### Abstract

**Objective**: Previous work from our laboratory has demonstrated that frog urotensin-II (UII), at a high concentration, inhibits glucose-induced insulin release in the rat pancreas. We have investigated the effect of rat UII and two structural analogs on insulin secretion and searched for the presence of UII-immunoreactivity in rat pancreatic extracts.

**Methods**: The study was performed in the perfused rat pancreas. UII as well as its analogs were synthesized by solid phase methodology. Pancreatic extracts were analyzed for UII by reversed-phase HPLC combined with a sensitive UII RIA.

**Results**: Infusion of synthetic rat UII inhibited glucose-induced insulin release in a dose-dependent manner (IC50: 0.12 nmol/l). UII (1 nmol/l) also inhibited the insulin responses induced by carbachol, glucagon-like peptide-1, and a calcium channel agonist (BA Y K 8644). The inhibitory effect of UII was mimicked by the potent G protein-coupled receptor (GPR14) agonist [3-iodo-Tyr6]UII(4 – 11). In contrast, [Ala8]UII(4 – 11), a UII analog devoid of contractile activity on rat aortic rings, did not affect glucose-induced insulin secretion. Analysis of rat pancreatic extracts revealed the presence of an immunoreactive peptide exhibiting the same retention time as synthetic rat UII.

**Conclusions**: Our results demonstrate that UII is a potent insulinostatic peptide. The observation that UII is actually present in the pancreas suggests that this peptide may play a physiological role in the control of insulin secretion. Concerning the two UII analogs tested, only [3-iodo-Tyr6]UII(4 – 11), reportedly possessing GPR14-mediated contractile activity, mimics the insulinostatic effect of UII. This finding would support the view that UII acts on the pancreatic beta cell through the GPR14 receptor.

### Introduction

Urotensin-II (UII) is a cyclic peptide originally isolated from the caudal neurosecretory organ (urophyse) of a teleost fish on the basis of its ability to contract smooth muscles (1). The sequence of UII has subsequently been determined in several species of fish (2), in frog (3), and in various species of mammals including mouse (4), rat (4), pig (5), monkey (6) and human (3). The sequence of the C-terminal cyclic hexapeptide of UII has been strongly preserved during evolution, and this C-terminal domain is the minimal sequence that retains full biological activity in fish (7) and mammals (8–10). The cyclic core of UII exhibits structural similarity to that of the biologically important central region of somatostatin (11). In mammals, the UII gene is primarily expressed in motorneurons of the brainstem and spinal cord (3, 4, 12–14), as well as in the cardiovascular system (15, 16). UII mRNA is also present, albeit at a much lower level, in various peripheral organs including the kidney, spleen, small intestine, thymus, prostate, adrenal gland and pancreas (3).

Using a reverse pharmacology approach, four groups have shown that UII is the cognate ligand for the orphan G protein-coupled receptor GPR14 (5, 15, 17, 18), a receptor that exhibits substantial structural similarity to that of the somatostatin 2 and 4 receptor subtypes (19, 20). In particular, the Asp residue that is found in transmembrane domain three (TMD3) of all somatostatin receptors is found at the same position in GPR14 (19, 20). This Asp residue has been shown to play an essential role in ligand binding by directly interacting with the Lys9 residue of somatostatin-14 (21–23). Structure–function relationship studies performed on human UII have revealed the crucial role of the Lys9 residue in the biological activity of the peptide (8–10), suggesting that the conserved Asp residue present in TMD3 of GPR14 may similarly interact with the Lys9 residue of UII. GPR14 mRNA is widely expressed in human cardiovascular tissues, including cardiac myocytes, vascular smooth muscle cells and...
endothelial cells, as well as in spinal cord and endocrine tissues (15, 17, 20).

Immunoreactive UII is readily measurable in human plasma, and the circulating levels of UII are elevated in patients with chronic renal failure (24) and heart failure (25, 26). Plasma UII levels are also increased in diabetic patients with or without proteinuria, but are not correlated with fasting blood glucose or HbA1c levels (27). Thus, it is unlikely that hyperglycemia stimulates the biosynthesis and/or the release of UII (27). Interestingly, it has recently been shown that the UII gene has a role in genetic susceptibility to type 2 diabetes mellitus in Chinese (28) and Japanese (29) subjects. On the other hand, we have previously reported that, in the rat pancreas, frog UII at a high concentration (100 nmol/l) inhibits glucose- and arginine-induced insulin responses (30). Taken together, these observations suggest a role for UII in the pathogenesis of diabetes mellitus.

