulin secretion

INS-1E cells (passages 45-56) were seeded at a density of $2x10^5$ cells in Falcon 24-well plates and used 5 days afterwards. Cells were maintained in "Complete" medium.

Before the experiment, cells were incubated for 2 hours in glucose-free RPMI 1640 supplemented with 0.1% BSA, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. After 2 hours the cells were washed twice and preincubated for 30 min at 37ºC in glucose-free Krebs-Ringer Bicarbonate HEPES buffer (KRBH: 135 mM NaCl, 3.6 mM KCl, 5 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 1.5 mM $CaCl_2$, 10 mM HEPES, pH 7.4, BSA 0.1%). Then, cells were washed once with glucose-free KRBH and afterwards incubated at 37ºC for 30 min in KRBH containing 20 mM glucose and in the absence or in the presence of treatments as indicated. Incubation was stopped by putting the plates on ice. The plates were then centrifuged at 4600 rpm for 5 min at 4ºC to avoid cell debris and the supernatants were collected and stored at –20ºC. Insulin released into the medium was assayed by using a rat/mouse insulin ELISA kit.

Each experiment was repeated at least three times, with 4 to 6 replicates each, with 15 to 35 total number of observations for each condition.

Results (mean ± SEM) are expressed as percentage of control, considering the mean of control values as 100 %. The control group was treated with KRBH containing 20 mM glucose.

RT-PCR

The expression of GHSR type 1a and ghrelin mRNA was investigated in INS-1E cells using RT-PCR assays. Total RNA was isolated and its quantity and quality was assessed using Nanodrop ND-1000 Spectrophotometer (Isogen Life Science B. V., The Netherlands). Total RNA (120 ng) underwent conventional RT reaction and cDNA was used for amplification, which was performed using Gene Amp® PCR system 9700 thermal cycler (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands).

PCR products were separated on 1.5 % agarose gel, stained with ethidium bromide and examined with UV light and visualized with Kodak Digital Science 1D and a DC120 camera (Scientific Imaging Systems, New Haven, CT, US).

RT-PCR analysis for ghrelin gene expression

Primer sequences specific for rat ghrelin were similar to those reported by Caminos *et al*. (32), as follows: forward 5'-TTGAGCCCAGAGCACCAGAAA-3', reverse 5'-AGTTGCAGAGGAGGCAGAAGCT-3'. The PCR conditions were similar to those described by Nanzer *et al*. (33), with an initial denaturation at 95ºC for 5 min; 40 cycles consisting of denaturation for 1 min at 94º C, annealing for 1 min at 55ºC, extension for 1 min at 72ºC; final elongation of 10 min at 72ºC.

RT-PCR and real time PCR analysis for GHS-R1a gene expression

Rat GHS-R1a specific intron-spanning primers were designed using Primer3 software and were found to be similar to those used by Nanzer *et al.* (33). Primer sequences were as follows: forward 5'-CTCATCGGGAGGAAGCTATG-3', reverse 5'-CAGGTTGCAGTACTGGCTGA-3'). The PCR conditions were: initial denaturation at 95ºC for 5 min; 40 cycles consisting of denaturation for 1 min at 94ºC, annealing for 1 min at 60ºC, extension for 1 min at 72ºC; final elongation for 10 min at 72ºC.

Rat stomach mRNA was used as positive control for both ghrelin and GHS-R1a. As negative controls we included cDNA samples whose RT reaction was performed in the absence of reverse transcriptase. To exclude contamination we also amplified samples without cDNA (H_2O controls).

For GHS-R1a a real-time PCR assay was also carried out using ABI PRISM™ 7700 Sequence detector (Applied Biosystems, The Netherlands). We used alternative primers and locked nucleic acid (LNA) probes (Exiqon A/S, Denmark). Primer sequences were as follows: forward 5'-AGGAAGCTATGGCGGAGAC-3'; reverse 5'-GAAAGCAAACACCACCACAG-3'; probe 5'-FAM-GCAGACAG–TAMRA-3' (probe library Rat#55). Rat hypoxanthine guanine phosphoribosyl transferase (HPRT) was used as housekeeping gene in all samples. HPRT specific primer and probe sequences were as follows: forward 5'-GACCGGTTCTGTCATGTCG-3'; reverse: 5'-ACCTGGTTCATCATCACTAATCAC-3', probe 5'-FAM-AGTCCCAG–TAMRA-3' (probe library Rat#43).

For both conventional PCR and real-time PCR each sample was assayed in duplicate in at least 2 different reactions and by using 2 different RNA extracts obtained from INS-1E cells at different passages.

RT-PCR analysis for CRF2 receptor gene expression

The CRF type 2 receptor (CRF2R) has recently been suggested to be involved in UAG actions (17) and the presence of CRF2R and its natural ligand urocortin III has been detected in pancreatic islets and in a mouse β- cell line, MIN6 (34).

Rat specific primer sequences were as follows: forward 5'- GCGGCCCCTCATCTC-CGTGAG-3'; reverse 5'- ACCTTCGGGGTCCGGGGGCTC -3'.

PCR conditions were: initial denaturation at 95ºC for 5 min; 40 cycles consisting of denaturation for 1 min at 94ºC, annealing for 1 min at 56ºC, extension for 1 min at 72ºC; final elongation of 10 min at 72ºC.

Rat ovary (diestrous phase) was used as positive control. Negative controls were included as previously described.

Immunocytochemistry

Cytospin preparations of INS-1E cells were fixed with acetone for 10 min at room temperature. After washing twice with PBS for 5 min, the cells were incubated overnight at 4ºC with primary antibody (dilution 1:2000) to human GHS-R1a. The cells were then incubated for 30 min at room temperature with PowerVision Poly-alkaline phosphatase-anti-Mouse/Rabbit IgG (Immunologic; Klinipath, Duiven, The Netherlands) that we used as secondary antibody. The cytospins were counterstained for 30 min at room temperature with Naphthol As-MX phosphate (Sigma) as substrate and New Fuchsin (Sigma) as chromogen.

For negative control experiments the antibody for GHS-R1a was preabsorbed with the peptide [Cys^0]-Growth Hormones Secretagogue Receptor type 1a (330-366) (6 µg/ml). HEK293 cells and CHO-A5 cells, both overexpressing GHS-R1a, were used as positive control.

Functional assay for acylated and unacylated ghrelin in CHO cells expressing GHS-R1a

In order to test the AG peptides used for the insulin secretion experiments and to confirm the inability of UAG to activate the GHS-R1a, we used a functional bioassay as previously described (20). Briefly, 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% bovine serum albumin, amphotericin, penicillin and streptomycin) at $5x10^6$ cells/mL, 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 $5x10^5$ cells/ml and 100µL was injected into wells of a 96-well plate containing 100µL of various concentrations of AG or UAG (range: 10^{-10}-10^{-7} M). Luminescence was measured for 20 seconds using a Victor2 1420 multilabel counter (Perkin-Elmer Wallac). EC_{50} was calculated from dose-response curves (GraphPad Prism, San Diego, CA, USA).

Statistical analysis

The results are expressed as percentage of control values. Statistical analysis was carried out with SPSS for Windows, release 10.0 (SPSS, Chicago, IL, USA). A few extreme points (those 3 SD above or below the interquartile range) have been identified and not included in the statistical analysis. The normal distribution of the data for each condition was assessed using Kolmogorov-Smirnov test. Data analysis was carried out using ANOVA, followed by least significant difference (LSD) post-hoc test. P values < 0.05 were considered significant.

RESULTS

Insulin secretion by INS-1E

Responsiveness to stimulatory and inhibitory stimuli

The INS-1E cells have been characterized thoroughly and their insulin secretory capacity in response to different stimuli has been shown to be maintained (31). To verify that the INS-1E cells in our hands could respond to non-nutrient pharmacological stimuli eliciting cell membrane depolarization we incubated the cells in the absence or the presence of tolbutamide 250 µM at 5.5 mM glucose concentration. Indeed tolbutamide strongly stimulated (mean ± SEM: 257.9 ± 27.0 % of control, $p<0.0001$) insulin secretion after 30 min incubation (data not shown). Responsiveness to inhibitory agents was tested by incubating the cells in the absence or the presence of diazoxide 250 µM at 20 mM glucose concentration. Diazoxide significantly inhibited the insulin output induced by 20 mM glucose concentration (37.2 ± 4.3 % of control, $p<0.0001$) (data not shown).

Acylated ghrelin

Rat AG (rAG) at 10 nM concentration stimulated insulin secretion by INS-1E cells after a short-term (30 min) static incubation at high glucose concentrations (20 mM) (300.8 ± 60.7 % *vs* control; $p<0.0001$; control absolute values: 51.3 ± 12.4 ng/ml) (Figure 1A). A similar effect was also exerted by equimolar concentrations of human

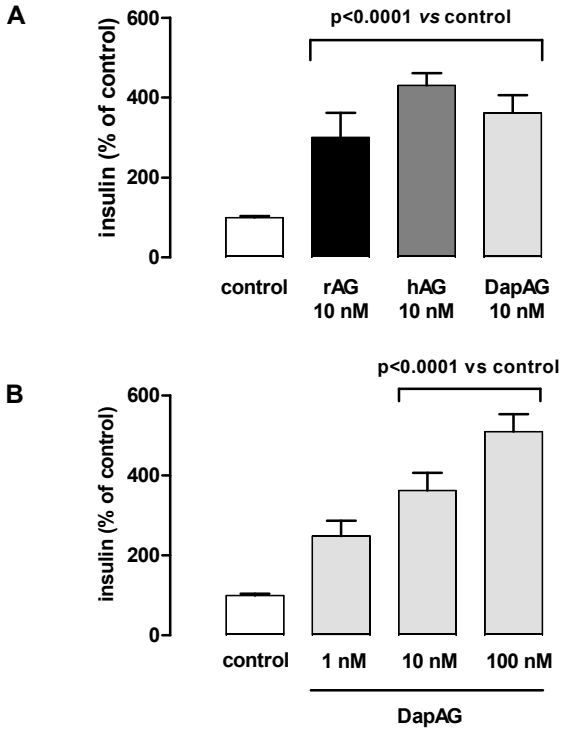

Figure 1. AG stimulates insulin release by INS-1E cells after 30 min static incubation in KRBH containing 20 mM glucose. A) Insulin output induced by rat, human or Dap-octanoyl (human) ghrelin (rAG, hAG and DapAG, respectively) at 10 nM concentrations is compared to control values. B) Insulin output after incubation with increasing concentrations of DapAG.

AG (hAG) (430.6 ± 30.6 % of control, p<0.0001), implying that a species-specific sequence of the ghrelin peptide was not required.

We also evaluated the insulin output after incubation of the INS-1E cells with Dap-octanoylated (human) ghrelin (DapAG), a ghrelin molecule where the octanoyl group on the third serine residue is stabilized by a α-,β- diaminopropanoic acid. DapAG at 10 nM concentration induced a potent insulin release (362.0 ± 44.6 % of control, p<0.0001 *vs* control), similar to those stimulated by equimolar concentrations of rat and human ghrelin. (Fig. 1A). Due to the consistency and the similarity of the responses to rat- and human-specific peptides, for the rest of the study we used DapAG because of its stability. The stimulatory effect exerted by DapAG was dose-dependent, being significant at a concentration of 10 nM and showing a further increase at 100 nM (509.0 ± 43.9 % of control, p<0.0001 *vs* control) (Figure 1B).

