REVIEW

Unraveling the role of the ghrelin gene peptides in the endocrine pancreas

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Abstract

The ghrelin gene peptides include acylated ghrelin (AG), unacylated ghrelin (UAG), and obestatin (Ob). AG, mainly produced by the stomach, exerts its central and peripheral effects through the GH secretagogue receptor type 1a (GHS-R1a). UAG, although devoid of GHS-R1a-binding affinity, is an active peptide, sharing with AG many effects through an unknown receptor. Ob was discovered as the G-protein-coupled receptor 39 (GPR39) ligand; however, its physiological actions remain unclear. The endocrine pancreas is necessary for glucose homeostasis maintenance. AG, UAG, and Ob are expressed in both human and rodent pancreatic islets from fetal to adult life, and the pancreas is the major source of ghrelin in the perinatal period. GHS-R1a and GPR39 expression has been shown in β -cells and islets, as well as specific binding sites for AG, UAG, and Ob. Ghrelin colocalizes with glucagon in α -islet cells, but is also uniquely expressed in ϵ -islet cells, suggesting a role in islet function and development. Indeed, AG, UAG, and Ob regulate insulin secretion in β -cells and isolated islets, promote β -cell proliferation and survival, inhibit β -cell and human islet cell apoptosis, and modulate the expression of genes that are essential in pancreatic islet cell biology. They even induce β -cell regeneration and prevent diabetes in streptozotocin-treated neonatal rats. The receptor(s) mediating their effects are not fully characterized, and a signaling crosstalk has been suggested. The present review summarizes the newest findings on AG, UAG, and Ob expression in pancreatic islets and the role of these peptides on β -cell development, survival, and function.

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Introduction

Ghrelin is a 28-aminoacid peptide initially isolated from human and rat stomach as an endogenous ligand for the GH secretagogue receptor type 1a (GHS-R1a; Kojima *et al.* 1999). Ghrelin peptides exist in two major molecular forms, acylated ghrelin (AG) and unacylated ghrelin (UAG). The acylation, catalyzed by ghrelin *O*-acyltransferase (GOAT; Gutierrez *et al.* 2008, Yang *et al.* 2008), occurs on the third residue (Ser), and is essential for binding to GHS-R1a (Fig. 1), which is responsible for ghrelin GH-releasing and orexigenic central activities (Howard *et al.* 1996, Sun *et al.* 2004). Circulating ghrelin is mainly produced in the stomach; however, lower amounts have been detected in other central and peripheral tissues, including the gastrointestinal tract, pancreas, brain, pituitary gland, kidney, lung, heart, and placenta. Similarly, GHS-R1a is expressed in a variety of tissues (suggesting that

AG may target them) besides the hypothalamus and pituitary (Kojima *et al.* 1999, Gnanapavan *et al.* 2002, van der Lely *et al.* 2004). Indeed, AG was demonstrated to act as an autocrine/paracrine factor, regulating cell proliferation and survival, apoptosis, inflammation, cardiovascular and gastric functions, metabolism, angiogenesis, development, and reproduction (Muccioli *et al.* 2007, Dezaki *et al.* 2008, Chanoine *et al.* 2009, van der Lely 2009).

UAG, which circulates in amounts far greater than AG, does not bind to GHS-R1a, and is devoid of effects on GH release (van der Lely *et al.* 2004, Muccioli *et al.* 2007). Initially thought to be nonfunctional, UAG was subsequently proven to exert specific biological activities, some of which are similar to and others opposed to or independent of AG. Interestingly, AG and UAG recognize common binding sites, and exert functions in cells and tissues that do not express the GHS-R1a, implying the existence of an as yet unidentified receptor

Figure 1 Ghrelin mRNA is translated into a 117-amino acid ghrelin precursor, prepro-ghrelin. The 28-aminoacid peptide ghrelin derives from cleavage of the N-terminal fragment of prepro-ghrelin. Obestatin originates from the C-terminal fragment of the ghrelin precursor. Ghrelin exists in two main forms: acylated and unacylated. Ghrelin acylation on serine 3, promoted by GOAT, is essential for binding to the GHS-R1a and for its endocrine functions. Unacylated ghrelin is devoid of GHS-R1a affinity, and its receptor remains to be identified. Obestatin was proposed as the natural ligand of the GPR39 orphan receptor; however, this finding has been questioned, and the identity of obestatin receptor(s) remains uncertain. GHS-R1a, GH secretagogue receptor type 1a; GPR39, G-protein-coupled receptor 39; GOAT, ghrelin acyl transferase.

for the ghrelin isoforms (Fig. 1; Soares & Leite-Moreira 2008, Inhoff *et al.* 2009, van der Lely 2009).