In the present study, we have investigated the effect of rat UII on insulin secretion in the perfused rat pancreas. In order to gain insight into the structure–activity relationships of UII in pancreatic beta cells, we have also studied the effects of two UII analogs that have previously been found to have either high or no contractile activity in a rat aortic ring assay (10). In addition, given that a number of insulinostatic peptides – somatostatin, aminlin, galanin, pancreastatin (10) – are present in pancreatic tissue (31), we have searched for the possible occurrence of UII in rat pancreatic extracts.

Materials and methods

Peptides

Rat UII (pOHGTATPFCFWKYCI), [3-iodo-Tyr\(^6\)]UII (4–11), and [Ala\(^8\)]UII(4–11) were synthesized (0.25 mmol scale) by the solid phase methodology on Fmoc-PEG-PS, Fmoc-Val-PEG-PS, and Fmoc-Ala-PEG-PS respectively, using a Pioneer Perseptice Biosystems peptide synthesizer (Applera-France, Courtaboeuf, France) and the standard Fmoc procedure as previously described (10). The synthetic peptides were purified by reversed-phase HPLC on a 2.2×25-cm Vydac 218TP1022 C18 column, using a linear gradient (10–50% over 50 min) of acetonitrile/trifluoroacetic acid (TFA) (99.9:0.1, v/v) at a flow rate of 10 ml/min. Analytical HPLC, performed on a 0.46×25-cm Vydac 218TP54 C18 column, showed that the purity of the peptides was >99.8%. The purified peptides were characterized by MALDI-TOF MS on a Voyager DE PRO (Applera-France) in the reflectron mode with a mass accuracy of ±0.1%. The peptides were dissolved in 0.9% NaCl, containing 0.1% bovine serum albumin (BSA, Fraction V; Sigma-Aldrich, St. Louis, MO, USA). Peptide solutions were prepared daily, immediately before experiments.

Animals

Male Wistar rats (200–225 g body weight) with food available ad libitum were used as tissue donors. The animals were maintained in accordance with the guidelines established by the European Union.

Experimental protocols

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the pancreata were dissected and perfused in situ as previously described (32). The pressure was monitored by means of a pressure transducer and amplifier (Presmeter 98, Cibertec S.A., Madrid, Spain). Insulin secretion was stimulated by increasing glucose concentration (from 5.5 to 9 mmol/l) in the perfusion medium or by infusion of 1 μmol/l carbachol (Peninsula Laboratories), 1 nmol/l GLP-1 or 10 μmol/l BA Y K 8644 (Sigma-Aldrich). The sequence of addition of compounds to the perfusate is indicated in the corresponding figures. Insulin secretion was analyzed by radioimmunoassay (RIA) (33). Anti-pig insulin serum (B8510, Sigma-Aldrich) and rat insulin standards (Novo Nordisk, Denmark) were employed. Inter- and intra-assay variation were 5.6 and 4% respectively. Experiments were randomized and all samples for each series of perfusions were analyzed within the same assay. Results are expressed as the mean ± S.E.M. Hormone response was calculated as the integrated area under the curve by using the trapezoidal method. Differences between values were tested for significance by repeated-measures analysis of variance for each group and by the Student’s t-test for unpaired samples.

Tissue extraction

Pancreata from 1.2 rats were collected and kept frozen at −70 °C. Each pancreas was extracted individually in ethanol/sulfuric acid (82.5:17.5, v/v) and homogenized by sonication. The homogenates were stored at 4 °C for 24 h, centrifuged (3500 × g, 4 °C; 5 min) and the supernatants were lyophilized. Nine pancreatic extracts were used for quantification of UII-like immunoreactivity and three pancreas extracts were used for HPLC analysis. The extracts were resuspended in water/TFA (99.88:0.12, v/v) and pre-purified on Sep-Pak C18 cartridges (Waters Associates, Milford, MA, USA). Bound material was eluted with acetonitrile/water/TFA (60:39:1.0, v/v/v) and acetonitrile was evaporated in a Speed-Vac concentrator (Savant, Hicksville, NY, USA). The pancreatic extracts were then processed for direct UII RIA or HPLC analysis.
Chromatographic analysis of UII in pancreatic extracts

Sep-Pak pre-purified extracts were redissolved in water/TF A (99.9:0.1, v/v) and chromatographed onto a 0.46 × 25-cm Vydac 219TP54 diphenyl reversed-phase HPLC column (Alltech) equilibrated with a solution of acetonitrile/water/TF A (7.0:92.9:0.1, v/v/v), at a flow rate of 1 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 35% over 60 min using a linear gradient. Eluting fractions were collected every minute and assayed for UII-like immunoreactivity. Synthetic rat UII, used as a reference peptide, was chromatographed in the same conditions as the rat tissue extracts.