Unacylated ghrelin

Interestingly, like AG, rat UAG (rUAG) at a concentration of 10 nM was also able to elicit a strong insulin release by the INS-1E cells during short-term static incubation (288.0 ± 29.5 % of control, p<0.0001 *vs* control) in the presence of 20 mM glucose. Equimolar concentrations of human UAG (hUAG) elicited significantly insulin output

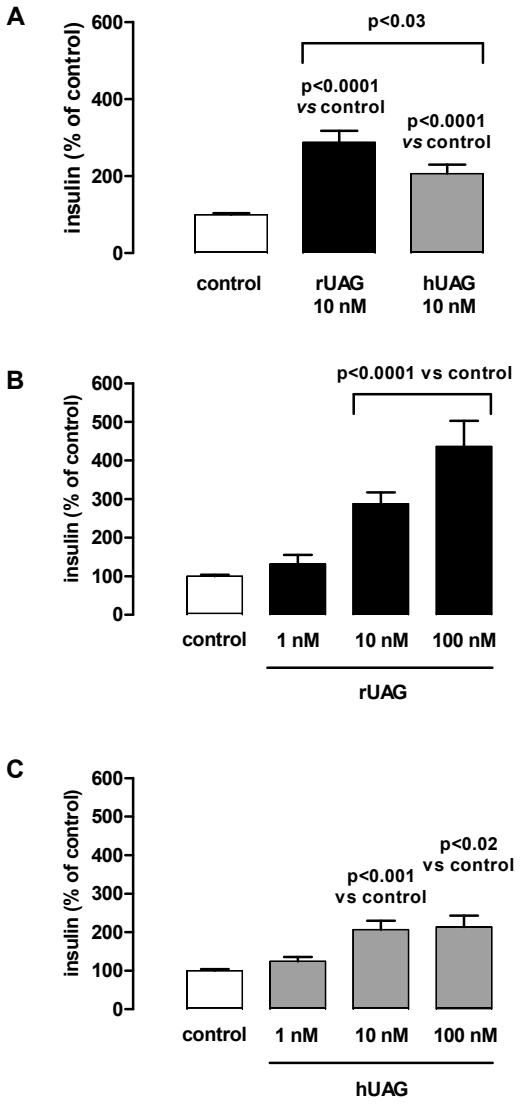

Figure 2. UAG stimulates insulin release by INS-1E cells after 30 min static incubation in KRBH containing 20 mM glucose. A) Insulin output induced by rat and human unacylated ghrelin (rUAG and hUAG, respectively) at 10 nM concentrations; B) dose response to rUAG; C) dose-response to hUAG .

by INS-1E cells (207.0 ± 23.1 % of control, p<0.0001 *vs* control), although this effect was lower than that elicited by rUAG (p<0.03) (Figure 2A).

The insulin response to rUAG showed dose-dependency in the nanomolar range, being significant at 10 nM and even higher at 100 nM (436.3 ± 82.4 % of control, p<0.0001 *vs* control) (Figure 2B), whereas the response to 10 nM hUAG showed no further increase at 100 nM (p<0.0001 and p<0.02 *vs* control, respectively) (Figure 2C).

GHS-R1a antagonists

To assess whether insulin secretion by INS-1E cells is regulated by endogenous ghrelin, we investigated the effects of two ghrelin GHS-R1a antagonists: [D-Lys^3]-GHRP-6 (28, 35) and BIM 28163 (36). We also investigated the effects of these two GH-secretagogue receptor antagonists on insulin secretion elicited by exogenous AG and UAG.

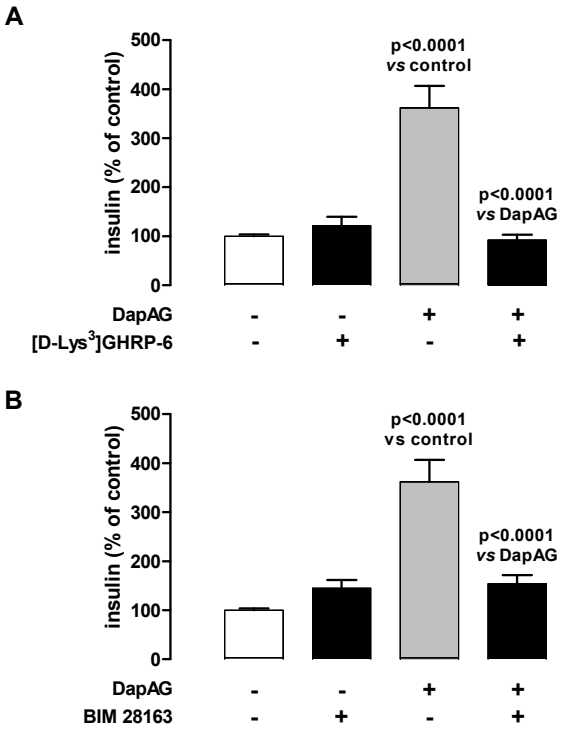

Figure 3. [D-Lys3]GHRP-6 (10 μM) and BIM28163 (1 μM), two GHS-R1a antagonists, prevent AG-induced increase in insulin secretion by INS-1E cells after 30 min static incubation KRBH containing 20 mM glucose. A) insulin output after 30 min incubation in the absence of treatment (control), in the presence of 10 μM [D-Lys3]GHRP-6, 10 nM DapAG, or 10 nM DapAG + 10 μM [D-Lys3]GHRP-6. B) insulin output after 30 min incubation in the absence of treatment (control), in the presence of 1 μM BIM28163, 10 nM DapAG, or 10 nM DapAG + 1 μM BIM28163.

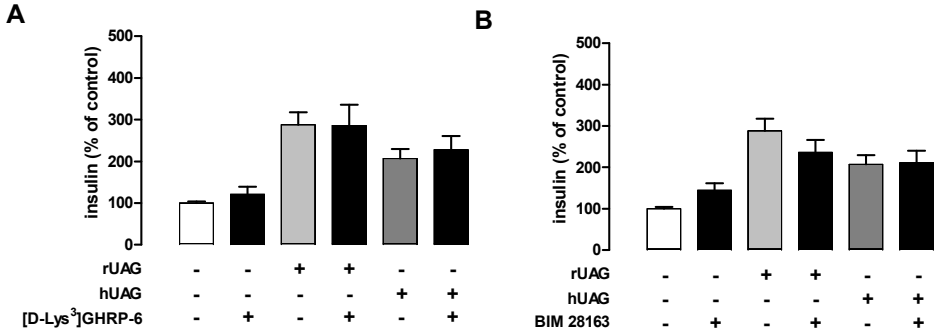

Figure 4. [D-Lys3]GHRP-6 (10 μM) and BIM28163 (1 μM), two GHS-R1a antagonists, do not modify UAG-induced increase in insulin secretion by INS-1E cells after 30 min static incubation KRBH containing 20 mM glucose. A) insulin output after 30 min incubation in the absence of treatment (control), in the presence of 10 μM [D-Lys3]GHRP-6, 10 nM rUAG or hUAG alone and after coincubation with 10 μM [D-Lys3]GHRP-6. B) insulin output after 30 min incubation in the absence of treatment (control), in the presence of 1 μM BIM 28163, 10 nM rUAG and hUAG alone and after coincubation with 1 μM BIM 28163.

Neither [D-Lys^3]GHRP-6 (10 μM) nor BIM 28163 (1 μM) had any significant independent effect on insulin secretion in high glucose conditions (Figures 3A and 3B).

Surprisingly, 10 μM [D-Lys^3]GHRP-6 and 1 μM BIM28163 were able to blunt insulin output induced by 10 nM DapAG (362.0 ± 44.6 % down to 92.2 ± 10.9 % and 154.1 ± 17.2 %, respectively, $p<0.0001$ *vs* DapAG treated group) (Fig. 3A and 3B). On the contrary, neither of the GHS-R1a antagonists could modify insulin secretion induced by rat or human UAG (Figures 4A and 4B).

CRF2 receptor antagonist

Astressin$_2$B (1 μM), a selective CRF2R antagonist, did not exert any independent effect on insulin release and it did not modify the insulin output induced by 10 nM hUAG (data not shown).

Insulin secretion in the absence of calcium

Since insulin release is a calcium dependent phenomenon, we examined insulin output after static incubation under calcium-free conditions (KRBH with 20 mM glucose without Ca^{2+}), in the absence and the presence of 10 nM DapAG and 10 nM hUAG, as negative control for our experiments.

In the absence of calcium the glucose-stimulated insulin secretion by INS-1E cells was hampered (52.0 ± 6.9 % of controls at 1.5 mM Ca^{2+}; absolute values, mean ± SEM (ng/ml): 26.7 ± 3.5 ng/ml *vs* 51.3 ± 12.4 ng/ml at 1.5 mM Ca^{2+}, $p<0.01$) and neither AG nor UAG stimulated insulin output by INS-1E cells (Figure 5).

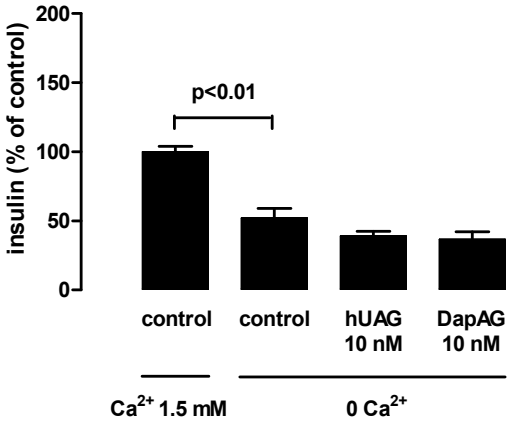

Figure 5. In the absence of calcium insulin secretion by the INS-1E cells is blunted and the stimulatory effect of AG and UAG is completely lost. Bars represent insulin secretion after 30 min incubation in KRBH containing 1.5 mM Ca^{2+} (controls at 1.5 mM Ca^{2+} = 100 %) or in the absence of calcium, without and with 10 nM DapAG or 10 nM hUAG, as indicated. Results are expressed as % (± SEM) of control samples at 1.5 mM Ca^{2+}.

Expression studies

By using RT-PCR analysis we found that GHS-R1a mRNA expression was detectable in INS-1E cell line as an amplicon of 216 bp as expected. The signal was lower than in the rat stomach, which we used as a positive control (Figure 6A). In each sample the expression of β-actin showed a high level of expression and the signal was constant among the different samples (Figure 6D).

A quantitative analysis was carried out using real-time PCR with a specific primer pair, amplifying a product of 61 bp. Because of the low level of expression the results are reported as Ct values, the detection threshold that depends on the starting template copy number and the efficiency of the reaction (the most efficient reaction has the lowest Ct).

In all INS-1E samples the level of GHS-R1a mRNA was extremely low (Ct (range): 36-39), when compared to the expression level of HPRT that was used as housekeeping gene (Ct: 17-18). The expression level of GHS-R1a in the stomach, which we used as a positive control, was also low (Ct: 33) when compared to HPRT (Ct: 20).

The quality of RNA used in the reaction was checked by amplification of HPRT as housekeeping gene. In INS-1E samples HPRT expression was even higher than in rat stomach (data not shown).

A specific immunoreactivity for GHS-R1a was detected only in the HEK293 and, in a lesser extent, in the CHO-A5 cells overexpressing human GHS-R1a (positive controls), but not in the INS-1E cells (data not shown).