The 23-aminoacid amidated peptide obestatin (Ob) is a novel ghrelin gene product, which was identified as the G-protein-coupled receptor 39 (GPR39) ligand and claimed to be a physiological opponent of AG (Zhang *et al.* 2005, 2008). However, these findings have lately been questioned, and Ob physiological relevance remains unclear (Fig. 1; Gourcerol & Tache 2007, Ren *et al.* 2008a, Tang *et al.* 2008). Ob is mainly produced in the stomach by the same endocrine cells as ghrelin, and at lower level in the pancreas (Granata *et al.* 2008, Gronberg *et al.* 2008, Volante *et al.* 2009). Central activities have been reported for Ob, i.e. inhibition of thirst, modulation of mnemonic functions, of anxiety and sleep, but also peripheral effects. At the cellular level, Ob regulates cell proliferation and survival (Granata *et al.* 2008, Tang *et al.* 2008), and was found to stimulate GH release from rat tumor somatotrope cells (Pazos *et al.* 2009).

Recently, most of the interest has focused on the role played by the ghrelin gene peptides in the modulation

of pancreatic function. Thus, in the present review, we will summarize the newest findings on AG, UAG, and Ob expression and their effects in endocrine pancreatic cells. In particular, we will consider the implication of these peptides for pancreatic β -cell development, survival, and secretory activity.

AG, UAG, and Ob expression in the endocrine pancreas

The pancreas is composed of exocrine cells essential for nutrient digestion, and of endocrine cells, regulating glucose homeostasis. The islets of Langerhans, which are the functional units of the endocrine pancreas, consist of five different cell types: glucagon-secreting alpha (α)-cells, insulin-secreting beta (β)-cells, somatostatin-producing delta (δ)-cells, pancreatic polypeptide (PP)-containing cells, and the recently identified ghrelin-producing epsilon (ϵ)-cells (Assmann *et al.* 2009).

The ghrelin and GHS-R1a mRNAs and proteins have been identified in both human and rat pancreatic islets (Date *et al.* 2002, Dezaki *et al.* 2004). GHS-R1a immunoreactivity colocalizes with glucagon in most rat α -cells and in some of the β -cells. Both ghrelin and GHS-R1a-like staining were instead detected in the same cells in the periphery of rat islets (Kageyama *et al.* 2005). Furthermore, in human pancreatic islets, GHS-R1a immunoreactivity partially overlaps with insulin-positive β -cells, as demonstrated by double immunofluorescence staining for both GHS-R1a and insulin (Granata *et al.* 2007), indicating that human β -cells might also be responsive to ghrelin stimulation. Like the two forms of ghrelin, GHS-R1a expression was shown in human and rat developing pancreatic islets, from early gestation to adult (Date *et al.* 2002, Chanoine & Wong 2004, Granata *et al.* 2007, Ueberberg *et al.* 2009).

In normal rats, ghrelin-immunoreactive cells have been observed in the peripheral region of the islet of Langerhans, whereas in diabetic rats, ghrelin immunoreactivity was discerned in the central region of the islets, with a more intense staining (Adeghate & Ponery 2002). Ghrelin and its receptor colocalization with other islet hormones are still a matter of debate. The existence of a new islet cell population expressing ghrelin was initially postulated by Wierup *et al.* They provided evidence for ghrelin absence from other islet endocrine cells, with the exception of a short perinatal period in rats, when a minor ghrelin cell subpopulation expressed glucagon and PP as well (Wierup *et al.* 2002, 2004). More recently, ghrelin and preproghrelin immunostaining have been shown to be either dependent or independent of glucagon staining, confirming the existence of at least two different ghrelin cells in the pancreas (Walia *et al.* 2009). The majority of ghrelin-producing cells represent a unique population, named the ε -cell type, which enlarges when genes essential for β -cell development are knocked down (Prado *et al.* 2004). Only one study reported β -cell restricted ghrelin expression in the human pancreas (Volante *et al.* 2002). Ghrelin expression in insulin-producing cells was, however, also demonstrated in rodent-derived β -cell lines (Granata *et al.* 2007). Interestingly, AG and UAG were found expressed both in the adult and perinatal rat pancreas (Date *et al.* 2002, Chanoine *et al.* 2006), suggesting autocrine/paracrine effects throughout the organ's development.

