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Des-Acyl Ghrelin Fragments and Analogues Promote Survival of Pancreatic β-Cells and Human Pancreatic Islets and Prevent Diabetes in Streptozotocin-Treated Rats

Abstract

Des-acyl ghrelin, although devoid of binding to ghrelin receptor (GRLN), exerts many biological effects, including regulation of glucose and lipid metabolism. Indeed, des-acyl ghrelin promotes pancreatic β-cell and human islet cell survival and prevents diabetes in streptozotocin (STZ) treated rats. We investigated whether des-acyl ghrelin fragments excluding serine^{3}, which is essential for binding to GRLN, would display similar actions. Among the different compounds tested, des-acyl ghrelin$_{(6–13)}$ and des-acyl ghrelin$_{(6–13)}$ with alanine substitutions or cyclization, but not with d-amino acid substitutions, showed the best survival effect, similar to des-acyl ghrelin. Des-acyl ghrelin$_{(6–13)}$ even prevented diabetes in STZ-treated rats and protected human circulating angiogenic cells from oxidative stress and senescence, similar to des-acyl ghrelin. These results suggest that not only full-length des-acyl ghrelin but also short des-acyl ghrelin fragments have clear beneficial effects on several tissues in vitro and in vivo.

Ghrelin is a 28 amino acid peptide mainly produced by the stomach but also in other tissues such as the gastrointestinal tract and the pancreas.(1, 2) Ghrelin potently stimulates growth hormone (GH) release from the pituitary and exerts orexigenic activities at the central level.(3) These neuroendocrine actions require acylation on the third serine residue by ghrelin O-acyl transferase (GOAT), and ghrelin acylaton is essential for binding to its receptor, the GH secretagogue receptor type 1a (GHS-R1a), lately designated GRLN.(1, 4-8)

Besides the hypothalamus–pituitary and other central areas, GRLN is distributed in peripheral tissues, including the endocrine pancreas and adipose tissue.(2, 3) Consistently, ghrelin elicits many peripheral actions, including regulation of pancreatic β-cell function and influence on glucose and lipid metabolism.(9-12) Mice deleted for both ghrelin and GRLN genes show improved glucose tolerance and insulin secretion and sensitivity under high-fat diet treatment. In obese, leptin-deficient (ob/ob) mice, ablation of ghrelin was found to increase insulin release and to reduce hyperglycemia, suggesting negative effects of ghrelin on insulin secretion and glucose metabolism.(13, 14) Notably, ghrelin infusion in humans induces acute insulin resistance and lipolysis, and ghrelin levels are strongly increased in insulin-resistant obese individuals, suggesting that ghrelin may contribute to insulin resistance in obesity.(15, 16)

At variance with ghrelin, des-acyl ghrelin is devoid of endocrine activities and GRLN binding. However, des-acyl ghrelin is the most abundant circulating form of ghrelin, exerting a variety of effects, including positive actions on glucose and lipid metabolism.(3, 9, 17) Indeed, des-acyl ghrelin has been shown to modulate the expression of metabolic genes in GRLN-deleted mice tissues, to inhibit lipolysis in adipocytes, and to counteract the ghrelin diabetogenic actions in animals and humans.(18-20)

On the basis of des-acyl ghrelin inabilit to bind GRLN, the existence of a different and yet unknown receptor involved in the effects of the peptide has been proposed. Indeed, common binding sites for both ghrelin and des-acyl ghrelin have been shown in pancreatic β-cells and in a variety of cell types, where both ghrelin and des-acyl ghrelin exert similar effects.(20-22)

Pancreatic β-cell survival is of major importance for maintaining normal glucose metabolism, and β-cell apoptosis is a critical event in both type 1 and type 2 diabetes.(23, 24) We have recently demonstrated that des-acyl ghrelin, like ghrelin, counteracts serum starvation-induced and cytokine-induced β-cell death and apoptosis.(22) In addition, des-acyl ghrelin, like ghrelin and obestatin, the other ghrelin gene-derived peptide, was found to prevent diabetes at adult age in streptozotocin (STZ) treated neonatal rats by reducing blood glucose, and increasing β-cell mass and insulin secretion.(25) These findings indicated that des-acyl ghrelin and ghrelin exert similar effects on β-cell survival. However, on the basis of its greater positive effects on insulin sensitivity and glucose

homeostasis, des-acyl ghrelin is a more appealing candidate for the treatment of metabolic dysfunctions such as insulin resistance and type 2 diabetes.
This study aimed at verifying whether des-acyl ghrelin fragments would mimic the actions of full length des-acyl ghrelin(1–28) on survival of β-cells and human pancreatic islets. These fragments either included or excluded serine3, the site for ghrelin acylation that is essential for GRLN binding.(3)

Results

Des-Acyl Ghrelin$_{(1–14)}$ (Compound **3**) and Des-Acyl Ghrelin$_{(1–18)}$ (Compound **2**), but Not Des-Acyl Ghrelin$_{(1–5)}$ (Compound **4**) and Des-Acyl Ghrelin$_{(17–28)}$ (Compound **5**), Promote HIT-T15 β-Cell Survival

Full length des-acyl ghrelin$_{(1–28)}$ (compound **1**) has been previously shown to protect pancreatic β-cells and human pancreatic islets from cell death induced by either serum starvation or inflammatory cytokines, whose synergism is involved in β-cell death in both type 1 and type 2 diabetes.(22, 26) Here, we investigated the survival effects of des-acyl ghrelin fragments in HIT-T15 β-cells and human pancreatic islets, under both serum starvation and interferon-γ (IFN-γ)/tumor necrosis factor α (TNF-α)/interleukin-1β (IL-1β) synergism. We initially examined the survival effects of fragments including the N-terminal region of des-acyl ghrelin (15–19 amino acid length), as well as its N-terminal and C-terminal regions (5–12 amino acid length).
Compound 1 and its fragments, compounds 2–5 (Table 1 and S1) were tested at increasing concentrations ranging from 1 to 100 nM. Compounds 2 and 3 displayed protective effects against both serum starvation- and cytokine-induced cell death at all the concentrations tested, similar to compound 1 (Figure 1A and C). Conversely, compounds **4** and **5** were inactive (Figure 1B and D).