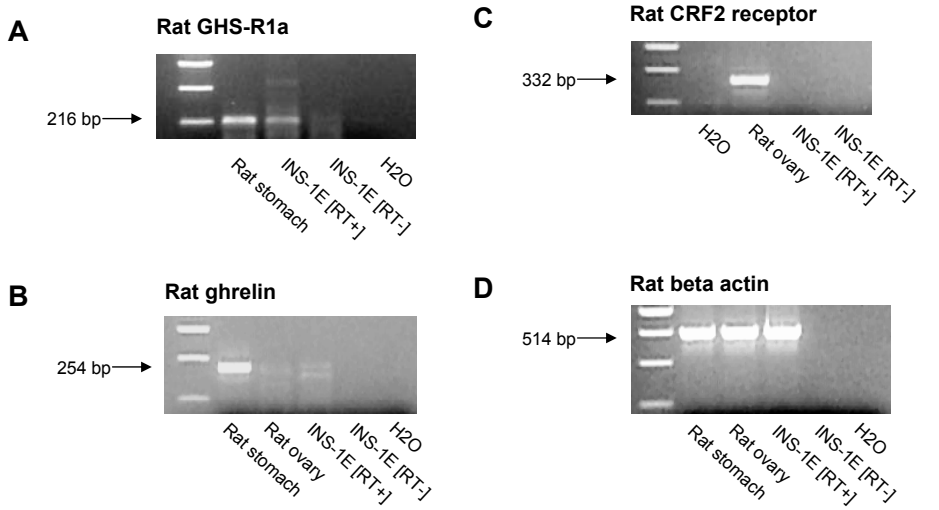

Figure 6. Gene expression evaluated by RT-PCR analysis. A) GHS-R1a; B) ghrelin; C) CRF2R; D) β-actin. Rat stomach mRNA was used as positive control for both ghrelin and GHS-R1a. For INS-1E cells cDNA samples whose RT reaction was performed in the absence of reverse transcriptase ([RT-]) were used as negative controls. Samples in which RT reaction was performed in the presence of the enzyme are indicated as [RT+]. To exclude contamination we also amplified samples without cDNA (H_2O).

By using RT-PCR analysis we found that ghrelin mRNA expression was detectable in the INS-1E cell line as an amplicon of 254 bp as expected, although the signal was very low when compared to the rat stomach RNA. (Figure 6B).

We found no amplification product for CRF2R in INS-1E cells, despite a positive signal in rat ovary cDNA (Figure 6C).

AG elicits $[Ca^{2+}]_i$ through GHS-R1a, whereas UAG does not

All the AG peptides used induced a characteristic stimulation of $[Ca^{2+}]_i$ with an EC_{50} of ~2 nM, whereas UAG was incapable of activating the GHS-R1a in this system (data not shown).

DISCUSSION

Our data showed a novel *in vitro* action of UAG in the regulation of insulin secretion by the INS-1E rat insulinoma cell line, as we found that UAG strongly and rapidly stimulated insulin output in this cell type. This UAG-induced insulin release was dose-dependent and significant at concentrations of UAG in the nanomolar range. Our results strongly suggest the expression of a sensitive UAG receptor, which is by

definition not the GHS-R1a for two reasons. Firstly because UAG does not activate the GHS-R1a receptor and secondly because the UAG induced insulin output was not inhibited by the two GHS-R1a antagonists [D-Lys^3]-GHRP-6 and BIM 28163.

We observed that also AG had strong and dose-dependent effect on insulin secretion by the INS-1E rat insulinoma cell line. We attribute this action to the whole (acylated) ghrelin molecule and not to deacylation products, although it has been reported that acylated ghrelin is rapidly degraded in the incubation medium by 75-80% (21, 37), because we performed the experiments using a deacylation-resistant ghrelin analog, which was as potent as the rat specific peptide.

Our data on the effects of AG on insulin secretion are not in agreement with the majority of reports in the literature that show a suppressive effect of ghrelin on glucose-induced insulin secretion (12, 26-28, 38). Nevertheless, a stimulatory effect has also been reported (29, 39) (24, 25, 30).

However, the stimulatory effect of AG that we observed was highly reproducible and dose dependent. We are aware that by using this model we cannot explain the metabolic effects of AG and UAG observed *in vivo* or other *in vitro* systems (*i.e.* isolated islets), because insulinoma cell lines have altered mechanisms that may not resemble physiology. We only used the endpoint of insulin secretion by this cell line as a highly sensitive and reproducible read-out for both AG and UAG receptor mediated actions, without any attempt to describe a possible role of these peptides in the regulation of the endocrine pancreas. However, we are convinced that the AG and UAG induced insulin secretion by the INS-1E cells is not an artifact. Indeed, in calcium free conditions the glucose-induced insulin release was dramatically hampered and the stimulatory effect of both AG and UAG was completely lost. The dependence of insulin output on the presence of calcium indicates that the hormone concentration measured in the incubation medium is more likely to be secreted than released by dead cells, whose number was negligible after the short incubation time. Moreover, we verified that the insulin secretion by INS-1E cell line maintained responsiveness not only to positive stimuli (*i.e.* tolbutamide), but also to pharmacological inhibition (*i.e.* diazoxide). Finally, the activity of the AG peptides used in the series of experiments presented here has been tested using the measurement of intracellular calcium as a functional assay. The concentration of the peptides used was verified with a radioimmunoassay for total and acylated ghrelin.

We also examined the presence of GHS-R1a that, in agreement with previous reports (27, 38), was detectable, although at a low level of expression. This is probably the explanation why we could only detect the presence of GHS-R1a receptors using sensitive techniques as conventional and real time PCR, whereas the less sensitive immunocytochemical staining could not confirm the presence of this receptor. The question is whether low levels of expression of GHS-R1a can mediate

the observed potent action of AG on insulin secretion. To answer this question we studied whether by using two different GHS-R1a antagonists, [D-Lys^3]-GHRP-6 and BIM 28163, we could modulate the biological activity of this receptor, *i.e.* in mediating AG- or UAG- induced insulin release. Surprisingly, both [D-Lys^3]-GHRP-6 and BIM 28136 significantly suppressed the AG induced insulin output, whereas they did not affect UAG stimulated insulin release.

Since the INS-1E cells express the GHS-R1a receptor, and the GHS-R1a antagonists block the induction of insulin secretion by AG, but not by UAG, in line with previous reports (38) we come to the conclusion that most likely AG effects on insulin release are GHS-R1a mediated. It has been suggested that BIM28163, besides acting as a GHS-R1a antagonist, is likely to activate an as yet unknown ghrelin receptor subtype (36). However, to date there is no evidence indicating that [D-Lys^3]-GHRP-6 could interact with GHS receptor subtypes different from GHS-R1a. Therefore, the hypothesis of the expression of a novel receptor able to bind both AG and the GHS-R1a antagonists in this system seems to be too speculative. The fact that two GHS-R1a antagonists alone did not modify glucose-induced insulin secretion, along with the observation that ghrelin mRNA was barely detectable in INS-1E cells, indicates that, most likely, in our system auto/paracrine endogenous ghrelin did not modulate insulin secretion significantly. Chan and coworkers showed that [D-Lys^3]-GHRP-6, in cells overexpressing the seabream GHS-R1a, behave as inverse agonist of the receptor constitutive activity (35). We hypothesize that the lack of activity of [D-Lys^3]-GHRP-6 alone in our experiments can be explained by species-related differences and/or by the fact that the coupling of low levels of expression of both GHS-R1a and endogenous ghrelin does not suffice in inducing measurable changes in insulin output.

Besides the role of GHS-R1a in mediating AG activity, we strongly suspect the existence of a separate receptor specific for UAG. It is known that UAG neither activates nor binds the GHS-R1a (1). Moreover, UAG does not displace radiolabeled AG from its hypothalamic or pituitary binding sites (40), whereas it does inhibit AG binding in human thyroid and breast tumors and related neoplastic cell lines and in the adipose tissue (15, 21). The existence of specific binding sites common to AG and UAG might explain some biological actions that UAG shares with AG (6, 7, 14, 15, 21, 22) and provides an explanation for the effects exerted by AG in systems in which the GHS-R1a is not expressed (20, 22).

However, the data available on the existence of common binding sites do not rule out the existence of receptors with specificity for UAG only. In fact, to date UAG has only been shown to compete with AG binding (7, 14), but binding of radiolabeled UAG has not been demonstrated by any of the groups involved in ghrelin research. Moreover, our findings that GHS-R1a antagonists can block AG but not UAG actions support the hypothesis that there might exist more than one ghrelin

receptor. Interestingly, this concept is strongly supported by a recent observation by Toshinai and coworkers, who showed that the administration of UAG induces an increase in food intake in GHS-R-deficient mice, despite the lack of AG-induced orexigenic effect (41). Chen and collaborators, demonstrated that UAG administration in rats displays activities (*i.e.* on food intake, on fasted motor activity of the stomach and *c-fos* expression in the hypothalamus) with a different pattern than AG (17), indicating AG-independent actions of UAG. Moreover, they reported that these AG-independent effects of UAG could be completely antagonized by two highly selective CRF subtype 2 receptor antagonists, strongly suggesting the involvement of the CRF2 receptor in UAG activity (17). In the INS-1E cell line we studied, however, we could not detect expression of CRF2R mRNA by using conventional PCR, nor was the UAG-induced insulin response modified by atressin$_2$B, a selective antagonist of CRF2 receptors. Therefore, an involvement of CRF2R in UAG-mediated effects on INS-1E cells is very unlikely.

Our data showed that both UAG and AG can stimulate insulin secretion by the INS-1E rat insulinoma cell line that expresses very low levels of the known ghrelin receptor GHS-R1a. Since AG induced insulin secretion in the presence of GHS-R1a expression, and the GHS-R1a antagonists [D-Lys^3]-GHRP-6 and BIM 28136 can block AG actions, we assume that already a low expression level of this receptor is sufficient enough to exert potent biological actions. However, as this receptor cannot bind UAG and UAG actions cannot be blocked by [D-Lys^3]-GHRP-6 and BIM 28136, we suspect the presence of a specific UAG receptor that does not bind AG. Apparently, UAG is certainly not an inert molecule but might have potent endocrine actions via this putative non-GHS-R1a UAG receptor, distinct from another non-GHS-R1a-UAG receptor, namely the CRF2R. Indeed, further studies are needed for a characterization this putative receptor, whose binding affinity, activity and regulation have to be defined.

In conclusion, we characterized a sensitive and reproducible assay for UAG action *in vitro*. We also found indirect, but strong evidence for the existence of a non-GHS-R1a ghrelin receptor that selectively binds the unacylated form of ghrelin. Characterization of this selective UAG receptor might have clinical implications, *e.g.* in those conditions in which glucose and insulin metabolism is disturbed.

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Chapter VI.2.

Unacylated ghrelin is not a functional antagonist but a full agonist of the type 1a growth hormone secretagogue receptor (GHS-R)

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ABSTRACT

Recent findings demonstrate that the effects of ghrelin can be abrogated by co-administered unacylated ghrelin (UAG). Since the general consensus is that UAG does not interact with the type 1a growth hormone secretagogue receptor (GHS-R), a possible mechanism of action for this antagonistic effect is via another receptor. However, functional antagonism of the GHS-R by UAG has not been explored extensively. In this study we used human GHS-R and aequorin expressing CHO-K1 cells to measure $[Ca^{2+}]_i$ following treatment with UAG. UAG at up to 10^{-5} M did not antagonize ghrelin induced $[Ca^{2+}]_i$. However, UAG was found to be a full agonist of the GHS-R with an EC_{50} of between 1.6 and 2 μM using this *in vitro* system. Correspondingly, UAG displaced radio-labeled ghrelin from the GHS-R with an IC_{50} of 13 μM. In addition, GHS-R antagonists were found to block UAG induced $[Ca^{2+}]_i$ with approximately similar potency to their effect on ghrelin activation of the GHS-R, suggesting a similar mode of action. These findings demonstrate in a defined system that UAG does not antagonize activation of the GHS-R by ghrelin. But our findings also emphasize the importance of assessing the concentration of UAG used in both *in vitro* and *in vivo* experimental systems that are aimed at examining GHS-R independent effects. Where local concentrations of UAG may reach the high nanomolar to micromolar range, assignment of GHS-R independent effects should be made with caution.

INTRODUCTION

Ghrelin was discovered through its ability to activate the type 1a growth hormone secretagogue receptor (GHS-R) and stimulate growth hormone release *in vivo* (1, 2). An evolutionarily conserved feature of ghrelin is the acylation of its third residue, usually with *n*-octanoic and, less commonly, with *n*-decanoic acid (3). Kojima *et al.* (4) were the first to describe the requirement that ghrelin be acylated on its third serine residue for activation of the GHS-R in the nanomolar range, with an EC_{50} for increased $[Ca^{2+}]_i$ of 2.5 x 10^{-9} M. In the circulation, ghrelin also occurs as a unacylated isoform (UAG) at 10-50 times the concentration of acylated ghrelin (5, and our unpublished observations).