Consistent with the assumption that ghrelin cells represent a specific islet cell type, Ob colocalizes with ghrelin in rat pancreatic islet periphery, its distribution differing from the other islet hormones, as demonstrated by immunohistochemical and electron microscopy studies (Zhao *et al.* 2008, Walia *et al.* 2009). Several other authors confirmed these findings in both fetal and adult human pancreas (Granata *et al.* 2008, Gronberg *et al.* 2008, Volante *et al.* 2009). Interestingly,

although not coexpressed with insulin-producing β -cells in the pancreas, Ob secretion has been shown in human pancreatic islet cells and in the INS-1E β -cell line, suggesting that, similarly to ghrelin, Ob may act in the pancreas through autocrine/paracrine mechanisms (Granata *et al.* 2008). Besides GPR39 potentially being the receptor of Ob (Zhang *et al.* 2005, 2008, Dong *et al.* 2009), its expression has been demonstrated both in human and rodent endocrine pancreas and in β -cell lines (Holst *et al.* 2009, Tremblay *et al.* 2009), raising the possibility of GPR39 ligands directly influencing pancreatic islet activity.

GOAT has been identified as the enzyme which transfers an octanoate group to ghrelin, a modification essential for its hormonal activities (Gutierrez *et al.* 2008, Yang *et al.* 2008). Notably, both the human stomach and particularly the pancreas express high levels of GOAT mRNA. As both the ghrelin and GOAT transcripts are expressed in the pancreas, it may be hypothesized that a fine tuning of pancreatic AG versus UAG levels may take place and affect pancreatic homeostasis, with particular regard to insulin secretion (Gutierrez *et al.* 2008).

Major genes controlling endocrine pancreatic development with respect to the ghrelin gene

Both in the rodent and human pancreas, ghrelin levels peak at late gestation and gradually decline after birth (Wierup *et al.* 2002, 2004). Indeed, ghrelin mRNA and protein are detectable in the mouse pancreas at different developmental stages (Prado *et al.* 2004). Interestingly, ghrelin onset in the pancreas precedes by far that in the stomach, although pancreatic ghrelin cell number remains lower in adult islets (Wierup *et al.* 2002, 2004). Furthermore, ghrelin content is markedly elevated in fetal pancreas compared with the stomach; thus, at this stage, the pancreas and not the stomach might be the major source of ghrelin (Chanoine & Wong 2004). With regard to Ob, although its concentration in the rat perinatal pancreas is lower than that of AG, a correlation between insulin and Ob levels has been observed in the postnatal pancreas, suggesting a role for it as well (Chanoine *et al.* 2006). Collectively, all the above studies lead to the hypothesis of a developmental function for the ghrelin gene products.

Islet cell differentiation is the end product of complex interactions among timely activated transcription factors during pancreas organogenesis (Fig. 2). The endocrine lineage develops according to the hierarchical expression of the homeodomain-containing transcription factor duodenal homeobox (pancreatic and duodenal homeobox 1, PDX1) in both endocrine and exocrine progenitor cells, followed

Figure 2 Simplified schematic representation of the hierarchical activation of genes involved in islet endocrine cell subtypes specification. Black arrows indicate well-established genetic paths responsible for the progression of a putative common pancreatic progenitor toward differentiation into a specialized hormone-secreting cell (Samson & Chan 2006, Bonal & Herrera 2008). Red arrows indicate the hypothesized paths guiding progenitors toward the ghrelin/obestatin-secreting cells fate (Prado *et al.* 2004, Heller *et al.* 2005, Bonal & Herrera 2008, Wang *et al.* 2008, Soyer *et al.* 2010), while dotted arrows refer to the recently highlighted roles of the transcription factor Rfx6 in pancreatic cell lineage specification (Soyer *et al.* 2010). Please note that the path followed by the ϵ -cell population becomes independent from the one leading to the mixed ghrelin+/glucagon+ population at relatively early stages of pancreatic progenitor differentiation (Heller *et al.* 2005). NEUROG3, neurogenin 3; PDX1, pancreatic and duodenal homeobox 1; NKX2.2, NK2 homeobox 2; NKX6.1, NK6 homeobox 1; PAX4, paired box gene 4; PAX6, paired box gene 6; NEUROD1, neurogenic differentiation 1; RFX6, regulatory factor X, 6; ARX, aristaless-related homeobox; ISL1, islet1.

by the basic helix-loop-helix transcription factors neurogenin 3 (NEUROG3) and the targeted NEUROD1, which initiate the endocrine progenitor cascade. The subsequent turning-on of transcription factors such as NK-homeobox factor 2.2 (NKX2.2), paired box gene 4 (PAX4), or NK-homeobox factor 6.2 (NKX6.2) is essential for cell-type specification in β -, δ - (NKX2.2 and PAX4), PP- (NKX2.2), and α -cells (NKX6.2, PAX6). But while PDX1 expression persists in the mature β -cell participating in insulin gene transcription, most of the above transcription factors tend to be down-regulated as differentiation progresses (Samson & Chan 2006, Oliver-Krasinski *et al.* 2009). Moreover, depending on the developmental day and on the cell type, these

factors may function as repressors or activators of downstream genes, as is the case for NKX2.2.