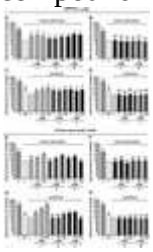

Figure 1. Survival effect of compounds **1**–**5** in HIT-T15 β-cells and human pancreatic islets. HIT-T15 β-cells (A–D) and human pancreatic islets (E–H) were cultured in the presence of serum(s) or serum starved for 24 h, then incubated in serum-free medium (SF) for a further 24 h with the fragments, at the concentrations indicated, either alone or in the presence of cytokines (100 ng/mL IFN-γ, 200 ng/mL TNF-α, and 10 ng/mL IL-1β). Cell survival was assessed by MTT. Results are expressed as percent of control (c, serum-free medium for parts A, B, E, and F; c, cytokines for parts C, D, G, and H) and are the mean ± SE of at least three independent experiments, each performed in quadruplicate ((∗) $P <$ 0.05, (∗∗) $P < 0.01$ vs c; ns, not significant).

Table 1. List of Des-Acyl Ghrelin Fragments

compd	peptide	sequence
1	des-acyl ghrelin$_{(1–28)}$	GSSFLSPEHQRVQQRKESKKPPAKLQPR-COOH
2	des-acyl ghrelin$_{(1–18)}$	GSSFLSPEHQRVQQRKES-NH_2
3	des-acyl ghrelin$_{(1–14)}$	GSSFLSPEHQRVQQ-NH_2
4	des-acyl ghrelin$_{(1–5)}$	GSSFL-NH_2

compd	peptide	sequence
5	des-acyl ghrelin$_{(17–28)}$	ESKKPPAKLQPR-COOH
6	des-acyl ghrelin$_{(6–13)}$	SPEHQRVQ-NH_2
7	des-acyl ghrelin$_{(8–13)}$	EHQRVQ-NH_2
8	des-acyl ghrelin$_{(8–12)}$	EHQRV-NH_2
9	des-acyl ghrelin$_{(8–11)}$	EHQR-NH_2
10	des-acyl ghrelin$_{(9–12)}$	HQRV-NH_2
11	des-acyl ghrelin$_{(9–11)}$	HQR-NH_2
12	retro-des-acyl ghrelin$_{(1–14)}$	QQVRQHEPSLFSSG-COOH

Compounds **2** and **3**, but Not Compounds **4** and **5**, Promote Human Pancreatic Islet Cell Survival

Compound **1** increased human islet cell survival at all the concentrations tested and in both conditions except for 1 nM in serum starved cells. Compound **3** was effective in serum deprived medium at both 10 and 100 nM, whereas under cytokine treatment the survival effect was observed only at 100 nM (Figure 1E and G). Compound 2 increased islet cell survival at 1 and 10 nM, but not at 100 nM, in both experimental conditions (Figure 1E and G). Compounds 4 and 5 were inactive at all the concentrations tested (Figure 1F and H).

These results indicate that compounds **2** and **3** increase islet cell survival in serum deprived medium, similar to compound **1**. Their survival action is instead reduced in the presence of cytokines, where des-acyl ghrelin-induced protection is maintained.

Effect of Des-Acyl Ghrelin$_{(6–13)}$ (Compound **6**), Des-Acyl Ghrelin$_{(8–13)}$ (Compound **7**), Des-Acyl Ghrelin$_{(8–12)}$ (Compound **8**), Des-Acyl Ghrelin$_{(8–11)}$ (Compound **9**), Des-Acyl Ghrelin$_{(9–12)}$ (Compound **10**), and Des-Acyl Ghrelin$_{(9–11)}$ (Compound **11**) on HIT-T15 β-Cell Survival

We next determined whether small fragments of eight to three amino acids, corresponding to the central part of compound **2** (Tables 1 and S1), would display biological effects.

The survival effect of compounds **6**–**11**, compared to that of compound **1**, was assessed in cytokine-treated HIT-T15 β-cells. As expected, cytokine synergism strongly reduced cell survival with respect to normal culture conditions (serum containing medium) (Figure 2A). Compound 6, at all the concentrations tested (1–100 nM) and particularly at 100 nM, potently inhibited cytokine-induced cell death by increasing cell survival to values similar to or even greater than those of serum. This effect was comparable to that of compound 1 (Figure 2A).