Of great interest to us has been the finding that in humans co-administration of UAG can antagonize the metabolic effects of ghrelin *in vivo*. Ghrelin administration causes hyperglycemia, hypoinsulinemia, increased circulating free fatty acids and worsening insulin sensitivity, but these effects are reversed or prevented by co-administration with UAG (6, 7). These effects seem to be specific to ghrelin's metabolic activity since UAG has no impact on GH, PRL or ACTH secretion (6). This suggested a direct action on the endocrine pancreas, and perhaps on hepatic glucose production. In relation to these *in vivo* findings, we have shown that UAG not only suppresses glucose output, but also blocks ghrelin induced glucose release by primary hepatocytes (8). In support of these findings, a recent report demonstrated the antagonistic effect in fish where ghrelin's orexigenic effects were blocked by administration of UAG. Furthermore, this effect appears to occur both centrally and peripherally (9). There are now many reports of direct biological activity of UAG *in vitro* that suggest a receptor mediated cellular response, perhaps via a specific receptor that is not GHS-R (10-17). Despite these findings, the current consensus appears to be that UAG is inactive as an agonist of the GHS-R. However, the possibility remains that UAG is somehow able to block the ghrelin response by antagonizing the GHS-R. Therefore, we have explored in more detail, in a defined *in vitro* system, the ability of UAG to antagonize activation of the GHS-R by ghrelin, the potency of UAG at the GHS-R, and the effects of GHS-R antagonists.

MATERIALS AND METHODS

Peptides

Human UAG was obtained from NeoMPS (Strasbourg, France) and Thera Technologies (Montreal, Canada). Human ghrelin, [D-Lys^3]GHRP-6, somatostatin28, obestatin and glucagon were obtained from NeoMPS. The ghrelin analog BIM28163, a potent antagonist of the GHS-R, was kindly provided by IPSEN Pharmaceuticals (Milford, MA). All peptides had been assessed for purity and integrity by high performance liquid chromatography and mass spectrometry.

Aequoscreen assay for ghrelin and UAG activity

Aequoscreen cells were kindly provided by Euroscreen s.a. (Gosselies, Belgium). These cells (CHO-A5) are stably transfected with a pIRES-puro (Clontech, Mountain View, CA) construct containing mitochondrially targeted apoaequorin which allows luminometric determination of $[Ca]_i$. An identical cell line was also provided that had also been stably transfected with a human GHS-R1a expression construct with a *Neo* cassette (CHO-A5-GHSR). These cell lines were maintained in HAM F12 containing 10% fetal calf serum, 2.5 µg/mL amphotericin, 100 IU/mL penicillin, 100 µg/mL streptomycin, 5 µg/mL puromycin. The GHS-R expressing cells were maintained in the same medium but with the addition of 400 µg/mL G418. On the day of the assay the cells were resuspended in BSA assay buffer (DMEM/HAM's F12, with HEPES, without phenol red, 0.1% BSA, 2.5 µg/mL amphotericin, 100 IU/mL penicillin, 100 µg/mL streptomycin) at $5x10^6$ cells/ml, and then coelenterazine h (Sigma, St Louis, MO) was added to a final concentration of 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 $5x10^5$ cells/ml, and 100 µL was injected into wells of a black 96-well plate containing 100 µL of various concentrations and combinations of ghrelin, UAG and other peptides. Luminescence was integrated for 15 s using a Victor2 1420 multilabel counter (Perkin-Elmer, Wellesley, MA, USA), a short enough time that it is very unlikely that modification of the UAG could occur during the assay. After collection of data, the residual response of the cells was measured by permeabilizing their membranes with 100 µL of 1% Triton X-100. Data were calculated as the fractional response to agonist relative to the total response of the cells to agonist and Triton X-100 (fractional response = $x(x + y)^{-1}$, where x = agonist response and y = residual response). In experiments with antagonists we have normalized the data as percentage of maximal response in the absence of antagonist. UAG was able to saturate the response of the aequoscreen cells in the presence of an EC_{75} concentration of ghrelin

(Fig1A). Therefore, we have set the maximal response of UAG at that of ghrelin in the correlation function used for regression analysis. Non-linear regression analysis was performed using Graphpad Prism version 3 (San Diego, CA).

Radioligand binding studies

Membranes were prepared from CHO-A5-GHSR cells using a protocol from Euroscreen. Briefly, cells at 80% confluence in monolayer culture in 75 cm^2 flasks, were scraped into PBS, then pelleted at 1500 *x g* for 3 min. The cell pellet was then resuspended in buffer A (15 mM Tris-HCl (pH7.4), 2 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA) at 4°C and homogenized in a glass/teflon homogenizer. The crude membrane fraction was collected by two consecutive centrifugation steps at 40,000 *x g* for 25 min separated by a washing step in buffer A. Membrane pellets were resuspended in buffer B (75 mM Tris-HCl (pH7.5), 12.5 mM $MgCl_2$, 0.3 mM EDTA, 1 mM EGTA, 250 mM sucrose) and stored at -80°C until use. Protein content was measured using the Biorad protein assay (Biorad, Hercules, CA).

Competitive binding dose curves for ghrelin and UAG were then run using 75 µg of membrane protein and 50,000 cpm (0.1 nM) of [His[^{125}I]]-ghrelin (NEX388, Perkin-Elmer, Boston, MA) in binding buffer (25 mM HEPES pH7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$) in a total volume of 100 µL. Binding was carried out at 21°C for 60 min, then membranes were collected by centrifugation, washed with ice-cold binding buffer, and radioactivity was counted. Samples were run in duplicate. Non-linear regression analysis was performed using Graphpad Prism.

RESULTS

The main reason for examining UAG modulation of GHS-R activity was to elucidate a mechanism for our finding that this peptide could antagonize the effects of ghrelin on hepatocyte glucose production *in vitro* (8). Therefore, the first experiment that we ran was designed to determine if UAG had any antagonistic activity in an *in vitro* system where we could examine its rapid effects directly. We have used Aequoscreen cells transfected with the human GHS-R (CHO-A5-GHSR) for this purpose, where the induction of $[Ca^{2+}]_i$ by exposure of the cells to ghrelin can be accurately and rapidly assessed by measuring aequorin luminescence. CHO-A5-GHSR cells are sensitive to ghrelin treatment, responding consistently and robustly with an EC_{50} of approximately 2 nM (Figure 1A). In initial experiments we treated the cells with 2.5 nM or 5 nM ghrelin (generating approximately 80 and 93% of their maximum luminescence response) together with increasing concentrations of UAG ranging from 10^{-10} to 10^{-5}

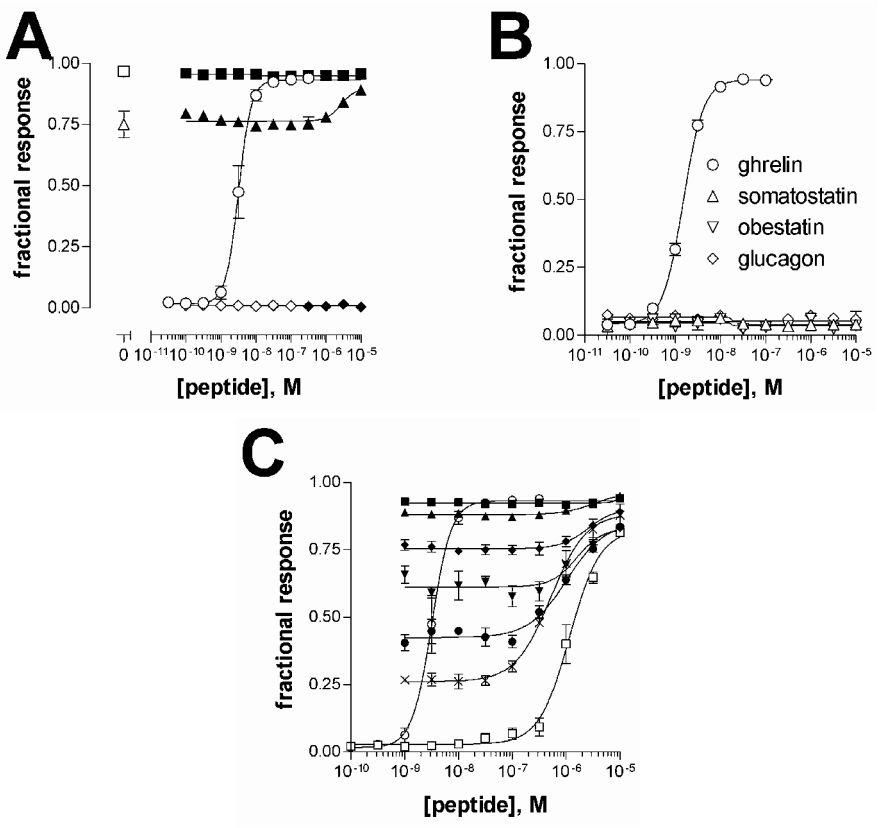

Figure 1. A. UAG does not antagonise ghrelin's action on the GHS-R. Open circles represent the $[Ca^{2+}]_i$ response of CHO-A5-GHS-R cells to a dose curve of ghrelin. The cells' response to 2.5 nM (triangles) and 5 nM ghrelin (squares), in the absence (open symbols) and presence (closed symbols) of dose curves of UAG are superimposed on the ghrelin dose response curve. UAG further stimulates the effects of 2.5 nM ghrelin, approaching the maximal response obtained with ghrelin alone (fractional response of 0.93). Neither ghrelin (open diamonds) nor UAG (closed diamonds) activate Aequorin cells that lack the GHS-R. B. Peptide ligands (somatostatin28, obestatin and glucagon) of similar molecular weight to UAG do not activate the GHS-R in aequoscreen cells in the 10^{-11} to 10^{-5} M range. A ghrelin dose response was run concurrently as a positive control. C. UAG (open squares), activates the GHS-R with an EC_{50} in the low micromolar range, but does not antagonize activation of the GHS-R by ghrelin in a range of concentrations corresponding to 25-100% of maximal response (closed symbols represent UAG dose response curves run in increasing concentrations of ghrelin from 0.75 to 9 nM). The dose response for ghrelin is shown for comparison (open circles). Error bars represent ±SEM.

M (Fig. 1A). We found that over this dose range UAG does not antagonise ghrelin's action on the GHS-R. In fact, under conditions of sub-maximal stimulation with 2.5 nM ghrelin, UAG in the low micromolar range was able to further stimulate the cells towards their maximal fractional response (in this case, 93%).

The possibility remained that the CHO-A5 cells themselves contained a receptor that was responsive to UAG, and that the response we were measuring was not

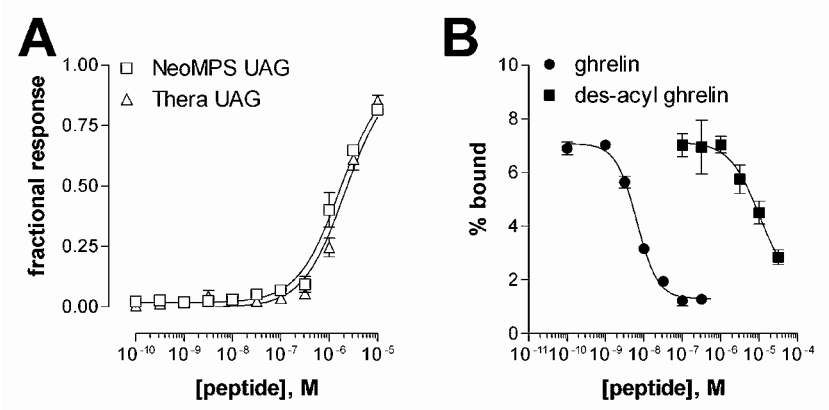

Figure 2. UAG is an agonist of the GHS-R. A. Dose response curves for UAG from two sources (NeoMPS, n=4, EC_{50}=1.6 μM; and Thera Technologies, n=15, EC_{50}= 2.1 μM) on the CHO-A5-GHS-R cells. Measured endpoint is aequorin luminescence B. Displacement curves for [His[^{125}I]]-ghrelin by ghrelin (IC_{50}, 7 nM) and UAG (IC_{50}, 13 μM). Error bars represent ±SEM.

derived from activation of the GHS-R but from another receptor. Therefore, we tested the response to UAG and ghrelin in Aequoscreen cells that had not been transfected with the GHS-R. Dose response curves for UAG (10^{-8} M to 10^{-5} M) and ghrelin (10^{-10} M to 10^{-7} M) caused no luminescent response from these cells (Fig. 1A), whereas combined treatment with 0.5 μM PMA and 100 nM A23187 elicited a maximal fractional response of 0.98 ± 0.003 (n = 4). These findings confirm that the response we have measured for UAG is not mediated by an alternative receptor system in CHO-A5 cells. There was also the possibility that UAG was having a non-specific effect on receptor activation in the micromolar range. To test this we ran dose response curves for three other peptides of similar size but unrelated amino acid sequence (somatostatin28, obestatin and glucagon) at between 5 x 10^{-11} and 10^{-5} M. None of these peptides generated a signal at any of the concentrations tested (Figure 1B).

