Unacylated ghrelin and obestatin increase islet cell mass and prevent diabetes in streptozotocin-treated newborn rats

Riccarda Granata, Marco Volante\textsuperscript{1}, Fabio Settanni, Carlotta Gauna\textsuperscript{2}, Corrado Ghé\textsuperscript{2}, Marta Annunziata, Barbara Deidda\textsuperscript{1}, Iacopo Gesmundo, Thierry Abribat\textsuperscript{3}, Aart-Jan van der Lely\textsuperscript{4}, Giampiero Muccioli\textsuperscript{2}, Ezio Ghigo and Mauro Papotti\textsuperscript{1}

Laboratory of Molecular and Cellular Endocrinology, Division of Endocrinology, Diabetology and Metabolism, Department of Internal Medicine, University of Turin, Corso Dogliotti 14, 10126 Turin, Italy

\textsuperscript{1}Department of Clinical and Biological Sciences, San Luigi Hospital, University of Turin, Regione Gonzole 10, 10043 Orbassano, Turin, Italy

\textsuperscript{2}Department of Anatomy, Pharmacology and Forensic Medicine, University of Turin, Via P. Giuria 13, 10125 Turin, Italy

\textsuperscript{3}Alizé Pharma, 15, chemin du Saquin Espace Européen, Building G, 69 130 Ecully, France

\textsuperscript{4}Department of Internal Medicine, Erasmus University Medical Center, Erasmus MC, ’s-Gravendijkwal 230, 3015 CE Rotterdam, The Netherlands

(Correspondence should be addressed to R Granata; Email: riccarda.granata@unito.it)

Abstract

The ghrelin gene products, namely acylated ghrelin (AG), unacylated ghrelin (UAG), and obestatin (Ob), were shown to prevent pancreatic β-cell death and to improve β-cell function under treatment with cytokines, which are major cause of β-cell destruction in diabetes. Moreover, AG had been described previously to prevent streptozotocin (STZ)-induced diabetes in rats; however, the effect of either UAG or Ob has never been examined in this context. In the present study, we investigated the potential of UAG and Ob to increase islet β-cell mass and to reduce diabetes at adult age in STZ-treated neonatal rats. One-day-old rats were injected with STZ and subsequently administered with either AG, UAG or Ob for 7 days. On day 70, plasma glucose levels, plasma and pancreatic insulin levels, pancreatic islet area and number, insulin and pancreatic/duodenal homeobox-1 ($Pdx1$) gene expression, and antiapoptotic BCL2 protein expression were determined. Similarly to AG, both UAG and Ob counteracted STZ-induced high glucose levels and improved plasma and pancreatic insulin levels, which were reduced by the diabetogenic compound. UAG and Ob increased islet area, islet number, and β-cell mass with respect to STZ treatment alone. Finally, in STZ-treated animals, UAG and Ob up-regulated insulin and $Pdx1$ mRNA and increased the expression of BCL2 similarly to AG. Taken together, our results suggest that in STZ-treated newborn rats, UAG and Ob improve glucose metabolism and preserve islet cell mass, granting a therapeutic potential in medical conditions associated with impaired β-cell function.

Journal of Molecular Endocrinology (2010) 45, 9–17

Introduction

Ghrelin, a 28-amino acid peptide, was identified as an endogenous ligand for the GH secretagogue receptor type 1a (GHS-R1a; Kojima et al. 1999). Ghrelin acylation, promoted by ghrelin O-acetyltransferase on the third serine residue (Gutierrez et al. 2008, Yang et al. 2008), is critical for GHS-R1a activation and for the peptide endocrine effects, including the stimulation of GH release from pituitary, promotion of food intake, and adiposity (van der Lely et al. 2004). Besides central effects, acylated ghrelin (AG) features many peripheral activities which engage metabolic, endocrine, cardiovascular, reproductive, and immune systems (van der Lely et al. 2004).