In mice lacking NKX2.2 (*Nkx2.2*^{-/-}), all β -cells and most α -cells are replaced with ϵ -cells; therefore, it was proposed that the ghrelin-producing ϵ -cells may derive from the same precursor of insulin-producing β -cells (Prado *et al.* 2004). Similar results were obtained in PAX4 mutant mice, suggesting that both genes negatively influence ghrelin expression in favor of insulin and glucagon (Prado *et al.* 2004). Recently, a similar increase in ϵ -cells at the expense of α - and β -cells was shown to result from the knockdown of the transcription factor *Irx3a* in zebrafish (Ragvin *et al.* 2010). Nevertheless, while PAX4 was confirmed to be a transcriptional

repressor of the ghrelin gene in pancreatic endocrine progenitors (Wang *et al.* 2008), a recent report demonstrated *Nkx2.2*^{−/−} direct binding to a promoter region of the ghrelin gene through which it fosters its transcription (Hill *et al.* 2010). Thus, in *Nkx2.2*^{−/−} mice, the cause of pancreatic islet population altered assortment may not be the lack of NKX2.2 repressive activity on the ghrelin gene. Rather, a more complex change in the regulatory mechanisms of the ghrelin gene transcription might have occurred, which needs further investigation. The *Nkx2.2*^{−/−} and *Neurod1*^{−/−} double knockout mice are only one example of how the interaction among a multiplicity of transcription factors determines islet cell type specification: compared with the *Nkx2.2*^{−/−} single knockout mice, the double knockout mice show reduced ϵ - and increased α -cell number, suggesting that the α -cell population is restored at the expense of the ghrelin-producing cells, and that NEUROD1 may be a determinant in ϵ -cell type specification (Chao *et al.* 2007). Only recently, a novel winged helix NEUROG3-dependent transcription factor was identified in zebrafish, regulatory factor X 6 (Rfx6), which is required for the differentiation of pancreatic progenitors into glucagon-, ghrelin-, and somatostatin-secreting cells (Soyer *et al.* 2010). The loose organization of β -cells in Rfx6 morphants suggests that Rfx6 controls genes responsible for β -cell clustering, and, perhaps, that glucagon, ghrelin, or somatostatin are somehow involved in this process. Based on all the above findings and on evidence that ghrelin regulates cell differentiation of several tissues (Kim *et al.* 2005, Filigheddu *et al.* 2007), ghrelin null mice were expected to have impaired endocrine pancreas development. However, ghrelin null mice showed no perturbation of islet cell populations and structure, indicating that ghrelin is not essential for normal islet formation. In addition, the elimination of ghrelin in *Nkx2.2*^{−/−} islets does not restore α - and β -cell populations, confirming that the event responsible for the loss of insulin- and glucagon-producing cells in *Nkx2.2* null mice is not the upregulation of ghrelin (Hill *et al.* 2009).

In the human fetus, ghrelin cells were shown to possess unique ultrastructural features (Wierup & Sundler 2005), and ontogenic and morphogenetic pattern different from that of other islet cells (Andralojc *et al.* 2009). However, at present, the temporal and cellular activation of the net of genes participating in ϵ -cell specification (with respect to the other hormone secreting cells) is still far from being completely unraveled (Fig. 2). Part of ghrelin-producing islet cells derive from NEUROG3-expressing precursors and require NEUROG3 activity for their development, a transcription factor necessary for the differentiation of the endocrine precursor into mature cells (Heller *et al.* 2005). NEUROG3-dependent genes *ARX* and *PAX4*, although playing unique roles in

shaping α -cell destiny, including one of the population coexpressing ghrelin and glucagon, do not participate in the ϵ -lineage specification. On the other side, ϵ -cell formation is antagonized by PAX6, which is also downstream of NEUROG3. PAX6-deficient mice, in fact, exhibit an excess of ϵ -cells at the expenses of α -cells (Heller *et al.* 2005). Thus, apart from those mentioned, most of the genes determining the progression from the earliest progenitors to the ϵ -cells population remain almost completely obscure (Fig. 2).

Survival effects of AG, UAG, and Ob in β -cells and human pancreatic islets

AG has been shown to promote β -cell regeneration and to increase insulin expression and secretion in newborn rats treated with streptozotocin (STZ; Irako *et al.* 2006), a compound traditionally used to study pancreatic regeneration which specifically induces β -cell destruction (Yamamoto *et al.* 1981). The same protective effects against STZ-induced diabetes in newborn rats were recently demonstrated for UAG and Ob (Granata *et al.* 2010). Indeed, in the pancreas of STZ-treated rats AG, UAG and Ob increase insulin expression and secretion, as well as *Pdx1* mRNA (Irako *et al.* 2006, Granata *et al.* 2010), which is essential for insulin transcription and maintenance of β -cell mass (Ackermann & Gannon 2007). UAG and Ob even increase the area and number of pancreatic islets in STZ-treated rats and reduce the expression of the *Bcl2* antiapoptotic gene (Granata *et al.* 2010).