UAG may antagonise ghrelin's action at concentrations other than ghrelin's EC_{90}. To test this possibility, we examined the response of the aequoscreen cells to UAG in the presence of ghrelin at concentrations that spanned its dose response curve. UAG had no antagonistic effect on any of these concentrations of ghrelin (Figure 1C), and the results are fully consistent with UAG acting agonistically on the GHSR in the 10^{-7} to low 10^{-6} M concentration range.

We also constructed dose response curves using two different sources of the peptide (NeoMPS and Thera Technologies). These experiments showed that UAG is a full agonist of the GHS-R with EC_{50} values of 1.6 μM (n = 4, R^2 = 0.95) for NeoMPS peptide, and 2.1 μM (n = 15, R^2 = 0.94) for Thera Technologies peptide (Figure 2A).

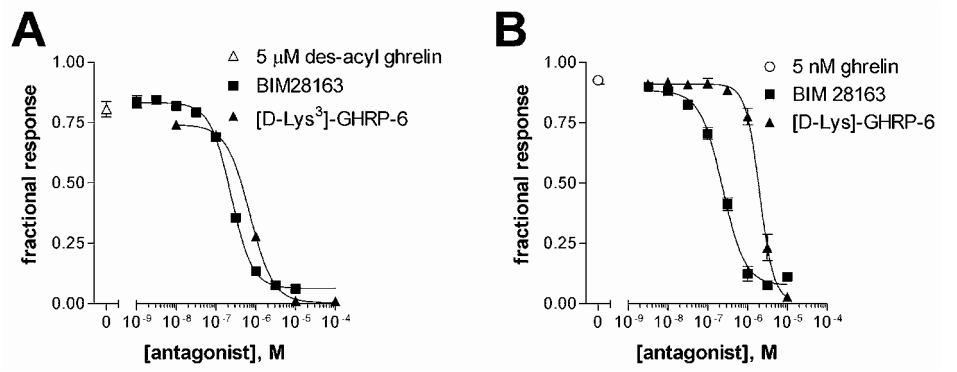

Figure 3. GHS-R antagonists antagonize both UAG and ghrelin with similar IC_{50} values. A. Inhibitory dose response curves against 5 µM UAG in CHO-A5-GHS-R cells for BIM28163 (IC_{50}, 240 nM) and [D-Lys^3]GHRP-6 (IC_{50}, 700 nM). B. Inhibitory dose response curves against 5 nM ghrelin in CHO-A5-GHS-R cells for BIM28163 (IC_{50}, 240 nM) and [D-Lys^3]GHRP-6 (IC_{50}, 1.98 µM). Error bars represent ±SEM.

Fractional response values of 0.84 and 0.89 were reached at 10 µM, which are close to the maximal response for ghrelin at 10 nM. The potency of UAG was further confirmed with membrane preparations of CHO-A5-GHSR cells. In radioligand binding experiments UAG displaced [His[^{125}I]]-ghrelin from the GHS-R with an IC_{50} of 13 µM (Figure 2B). Ghrelin demonstrated a typical displacement curve using the same membrane preparations (Fig. 2B; IC_{50}, 7 nM).

As additional evidence that UAG was activating the GHS-R, we tested the ability of two GHS-R antagonists to block its effect. BIM28163 is an analog of full-length ghrelin (18), whereas [D-Lys^3]GHRP-6 is an antagonistic analog of the hexapeptide growth hormone secretagogue GHRP-6 (19). Both have been demonstrated to have antagonistic activity *in vitro* and *in vivo* (18, 19). Inhibitory dose response curves against UAG in GHS-R Aequoscreen cells demonstrated that both peptidyl antagonists were capable of blocking the UAG response (Figure 3A; BIM28163 and [D-Lys^3] GHRP-6; 238 nM and 700 nM, respectively). The potencies of these antagonists was similar to those found against ghrelin using the same *in vitro* system (Figure 3B; BIM28163 and [D-Lys^3]GHRP-6; 240 nM and 1.98 µM, respectively). These data suggest that UAG activates the GHS-R with a similar mechanism of action.

DISCUSSION

Overall, our observations in a cell line in which we can measure the effects of peptides exclusively on GHSR activation suggest that UAG can reinforce the activity of ghrelin, depending on effective concentrations. However in other *in vitro* models,

and *in vivo*, the situation appears to be more complex. Previous studies show that UAG can displace radiolabelled ghrelin from membrane extracts of cell-lines that lack expression of GHS-R1a mRNA (*e.g.* 15, 20), and that UAG can antagonise ghrelin induced glucose release in hepatocytes (8) that probably do not express GHS-R1a. These studies present the argument that there is a separate UAG receptor that is involved in modulating the metabolic effects of ghrelin *in vivo*, for example on glucose homeostasis (6, 7). In these instances, UAG could modulate the metabolic effects of ghrelin directly in the liver or pancreas directly on a receptor that is shared by ghrelin and UAG, or indirectly via a UAG-specific receptor. It is likely that a direct antagonistic effect of UAG on ghrelin activity at the GHSR is ruled out by our findings, strengthening the argument for an alternate receptor that is involved in the antagonistic effects of UAG.

However, we also demonstrate that UAG is capable of activating the GHS-R in the high nanomolar to low micromolar range in the Aequoscreen *in vitro* system. These are lower concentrations than previously determined, suggesting that UAG could activate GHS-R in other cellular systems, perhaps even *in vivo*. An important issue is whether local concentrations of UAG ever reach the levels needed for agonistic action on GHS-R. Hormone concentrations can reach levels in tissues that are not attained in the circulation, through local production. Examples of this include interleukin-6, and the peptide hormones angiotensin-I and angiotensin-II which have been demonstrated to attain tissue concentrations 100-fold higher than circulating levels (21, 22). It has recently been demonstrated that UAG concentrations are in the nanomolar range in the pancreatic (splenic) vein resulting from high levels of local production in the pancreas (23). If, like the similarly sized angiotensins, pancreatic tissue levels of UAG were 100-fold greater than in the circulation, then this would bring local concentrations into the range required to activate the GHS-R. Ghrelin appears to be synthesized in numerous tissues (24), in each of which local concentrations of both acylated and UAG may reach concentrations higher than those in the blood. Additionally, high local concentrations of UAG could also occur in certain experimental conditions, such as at the site of infusion of the peptide.

Bednarek *et al.* (25) examined the requirement for a bulky hydrophobic group in the side chain of the Ser^3 residue of ghrelin for its ability to activate GHS-R1a in a similar aequorin reporter system. This study found that human UAG activated the GHSR to 40% of maximal values at 10 μM. A similar structure-function study of rat ghrelin that measured $[Ca^{2+}]_i$ mobilization using Fura2 has been described by Matsumoto *et al.* (26), with UAG activating the GHS-R with an EC_{50} of 3.5 μM. Although these studies hinted at the activity of the des-octanoylated form of the peptide, they did not directly address the possibility of a functional antagonistic effect of UAG on this receptor. We

find that human UAG activates the human GHS-R with similar or greater potency to that of rat UAG, and that its mechanism of action is similar to that of ghrelin since GHS-R antagonists block its action. Our data suggest UAG may act on the GHS-R in a potentially physiological range of concentrations. At the very least, our findings illustrate that peptide concentration must be carefully considered in experiments where local concentrations of UAG may reach the low micromolar range *in vivo*, or in *in vitro* experiments.

In conclusion, we find in a defined *in vitro* system that UAG cannot functionally antagonize the action of ghrelin on the GHS-R. This suggests that the apparent antagonistic activity of UAG is mediated by an indirect mechanism, perhaps involving a specific UAG ghrelin receptor. UAG is a full agonist of the GHS-R, with activity in the high nanomolar range. The ability of UAG to interact with the GHS-R makes it necessary to interpret GHS-R independent activity of this peptide with caution in *in vitro* and *in vivo* experiments where the local peptide concentrations following treatment may reach the high nanomolar range.

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Chapter VII

General discussion

In the last decade the discovery of novel gut hormones brought a new perspective to the classical view of the regulation of glucose homeostasis. Ghrelin is a gut peptide predominantly produced by the stomach, but also by other regions of the gastrointestinal tract (1-5). Ghrelin circulates in the bloodstream in two forms: acylated and unacylated. Acylated ghrelin (AG), which accounts for approximately 10% of total ghrelin, has a fatty-acid modification that allows binding to and activation of the growth hormone secretagogue receptor type 1a (GHS-R1a), the only known ghrelin receptor so far (1). The GHS-R1a mediates some of AG biological actions, such as the orexigenic and the neuroendocrine effects. However, both AG and UAG exert pleiotropic actions that involve also receptors, not yet identified, different from the GHS-R1a (5).

AG and UAG secretion is mainly under metabolic control, being modulated by glucose, insulin and feeding (5). On the other hand, AG and UAG influence energy metabolism by acting on central and peripheral circuits that control energy homeostasis, feeding behavior and fuel storage and availability (5). Thus, AG, UAG and their known and unknown receptors identify a very complex system, deeply involved in the modulation of metabolic functions, whose complete understanding will probably increase our knowledge about those pathological conditions characterized by disruption of glucose homeostasis, such as metabolic syndrome and diabetes mellitus.

The majority of reports in the literature examined the effects of exogenously administered AG and UAG, thereby elucidating their pharmacological properties. The physiological implications of the endogenous peptides are much less clear, in part because of the complexity of the ghrelin system itself (*i.e.* both AG and UAG are encoded by a single gene, they are produced by several tissues and the mechanisms of acylation/deacylation are still largely unknown), but also due to the fact that assays available to detect the AG and UAG fractions have been only recently developed and still present technical difficulties.

This thesis is focused on the pharmacological and physiological implications of the ghrelin system on peripheral glucose homeostasis. In this chapter I give an updated overview on the subject and, while considering some methodological limitations, I look ahead to potential clinical applications of interfering with the ghrelin system.