Unacylated ghrelin (UAG), which circulates in amounts far greater than those of AG, does not bind to GHS-R1a, and is devoid of central effects on GH release (van der Lely et al. 2004, Muccioli et al. 2007). Initially thought to be nonfunctional, UAG was then proven to exert several biological activities, some of which are similar to and other opposed or independent of those of AG (Baldanzi et al. 2002, Broglio et al. 2004, Thompson et al. 2004, Toshinai et al. 2006). 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 a yet unidentified ghrelin receptor (Baldanzi et al. 2002, Filigheddu et al. 2007, Granata et al. 2007).

Although mainly produced by the stomach, ghrelin has been detected in other central and peripheral tissues (Kojima et al. 1999, van der Lely et al. 2004). In the endocrine pancreas, it localizes to glucagon-producing α-cells and to the ghrelin-producing ε-cells, suggesting a role in islet function (Date et al. 2002, Prado et al. 2004, Wierup et al. 2004, Andralojc et al. 2009).
β-cell survival is essential for the maintenance of normal glucose metabolism, and apoptotic β-cell death is a critical event in both type 1 and type 2 diabetes (Donath et al. 2003). We recently reported that AG and UAG promote proliferation and inhibit serum starvation- and cytokine-induced apoptosis of β-cells and human pancreatic islets (Granata et al. 2006, 2007). Furthermore, both peptides stimulated glucose-induced insulin secretion by β-cells (Granata et al. 2007). However, data regarding ghrelin influence on insulin secretion are still contradictory; both inhibitory and stimulatory effects having been reported (Date et al. 2002, Dezaki et al. 2004).

The 23-amino acid amidated peptide obestatin (Ob), a new ghrelin gene product, was identified as the G-protein-coupled receptor 39 (GPR39) ligand (Zhang et al. 2005, 2008a). Ob was found to reduce food intake and body weight, and to counteract many central and peripheral effects of ghrelin (Zhang et al. 2005). However, these findings, as well as binding to GPR39, have lately been questioned (Lauwers et al. 2006, Bassil et al. 2007, Gourcerol & Tache 2007), and Ob physiological functions are still unclear. Like ghrelin, Ob is mainly produced in the stomach and expressed in the same endocrine cells (Gronberg et al. 2008, Volante et al. 2009). In pancreatic islets, Ob colocalizes with ghrelin, probably in ε-cells, suggesting a role in β-cell function (Gronberg et al. 2008, Volante et al. 2009). Indeed, we recently showed that, similarly to ghrelin, Ob promotes proliferation and survival of β-cells and human pancreatic islets, stimulates insulin secretion and expression in human islet cells, and increases the mRNA of genes involved in β-cell function, survival, and differentiation (Granata et al. 2008).

AG had been reported to prevent diabetes in streptozotocin (STZ)-treated rats (Irako et al. 2006), an animal model of diabetes traditionally used to study pancreatic regeneration (Tsuij et al. 1988). Based on this evidence and our previous findings, we tested the hypothesis that UAG and Ob would also prevent pancreatic islet destruction and improve β-cell function at adult age in newborn STZ-treated neonatal rats. To this end, plasma glucose, plasma and pancreatic insulin levels, and pancreatic islet area and number were investigated in this animal model, following administration of either UAG or Ob, as well as of AG. Furthermore, we analyzed the mRNA expression of insulin and of the transcription factor pancreatic/duodenal homeobox-1 (PDX1), which is essential for pancreatic development, maintenance of β-cell mass, and insulin transcription (Li et al. 2005, Ackermann & Gannon 2007, Kaneto et al. 2008). Finally, expression levels of the antiapoptotic protein BCL2 were investigated.