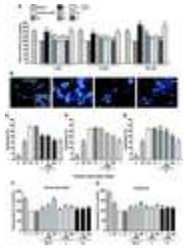

Figure 2. Survival and antiapoptotic effects of small des-acyl ghrelin fragments in HIT-T15 β-cells and human pancreatic islets. HIT-T15 β-cells were starved for 24 h and incubated for further 24 h in the presence or absence of cytokines (CK) (100 ng/mL IFN-γ, 200 ng/mL TNF-α, and 10 ng/mL IL-1β) either alone or with the indicated compounds. Human islet cells were incubated for 72 h in the presence of serum or in serum-free medium either alone or with IFN-γ/TNF-α/IL-1β (5 ng/mL each) and the indicated peptides. (A) HIT-T15 β-cell survival assessed by MTT. The peptides were used at the concentrations indicated. Compound **12** was used as control peptide. Results are the mean ± SE of at least three independent experiments, each performed in quadruplicate ((*) $P < 0.05$, (**) $P < 0.01$ vs control (ns, not significant). (B) Hoechst 33258 nuclear staining (magnification ×200) of HIT-T15 β-cells treated with cytokines either alone or with the indicated

fragments (100 nM). (C–E) HIT-T15 β-cell apoptosis assessed by counting condensed/fragmented Hoechst-stained nuclei. Compounds **1** and **3** were used at 100 nM (s, serum; SF, serum-free medium; CK, cytokines). Values are expressed as percent of apoptotic cells and are the mean ± SE of duplicate determinations (500 cells each) ($n = 3$) ((*) $P < 0.05$, (**) $P < 0.01$ vs CK; ns, not significant). (F, G) Cell survival assessed by MTT in human pancreatic islets. Compound **12** (100 nM) was used as control peptide. Results are the mean ± SE of at least three independent experiments, each performed in quadruplicate ((*) $P < 0.05$, (**) $P < 0.01$ vs control; ns, not significant).

Compound **7**, although less than compound **6**, increased cell survival at all the concentrations examined. Compound **8** and to a similar extent compound **10** displayed significant, although reduced, survival action at 10 and 100 nM only. Compounds **9** and **11** showed reduced or no effect. Retro des-acyl grelin$_{(1–14)}$ (compound **12**) used as negative control in these experiments was inactive.

These results suggest that among the different des-acyl ghrelin fragments examined, compound **6** is the most potent in counteracting cytokine-induced β-cell death.

Compounds **6**–**8** Inhibit Cytokine-Induced Apoptosis in HIT-T15 β-Cells

The antiapoptotic effect of compounds **6**–**8** was next investigated in cytokine-treated HIT-T15 β-cells. As previously reported,(22) apoptosis, assessed by Hoechst staining of apoptotic nuclei, increased under cytokine treatment with respect to serum starvation alone, the cells appearing smaller, scattered, and with fragmented and condensed nuclei (Figure 2B). Compound 6 strongly increased the amount of cells, preserved their shape, and increased small islet formation with respect to cytokine treatment alone (Figure 2B). Moreover, it reduced cytokine-induced apoptosis at 1 and 10 nM and, particularly, at 100 nM, where the antiapoptotic effect was even stronger than that displayed by compound 1 (Figure 2C).

Compound 7, although less than compound 6, reduced apoptosis at 10 and 100 nM (Figure 2B and D), whereas compound 8 was effective only at 100 nM (Figure 2B and E). Compound 12, used as negative control, was inactive (Figure 2C–E).

These results indicate that, as for cell survival, compound **6** is the most potent antiapoptotic fragment in cytokine-treated β-cells.

Compound **6** Promotes Human Pancreatic Islet Cell Survival

In human pancreatic islets, the survival effect of compound **6** was investigated in both serum starved conditions and cytokine synergism. Exendin-4 (Ex-4), an analogue of glucagon-like peptide-1 (GLP-1), which is known to exert survival and antiapoptotic effects in β-cells and human islets,(27) was used as positive control. In both conditions compound 6 increased islet cell survival at 10 and 100 nM with respect to control. These effects were similar to those of compound 1, although reduced compared to Ex-4. Compound 12, used as negative control, was inactive (Figure 2F and G).

Effect of Des-Acyl Ghrelin$_{(6–13)}$ with Either Alanine- or d-Amino Acid Substitutions on HIT-T15 β-Cell Survival

Alanine (Ala) substitutions have been used to determine the contribution of each amino acid to the biological activity of peptide.(28) Moreover, D-scans, substitution of l- by d-amino acids, were principally used to probe conformational effects when conducted as part of a structure–activity relationship (SAR). Indeed, amino acid configuration scan as a means of revealing the stereochemical SAR is an important facet in developing peptides.(28, 29)

The contribution of the amino acid side chains to the survival action of compound 6 was evaluated by systematic alanine replacement of each residue of the peptide sequence (compounds 13–20, Tables 2 and S1). As expected, compound 6 increased β-cell survival in serum-free medium (Figure 3A). All the peptides with Ala replacement, except [Ala^{10}]des-acyl ghrelin$_{(6–13)}$ (compound **17**) and [Ala^{11}]des-acyl ghrelin$_{(6–13)}$ (compound **18**), showed survival action comparable to that of

compound **6** at both the concentrations tested (1 and 100 nM) (Figure 3A). Similar effects were observed under cytokine treatment, where all compounds with Ala substitutions, except compounds **17** and **18**, increased cell viability (Figure 3B). These results suggest that Ala replacement at positions 6–9 (compounds **13**–**16**) and 12 and 13 (compounds **19** and **20**) does not substantially affect the activity of des-acyl ghrelin$_{(6–13)}$, whereas Ala substitution at position 10 (Gln^{10}) or 11 (Arg^{11}) (compounds **17** and **18**) results in a complete loss of activity.