PHARMACOLOGICAL ACTIONS OF AG AND UAG ADMINISTRATION

The first evidence of an effect of the ghrelin system on glucose and insulin handling came from clinical studies in which AG, but not UAG or synthetic GHSs, was

able to induce a modulation of fasting glucose and insulin systemic levels, besides having a strong GH-releasing effect (5-7). In these studies the GH-independency of this phenomenon had been (indirectly) suggested (5-7). We further explored a possible direct (*i.e.* GH-independent) role of AG and UAG in the regulation of glucose metabolism by administering AG and UAG in GH deficient subjects (GHD). We found that in conditions of low circulating levels of GH, not only AG, but also UAG slightly increases glucose levels in fasting conditions (chapter II). However, the most stunning observations were related to the post-prandial effects of these peptides. In fact, we found that AG administration markedly worsens insulin sensitivity, whereas the co-administration of UAG with AG completely blocks this effect and also improves insulin sensitivity *per se*. An involvement of ghrelin in the modulation of insulin sensitivity had been previously suggested by the existence of an association between insulin and ghrelin levels in physiological and pathological conditions (5). Subsequently Heijboer *et al.* (8) clarified, using a euglycemic-hyperinsulinemic clamp technique, that AG induces insulin resistance by suppressing the inhibition that insulin exerts on glucose production in the liver. The co-administration of UAG blocked this suppressive effect of AG (8). This finding was in accordance with a previous *in vitro* study, where we demonstrated that AG and UAG, alone and in combination, affect glucose output by the liver. Thus, AG stimulates, whereas UAG inhibits, glucose output by primary hepatocytes in suspension culture. In this *in vitro* system again the combination of AG with UAG was able to abolish this "metabolic effect" of AG (or UAG) when administered alone. Interestingly, UAG also partially counteracted the glucagon-induced glucose release (chapter III).

Besides their effects on insulin sensitivity, AG and UAG have been shown to regulate insulin secretion. Exogenous AG suppresses basal and glucose-stimulated insulin release in different *in vivo* and *in vitro* models (6, 9-11), although a stimulatory effect has also been reported (3, 12, 13). Conversely, UAG did not induce significant changes in systemic insulin levels *in vivo*, in fasting as well as in meal- or glucose-stimulated conditions (7, 9, 10, 14), nor did it alter glucose-induced insulin release by perfused pancreas (10). However, we and others (15) found that UAG increases insulin release by rat insulinoma cell lines exposed to high glucose concentrations (chapter VI). The evidence that UAG blocks the AG-induced modulation of insulin release and hepatic insulin sensitivity *in vivo*, and that UAG alone has effects on glucose output from the liver allowed us to hypothesize that UAG might modulate insulin secretion into the portal vein and that somehow this effect is masked by measurement of systemic insulin levels. This hypothesis was verified by studying the effects of UAG administration on portal and systemic glucose-induced insulin release in anesthetized rats. We observed that UAG potently enhances the glucose-induced

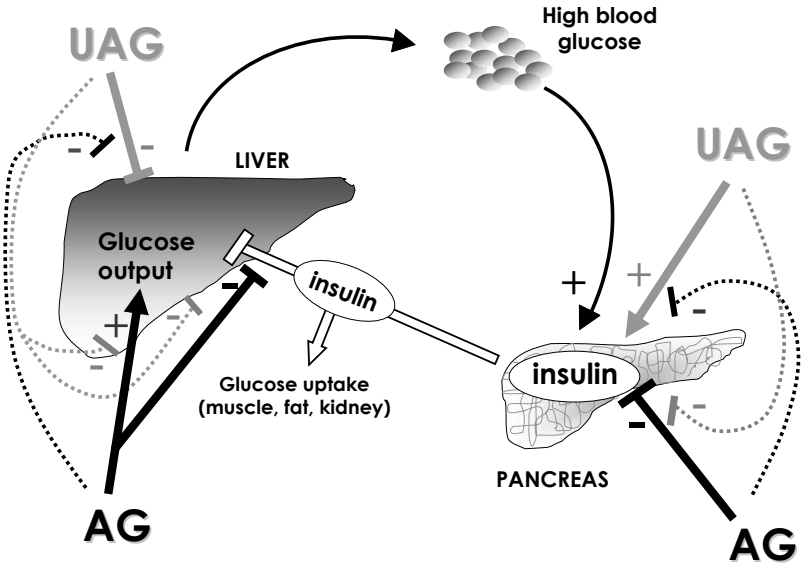

Figure 1. Schematic representation of the pharmacological actions of AG (black lines) and UAG (gray lines) in glucose-stimulated conditions. Dotted lines indicate the antagonistic effect that AG (black) and UAG (gray) have on each-other's actions when administered together. The white line indicates the hepatic action of insulin secreted into the portal vein. + : stimulatory effect; -: inhibitory effect.

insulin release into the portal vein, but this effect is severely blunted in the systemic circulation. Moreover, once again we found that the co-administration of AG with UAG completely abolishes the UAG-induced stimulation on the insulin response to glucose (chapter IV).

Interestingly, recent reports showed that AG promotes regeneration of β-cells in streptozotocin-treated newborn rats (16) and that both AG and UAG promoted cell growth and counteracted apoptosis in β-cells *in vitro* (15). These findings suggest that AG and UAG might also play a protective role on β-cell mass and function in type 1 diabetes.

Figure 1 shows our current hypothesis on the pharmacological actions of AG and UAG in glucose-stimulated conditions.

PHYSIOLOGICAL ACTIONS OF ENDOGENOUS AG AND UAG

Endogenous AG and UAG are produced in the endocrine pancreas, which also expresses the GHS-R1a (3, 4, 9, 17, 18). It is currently accepted that endogenous AG, systemic and of pancreatic origin, exerts a tonic inhibition on glucose-induced

insulin release in pancreatic islets, probably via the GHS-R1a (9), as demonstrated by the marked increase of insulin response to glucose after blockade of endogenous AG action using GHS-R1a antagonism and anti-AG antiserum (9-11). A similar stimulation of the glucose-induced insulin release is observed after deletion of the ghrelin gene in mice (11). Moreover, ablation of the ghrelin gene in states of insulin resistance, such as in genetic (*i.e.* in leptin-deficient (ob/ob) mice) or in high-fat diet induced obesity, improves insulin secretion and insulin sensitivity, resulting in an improved glucose tolerance (10, 11). It is assumed that the phenotype of ghrelin knock out mice reflects the lack of endogenous AG actions, because the administration of exogenous AG restores glucose-induced insulin release to the same extent as in control animals. However, ablation of the ghrelin gene implies the deletion not only of AG, but also of UAG, since AG and UAG are encoded by the same gene, as also demonstrated by undetectable total ghrelin levels (19). Since we showed that exogenous UAG has opposite actions than AG on insulin secretion and sensitivity as well as on hepatic glucose metabolism, we propose that the (inter-)action of endogenous AG and UAG, rather than AG alone, should be considered as a novel regulator of glucose homeostasis. In accordance with this, mice over-expressing UAG in pancreatic islets may have improved insulin sensitivity (20).

However, the regulation of endogenous AG and UAG levels and their relevance in physiological conditions and/or in disorders in which insulin sensitivity is disturbed have not been fully elucidated. It is known that circulating ghrelin levels are regulated by nutritional states. In particular, insulin and glucose are the major regulators of circulating total ghrelin levels. Acute (*i.e.* postprandial or induced by euglycemic hyperinsulinemic clamp technique) and chronic (*i.e.* insulin resistance) increase of insulin levels are associated with a reduction of total ghrelin (5, 21, 22). However, the majority of reports in the literature about the nutritional regulation of ghrelin refer to systemic total ghrelin levels, which may differ from the AG and UAG fractions released by the gut into the local circulation. In fact, since the main source of ghrelin is the gastrointestinal tract, ghrelin is secreted into the portal vein and has to pass the liver before it reaches the peripheral circulation. It is known that the liver passage is crucial for clearance of other hormones secreted into the hepatic portal vein (such as insulin and glucagon) (23-26). We hypothesized that the liver also regulates the ratio between AG and UAG and thereby the physiological levels of these hormones. By using a rat *in vivo* model we showed that AG and UAG levels are higher in the portal vein than in the systemic circulation and that they are differentially regulated by glucose. In fact, an intravenous glucose load inhibits UAG levels in the portal and the systemic circulation, whereas it blunts only pre-hepatic but not systemic AG concentrations. Moreover, our data suggest that the liver clears a significant portion of gut-derived ghrelin, favoring removal of AG over UAG.

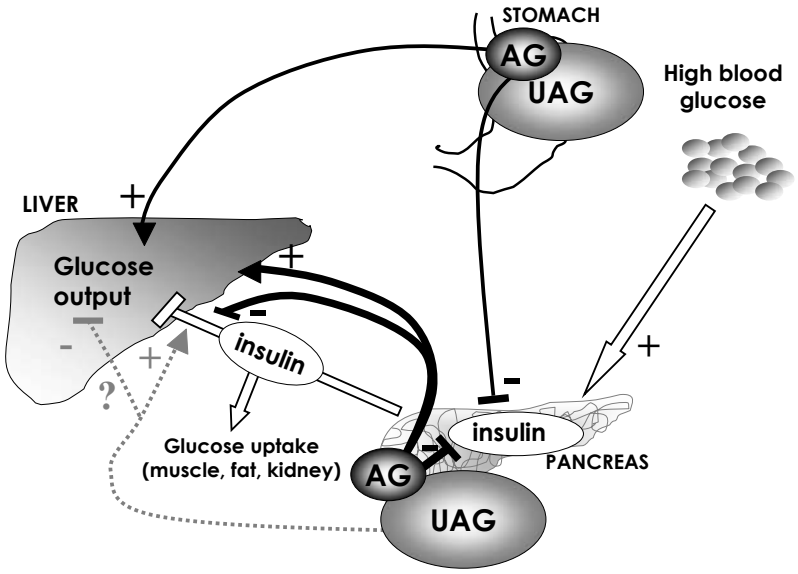

Figure 2. Schematic representation of the physiological actions of endogenous AG (black arrows and lines) in glucose-stimulated conditions. Dotted gray lines indicate the (suggested) improvement of (hepatic) insulin sensitivity by an excess of (pancreatic) UAG. The white line indicates the glucose-stimulated insulin secretion. + : stimulatory effect; -: inhibitory effect.

In obesity, characterized by decreased total ghrelin levels, the presence and the extent of insulin resistance are associated with a relative increase of endogenous AG and therefore with a higher ratio of AG/UAG (27). However, whether in states of insulin resistance the AG and UAG portal concentrations and/or hepatic clearance are altered has not been clarified to date and more studies are needed.

Figure 2 shows our current hypothesis on the physiological actions of endogenous AG in glucose-stimulated conditions.

AG AND UAG RECEPTORS

A full comprehension of the mechanisms of action of AG and UAG would require the identification of the receptor(s) that mediate their action on insulin secretion and sensitivity.

AG is a natural ligand of the growth hormone secretagogue receptor type 1a (GHS-R1a), also called ghrelin receptor (GRLN-R). Concerning the metabolic effects of AG, it is currently accepted that the GHS-R1a mediates the modulation of insulin secretion, since it is expressed in the endocrine pancreas (4, 17) and the use of

[D-Lys^3]GHRP-6, a GHS-R1a (ant-)agonist, improves the insulin response to glucose to the same extent as the use of anti-AG antiserum (10). On the other hand, the AG actions on (hepatic) insulin sensitivity are likely to be mediated by a receptor different from GHS-R1a, since they are not exerted by synthetic GHSs and the GHS-R1a is not expressed in the liver (28-30). Since UAG has been shown not to bind or activate the GHS-R1a, the existence of a putative receptor specific for UAG has been hypothesized (5). The presence of binding sites common to AG and UAG in systems that do not express GHS-R1a indicate that AG and UAG share a common receptor (5), which could also be involved in the antagonistic activity of UAG on AG actions, *e.g.* in the liver.

However, in systems that express the GHS-R1a, such as the pancreas, UAG might be able, somehow, to functionally block AG effects by antagonizing the GHS-R1a. Interestingly, in cells overexpressing this receptor, we observed that UAG not only blocks the AG-induced activation of GHS-R1a, but also behaves as a full agonist when used at concentrations in the high nanomolar range (chapter VI.2.). However, in our *in vitro* and *in vivo* systems the concentration of UAG able to stimulate the glucose-induced insulin release was in the (low) nanomolar range. Moreover, this action was not blocked by the GHS-R1a antagonist. This makes it very unlikely that the GHS-R1a mediates UAG effects in the pancreas.

Taken together, our data further strengthen the hypothesis of the existence of a putative UAG receptor.