Materials and methods

Reagents

Rat AG, UAG, and Ob were purchased from Chem Progress (San Giuliano Milanese, Milan, Italy). STZ and Mammalian Cell Lysis Kit were obtained from Sigma Life Science. Real-time PCR reagents were obtained from Applied Biosystems (Monza, Italy). Insulin RIA kit was obtained from DiaSorin (Saluggia (VC), Italy). Anti-insulin antibody was purchased from DAKO Cytomation (Milan, Italy); anti-BCL2 antibody was purchased from Upstate (Milan, Italy); β-actin antibody was purchased from (Sigma); and HRP-conjugated antibody was purchased from Bethyl Laboratories (Bologna, Italy). Enhanced chemiluminescence (ECL) reagent was purchased from Perkin Elmer Life Sciences (Milan, Italy).

Animals

The animals received human care in compliance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, revised 1996), and in accordance with the Italian law (DL-116, 27 January 1992). The scientific project was supervised and approved by the local ethical committee. Pregnant female Sprague–Dawley rats (day 14–15th of pregnancy) were purchased from Harlan Srl (Milan, Italy), caged allowing free access to water, and fed with a standard diet. Natural birth occurred 6–7 days later.

The following groups were studied: 1) Control: rats received a single i.p. injection of citrate buffer (0-05 mmol/l, pH 4-5) on day 1 of birth; 2) AG: rats received single i.p. injection of citrate buffer (day 1) followed by AG administration (100 μg/kg s.c., twice daily), from day 2 to 8; 3) UAG: rats received single i.p. injection of citrate buffer (day 1) followed by UAG administration (100 μg/kg s.c., twice daily), from day 2 to 8; 4) Ob: rats received single i.p. injection of citrate buffer (day 1) followed by Ob administration (100 μg/kg s.c., twice daily), from day 2 to 8; 5) STZ: rats received single i.p. injection of STZ (100 mg/kg body weight) dissolved in citrate buffer (day 1); 6) STZ-AG: rats received single i.p. injection of STZ (day 1) followed by AG administration (100 μg/kg s.c., twice daily), from day 2 to 8; 7) STZ-UAG: rats received single i.p. injection of STZ (day 1) followed by UAG administration (100 μg/kg s.c., twice daily), from day 2 to 8; 8) STZ-Ob: rats received single i.p. injection of STZ (day 1) followed by Ob administration (100 μg/kg s.c., twice daily), from day 2 to 8.

Dams were randomly assigned to the eight groups, and pups from the same litter were assigned to the same group. Pups were left with their mothers. All neonates
were tested on day 2 for glycosuria with Glucofix (Menarini, Firenze, Italy). Only those animals that were glycosuric on day 2 after birth were included in the study. Number of animals in each group was as follows: 12 (Control), 10 (AG), 10 (UAG), 10 (Ob), 10 (STZ), 8 (STZ–AG), 10 (STZ–UAG), and 10 (STZ–Ob). Animals were killed on day 70 after birth by decapitation, and blood samples were immediately collected and centrifuged at 20,000 g for 2 min at 4 °C, and then stored at −20 °C until assayed.

### Pancreas extraction

The pancreatic tissues were removed after killing the rats, and weighed. To assess insulin content, 80 mg of each pancreas were homogenized and centrifuged in 5 ml acid–ethanol solution (75% ethanol, 1.5% 12 M HCl, and 23.5% distilled water). Supernatants were stored at −80 °C. Different pancreatic sections were fixed in 4% paraformaldehyde for immunohistochemical studies.

### Glucose and insulin analysis

Plasma glucose levels were determined using a colorimetric assay (Glucofix, Menarini) in nonfasted animals. Insulin was measured in the pancreas (80 mg) and plasma by RIA as described previously (Granata et al. 2008).

### Morphology and immunohistochemistry

After fixation in 4% paraformaldehyde, pancreases were fixed for 24 h in 10% buffered formalin and embedded in paraffin. Hematoxylin and eosin staining was performed, and the slides were visualized using a digital microscope (DMD108, Leica MicroSystems, Milan, Italy). Islet morphology and the presence/absence of inflammatory infiltrate were recorded. Morphometric analysis included mean number of pancreatic islets (counted in ten fields at medium power) and mean islet area (evaluated in up to ten islets for each case). Immunohistochemical analysis was performed in parallel sections using a specific anti-insulin antibody. Antibody specificity was confirmed on additional rat tissues, including spleen, liver, stomach, and intestine.