Urotensin-II radioimmunoassay

The concentrations of UII-like immunoreactivity in Sep-Pak pre-purified pancreatic extracts or HPLC fractions were measured by RIA. Briefly, synthetic human UII (3 μg) was iodinated by means of the lactoperoxidase technique and the radiiodinated peptide was purified by reversed-phase HPLC. Monoiodinated UII was used as a tracer. The RIA was performed at 4°C in 0.02 mol/l veronal buffer (pH 8.6) containing 0.4% BSA using monoiodinated UII as a tracer and synthetic rat UII as a reference standard. The final dilution of the UII antiserum (code #102.171201) was 1:200 000 and the total amount of tracer was 7000 c.p.m./tube. The bound fraction was immunoprecipitated by a double-antibody method (34). The sensitivity of the assay was 5 pg/tube and 50% displacement was achieved at 130 pg. The cross-reactivity of somatostatin-14 and somatostatin-28 in the assay was lower than 0.002% (35).

Results

In a first series of experiments (Fig. 1), we compared the effects of the same concentration (10 nmol/l) of frog UII and rat UII on the insulin response to an increase in perfusate glucose concentration from 5.5 to 9 mmol/l in the perfused rat pancreas. Solid bold line corresponds to control experiments: from 10–25 min, 9 mmol/l glucose infusion (n = 6). Solid thin line corresponds to frog UII experiments: from 0–10 min, frog UII infusion; from 10–25 min, 9 mmol/l glucose + frog UII infusion (n = 6). Broken line corresponds to rat UII experiments: from 0–5 min, rat UII infusion; from 10–25 min, 9 mmol/l glucose + rat UII infusion (n = 6). Results are means ± S.E.M.

Figure 2 demonstrates that coinfusion of 1 nmol/l rat UII inhibits both phases of the insulin response to 1 μmol/l carbachol (incremental area: 69 ± 20 ng/15 min vs. 186 ± 38 ng/15 min in controls; P < 0.05). The inhibitory effect of rat UII on insulin release was also observed when beta cell secretion was stimulated.

Figure 1 Effect of frog UII and rat UII at 10 nmol/l on basal as well as on the insulin response to an increase in perfusate glucose concentration from 5.5 to 9 mmol/l in the perfused rat pancreas. Solid bold line corresponds to control experiments: from 10–25 min, 9 mmol/l glucose infusion (n = 6). Solid thin line corresponds to frog UII experiments: from 0–10 min, frog UII infusion; from 10–25 min, 9 mmol/l glucose + frog UII infusion (n = 6). Broken line corresponds to rat UII experiments: from 0–5 min, rat UII infusion; from 10–25 min, 9 mmol/l glucose + rat UII infusion (n = 6). Results are means ± S.E.M.

Figure 2 Effect of graded concentrations of rat UII on glucose-induced insulin release in the perfused rat pancreas. The experimental values were obtained from data similar to those presented in Fig. 1 and were calculated as the areas under the curves.

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by 1 nmol/l GLP-1 (Fig. 4) (incremental area: 5.6 ± 3.7 ng/16 min vs. 19 ± 3 ng/16 min in controls; P < 0.025).

Rat UII blocked the first insulin secretory phase elicited by 10 μmol/l BAY K 8644 (incremental area: 17 ± 2 ng/5 min vs. 39 ± 6 ng/5 min in controls; P < 0.025) (Fig. 5).

Infusion of 1 nmol/l [3-iodo-Tyr⁶]UII(4–11), a GPR14 agonist (10), clearly blocked the insulin response to an increase in perfusate glucose concentration from 5.5 to 9 mmol/l (incremental area: 19 ± 4 ng/15 min vs. 43 ± 7 ng/15 min in controls; P < 0.005) (Fig. 6). However, 1 nmol/l [Ala⁸]UII(4–11), a UII analog that is devoid of contractile activity on rat aortic rings (10), did not significantly affect insulin secretion (Fig. 7).

The amount of UII in Sep-Pak pre-purified pancreatic extracts was 288 ± 78 pg/pancreas. Reversed-phase HPLC analysis of pancreatic extracts revealed the existence of a single immunoreactive peak that exhibited the same retention time as synthetic rat UII (Fig. 8).