CLINICAL IMPLICATIONS AND FUTURE PERSPECTIVES

In today's industrialized world insulin resistance is a common metabolic dysfunction associated with several high-prevalence physiopathological conditions (*i.e.* obesity), with serious clinical consequences, most importantly type 2 diabetes, which affects over 150 million people worldwide (31). Early in the pathogenesis of type 2 diabetes, insulin resistance is well established, whereas glucose tolerance remains normal because of a compensatory increase in insulin secretion. In people with normal-functioning β-cells, the pancreas is able to "read" the severity of insulin resistance and adjust its secretion of insulin to maintain normal glucose tolerance. Therefore, the pancreas offsets the deterioration in glucose homeostasis by progressively increasing fasting plasma insulin levels. However, when β-cell function cannot compensate the prevailing severity of insulin resistance anymore, a defect in insulin secretion occurs and glucose tolerance is impaired. The progression from this state of impaired glucose tolerance to type 2 diabetes is then heralded by an inability of the β-cells to maintain its previously high rate of insulin secretion in response to a

glucose challenge, without any further or minimal deterioration in tissue sensitivity to insulin.

Potential therapeutic implications of UAG in diabetes mellitus

Looking at the natural history of type 2 diabetes, it becomes clear that the development of new compounds that intervene with the physiopathological mechanisms involved in type 2 diabetes would be extremely helpful in preventing the development of overt disease.

In the last few years a new class of agents has been developed and recently approved (in the USA) as an additional treatment for type 2 diabetes: the incretin mimetics, such as the glucagon-like peptide-1 (GLP-1) mimetic exenatide. This new class of agents exhibits a series of "anti-diabetogenic" actions, including stimulation of glucose-induced insulin release, increase in beta cell mass, maintenance of β-cell function and improvement of insulin sensitivity and also inhibition of glucagon secretion (32).

As revised in this thesis, UAG seems to share with GLP-1 some features, such as the beneficial effects on glucose-induced insulin secretion, insulin sensitivity and hepatic glucose production. If these UAG actions will be further confirmed, UAG might represent another molecule with good premises for (long-term) future clinical applications in type 2 diabetes. Moreover, if future studies verify that UAG stimulates regeneration of β-cells and inhibits their apoptosis, a possible interest for UAG in the field of type 1 diabetes, which is characterized by destruction of pancreatic islets, cannot be excluded. However, before this perspectives become real, several steps will need to be taken including more detailed understanding of the mechanisms of action that mediate UAG effects, identification of the putative UAG receptor(s), development of UAG analogues with low molecular weight and/or UAG receptor agonists with high potency and long half life and, finally, definition of their pharmacological profile.

Although the physiological and pathological regulation of endogenous AG requires far more research, it is possible that antagonism of the metabolic actions of endogenous AG might also become of interest in a therapeutic perspective.

Potential diagnostic implications of the ratio of AG/UAG

The current knowledge on endogenous ghrelin levels suggests that AG is "diabetogenic", whereas UAG might act as an "anti-AG/anti-diabetogenic" hormone.

It is well established that obesity and insulin resistance are associated with decreased systemic total ghrelin levels (5, 27, 33-35). Most of the systemic ghrelin levels are stomach-derived, as demonstrated by a decrease of approximately 80% after gastrectomy (36). However, a recent report shows that such low systemic total ghrelin levels do not significantly affect β-cell function and that insulin secretion by the pancreas is mainly (down)regulated by local, pancreas- and intestine- derived, (acylated) ghrelin (10). In fact, administration of a GHS-R1a antagonist is able to increase peripheral insulin concentration in gastrectomized rats to the same extent as in normal rats, despite the fact that gastrectomized rats have markedly reduced AG systemic levels (10).

It has been found that in obesity the presence and severity of insulin resistance is associated with increased endogenous AG and/or elevated ratio of AG/UAG (27). This is an intriguing observation and it is tempting to speculate on a future potential use of these parameters as markers of insulin resistance.

However, it remains largely unknown which are the local, *i.e.* pancreatic and/or intestinal, concentrations and mechanisms of regulation of AG and UAG. Moreover, the validity of the measurement of endogenous AG and UAG is still a serious issue, since it involves technical differences and difficulties, as revised in chapter I, paragraph 3.1.2. of the present thesis. Therefore, a potential diagnostic usefulness of AG and UAG endogenous levels seems to be unlikely, at least in the short term.

CONCLUSIVE REMARKS

In the last few years it has emerged that the gut peptides AG, UAG, and their receptor(s) identify a very complex system, which plays a pivotal role in the central and peripheral regulation of energy homeostasis. This thesis focuses on the glucometabolic aspects of the ghrelin system and highlights some physiological and pharmacological mechanisms that may be of help in the understanding and (perhaps) treatment of those physiopathological conditions characterized by insulin resistance and its evolution toward (type 2) diabetes.

Summary/Samenvatting

Summary

The ghrelin system is a gut-endocrine system discovered in the last decade. Ghrelin is a gut peptide predominantly produced by the stomach, but also by other regions of the gastro-intestinal tract. Ghrelin circulates in the bloodstream in two forms: acylated and unacylated. Acylated ghrelin (AG), which accounts for approximately 10% of total ghrelin, has a fatty-acid modification that allows binding to and activation of the growth hormone secretagogue receptor type 1a (GHS-R1a), the only known ghrelin receptor so far. The GHS-R1a mediates some of AG biological actions, such as the orexigenic and the neuroendocrine effects. However, both AG and UAG exert pleiotropic actions that involve also receptors, not yet identified, different from the GHS-R1a.

The aim of this thesis was to further elucidate the role of AG, UAG and their receptors on the peripheral regulation of glucose homeostasis.

Chapter I is an overview of the historical background of the ghrelin system. The discovery of ghrelin is an example of reverse pharmacology, it started with the cloning of the GHS-R1a and developed with the identification of ghrelin (AG) as its endogenous ligand. In this chapter the several aspects of the GHS-R, the regulation of ghrelin secretion and the biological activities of AG and UAG are described and give a comprehensive view of the metabolic implications of the ghrelin system. In the last part of this chapter the aim of the present thesis is clarified and refers to the different approaches that are described in details in the following chapters.

Chapter II is a study in which the effects of AG, UAG and their combination on basal and post-prandial glucose homeostasis in GH deficient subjects are investigated. GH deficient individuals were chosen in order to rule out the possible involvement of the GH in the modulation of the metabolic parameters measured in the study (insulin, glucose, free fatty acids). AG and UAG acutely increased fasting glucose and insulin levels. Post-prandially, AG induced a state of insulin resistance, which was completely blocked by the co-administration of UAG. Moreover, the combination of AG with UAG dramatically improved insulin sensitivity *per se*. Interestingly, these long-lasting effects of AG and UAG were observed several hours after the administration, despite the very rapid disappearance of these peptides from the circulation.

In **chapter III** both AG and UAG were showed to have a direct effect on glucose handling in the liver. AG stimulated the glucose output by primary porcine hepatocyte suspension cultures, whereas UAG had an opposite effect. Also in this system the combination of AG with UAG blocked the AG- stimulated (or the UAG-suppressed) glucose release by the hepatocytes. Moreover, UAG partially counteracted

the glucagon-enhanced glucose release. Since the GHS-R1a is not expressed in the hepatocytes, these findings suggest the existence of receptor(s), different from the known GHS-R1a, which mediates AG and UAG effects in the liver.

The study presented in **chapter IV** shows the effects of the ghrelin system on insulin release. Since insulin is secreted into the portal vein and it has to pass the liver before it reaches the systemic circulation, whether AG and UAG have differential effects on portal and systemic insulin response to an intravenous glucose load was evaluated in anesthetized rats. Interestingly, it was observed that UAG is a potent insulin secretagogue. This insulin-releasing effect was marked in the portal vein, but severely blunted in the systemic circulation, suggesting that UAG metabolic action is mainly targeted to the liver. The insulin-secretagogue effect of UAG was very similar to that exerted by a GHS-R1a antagonist and was abolished by the co-administration of AG. AG alone had no effects on glucose-induced portal or systemic insulin levels. Basing on these latter observations, it was speculated that UAG may antagonize the inhibitory tone exerted by endogenous AG on glucose-induced insulin release by pancreatic β-cells.

Taking into account the previous findings of opposite effects of AG and UAG on liver and pancreas, the endogenous AG and UAG concentrations in the portal vein and in the systemic circulation were evaluated in anesthetized rats, both in fasting and in glucose-stimulated conditions. This is described in **chapter V.** Portal concentrations of AG and UAG were found to be higher than the systemic ones. In the portal vein AG represented only 7% of total ghrelin levels and this fraction was further decreased in the systemic circulation. Therefore, the systemic ratio of AG/UAG was lower than the portal one. This portal-systemic gradient of the AG/UAG ratio may be explained by the fact that the liver clears approximately 38% of AG, but only about 11% of UAG. It is known that glucose administration suppresses total ghrelin levels. This study shows that an intravenous glucose load inhibits UAG in the portal and the systemic circulation, whereas it blunts only pre-hepatic, but not systemic, AG concentrations. Moreover, these data suggest that glucose reduces the fraction of UAG cleared by the liver to zero, but it does not change the hepatic clearance of AG.

The studies presented in chapters III-V, as well as other data in the literature, led to hypothesize the existence of a putative UAG receptor, which is the focus of the last part of this thesis (chapter VI).

Chapter VI.1. is a study in which the effects of AG and UAG on INS-1E rat insulinoma cells were evaluated, using insulin secretion after 30 min static incubation as a read-out. In this cell line both UAG and AG stimulated insulin release dose-dependently in the nanomolar range. The AG-induced insulin output was antagonized by two GHS-R1a antagonists, which did not block UAG actions. These

effects occurred in the presence of low levels of GHS-R1a mRNA. The corticotropin-releasing factor 2 receptor (CRF2R), which had been suggested as a putative UAG receptor, was not expressed by INS-1E cell, nor did a CRF2R antagonist modulate the UAG-induced insulin output. Therefore, with this study a sensitive and reproducible assay for specific effects of UAG, *i.e.* insulin release by INS-1E cells, is provided. These data strongly support the existence of a specific receptor for UAG, other than the CRF2R and GHS-R1a.

Furthermore, since UAG counteracts the AG-induced insulin secretion and the pancreas expresses the GHS-R1a, whether UAG might antagonize the AG-induced metabolic effects by blocking the GHS-R1a was verified. This study is described in **chapter VI.2**. Unexpectedly, it was observed that UAG, at concentrations in the high nanomolar to low micromolar range, stimulates $[Ca^{2+}]_i$ flux in cells overexpressing the GHS-R1a and aequorin. Correspondingly, UAG displaced radio-labeled AG from binding to membranes of these cells (IC_{50} of 13 µM). Moreover, the UAG-induced $[Ca^{2+}]_i$ response was prevented by two GHS-R1a antagonists and was abolished in cells expressing the $[Ca^{2+}]_i$ reporter, but not the GHS-R1a. Therefore, at concentrations in the micromolar range, UAG acts as a full agonist of the GHS-R1a. However, these concentrations are far higher than those present in systemic or even portal circulation and than those able to modulate insulin release *in vivo* and *in vitro*. Therefore, an involvement of the GHS-R1a in UAG metabolic effects is very unlikely.

Chapter VII contains a general discussion in which the findings described in this thesis are put in a broader perspective. The beneficial effects of UAG on glucose-induced insulin secretion, insulin sensitivity and hepatic glucose production represent a remarkable novelty in the ghrelin field and might suggest potential implications for treatment of insulin resistant states and type 2 diabetes.