### Evaluation of apoptotic cells in pancreatic islets

Five-micron-thick sections, serial to those used for conventional morphological evaluation for the presence of apoptotic bodies in pancreatic islets, were analyzed by means of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) by applying the In Situ Cell Death Detection Kit, POD (Roche Applied Science) according to the manufacturer’s instructions. In addition, immunohistochemical evaluation of activated caspase-3 expression was performed using a rabbit monoclonal antibody (clone 5A1E; diluted 1:200 following pressure-cooker antigen retrieval; Cell Signaling Technology, Boston, MA, USA).

### Real-time PCR

Total RNA extraction and reverse transcription to cDNA from 3 μg RNA were performed as described (Granata et al. 2007). Real-time PCR was carried out on ABI-Prism 7300 (Applied Biosystems) in a 25-μl volume solution containing 2 μl of cDNA using specific TaqMan Gene Expression Assays for rat insulin and Pdx1 under the conditions recommended by the supplier. Results were normalized to 18S rRNA, and relative quantification analysis was performed with ABI Prism 7900 SDS-software, using the comparative Ct method. mRNA levels were expressed as fold induction over control (vehicle).

### Western blotting

Seventy-microgram proteins were resolved in 13% SDS-PAGE. Proteins were treated as described (Granata et al. 2007), and incubated with the specific anti-BCL2 antibody. Blots were reprobed with an antibody against β-actin for normalization. Immunoreactive proteins were visualized using HRP-conjugated anti-rabbit antibody (1:1000) by ECL.

### Statistical analysis

Results are expressed as means ± s.e.m. Statistical analysis was performed using Student’s t-test or one-way ANOVA. The Newman–Keuls test was used for post hoc comparison among individual means. Significance was established when P<0.05.

### Results

#### UAG and Ob reduce blood glucose levels in STZ-treated rats

STZ is diabetogenic, and induces hyperglycemia in rats after 8–10 weeks of administration (Tsuji et al. 1988); therefore, we examined the long-term effects of early treatment with either AG, UAG or Ob in neonatal rats treated with STZ on day 1 of birth. The STZ group showed reduced body weight compared with the control group. Like AG, UAG and Ob, both in the absence and presence of STZ, increased body weight, although this effect was not statistically significant. Pancreas weight was slightly increased by AG and UAG,
with respect to the control, and was significantly increased by Ob. UAG and Ob, as well as AG, counteracted STZ-induced pancreas weight reduction, which was restored to control values (Table 1).

In the STZ group, UAG and Ob, like AG, reduced glucose levels. No effect was observed using the peptides alone (Fig. 1).

### UAG and Ob increase plasma and pancreatic insulin levels in STZ-treated rats

STZ strongly reduced both plasma and pancreatic insulin levels (Fig. 2A and B respectively). UAG and Ob blocked STZ effect by restoring plasma insulin to basal levels, similarly to AG. Moreover, they increased pancreatic insulin in STZ-treated rats. AG, UAG, and Ob groups alone did not differ from the control group receiving saline.

**Table 1** Body and pancreas weights of nonfasted rats on day 70. Data are the means ± S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AG</th>
<th>UAG</th>
<th>Ob</th>
<th>STZ</th>
<th>STZ–AG</th>
<th>STZ–UAG</th>
<th>STZ–Ob</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>278 ± 10.4 (12)</td>
<td>309 ± 14.7 (10)</td>
<td>302 ± 9.4 (10)</td>
<td>307 ± 11.8 (10)</td>
<td>258 ± 8.8 (10)</td>
<td>277 ± 10.9 (8)</td>
<td>270 ± 11.6 (10)</td>
<td>268 ± 13.2 (10)</td>
</tr>
<tr>
<td>Pancreas weight (g)</td>
<td>844 ± 37.7 (12)</td>
<td>885 ± 27.6 (10)</td>
<td>876 ± 31.8 (10)</td>
<td>943 ± 34.6 (10)</td>
<td>817 ± 52.5 (10)</td>
<td>845 ± 32.5 (8)</td>
<td>859 ± 41.3 (10)</td>
<td>837 ± 38.9 (10)</td>
</tr>
</tbody>
</table>

*P<0.05.