Both AG and UAG promote HIT-T15 and INS-1E β -cell proliferation, and inhibit apoptotic events induced by serum starvation and treatment with cytokines (Granata *et al.* 2006, 2007), a major cause of β -cell loss particularly in type 1 diabetes, but also in type 2 diabetes (Mandrup-Poulsen 2001). These effects involve a receptor differing from the AG canonical receptor, as demonstrated by the absence of both GHS-R1a mRNA and protein on HIT-T15 cells, and by evidence of AG/UAG common high-affinity binding sites on these cells. The signaling pathways elicited by AG and UAG stimulation involve the recruitment of G α protein-coupled receptor/adenylyl cyclase/3',5'-cAMP/protein kinase A pathway, the activation of phosphatidylinositol-3 kinase (PI3K)/AKT, and extracellular signal-regulated kinase (ERK)1/2 (Granata *et al.* 2007; Fig. 3), all of which control β -cell survival, antiapoptotic, and proliferative effects, and ultimately, insulin secretion (Jhala *et al.* 2003, Costes *et al.* 2006).

Either AG or UAG is expressed and secreted from HIT-T15 β -cells, and treatment with antighrelin antibodies increases apoptosis, supporting the survival potential of ghrelin. Also, AG and UAG reduce cytokine-induced nitric oxide (NO) production (Granata *et al.* 2007), which is involved in β -cell dysfunction and death

Figure 3 Main intracellular signaling pathways associated with the survival effects of the ghrelin gene peptides in β -cells and human pancreatic islets subjected to serum deprivation or cytokine treatment. Presumably, AG, UAG, and Ob bind to a GPCR which, through the $G_s\alpha$ subunit of the associated G-protein complex, stimulates AC, resulting in increased cAMP production. The involved receptors are GHS-R1a for AG, an unknown receptor(s) for UAG, and likely GPR39 or GLP1R for Ob. In addition, the existence of common receptor(s) for the three peptides has been suggested. In the case of Ob-induced cAMP elevation, the cAMP-dependent enzyme PKA phosphorylates and activates the transcription factor CREB. The effect of AG and UAG on CREB phosphorylation remains to be studied. The antiapoptotic, proliferative, and survival effects of AG, UAG, and Ob involve the canonical PI3K/AKT and MAPK (ERK1/2) pathways. Furthermore, AG and UAG also decrease NO, which has been associated with β -cell dysfunction and death; this effect was not examined for Ob. All these events contribute to reduced apoptosis, increased β -cell, and human islet cell proliferation and survival. Treatment with the ghrelin gene peptides determines increased glucose-induced insulin secretion in both rat β -cells and human pancreatic islets. Both the survival and insulinotropic actions of Ob are supported by its capacity to increase IRS2 phosphorylation and *IRS2*, *PDX1*, *GK*, and *GLP1R* mRNAs in human islets. (AC, adenylyl cyclase; CREB, cAMP response element-binding protein; ERK1/2, extracellular signal-regulated kinase 1/2; GHS-R1a, GH secretagogue receptor type 1a; GK, glucokinase; GLP1R, glucagon-like peptide-1 receptor; GPR39, G-protein-coupled receptor 39; $G_s\alpha$, G-coupled receptor stimulatory protein; IRS2, insulin receptor substrate-2; NO, nitric oxide; PDX1, pancreatic and duodenal homeobox-1; PI3K, phosphatidylinositol 3-kinase; PKA, protein kinase A.

(McCabe *et al.* 2006). Subsequently, others confirmed the antiapoptotic effects of AG in HIT-T15 β -cells under serum deprivation or treatment with doxorubicin. The pathways involved in these effects were the same as those previously observed; in addition, AG increased the antiapoptotic protein BCL2, decreased the proapoptotic protein BAX, and prevented cytochrome *c* release from mitochondria (Zhang *et al.* 2007). Moreover, AG was recently found to prevent lipotoxicity-induced apoptosis in MIN6 β -cells through the activation of protein kinase B, inhibition of jun N-terminal kinase (JNK) and of the mitochondrial pathway, and reduction of the nuclear translocation of the transcription regulator forkhead box O1 (FOXO1; Wang *et al.* 2010a,b).