Samenvatting

Ghreline maakt deel uit van het gastrointestinale endocriene systeem, en is tijdens het laatste decennium ontdekt. Ghreline is een darm peptide dat hoofdzakelijk geproduceerd wordt door de maag, maar ook andere gebieden van het maagdarmkanaal kunnen ghreline produceren. Ghreline circuleert in de bloedbaan in twee vormen: geacyleerd ("acylated" ghreline, AG) en ongeacyleerd ("unacylated" ghreline, UAG). AG, welke voor ongeveer 10% bijdraagt aan de totale hoeveelheid ghreline, heeft een vetzuur modificatie die noodzakelijk is voor binding en activering van de groeihormoon secretagoog receptor type 1a (GHS-R1a). GHS-R1a, de enige bekende ghreline receptor tot nu toe, medieert enkele belangrijke biologische effecten van AG, zoals de orexigene en neuroendocriene effecten. Echter, zowel AG als UAG hebben pleiotrope effecten waarbij receptoren, anders dan de GSH-R1a, betrokken zijn. Deze receptoren zijn nog niet geïdentificeerd.

Het doel van dit proefschrift was om de rol van AG, UAG, en hun receptoren in de perifere regulatie van glucose homeostase verder te verklaren.

Hoofdstuk I geeft een historische overzicht van het ghreline systeem. De ontdekking van ghreline is een voorbeeld van "omgekeerde" farmacologie, die begon met het klonering van GHS-R1a, gevolgd door de identificatie van ghreline (AG) als het endogene ligand. In dit hoofdstuk worden de werking van GHS-R, de regulatie van ghreline secretie, en de biologische effecten van AG en UAG beschreven. Verder geeft dit hoofdstuk een uitgebreid beeld van de implicaties van het ghreline systeem op metabolisme. In het laatste deel van dit hoofdstuk wordt het doel van dit proefschrift toegelicht, verwijzend naar de verschillende benaderingen welke in detail beschreven worden in de volgende hoofdstukken.

Hoofdstuk II beschrijft een studie waarin de effecten van AG, UAG, en de combinatie van beide, op basale en postprandiale glucose homeostase worden onderzocht in groeihormoon (GH) deficiënte personen. GH deficiënte personen werden gekozen om een mogelijke betrokkenheid van GH in de modulatie van metabolische parameters gemeten in deze studie (insuline, glucose, vrije vetzuren) uit te sluiten. Er werd gevonden dat AG en UAG een acute toename in glucose en insuline spiegels induceren tijdens vasten. Postprandiaal induceert AG een toestand van insulineresistentie, hetgeen volledig geblokkeerd kan worden door gelijktijdige toediening van UAG. Daarnaast zorgt de gecombineerde toediening van AG en UAG voor een drastische verbetering van de insuline gevoeligheid *an sich*. Een interessante bevinding was dat deze langdurende effecten van AG en UAG enkele uren na toediening waargenomen werden, ondanks de zeer snelle afname van deze peptiden in de circulatie.

Hoofdstuk III laat zien dat zowel AG als UAG een direct effect hebben op het glucosemetabolisme in de lever. Er werd gevonden dat AG de glucoseproductie stimuleert in een primaire kweek van (varkens)-hepatocyten, terwijl UAG een tegenovergesteld effect heeft. Ook werd geconstateerd we dat in dit systeem de gecombineerde toediening van AG en UAG de AG-gestimuleerde (of UAG-onderdrukte) glucose afgifte door hepatocyten tegengaat. Bovendien zorgt UAG voor het gedeeltelijke neutraliseren van de door glucagon-gestimuleerde glucose afgifte. Omdat de GHS-R1a niet in hepatocyten tot expressie komt, suggereren de resultaten dat de AG en UAG effecten in de lever door een receptor, anders dan de reeds bekende GHS-R1a, gemedieerd worden.

De studie beschreven in **hoofdstuk IV** laat de effecten van het ghreline systeem op de insuline afgifte zien. Omdat insuline wordt afgegeven aan het bloed in de poortader en het dus de lever moet passeren voordat het de systemische circulatie bereikt, werd onderzocht of AG en UAG een verschillend effect hebben op de portale en systemische insuline respons na een intraveneuze glucose belasting van verdoofde ratten. Een interessante bevinding was dat UAG een sterke insuline secretagoog is. Dit insuline-releasing effect is aanzienlijk in de poortader, maar is sterk afgezwakt in de systemische circulatie, hetgeen suggereert dat het metabolische effect van UAG voornamelijk gericht is op de lever. Het insuline secretagoge effect van UAG kwam sterk overeen met het effect van een GSH-R1a antagonist, en werd opgeheven door gelijktijdige toediening van AG. Toediening van AG alleen had geen effect op de glucose-geïnduceerde portale of systemische insuline spiegels. Op basis van deze laatstgenoemde waarnemingen veronderstellen we dat UAG het remmend effect van endogene AG op glucose-geïnduceerde insuline afgifte door de β-cellen van de pancreas kan tegenwerken.

Op basis van deze tegengestelde effecten van AG en UAG op de lever en de pancreas, werd in verdoofde ratten de endogene AG en UAG concentraties in de poortader en in de systemische circulatie gemeten, tijdens vasten en onder glucose-gestimuleerde condities. Deze resultaten zijn beschreven in **hoofdstuk V**. De concentraties van AG en UAG in de poortader bleken hoger te zijn dan in de systemische circulatie. In de poortader bedraagt AG slechts 7% van de totale ghreline spiegels en deze fractie is verder verlaagd in de systemische circulatie. Vandaar dat de systemische ratio van AG/UAG lager is dan de portale ratio. Deze portale-systemische gradiënt van de AG/UAG ratio zou verklaard kunnen worden door het feit dat de lever ongeveer 38% van het AG, maar slechts 11% van het UAG, klaart. Het is bekend dat toediening van glucose de totale ghreline spiegels onderdrukt. Er werd vastgesteld dat een intraveneuze glucose belasting zowel de UAG concentratie in de poortader als in de systemische circulatie onderdrukt, terwijl het alleen de AG concentraties voor de lever, maar niet de systemische concentraties, onderdrukt.

Bovendien suggereren de data dat glucose de fractie UAG geklaard door de lever reduceert tot nul, terwijl het de klaring van AG door de lever niet verandert. De studies beschreven in de hoofdstukken III-V, naast data beschreven in de literatuur, suggereren het bestaan van een mogelijke UAG receptor, hetgeen de focus is van het laatste deel van dit proefschrift (hoofdstuk VI).

Hoofdstuk VI.1. is een studie naar de effecten van AG en UAG op rat insulinoma cellen (INS-1E), waarbij de insuline secretie na 30 min incubatie als uitleeswaarde gebruikt wordt. In deze cellijn stimuleren zowel UAG als AG de insuline afgifte op een dosis afhankelijk manier in de nanomolaire range. De AG-geïnduceerde insuline productie werd geblokkeerd door twee GHS-R1a antagonisten, welke de UAG werking niet remden. Deze effecten vonden plaats in de aanwezigheid van lage GHS-R1a mRNA concentraties. De corticotropin-releasing factor 2 receptor (CRF2R), waarvan verondersteld wordt dat het een mogelijke UAG receptor is, kwam niet in de INS-1E cellen tot expressie. Bovendien had een CRF2R antagonist geen effect op de UAG-geïnduceerde insuline productie. Deze studie biedt dus een gevoelige en reproduceerbare assay voor specifieke effecten van UAG, namelijk insuline afgifte door INS-1E cellen. Deze resultaten bieden een sterke ondersteuning voor het bestaan van een specifieke UAG receptor, naast de CRF2R en GHS-R1a.

Omdat UAG de AG-geïnduceerde insuline secretie tegengaat en de pancreas de GHS-R1a tot expressie brengt, is er vervolgens gecontroleerd of UAG een antagonistische werking heeft op de AG-geïnduceerde metabolische effecten door de GHS-R1a te blokkeren. Deze studie wordt beschreven in **hoofdstuk VI.2**. Onverwachts is vastgesteld dat UAG, bij concentraties in de hoog nanomolaire tot laag micromolaire range, de $[Ca^{2+}]_i$ flux stimuleert in cellen waarin GHS-R1a en aeqourin tot overexpressie komen. Bovendien verdringt UAG radioactief gelabeld AG gebonden aan membranen van deze cellen (IC_{50} = 13 µM). Daarnaast kon deze UAG-geïnduceerde $[Ca^{2+}]_i$ respons geremd worden door twee GHS-R1a antagonisten, en was deze respons afwezig in cellen waarin alleen de $[Ca^{2+}]_i$ reporter, maar niet de GHS-R1a, tot expressie kwam. Dus, UAG werkt als een volledige agonist van de GHS-R1a bij concentraties in de micromolaire range. Echter deze concentraties zijn veel hoger dan de concentraties aanwezig in de systemische of zelfs portale circulatie, en de concentraties die in staat zijn om de insuline afgifte *in vivo* en *in vitro* te moduleren. Een rol voor de GHS-R1a in de metabolische effecten van UAG lijkt daarom zeer onwaarschijnlijk.

Hoofdstuk VII bevat een algemene discussie, waarin de resultaten beschreven in dit proefschrift in een breder perspectief worden geplaatst. De gunstige effecten van UAG op glucose-geïnduceerde insuline secretie, insuline gevoeligheid, en glucose productie in de lever, zijn opmerkelijke nieuwe bevindingen in het ghreline veld. Deze resultaten hebben mogelijk gevolgen voor de behandeling van insuline resistentie condities en type 2 diabetes.

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This thesis is dedicated to my parents and my sister: Paola, Piero and Chiara.

Curriculum vitae

Carlotta was born in Turin, Italy, the 13th December 1972.

After completing the Scientific Lyceum in Turin in 1991, she was admitted at the Faculty of Medicine, University of Turin, for its 6-year duration. In 1995, as a medical student, she joined the Division of Endocrinology and Metabolism, Department of Internal Medicine, University of Turin (Chairs: Prof. Ezio Ghigo and Prof. Franco Camanni), where she was involved in clinical research on neuroendocrine and metabolic abnormalities in obesity, with particular attention to the role of GABA- and growth-hormone secretagogue (GHS)- receptors on the hypothalamo-pituitary-adrenal (HPA) axis and the GH-IGF-I axis. In October 21st 1997, she obtained the Medical Degree, discussing the thesis "Hypothalamo-Pituitary Adrenal (HPA) axis in obesity. Effects of CRH, hexarelin and alprazolam on ACTH and cortisol release". In April 1998 she obtained the license to practice as a medical doctor from the University of Turin. Between 1997 and 1998, as a post-graduate fellow, she further investigated the neuroendocrine and metabolic alterations in simple obesity and in Cushing's disease (supported by a grant from the Italian foundation for the study of Endocrine and Metabolic Diseases). In 1998 she was admitted at the School of Specialization in Endocrinology and Metabolism in Turin, for its 5-year duration. In November 28th 2003 she obtained the post-graduate degree in Endocrinology and Metabolism, magna (70/70) *cum laude*, discussing the thesis "Effects of ghrelin on glucose metabolism". In 2000 she approached the basic research at the Laboratory of Molecular and Cellular Endocrinology, Department of Internal Medicine (Chair: Prof. Ghigo), where she collaborated to the main lines of research: i) IGF/IGFBP system in cardiomyocytes and endothelial cells and ii) effects of somatostatin and its synthetic analogues on cardiovascular system. In 2002 she joined the Division of Endocrinology (Chair: Prof.dr. Aart Jan van der Lely), Erasmus MC, Rotterdam, The Netherlands, as part of a collaboration project between the Universities of Turin and Rotterdam (Prof. Ghigo and Prof.dr. van der Lely, respectively), to study the basic aspects of the role of ghrelin on glucose homeostasis. From 2003 to 2007 she worked as a PhD fellow under the leadership of Prof.dr. van der Lely to clarify the gluco-metabolic aspects of the ghrelin system.

Since june 2007 she works as a scientific researcher at the University of Turin, Department of Anatomy, Pharmacology and Forensic Medicine, Section of Pharmacology, under the leadership of Prof. Giampiero Muccioli, to further investigate the role of the ghrelin system in diabetes mellitus.

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by Carlotta Gauna

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