### UAG and Ob increase the area and number of pancreatic islets in STZ-treated rats

Based on the evidence of insulin increase and on our recent findings showing the survival action of the ghrelin gene products in β-cells and human islets (Granata et al. 2007, 2008), we next analyzed the area and number of pancreatic islets, which were strongly decreased by STZ treatment (Fig. 3A and B respectively). In the STZ group, UAG and Ob, like AG, increased islet area. UAG, the most effective, also showed a significant effect alone with respect to the control (Fig. 3A). All the peptides counteracted STZ-induced islet number reduction, particularly Ob (Fig. 3B).

Morphometric analysis showed total islet area reduction in the STZ group, which was counteracted by treatment with AG, UAG, and Ob (Fig. 4a–e). Immunoreactive insulin was found in β-cell cytoplasm in all groups. Interestingly, whereas insulin immunoreactivity in the STZ group (Fig. 4g) was relatively weak, islets from the STZ–AG, STZ–UAG, and STZ–Ob groups showed strong insulin staining in many cells (Fig. 4h–j).

Ki-67 staining showed occasional proliferating islet cells, without difference among groups (data not shown). No case showed signs of inflammation, even in the STZ group, except for occasional lymphocytes that were randomly observed, especially in periductal spaces (data not shown).

These results provide evidence that UAG and Ob, like AG, preserve pancreatic islet cell mass in STZ-treated rats and counteract STZ effect on β-cell destruction.

### Effect of UAG and Ob on β-cell gene expression

UAG and Ob effects, along with those of AG, were next determined on the expression of pancreatic insulin and Pdx1. Insulin mRNA was strongly down-regulated in pancreases of the STZ group, and similarly to AG, which displayed the strongest effect, UAG and Ob counteracted STZ inhibitory effect. Insulin mRNA levels were unchanged using the peptides alone (Fig. 5A).

Similarly, Pdx1 mRNA was reduced by STZ. UAG, Ob, and particularly AG blocked this effect and strongly increased Pdx1 expression. AG even increased Pdx1 when administered alone (Fig. 5B).

These results suggest that UAG and Ob, like AG, prevent STZ-induced down-regulation of genes that are the main regulators of β-cell function and survival.

### UAG and Ob promote BCL2 increase in the pancreas of STZ-treated rats

Based on the ability to prevent apoptosis in pancreatic β-cell lines and human islets (Granata et al. 2007, 2008), we determined the effect of UAG and Ob, along with
in STZ-treated newborn rats. As expected, the STZ group showed reduced body and pancreas weight, hyperglycemia, and reduced plasma and pancreatic insulin levels. Treatment with either UAG or Ob counteracted all the STZ diabetogenic effects, by reducing glucose and enhancing insulin levels. Furthermore, similarly to AG, UAG and Ob increased pancreatic islet area and number, which were strongly reduced by STZ, suggesting that these peptides may either prevent β-cell death or stimulate β-cell regeneration. Indeed, the STZ–UAG and STZ–Ob groups, like STZ–AG group, showed increased pancreatic insulin immunoreactivity with respect to STZ alone, where insulin staining was weak and insulin-positive β-cells were strongly reduced.

UAG and Ob, as well as AG, even up-regulated insulin mRNA in STZ-treated animals, suggesting that increased insulin secretion and immunoreactivity may be due to either enhanced β-cell survival and/or regeneration, de novo insulin production or both. Consistently, we described previously that AG, UAG, and Ob stimulate glucose-induced insulin secretion in β-cell lines and human islets (Granata et al. 2007, 2008).

that of AG, on the antiapoptotic protein BCL2 expression. All the ghrelin gene products increased pancreatic BCL2, which was reduced by STZ (Fig. 6); however, by both TUNEL staining of apoptotic islet cells and western blot analysis of caspase-3 activity, we could not observe an increase in islet cell apoptosis (data not shown).