In human islets, similarly to β -cell lines, both AG and UAG recognize still unknown common specific binding sites on cell membranes, and GHS-R1a partially colocalizes with some insulin-expressing cells (Granata *et al.* 2007). Likely through these receptors, AG and UAG elicit protective effects similar to those described in pancreatic-derived cell lines against serum starvation- and cytokine-induced apoptosis (Fig. 3).

Thus, based on the above findings and on the presence of the ghrelin-producing ϵ -cell population within the islet, the regulation of β -cell function and survival by the endogenous ghrelin gene products appear to be a most likely event.

It was recently reported that in 90% pancreatectomized rats that showed high hyperglycemia, and decreased β -cell mass and insulin levels, AG administration strongly reduced glucose levels, increased insulin-producing β -cell number, and insulin secretion. On the contrary, ghrelin receptor antagonist administration in pancreatectomized rats worsens glucose levels and β -cell mass, in support of the involvement of GHS-R1a or of endogenous ghrelin in islet cell survival and function (Kerem *et al.* 2009).

Like ghrelin, Ob promotes β -cell proliferation, and reduces cell death and apoptosis induced by serum starvation and cytokine synergism (Granata *et al.* 2008). Furthermore, Ob is secreted by β -cells and human islets (Granata *et al.* 2008). Owing to the uncertainty of GPR39 being the receptor for Ob (Gourcerol & Tache 2007), binding studies were conducted demonstrating that Ob recognizes specific sites on HIT-T15 and INS-1E β -cell membranes, and also interacts with receptors recognized by AG, suggesting crosstalk among the ghrelin gene peptides to produce their pancreatic actions. The signaling pathways involved in Ob antiapoptotic effects are also similar to ghrelin. Indeed, Ob increases cAMP levels, and promotes ERK1/2 and PI3K/AKT phosphorylation (Fig. 3), while the inhibition of these pathways reduces Ob antiapoptotic effects. As Ob survival actions and signaling mechanisms in β -cells are similar to those elicited by the receptor of glucagon-like peptide 1 (GLP1R), it was hypothesized that Ob might also trigger

the GLP1R signaling cascade in β -cells. Indeed, Ob increases *GLP1R* mRNA in INS-1E cells and promotes survival to an extent equal to that of the GLP1 analog exendin-4 (Ex-4), and its survival action is prevented by the GLP1R antagonist Ex-9. Also, like Ex-4, Ob increases gene expression and phosphorylation of insulin receptor substrate 2 (IRS2; Granata *et al.* 2008), which is implicated in either the insulin receptor or GLP1R signaling to regulating β -cell survival and insulin secretion (Park *et al.* 2006). These findings are strengthened by evidence for Ob ability to bind the GLP1R, although at lower affinity than its natural ligand GLP1 or its antagonist Ex-9 (Granata *et al.* 2008).

Ob is secreted by human islet cells and, as already mentioned, is expressed in both fetal and adult human islets, colocalizing with ghrelin, likely in ϵ -cells, but not with other pancreatic hormones (Granata *et al.* 2008, Gronberg *et al.* 2008, Volante *et al.* 2009). Ob treatment of human islets promotes the phosphorylation of cAMP response element-binding protein, required for the maintenance of glucose homeostasis and β -cell survival (Jhala *et al.* 2003), and decreases the phosphorylation of Src, whose expression in β -cells has been related to an inhibitory role on Ca^{2+} -dependent insulin secretion (Cheng *et al.* 2007). Furthermore, in human islets, it upregulates *PDX1*, *IRS2*, and *GLP1R* mRNAs together with glucokinase mRNA, a glucose sensor for insulin secretion which also regulates β -cell mass and function (Terauchi *et al.* 2007; Fig. 3). Thus, in human islets, Ob elicits survival and antiapoptotic effects resembling those observed in β -cells, involving identical signaling pathways. In agreement with this, a recent report demonstrated that in rats, Ob reduces the damage severity of cerulein-induced acute pancreatitis, partly through improving pancreatic blood flow, by inhibiting the proinflammatory cytokine interleukin-1 β release and partly by increasing pancreatic cell viability (Ceranowicz *et al.* 2009). Therefore, like ghrelin, Ob is secreted by β -cells, and acts on them and on their surroundings, suggesting that the endogenous peptide might be part of pancreatic hormones autocrine/paracrine systems.

Effect of AG, UAG, and Ob on insulin secretion in pancreatic islets and β -cells

The role of the ghrelin gene-derived peptides in the regulation of insulin secretion and insulin action remains a controversial topic. AG, UAG, and Ob have been shown to either stimulate or inhibit insulin secretion, depending upon the experimental condition (Salehi *et al.* 2004, Sun *et al.* 2007, Dezaki *et al.* 2008, Soares & Leite-Moreira 2008).