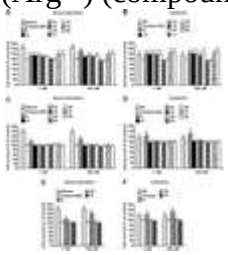

Figure 3. Effect of des-acyl ghrelin$_{(6–13)}$ with Ala or d-amino acid substitutions at positions 6–13 on HIT-T15 β-cell survival. The indicated peptides were tested by MTT at either 1 or 100 nM in both serum deprived medium and in the presence of cytokines. Cells were cultured in serum-free medium for 24 h and for further 24 h in serum starved medium alone or with cytokines (100 ng/mL IFN-γ, 200 ng/mL TNF-α, and 10 ng/mL IL-1β): (A, B) des-acyl ghrelin$_{(6–13)}$ with Ala substitutions; (C, D) des-acyl ghrelin$_{(6–13)}$ with d-amino acid substitutions; (E, F) enantiomer of des-acyl ghrelin$_{(6–13)}$ (rev, reverse fragment). Results are expressed as percent of the control (serum starvation for parts A, C, and E; cytokines for parts B, D, and F) and are the mean ± SE of three independent experiments, each performed in quadruplicate ((*) $P < 0.05$, (**) $P < 0.01$).

Table 2. Des-Acyl Ghrelin$_{(6-13)}$ Derivatives and Analogues

chemical modification	compd	peptide	sequence
alanine substitution	**13**	[Ala^6] des-acyl ghrelin$_{(6–13)}$	APEHQRVQ-NH_2
	14	[Ala^7] des-acyl ghrelin$_{(6–13)}$	SAEHQRVQ-NH_2
	15	[Ala^8]des-acyl ghrelin$_{(6–13)}$	SPAHQRVQ-NH_2
	16	[Ala^9]des-acyl ghrelin$_{(6–13)}$	SPEAQRVQ-NH_2
	17	[Ala^{10}]des-acyl ghrelin$_{(6–13)}$	SPEHARVQ-NH_2
	18	[Ala^{11}]des-acyl ghrelin$_{(6–13)}$	SPEHQAVQ-NH_2
	19	[Ala^{12}]des-acyl ghrelin$_{(6–13)}$	SPEHQRAQ-NH_2
	20	[Ala^{13}]des-acyl ghrelin$_{(6–13)}$	SPEHQRVA-NH_2
d-amino acid substitution	**21**	[d-Ser^6]des-acyl ghrelin$_{(6–13)}$	sPEHQRVQ-NH_2
	22	[d-Pro^7]des-acyl ghrelin$_{(6–13)}$	SpEHQRVQ-NH_2
	23	[d-Glu^8]des-acyl ghrelin$_{(6–13)}$	SPeHQRVQ-NH_2
	24	[d-His^9]des-acyl ghrelin$_{(6–13)}$	SPEhQRVQ-NH_2
	25	[d-Gln^{10}]des-acyl ghrelin$_{(6–13)}$	SPEHqRVQ-NH_2
	26	[d-Arg^{11}]des-acyl ghrelin$_{(6–13)}$	SPEHQrVQ-NH_2
	27	[d-Val^{12}]des-acyl ghrelin$_{(6–13)}$	SPEHQRvQ-NH_2
	28	[d-Gln^{13}]des-acyl ghrelin$_{(6–13)}$	SPEHQRVq-NH_2
cyclization	**29**	cyclo(des-acyl ghrelin$_{(6–13)}$)	c(SPEHQRVQ)
full length d-amino acid substitutions	**30**	d-des-acyl ghrelin$_{(6–13)}$	spehqrvq-NH_2
	31	retro-d-des-acyl ghrelin$_{(6–13)}$	qvrqheps-NH_2

Des-acyl ghrelin$_{(6–13)}$ fragments with d-amino acid substitution at different amino acid positions (6–13) were also tested on HIT-T15 β-cell survival (compounds 21–28, Tables 2 and S1). d-Amino replacement at positions 6–13 (Tables 2 and S1) led to analogues totally devoid of survival effect in both experimental conditions (Figure 3C and D). Similar effects were observed with enantiomer of des-acyl ghrelin$_{(6–13)}$ (d-des-acyl ghrelin$_{(6–13)}$, compound **30**) and the reverse enantiomer of des-acyl ghrelin(6–13) (retro d-des-acyl ghrelin(6–13), compound 31) (Tables 2 and S1 and Figure 3E and F).

These findings suggest that, at variance with Ala substitutions, d-amino acid replacements are detrimental for the biological activity of compound 6. Moreover, they indicate that there is a pronounced stereochemical SAR in that peptide, based on the d-amino acid scan results.

Effect of Des-Acyl Ghrelin(6–13) with Cyclization on HIT-T15 β-Cell Survival

Cyclization has been previously shown to increase the stability of small peptides;(30, 31) therefore, we assessed the survival effect of cyclo-des-acyl ghrelin(6–13) (compound 29) (Tables 2 and S1) (head-to-tail cyclization) in HIT-T15 β-cells cultured in both serum starved and cytokine synergism, compared to that of compounds 1 and 6.

Compound 29 dose-dependently increased cell survival in both experimental conditions and at all concentrations tested (1–100 nM), similar to compounds 1 and 6 (Figure 4). These results suggest that the in vitro survival action of des-acyl ghrelin$_{(6–13)}$ is maintained after cyclization. No effect was observed with compound **12** (100 nM) at any condition tested.

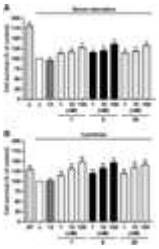

Figure 4. Survival effects of des-acyl ghrelin$_{(6–13)}$ with cyclization. The peptides were tested by MTT at the indicated concentrations in HIT-T15 β-cells in serum-free medium for 24 h (A) or for further 24 h in serum-free medium (SF) with cytokines (100 ng/mL IFN-γ, 200 ng/mL TNF-α, and 10 ng/mL IL-1β) (B). Results are expressed as percent of the control (c, serum starvation and cytokines for A and B, respectively) (n = 3; *, $P < 0.05$; **, $P < 0.01$).