Discussion

This study firstly shows that UAG and Ob preserve islet cell mass and prevent diabetes at adult age in newborn rats treated with STZ. Either UAG or Ob reduced plasma glucose and increased plasma and pancreatic insulin levels. Moreover, they increased islet area, islet number, and β-cell mass, and up-regulated insulin and Pdx1 mRNA and BCL2 protein expression. All these effects were similar to those displayed by AG.

AG had been shown previously to prevent diabetes at adult age in newborn rats treated with STZ (Irako et al. 2006), a toxic compound commonly used to induce both insulin-dependent and noninsulin-dependent diabetes, via pancreatic β-cell destruction (Yamamoto et al. 1981, Tsuji et al. 1988, Schnedl et al. 1994). However, the effects of the other ghrelin gene products UAG and Ob have never been studied in this animal model. Therefore, we determined the long-lasting effect of UAG and Ob, together with those of AG,

Figure 2 Effect of UAG and Ob on plasma and pancreatic insulin levels. Plasma (A) and pancreatic (B) insulin concentrations were measured in 70-day-old rats treated with citrate buffer alone (Control), AG, UAG or Ob, or with STZ, either in the absence or presence of the peptides. Groups: Control (n = 12); AG (n = 10); UAG (n = 10); Ob (n = 10); STZ (n = 10); STZ–AG (n = 8); STZ–UAG (n = 10); STZ–Ob (n = 10). Results are expressed as the means ± S.E.M. *P < 0.01 versus Control; **P < 0.001 versus STZ.

Figure 3 Effect of UAG and Ob on pancreatic islet area and number in STZ-treated rats (day 70). (A) The mean islet area was evaluated by morphometric analysis in at least ten islets for each treatment. Results are expressed as the means ± S.E.M. (n = 4). ##P < 0.001 versus Control; *P < 0.05; **P < 0.01 versus STZ; NS, not significant. (B) Calculation of the mean number of pancreatic islets by morphometric analysis (ten fields were counted). Results, expressed as the number of islets per µm² of pancreatic area, are the means ± S.E.M. (n = 4). ##P < 0.001 versus Control; **P < 0.01 versus STZ; NS, not significant.
This finding is either in agreement (Date et al. 2002, Gauna et al. 2007, Egido et al. 2009) or in disagreement (Dezaki et al. 2004, Wierup et al. 2004, Qader et al. 2008) with other reports and, at present, the role of the ghrelin gene peptides in insulin secretion remains a matter of debate.

Besides the survival effects on β-cells, AG has been reported to possess diabetogenic properties (Dezaki et al. 2008, Vestergaard et al. 2008) which, in some cases, are counteracted by UAG (Broglio et al. 2004, Gauna et al. 2005). Indeed, AG was found to induce hyperglycemia and to reduce insulin secretion (Gauna et al. 2005, Dezaki et al. 2008, Vestergaard et al. 2008), whereas UAG over-expression in mice reduced white fat mass and increased insulin sensitivity (Zhang et al. 2008). These findings strongly suggest the existence of different receptors, probably GHS-R1a for AG and a yet unknown receptor for UAG. Intriguingly, in a number of cell types that do not express GHS-R1a, AG and UAG have been shown to display high affinity binding for a common receptor (Baldanzi et al. 2002, Filigheddu et al. 2007, Granata et al. 2007, Muccioli et al. 2007). Therefore, either the same or the opposite effects exhibited by AG and UAG could be due to their binding to more than one receptor, or to different sites on a common receptor.