In rat β -cells, AG was found to increase cytosolic free Ca^{2+} concentration, and an insulinotropic effect of the peptide was observed in isolated rat pancreatic islets

(Date *et al.* 2002). Similarly, AG increases insulin secretion from the pancreas of both normal and diabetic rats, at concentrations ranging from 10^{-12} to 10^{-6} M (Adeghate & Ponery 2002). In pancreatectomized rats, administration of AG increases insulin positive β -cell number and plasma insulin levels (Kerem *et al.* 2009). Similarly, in newborn rats treated with STZ, either AG, UAG, or Ob increase β -cell mass and pancreatic insulin expression and secretion from β -cells (Irako *et al.* 2006, Granata *et al.* 2010). In rat INS-1E β -cells, both AG and UAG stimulate insulin release dose dependently in the nanomolar range. AG insulinotropic effect in β -cells may involve GHS-R1a, as indicated by experiments performed in the presence of GHS-R1a antagonists, whereas for UAG, the existence of a different receptor mediating its effect was proposed (Gauna *et al.* 2006). Accordingly, both AG and UAG were found to increase glucose-induced insulin secretion in HIT-T15 β -cells, which do not express the GHS-R1a, suggesting the existence of a different receptor mediating the effects of both peptides (Granata *et al.* 2007).

On the other side, GHS-R1a blockade in isolated rat islets using specific antagonists or through AG inactivation using an antiserum, enhanced glucose-induced insulin release, and intracellular Ca^{2+} concentration. Interestingly, whereas relatively high concentration of AG (10^{-8} M) suppressed insulin release, low concentrations could not (Dezaki *et al.* 2004). Later, the same authors showed that both AG and UAG are released from pancreatic islets, and that AG, but not UAG, reduces glucose-induced insulin release (Dezaki *et al.* 2006). Ghrelin knockout mice were found to escape high-fat diet-induced glucose intolerance because of enhanced insulin release; however, the density and average size of the islets were not significantly different between wild-type and ghrelin knockout mice (Dezaki *et al.* 2006). As opposed to the previously mentioned studies, these latter ones indicate that ghrelin serves as a downward regulator of insulin release and consequently upward regulator of glycemia. The insulinostatic effects of endogenous and exogenous ghrelin are blunted in rat islets pretreated with pertussis toxin, a specific inhibitor of G_i and G_o subtypes of trimeric GTP-binding proteins (Dezaki *et al.* 2007), suggesting signaling through an inhibitory GPR. Interestingly, in brain, pancreas and β -cell line, AG induces the expression of β -cell autoantigen for type 1 diabetes (IA-2 β). AG administration or overexpression of IA-2 β inhibits glucose-stimulated insulin secretion in β -cells, while the downregulation of IA-2 β expression by RNA interference reduced AG inhibitory effects on insulin release, suggesting a functional link among AG, IA-2 β , and insulin secretion (Doi *et al.* 2006).

In keeping with the above findings, other authors reported the inhibitory effects of exogenous AG on

insulin secretion in isolated mouse pancreas (Egido *et al.* 2002), in mouse and rat isolated islets (Colombo *et al.* 2003, Reimer *et al.* 2003, Qader *et al.* 2008), and in β -cell lines (Wierup *et al.* 2004).

It has recently been reported that, at a concentration ten times higher than that of AG, UAG abolishes the inhibitory effect of AG on insulin secretion from mouse and rat pancreatic islets, likely interacting with a receptor other than GHS-R1a (Qader *et al.* 2008). The same group previously showed that in rat pancreatic islets, AG reduces insulin secretion while increasing glucagon release and NO production (Qader *et al.* 2005).

Transgenic mice overexpressing ghrelin under the rat insulin II promoter (RIP-GTg) had a pancreatic UAG content that was 1000 times higher than the control littermates. Such mice showed reduced glucose-stimulated insulin secretion and lower blood glucose levels and triglyceride levels during insulin tolerance test. Insulin secretion from isolated islets was instead indistinguishable from that of nontransgenic littermates (Iwakura *et al.* 2005). These results imply that UAG, differently from AG, has a positive influence on insulin sensitivity.

The understanding of effects of Ob on insulin secretion from pancreatic β -cell lines and pancreatic islets is still limited and controversial, as either stimulation (Granata *et al.* 2008, 2010) or inhibition (Qader *et al.* 2008, Ren *et al.* 2008b, Unniappan *et al.* 2008) has been reported, based also on the peptide concentrations used in the experiments. In the perfused rat pancreas, Ob potentiates glucose-induced insulin release and, when infused at a high concentration, it inhibits glucose-induced insulin release. Furthermore, Ob potentiates the insulin responses elicited by arginine and tolbutamide (Egido *et al.* 2008).