Effect of Compound **6** in Streptozotocin (STZ) Induced Diabetes

Both ghrelin and des-acyl ghrelin have been shown to prevent diabetes in STZ-treated rats.(25, 32) Therefore, we examined the long-term effects of early treatment with compound 6, compared to those of compound 1, in neonatal rats treated with STZ at day 1 of birth. Compound 6 was tested at concentrations that were either equal to or higher than that of compound **1** (30 and 100 nmol/kg, respectively). At day 9 after STZ injection, the animal survival rate, which was decreased by STZ with respect to the control group (∼52%), was strongly increased by compound **1** (∼72%), as well as by both concentrations of compound **6** (∼71% and ∼89% for 30 and 100 nmol/kg, respectively) (Figure 5A). At day 70, plasma glucose was markedly increased in the STZ group. Compound 1, as expected, reduced STZ-induced glucose increase, and compound 6 showed a similar effect, although the higher concentration tested was found to be less effective (Figure 5B). Compound 6, like compound 1, also counteracted STZ-induced reduction of plasma and pancreatic insulin (Figure 5C and D). In untreated animals receiving saline, the peptides alone had no effect on glucose and insulin levels (data not shown).

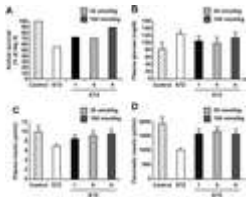

Figure 5. Effects of compound **6** in rats treated with STZ (100 mg/kg body weight) with or without 30 nmol/kg compound **1**, compound **6** at 30 nmol/kg, or compound **6** at 100 nmol/kg. (A) Animal survival at day 9. Data are expressed as percent of control (animals injected with a single dose of citrate buffer). Plasma glucose (B), plasma insulin (C), and pancreatic insulin (D) levels in 70-day-old rats. Control, rats treated with citrate buffer. Results are expressed as the means ± SE: control group (n = 11); STZ group (n = 11); STZ + compound **1** group (n = 16); STZ + compound **6**, 30 nmol/kg group (n = 21); STZ + compound **6**, 100 nmol/kg group (n = 15). ⁎⁎, $P < 0.01$.

These results suggest that compound **6** counteracts the diabetogenic effects of STZ in rats as potently as compound **1**.

Compounds **6** and **29** Protect Human Circulating Endothelial Progenitor Cells (EPCs) from Oxidative Stress and Prevent EPC Senescence

Several lines of evidence indicate that overproduction of reactive oxygen species (ROS) contributes to the impaired EPC bioavailability in diabetes.(33, 34) We have previously reported that des-acyl ghrelin protects human EPC from ROS generation;(17) therefore, the protective effect of compounds 6 and 29 on hydrogen peroxide (H2O2) treated cells was also evaluated. Figure 6A shows that, as for compound 1, compounds 6 and 29 drastically reduced ROS production in response to H2O2. Consistent with our previous findings,(17) treatment with either compound 6 or 29 also prevented Rac1 activation, which has been shown to be required for Nox2-mediated ROS production (data not shown).(35)

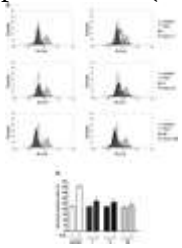

Figure 6. Effects of compounds **6** and **29** on ROS production and senescence in EPCs. (A) ROS production assessed by flow cytometry (DCF-DA assay) in cells treated with H_2O_2 for 2 h: left panels, fragments alone; right panels, fragments with H_2O_2. (B) Cell senescence evaluated by measuring the acidic β-gal (SA-β-gal) activity 24 h after addition of the indicated stimuli. Results are expressed as the percentage of SA-β-gal-positive cells ((⁎) $P < 0.05$, H_2O_2 vs untreated; (#) $P < 0.05$ vs H_2O_2 control. Results are the mean ± SE from three independent investigators.

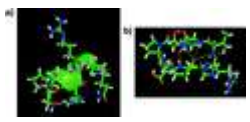

Figure 7. Model of the predicted structure of compounds 6 (A) and 29 (B).

The above results prompted us to evaluate whether the increased ROS production, generally considered as an upstream signal, translates into an accelerated onset of senescence and whether compounds **6** and **29** could rescue this effect. As for compound **1**, compounds **6** and **29** reduced the number of senescence-associated β-galactosidase (SA-β-gal) positive EPCs in response to H_2O_2 (Figure 6B).

Peptide Molecular Modeling

Ghrelin and des-acyl ghrelin were first considered to not possess a well-defined secondary and tertiary structure in solution as defined by circular dichroism (CD) and nuclear magnetic resonance spectroscopy.(36) However, more recent studies performed using molecular dynamic simulation(37) and in silico 3D model or CD spectroscopy(38) showed a clear formation of a short α-helix from Pro^7 to Glu^{13} and from Pro^7 to Ser^{18}, respectively. Currently, the molecular structure of compound **6** has not been analyzed. A blast search on Protein Data Bank (PDB) sequences was performed on compound **6**. No similar structural motif was found. Basic computer molecular modeling of compounds **6** and **29** was performed using Insight II, a comprehensive graphic molecular modeling program, minimized by the use of conjugate gradient after molecular dynamics. The model of compound 6 (Figure 7A) suggests that this compound has an α-helix structure as previously described for the full length des-acyl ghrelin peptide using molecular dynamic simulation(37) and in silico 3D model and CD spectroscopy.(38) The model of compound 29 (Figure 7B) seems to have a more constrained structure with a turn at Arg^{11}-Val^{12}. This structure could potentially be stabilized by interactions between the carbonyl group of Gln^{10} and the amine group of Val^{12}, Gln^{13}, and Ser^6.