With regard to Ob, its binding to GPR39 is still debated (Zhang et al. 2005, 2008a, Lauwers et al. 2006). Recent reports have highlighted GPR39 involvement in islet cell function, insulin secretion, and β-cell gene expression (Holst et al. 2009, Tremblay et al. 2009), which is in agreement with our previous results on the positive effects on β-cell survival and function of Ob, the putative GPR39 ligand (Granata et al. 2008). Identifying UAG receptor and unraveling the controversial issue of Ob receptor are of major importance for understanding the biology of the ghrelin gene peptides. The fact that AG, UAG, and Ob exert similar protective effects both in vitro, in pancreatic β-cells and islets, and in vivo, in the pancreas, strongly suggest the involvement of functionally similar receptors.

In rodent models of diabetes, proliferation and differentiation of pancreatic exocrine duct cells have been implicated in islet cell regeneration and β-cell differentiation (Sharma et al. 1999). Here, UAG and Ob and, similarly, AG increased the expression of the transcription factor PDX1, which is involved in the maintenance of β-cell mass and differentiation of pancreatic duct cells into β-cells (Sharma et al. 1999, Li et al. 2005, Suarez-Pinzon et al. 2005, Ackermann & Gannon 2007). Further studies are required to ascertain whether, besides regulating β-cell mass and function, the ghrelin gene peptides promote differentiation of endocrine progenitors into β-cells. In agreement with the present findings, we showed previously that Ob up-regulates insulin and PDX1 mRNA in human islets (Granata et al. 2008), and the same effect was described for AG in the pancreas of STZ-treated rats (Irako et al. 2006). Notably, this is the first report to show UAG stimulatory effect on pancreatic insulin and Pdx1 gene expression.

STZ causes diabetes by inducing β-cell destruction, partly through stimulation of apoptosis (Yamamoto et al. 1981, Schnedl et al. 1994). Here, either AG, UAG or Ob increased the antiapoptotic protein BCL2 in STZ-treated rats, suggesting that they may prevent β-cell death by enhancing the activity of the antiapoptotic cell machinery. Indeed, several studies have demonstrated the requirement of BCL2 in protection against β-cell apoptosis (Rabinovich et al. 1999, Kim et al. 2008).
UAG, and Ob promote protein (CREB; Pugazhenthi et al. 1999) expression through cAMP-response element-binding protein (CREB; Morimoto et al. 2005). This is in agreement with our previous findings showing that AG, UAG, and Ob promote β-cell and human islet cell survival through AKT and CREB phosphorylation (Granata et al. 2007, 2008). Therefore, we suggest that although BCL2 is up-regulated by the ghrelin gene products on day 70, its protective role against STZ-induced β-cell loss probably occurs at an early phase.

We suggested previously that AG, UAG, and Ob released by β-cells would exert autocrine/paracrine survival action (Granata et al. 2007, 2008). In fact, AG, UAG, and Ob are secreted by and localized in many cells and tissues, including the pancreas (Kojima et al. 1999, van der Lely et al. 2004, Prado et al. 2004, Wierup et al. 2004, Zhang et al. 2005, Granata et al. 2008, Gronberg et al. 2008, Volante et al. 2009). Moreover, GHS-R1a and specific binding sites for the peptides were found in the pancreas and in different cell types and tissues (Baldanzi et al. 2002, Filigheddu et al. 2007, Granata et al. 2007, 2008, Muccioli et al. 2007). Thus, we cannot exclude that in the STZ model, endogenous production of the ghrelin gene products may contribute to protection against β-cell destruction, probably through a regulated network that involves other pancreatic hormones and signaling pathways.

In conclusion, this study firstly shows that UAG and Ob exert long-term beneficial effects on β-cell destruction and glucose homeostasis in STZ-treated newborn rats. Therefore, the in vitro actions of these peptides on β-cells and human islets may be translated into in vivo effectiveness in a model of diabetes.

In terms of clinical application, the ghrelin gene products may be considered as promising lead candidates to treat diabetes.