Ob was initially identified as the GPR39 ligand (Zhang *et al.* 2005); however, this finding has been questioned recently, and the receptor for Ob has yet to be determined (Gourcerol & Tache 2007, Tang *et al.* 2008). Interestingly, isolated, perfused islets from GPR39 null mice have recently been shown to secrete less insulin in response to glucose stimulation than islets from wild-type littermates (Holst *et al.* 2009). Moreover, GPR39 null mice fed with either a low-fat/high-sucrose or high-fat/high-sucrose diet show decreased serum insulin levels during an oral glucose tolerance test in the face of unchanged insulin tolerance (Tremblay *et al.* 2009), suggesting that GPR39 is required for insulin secretion and may be a potential target for the treatment of diabetes. Hypothesizing that GPR39 is the Ob receptor, these findings would be in agreement with the stimulatory effects of Ob observed in β -cell lines and human pancreatic islets (Granata *et al.* 2008). The Ob positive effect on insulin secretion is further supported by its capacity to bind to GLP1R (Granata *et al.* 2008), which

mediates the insulinotropic effects of GLP1 and its analog Ex-4 (Drucker 2006).

By now it should be apparent that all three peptides influence pancreatic function at multiple levels. Beside the recruitment of distinct receptors, part of their effects may also be explained by promiscuous receptor coupling to G-proteins. This has already been demonstrated for GHS-R1a (Muccioli *et al.* 2007), for GRP39 (Holst *et al.* 2007), and GLP1R (Montrose-Rafizadeh *et al.* 1999), although it has not been specifically investigated in endocrine pancreatic cells. Only indirect evidence suggests that AG/UAG common unknown receptor expressed in β -cells could potentially activate G_i -dependent pathways (Dezaki *et al.* 2007) as well as G_s -dependent ones (Granata *et al.* 2007).

Concluding remarks

Since ghrelin has joined the pool of 'pancreatic hormones' as a result of the pancreatic ϵ -cell population discovery, much of the interest has focused on implications for diabetes. Indeed, all three ghrelin gene-derived peptides seem to participate in glucose balance by directly influencing insulin secretion. Nevertheless, as both AG and UAG have been shown to either stimulate or inhibit insulin secretion depending upon the experimental condition, the effective role of the ghrelin gene-derived peptides in the regulation of insulin secretion and action remains controversial. The only certain evidence is that systemic AG infusion elevates blood glucose level, while UAG does not. It is now well accepted that AG and UAG pleiotropic activities depend on their affinity for multiple receptors, some of which they share, but are still unknown, some others which they do not, as with GHS-R1a, which probably accounts for most of the differences. For Ob, the studies in this sense are still too limited to draw any conclusions. It seems clear that it stimulates insulin secretion *in vitro*, partly through the binding to GLP1R, possibly but not certainly to GPR39, which may or may not be the high-affinity binding site.

AG, UAG and Ob, GOAT, GHS-R1a, AG/UAG common uncloned receptors and Ob-binding sites, are all expressed at least in rodent pancreatic islets from early gestation to adult. Thus, hypothesizing the presence of a ghrelin autocrine pancreatic system is consequential. Indeed, in pancreatic islets and derived β -cell lines, AG, UAG, and Ob elicit similar protective effects against serum starvation- and cytokine-induced apoptosis *in vitro*, while they promote β -cell proliferation/regeneration and increase insulin expression and secretion in STZ-treated newborn rats. Although the recruited intracellular signaling pathways appear to

be comparable, the direct involvement of the GLP1R machinery may favor Ob for therapeutic exploitation, as it has been for Ex-4 (Exenatide). The same possibility accounts for UAG, which was found to counteract many of the AG diabetogenic effects.

Concerning pancreatic development, it is now established that ϵ -cells are present in the pancreas in large numbers during fetal life and early postnatal period, being reduced after birth. The early appearance of both peptides and receptors might imply an involvement in pancreas ontogenesis. However, at first glance, the ghrelin gene KO mice have no altered phenotype in this sense, suggesting that the three peptides are not essential for pancreas development. Nevertheless, it cannot be excluded that the timely and/or cellular-restricted selective secretion of the three peptides during pancreas development shapes the phenotype of cellular populations, which remain quiescent in normal conditions, but may be recruited or sensitized at need, i.e. in pathological settings involving pancreas inflammation and β -cell dysfunction.

In conclusion, many questions remain unanswered. Among these, may ghrelin secretion within the pancreas influence the physiology of other islet cell populations or vice versa? If so, what are the mechanisms involved in these effects? Which/how many receptors mediate protective effects of these peptides? What would be the role of changes in pancreatic ghrelin production under pathological conditions?

Declaration of interest

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

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