Discussion
We have previously reported that frog UII, at a high concentration (100 nmol/l), attenuates glucose-induced insulin secretion in the perfused rat pancreas (30). We now show that, in this model, rat UII inhibits...
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Figure 7 Effect of [Ala8]UII(4–11) (Ala-U-II) at 1 nmol/l on the insulin response to an increase in perfusate glucose concentration from 5.5 to 9 mmol/l in the perfused rat pancreas. Solid line corresponds to control experiments: from 5–20 min, 9 mmol/l glucose infusion \( (n = 5) \). Broken line corresponds to [Ala8]UII(4–11) experiments: from 0–5 min, [Ala8]UII(4–11) infusion; from 5–20 min, 9 mmol/l glucose + [Ala8]UII(4–11) infusion \( (n = 6) \). Results are means \( \pm \) S.E.M.

Figure 8 Reversed-phase high performance liquid chromatography analysis of UII-like immunoreactivity in rat pancreas. Sep-Pak pre-purified tissue extracts were chromatographed on a Vydac diphenyl column, and the immunoreactive material contained in the elution fractions was quantified by radioimmunoassay. The dashed line shows the concentration of acetonitrile in the eluting solvent. The arrow indicates the elution position of synthetic rat UII. URP, urotensin-related peptide.

insulin secretion, in a concentration-dependent manner, with an IC50 of 0.12 nmol/l. It has recently been observed that the mRNA encoding the UII receptor GPR14 is expressed in the human (15, 16), monkey and mouse (6) pancreas. Taken together, these observations allow the suggestion that UII might block insulin secretion by interacting with GPR14 receptors present in rat pancreatic beta cells. Nevertheless, in view of the structural similarities of UII and somatostatin molecules, the possibility of UII interacting with somatostatin receptors must be considered. Concerning this point, on the basis of the sequence homology between the GPR14 and members of the somatostatin receptor family, Carotenuto et al. (36) have hypothesized that UII and somatostatin may activate a common evolutionary pathway for the signal transmission system.

Given that UII has been described as a potent vasoconstrictor agent (15, 16, 37), the inhibitory effect of UII might have been ascribed to a reduction of the perfusate flow through the pancreas. However, no changes either in the perfusate flow rate or in the pressure of the perfusion system were observed during UII infusion. An effect to selectively reduce the perfusate supply to the islets could not only reduce insulin output but could also affect the overall islet secretion and, as described in our previous work (30), UII infusion did not modify glucagon or somatostatin output. Furthermore, in the herein reported experiments UII did not affect basal insulin release, thus excluding vasoconstriction as a factor hampering the secretory activity of the pancreatic islet.

The insulinostatic effect of UII was also evidenced when insulin secretion was triggered by various secretagogues that act on the beta cell through distinct signaling mechanisms, such as carbachol that activates phosphoinositide turnover (38, 39), GLP-1 which stimulates the adenylyl cyclase/cyclic AMP pathway (40, 41), or the dihydropyridine BAY K 8644 that causes Ca2+ influx through L-type Ca2+ channels (42, 43). In agreement with these data, it has previously been reported that UII inhibits carbachol-induced contraction of the anococcygeous muscle (44) and that dbcAMP and forskolin counteract the contractile response evoked by UII in rat aortic strips (45). UII has also been shown to lower cytoplasmic free Ca2+ concentration in goby enterocytes (46). It should be taken into account that inhibition of insulin secretion by somatostatin, a potent insulinostatic peptide that exhibits structural similarities with UII, has been associated with both decreased formation of cAMP coupled with a pertussin toxin-sensitive G-protein (47) and inhibition of the intracellular Ca2+ increase induced by a variety of insulin secretagogues (48).

The structure–activity relationships of a series of UII analogs have recently been determined by measuring their binding affinity on GPR14-transfected CHO cells and their contractile activity in a rat aortic ring assay (8–10). Here, we show that the potent GPR14 agonist [3-iodo-Tyr6]UII(4–11) (10) mimics the insulinostatic effect of UII while the [Ala8]UII(4–11) analog, that is totally devoid of contractile activity on rat aortic rings (10), has no effect on glucose-induced insulin secretion. These correlations suggest that the pharmacological profile of the receptor mediating the actions of UII on the pancreatic beta cell shares similarities with that of GPR14. However, it cannot be ruled out that replacing Lys8 by Ala would abolish reactivity towards GPR14 as well as to somatostatin receptors.

Reversed-phase HPLC analysis of pancreatic extracts combined with a sensitive and specific RIA that did not detect somatostatin, revealed the presence of an
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