Discussion

This study shows that des-acyl ghrelin fragments, particularly compound **6** and its analogues, exert survival effects in pancreatic β-cells and human pancreatic islets, which are comparable to those of their parent molecule. Compound **6** also inhibits STZ-induced diabetes in rats and reduces oxidative stress and senescence in human circulating angiogenic cells.

We focused on des-acyl ghrelin and not on ghrelin because of the previously reported positive effects of des-acyl ghrelin on glucose and lipid metabolism, which are opposed to the insulinostatic actions of ghrelin.(12-15, 15-17, 23) Des-acyl ghrelin fragments were tested in HIT-T15 β-cells and human pancreatic islets, which were previously used to demonstrate the survival and antiapoptotic effects of all the ghrelin gene products (ghrelin, des-acyl ghrelin, and obestatin).(22, 39) β-Cells and human islets were cultured in either serum deprived medium or in the presence of cytokines whose synergism causes β-cell destruction in both type 1 and type 2 diabetes.(23, 24)

Among the different fragments, those including amino acids at positions 1–14 and 1–18 increased survival in both HIT-T15 β-cells and human pancreatic islets, under either serum starvation or cytokine synergism. Conversely, those comprising amino acids 1–5 and 17–28 (N-terminal and C-terminal, respectively) were totally devoid of activity. These results initially suggested that the biologically active domain of des-acyl ghrelin is located in its N-terminal part of the peptide, likely including amino acids 1–18.

When testing a number of small fragments, comprising eight to three amino acids, we observed that the most potent survival effect against cytokine-induced β-cell destruction was harbored by the central region of des-acyl ghrelin, particularly that including amino acids at positions 6–13. Indeed, compound **6**, more than any other, not only increased cell survival but also potently inhibited cytokine-induced apoptosis in HIT-T15 β-cells. In addition, compound **6** even increased survival in human pancreatic islets under both serum starvation and cytokine synergism, and this effect resembled that of compound **1**.

Interestingly, compound **4**, which includes Ser^3, the site for octanoylation responsible for binding to GRLN and for the endocrine activities of ghrelin, was found here to be inactive when not acylated

with *n*-octanoic acid. Conversely, compound **6** displayed strong biological activity, comparable to that of compound **1**. These opposite findings further suggest the existence of different receptors involved in the effects of either ghrelin or des-acyl ghrelin/des-acyl ghrelin fragments. Interestingly, a recent study from Bednarek et al. confirmed that the *n*-octanoyl group of ghrelin is one of the principal structural features determining its potency for GRLN binding.(40) This paper even showed that Ser2 octanoylation is similar to Ser3 in terms of binding to GRLN, suggesting that GRLN does not distinguish between Ser(*n*-octanoyl) in position 2 or 3.

Naturally occurring cyclic proteins, which have been found in bacteria, plants, and animals, show improved activity and exceptional stability; therefore, protein cyclization to improve their in vivo half-life is of potential great importance for proteins of therapeutic value.(30, 31) Here, we tested the effects of compound 29, which normally occurs in linear form. Interestingly, the in vitro survival effect of des-acyl ghrelin(6–13) was fully maintained after cyclization, suggesting the possibility of synthesizing cyclic fragments with increased stability and improved pharmacokinetic properties for clinical use. However, in the present study, des-acyl ghrelin analogues were often observed to be without dose response effect. Whether or not this is due to lack of power of the study design (cell-specific response, noise in the experiments) or that another factor causes it is difficult to answer. Moreover, the lack of dose-dependent effects is not supportive of a receptor specific to des-acyl ghrelin or the foregoing fragments thereof. Definitely, there is a need for more in vitro and in vivo data that give a broader insight into the mechanisms and the pharmacodynamics of des-acyl ghrelin and its potential analogues.

The biological activity of compound **6** was also assessed in vivo in adult diabetic rats that were treated with STZ at neonatal age. We have previously demonstrated the antidiabetogenic actions of des-acyl ghrelin in this animal model of type 1 diabetes and suggested that these effects were at least partly due to the des-acyl ghrelin survival actions in pancreatic β-cells.(22, 25) Here, the effects of compound 6 were tested at concentrations that were either equivalent or higher than that of compound 1, which was used as control peptide. The parameters selected comprised plasma glucose levels, which were reduced by compound 6 in STZ-treated rats, and plasma and pancreatic insulin levels, both increased by compound **6** in the same conditions. These results suggest that compound **6**, like compound **1**, prevents STZ-induced diabetes. Moreover, on the basis of the strong survival effects in β-cells, compound **6** likely elicits its antidiabetogenic actions in vivo, at least partly by reducing the detrimental effects of STZ on β-cell mass.

Interestingly, as previously demonstrated for des-acyl ghrelin, compounds **6** and **29** were found here to protect EPCs from oxidative stress and senescence, further supporting the hypothesis that the des-acyl ghrelin biological activity may reside in its central region, excluding the site for octanoylation.

Likely, the effects observed for both des-acyl ghrelin and active des-acyl ghrelin fragments do not involve GRLN. In addition, although specific des-acyl ghrelin binding sites have been previously described in different cell types,(20, 21) including β-cells(22) and EPCs,(17) the lack of dose–response effect often observed in the present study suggests that des-acyl ghrelin and its analogues may bind as well to nonspecific receptor(s).

Interestingly, previous studies have shown that small peptide or non-peptide agonists may act as allosteric modulator, binding to a different site of GRLN and either increasing or reducing the sensitivity of the receptor for the natural ligand.(41) However, in the present study the cells were incubated only with des-acyl ghrelin fragments in the absence of ghrelin, the natural ligand of GRLN; therefore, allosterism could not be considered.