Our results are consistent with previous findings on the antiapoptotic action of AG, UAG, and Ob in a variety of cell types, including pancreatic β-cells (Baldanzi et al. 2002, van der Lely et al. 2004, Granata et al. 2006, 2007, 2008, Granado et al. 2009). However, we could not detect apoptosis in the islets of STZ-treated rats after 70 days of treatment, probably because STZ-induced β-cell apoptosis is an early event, occurring within few hours of STZ administration (Yamamoto et al. 1981, Morimoto et al. 2005). AG, UAG, and Ob may promote BCL2 increase as a result of specific signaling cascade activation. Indeed, it has been demonstrated previously that metabolic and survival pathways, such as phosphatidylinositol-3-kinase/AKT, up-regulate BCL2 expression through cAMP-response element-binding protein (CREB; Pugazhenthi et al. 2000). This is in agreement with our previous findings showing that AG, UAG, and Ob promote β-cell and human islet cell survival through AKT and CREB phosphorylation (Granata et al. 2007, 2008). Therefore, we suggest that AG, UAG, and Ob prevent STZ-induced diabetes.

**Figure 5** Effect of UAG and Ob on pancreatic gene expression. Insulin (A) and Pdx1 (B) mRNA levels were determined in the pancreas of 70-day-old STZ-treated rats by real-time PCR. Results, normalized to 18S rRNA transcript, are expressed as fold increase over Control and are the mean ± S.E.M. of three independent experiments performed in all pancreases of each group. Control (n = 12); AG (n = 10); UAG (n = 10); Ob (n = 10); STZ (n = 10); STZ–AG (n = 8); STZ–UAG (n = 10); STZ–Ob (n = 10). (*P < 0.05, **P < 0.001 versus Control; *P < 0.05, **P < 0.01 versus STZ; NS, not significant).

**Figure 6** Effect of UAG and Ob on BCL2 expression in the pancreas of STZ-treated rats. Western blot analysis was performed on protein extracts obtained from pancreatic tissues of the groups STZ, AG, UAG, Ob, STZ–AG, STZ–UAG, or STZ–Ob on day 70, using a specific anti-BCL2 antibody. Equal protein loading was determined by reprobing with antibodies to β-actin. A representative blot from three independent experiments (n = 3 for each group) is shown. Graphs represent the densitometric analysis of BCL2 normalized to β-actin and reported as percent of STZ (*P < 0.05 versus Control; **P < 0.001 versus STZ).
compounds for the development of novel therapeutic strategies in the cure of diabetes. Notably, due to the diabetogenic effects of AG (Gauna et al. 2005, Dezaki et al. 2008, Vestergaard et al. 2008), the best therapeutic candidate would be UAG and its analogs. Indeed, UAG counters the diabetogenic effects of AG (Broglio et al. 2004, Gauna et al. 2005) and has shown positive metabolic profile (Zhang et al. 2008b), besides increasing β-cell function and survival (Granata et al. 2007). Finally, although its anti-diabetogenic action requires further investigation, the results obtained in this and other studies (Granata et al. 2008, Egido et al. 2009) suggest that Ob may also be a promising therapeutic molecule.

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.

Funding

This work was supported by grants to R Granata (University of Turin: Ex-50% 2007) and to E Ghigo (Regione Piemonte 2007; Compagnia di San Paolo 2008), by Alizé Pharma, and by Studio delle Malattie Endocrinio Metaboliche (S MEM) Foundation, Turin, Italy.

Acknowledgements

The authors wish to thank Dr Alessandra Baragli, Dr Soraya Allas, Dr Davide Gallo, Dr Cristina Grande, Mrs Marina Taliano, Dr Francesca Scarlatti, and Dr Letizia Trovato for their contribution to the study.

References


Dezaki K, Sone H & Yada T 2008 Ghrelin is a physiological regulator of insulin release in pancreatic islets and glucose homeostasis. Pharmacology and Therapeutics 118 239–249.


Received in final form 12 February 2010
Accepted 9 April 2010
Made available online as an Accepted Preprint 9 April 2010