Furthermore, although the effects on survival and apoptosis were found to be statistically significant, our data do not automatically imply that des-acyl ghrelin or its analogues will have these beneficial effects in a clinical setting. Des-acyl ghrelin analogues for the use in (pre)clinical studies are not available to date, and no data exist on prolonged administration of des-acyl ghrelin (analogues) in the endocrine pancreas.

Conclusions

Collectively, the results of this study show that like their parent molecule, des-acyl ghrelin fragments, particularly compound **6**, increase survival of β-cells and human pancreatic islets, prevent STZ-induced diabetes in rats, and reduce oxidative stress and senescence in human EPCs. Therefore, these findings suggest that des-acyl ghrelin fragments exhibit the same pharmacological profile and therapeutic potential of their parent molecule and may represent promising tools for preservation of β-cell mass and impaired vascular growth in metabolic dysfunctions and diabetes.

Experimental Section

Materials

Compounds **1**–**5** and exendin-4 were purchased from Phoenix Pharmaceuticals (Belmont, CA). All other fragments were obtained from Tib MolBiol (Genova, Italy). Cell culture reagents were purchased from Invitrogen (Milano, Italy).

Peptide Synthesis, Cleavage, and Purification

The different peptides were synthesized on solid support on a Symphony peptide synthesizer (Protein Technologies Inc., U.S.) at room temperature using the standard manufacturer's procedure.(42)

Linear Peptides

C-Terminal free was synthesized (0.8 mmol scale) on a Fmoc-Arg(Pbf)-MPPA(Wang)-MBHA resin. All Fmoc-amino acids (8 mmol, 10 equiv) were coupled with PyBOP/HOBt in DMF. Reactive side chains were protected as follows: Thr, Ser, and Tyr, *tert*-butyl (^{t}Bu) ether; Lys, *tert*-butyloxycarbonyl (Boc) carbamate; Glu, *O*-*tert*-butyl (O^{t}Bu) ester; Asn, His trityl (Trt), and Arg, pentamethyldihydrobenzofuran (Pbf) sulfonylamide. All coupling reactions were monitored by Kaiser's test (or chloranile test for secondary amine). After completion of the chain assembly, the peptides were cleaved from the resin and deprotected by adding 40 mL of mixture TFA/phenol/H_2O/thioanisole/ethanedithiol (82.5:5:5:5:2.5, v/v/v/v/v) agitated at room temperature for 3 h. Crude peptides were purified by preparative reverse-phase HPLC (RP-HPLC) on an Ultrasep ES column (RP-18, 10 μm, 250 mm × 20 mm) using a linear gradient (0–24% over 30 min) of acetonitrile/H_2O + Et_3N + H_3PO_4 (99.8:0.1:0.1, v/v/v). The peptide was desalted on a C18 column using 0.1% TFA buffer.

Synthesis of Compound **29**

Peptide was synthesized (1.5 mmol scale) on Fmoc-Pro-chlorotrityl resin. All Fmoc-amino acids (3 equiv) were coupled with PyBOP/HOBt/DIEA or DIC/HOBt in DMF. Reactive side chains were protected as follows: Ser, *tert*-butyl (^{t}Bu) ether; Glu, *O*-*tert*-butyl (O^{t}Bu) ester; Gln and His, trityl (Trt); and Arg, pentamethyldihydrobenzofuran (Pbf) sulfonylamide. All coupling reactions were monitored by Kaiser's test (or chloranile test for secondary amine). After completion of the chain assembly the peptide–resin was treated with HFIP/DCM (15:85, v/v) for 1 h under gentle agitation. After filtration of the resin, the filtrate was evaporated under vacuum and precipitated in diisopropyl ether. The cyclization was performed in DMF with HATU/NMM. After concentration of DMF, the peptide was washed with H_2O. Cyclic peptide was deprotected by adding a mixture TFA/H_2O/ethanedithiol (90:5:5, v/v/v) agitated 3 h at room temperature. Crude peptide was purified by three successive preparative reverse-phase purifications using an Ultrasep ES column (RP-18, 10 μm, 250 mm × 20 mm) and a linear gradient of acetonitrile: first purification with 0.1% TFA buffer, second purification with TEA/H_3PO_4, 0.1%, buffer, third purification with 0.1% TFA buffer.

Peptide Analysis

The purified peptides were at least 95% pure as determined by analytical RP-HPLC analysis performed on an Ultrasep ES column (RP-18.7 μm, 250 mm × 3.0 mm) using a linear gradient (0–

50% over 20 min) of 0.005% TFA/TFA + acetonitrile + H2O (0.05:80:19.95, v/v/v) (Table S1). The peptides were further characterized by MALDI-TOF mass spectrometry on a MALDI 2 DE instrument (Shimadzu, Japan).

Cell Culture

Hamster HIT-T15 β-cells were obtained and cultured as previously described.(22)

Human Islet Isolation

Human islets were obtained from pancreases of multiorgan donors as described.(22, 39) Islet preparations with purity of >70%, not suitable for transplantation, were used after approval by the local ethical committee. Islets (10 000) were cultured in CMRL (Invitrogen) with 10% FBS.

Cell Survival Assay

Cell survival was assessed by 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) as previously described.(22) Cells were seeded on a 96-well plate at a density of 5 × 103 cells/well and 25 islets/well for HIT-T15 cells and human pancreatic islets, respectively. After treatments, cells were incubated with 1 mg/mL MTT for ~1 h. The medium was aspirated and the formazan product solubilized with 100 μL of DMSO. Viability was assessed by spectrophotometer at 570 nm absorbance using a 96-well plate reader.

Hoechst Staining of Apoptotic Cells

Morphological changes in the nuclear chromatin of apoptotic cells were detected by Hoechst 33258 staining. HIT-T15 cells, harvested by PBS–EDTA, were pooled with the cells from conditioned medium, fixed with 4% formaldehyde in PBS for 15 min at 4 °C, washed, resuspended in 70% ethanol, and stored at −20 °C until use. Cells were then washed twice in PBS and stained in 50 μL of PBS containing 10 μg/mL Hoechst 33258. Following 15 min of incubation at room temperature, a 15 μL aliquot was placed on a glass slide and 500 stained nuclei were double counted under a fluorescence microscope (DAPI filter).

Isolation, Characterization, and Culture of EPCs

Peripheral blood mononuclear cells were recovered and cultured onto collagen type 1 coated dishes for 21 days in EGM-2 medium (Cambrex, Walkersville, MD) as described by Yoder et al.(43) Fluorescence activated cell sorter analysis was used to characterize EPC surface markers: CD45, CD14, CD34, CD31, Tie-2, KDR, vWF.

Detection of ROS

Kinetic analysis of ROS production was performed by using DCF-DA (5(and 6)-carboxy-2′,7′-dichlorofluorescein diacetate, 0.5 μM final concentration) (Molecular Probe, Invitrogen) assay. The majority of experiments using the DCF-DA assay were performed after 2 h of the indicated treatments, as previously described.(17) H2O2 (150 μM) was used as positive control.

EPC Senescence Assay

Senescence was evaluated by measuring the acidic β-gal activity, as described by Togliatto et al.(17) on EPCs treated with or without compound 1 (1 μM), compound 6, or compound 29 (10 μM). Briefly, EPCs were washed in phosphate-buffered saline (PBS), fixed for 3 min at room temperature in 2% paraformaldehyde, washed, and incubated for 24 h at 37 °C with fresh SA-β-gal stain solution: 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside (X-gal), 5 mM potassium ferrocyanide, 5 mM ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet P-40. Senescence was expressed as the percentage of SA-β-gal-positive cells over a total of 100 cells, manual count at 20× magnification by three independent investigators. The acidic β-galactosidase staining kit was from Invitrogen.

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, January 27, 1992). The scientific project was supervised and approved by the local ethical committee. Pregnant female Sprague–Dawley rats (days 14 and 15 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.

Five experimental groups were studied, following a previously described protocol:(25) (1) control, rats received a single ip injection of citrate buffer (0.05 mM, pH 4.5) at day 1 of birth; (2) STZ, single ip injection of STZ (100 mg/kg body weight), dissolved in citrate buffer (day 1); (3) STZ + compound **1**, single ip injection of STZ (day 1) followed by compound **1** (30 nmol/kg sc, twice daily), from day 2 to day 8; (4) STZ + compound **6**, single ip injection of STZ (day 1) followed by compound **6** (30 nmol/kg sc, twice daily), from day 2 to day 8; (5) STZ + compound **6**, single ip injection of STZ (day 1) followed by compound **6** (100 nmol/kg sc, twice daily), from day 2 to day 8. Dams were randomly assigned to the five 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 at day 2 after birth were included in the study. Number of animals in each group was as follows: 11 (control), 11 (STZ), 16 (STZ + compound **1**), 21 (STZ + compound **6**, 30 nmol/kg), and 15 (STZ + compound **6**, 100 nmol/kg). Animals were killed at day 70 after birth by decapitation and blood samples immediately collected and centrifuged at 20000*g* for 2 min at 4 °C, then stored at −20 °C until assayed.

Glucose and Insulin Analysis

Plasma glucose levels were determined using a colorimetric assay (Glucofix, Menarini, Firenze, Italy) in nonfasted animals. Insulin was measured from pancreas (80 mg) and plasma by RIA, as previously described.(39)

Statistical Analysis

Statistical analyses were performed with Student's *t* test for independent samples or by two-way ANOVA followed by Newman–Keuls post hoc test for multiple comparisons, using GraphPad Prism, version 5.0, software (GraphPad Software, Inc., San Diego, CA). Results are presented as the mean ± SE and considered significant for $P < 0.05$.

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Abbreviations Used

CD	circular dichroism
DCF-DA	5(and 6)-carboxy-2′,7′-dichlorofluorescein diacetate
DCM	dichloromethane
DIC	*N*,*N*′-diisopropylcarbodiimide
DIEA	*N*,*N*-diisopropylethylamine
DMF	*N*,*N*-dimethylformamide
DMSO	dimethyl sulfoxide
EDTA	ethylenediaminetetraacetic acid
EPC	endothelial progenitor cell
Ex-4	exendin-4
FBS	fetal bovine serum
Fmoc	fluorenylmethyloxycarbonyl
GH	growth hormone

GRLN	ghrelin receptor
HATU	2-(1*H*-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluranium hexafluorophosphate methanaminium
HFIP	hexafluoroisopropanol
HOBt	hydroxybenzotriazole
STZ	streptozotocin
IFN-γ	interferon γ
IL-1β	interleukin-1β
MBHA	4-methylbenzhydrylamine
MPPA	methylphenoxypropionic acid
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide
NMM	*N*-methylmorpholine
PBS	phosphate buffered saline
PyBOP	benzotriazol-1-yloxytrispyrrolidinophosphonium hexafluorophosphate
RIA	radioimmunoassay
ROS	reactive oxygen species
RP-HPLC	reverse-phase high-performance liquid chromatography
SA-β-gal	senescence-associated β-galactosidase
STZ	streptozotocin
TEA	triethylamine
TFA	trifluoroacetic acid
TNF-α	tumor necrosis factor α

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