EXPERIMENTAL STUDY

Urotensin-II is present in pancreatic extracts and inhibits insulin release in the perfused rat pancreas

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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 (BAY 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.

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Introduction

Urotensin-II (UII) is a cyclic peptide originally isolated from the caudal neurosecretory organ (urophysis) 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 motoneurons 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 Lys⁸ 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 Lys⁸ 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, amilin, galanin, pancreastatin – 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 (*p*QHGTAPCEFWKYCI), [3-iodo-Tyr⁶]UII (4–11), and [Ala⁸]UII(4–11) were synthesized (0.25-mmol scale) by the solid phase methodology on Fmoc-Ile-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 (Alltech, Templemars, France), 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 α -cyano-4-hydrocinnamic acid as a matrix. Frog (*Rana ridibunda*) UII and rat glucagon-like peptide-1 (GLP-1) were purchased from Peninsula Laboratories (San Carlos, CA, USA).

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 9S, 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 μ mol/l carbachol (Peninsula Laboratories), 1 nmol/l GLP-1 or 10 μ mol/l BAY 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 (I8510, 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 $\pm$ 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 12 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 (60:39.9:0.1, 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/TFA (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/TFA (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 radioiodinated 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). Coinfusion of frog UII only inhibited the first insulin secretory phase (incremental area: 22.6 ± 2.3 ng/5 min vs. 33 ± 2.8 ng/10 min in controls; $P < 0.05$) while rat UII curbed the whole insulin response (incremental area: 26 ± 3.8 ng/15 min vs. 64 ± 5.7 ng/15 min in controls; $P < 0.01$). Neither rat UII nor frog UII modified basal insulin secretion ($F_{10,30} = 0.59$ and $F_{10,30} = 0.35$ respectively; not significant).

The insulinostatic effect of 10 nmol/l rat UII (incremental area: 25 ± 5 ng/15 min vs. 79 ± 6.9 ng/15 min in control perfusions; $P < 0.01$) was also observed at lower concentrations of this peptide: at 1 nmol/l (26 ± 7 ng/15 min vs. controls; $P < 0.05$), and at 0.314 nmol/l (33 ± 11 ng/15 min vs. controls; $P < 0.05$). Infusion of UII at 0.1 nmol/l reduced by 40%

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 $\pm$ S.E.M.

the insulin response to glucose (48 ± 10 ng/15 min) although this difference did not attain the 5% level of statistical significance. The dose–response curve fits a sigmoidal curve ($R^2 = 0.9851$) with an IC_{50} of 0.12 nmol/l (Fig. 2).

Figure 3 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 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.

Figure 3 Effect of 1 nmol/l rat UII on the insulin response to 1 μ mol/l carbachol in the perfused rat pancreas. Solid line corresponds to control experiments: from 5–20 min, 1 μ mol/l carbachol infusion ($n = 5$). Broken line corresponds to rat UII experiments: from 0–5 min, rat UII infusion; from 5–20 min 1 μ mol/l carbachol + rat UII infusion ($n = 6$). Results are means $\pm$ S.E.M.

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 μ mol/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; $P < 0.05$) (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).

Figure 4 Effect of 1 nmol/l rat UII on the insulin response to 1 nmol/l GLP-1 in the perfused rat pancreas. Solid line corresponds to control experiments: from 5–20 min, 1 nmol/l GLP-1 infusion ($n = 5$). Broken line corresponds to rat UII experiments: from 0–5 min, rat UII infusion; from 5–20 min 1 nmol/l GLP-1 + rat UII infusion ($n = 6$). Results are means $\pm$ S.E.M.

Figure 5 Effect of 1 nmol/l rat UII on the insulin response to 10 μ mol/l BAY K 8644 in the perfused rat pancreas. Solid line corresponds to control experiments: from 5–20 min, 10 μ mol/l BAY K 8644 infusion ($n = 5$). Broken line corresponds to rat UII experiments: from 0–5 min, rat UII infusion; from 5–20 min, 10 μ mol/l BAY K 8644 + rat UII infusion ($n = 6$). Results are means $\pm$ S.E.M.

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

Figure 6 Effect of [3-iodo-Tyr⁶]UII(4–11) (1-Tyr-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 [3-iodo-Tyr⁶]UII(4–11) experiments: from 0–5 min, [3-iodo-Tyr⁶]UII(4–11) infusion; from 5–20 min, 9 mmol/l glucose + [3-iodo-Tyr⁶]UII(4–11) infusion ($n = 6$). Results are means $\pm$ S.E.M.

Figure 7 Effect of [Ala⁸]U-II(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]U-II(4-11) experiments: from 0–5 min, [Ala⁸]U-II(4-11) infusion; from 5–20 min, 9 mmol/l glucose + [Ala⁸]U-II(4-11) infusion ($n = 6$). Results are means $\pm$ S.E.M.

insulin secretion, in a concentration-dependent manner, with an IC_{50} of 0.12 nmol/l. It has recently been observed that the mRNA encoding the U-II receptor GPR14 is expressed in the human (15, 16), monkey and mouse (6) pancreas. Taken together, these observations allow the suggestion that U-II might block insulin secretion by interacting with GPR14 receptors present in rat pancreatic beta cells. Nevertheless, in view of the structural similarities of U-II and somatostatin molecules, the possibility of U-II 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 U-II and somatostatin

Figure 8 Reversed-phase high performance liquid chromatography analysis of U-II-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 U-II. URP, urotensin-related peptide.

may activate a common evolutionary pathway for the signal transmission system.

Given that U-II has been described as a potent vasoconstrictor agent (15, 16, 37), the inhibitory effect of U-II 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 U-II 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), U-II infusion did not modify glucagon or somatostatin output. Furthermore, in the herein reported experiments U-II did not affect basal insulin release, thus excluding vasoconstriction as a factor hampering the secretory activity of the pancreatic islet.

The insulinostatic effect of U-II 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 polyphosphoinositide turnover (38, 39), GLP-1 which stimulates the adenylyl cyclase/cyclic AMP pathway (40, 41), or the dihydropyridine BAY K 8644 that causes Ca^{2+} influx through L-type Ca^{2+} channels (42, 43). In agreement with these data, it has previously been reported that U-II inhibits carbachol-induced contraction of the anococcygeous muscle (44) and that dbcAMP and forskolin counteract the contractile response evoked by U-II in rat aortic strips (45). U-II has also been shown to lower cytoplasmic free Ca^{2+} 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 U-II, has been associated with both decreased formation of cAMP coupled with a pertussin toxin-sensitive G-protein (47) and inhibition of the intracellular Ca^{2+} increase induced by a variety of insulin secretagogues (48).

The structure–activity relationships of a series of U-II 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]U-II(4-11) (10) mimics the insulinostatic effect of U-II while the [Ala⁸]U-II(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 U-II 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

immunoreactive peptide that exhibited the same retention time as synthetic rat UII. The concentration of UII measured in crude pancreatic extracts (288 ± 78 pg/pancreas i.e. about 189 pg/g fresh tissue) is 7–27 times higher than the plasma UII level in normal subjects (7–28 pg/ml) (27), suggesting that UII is produced locally in the rat pancreas. In support of this hypothesis, the mRNA encoding the UII precursor has been detected in rat (49) and human (3) pancreas, a finding not observed by Nothacker *et al.* (18) using a similar mRNA analysis of human pancreatic tissue. Whether UII acts as a paracrine factor controlling beta cell function remains to be established.

Alternatively, in certain pathological conditions including diabetes (24–27, 50), the plasma concentrations of UII may attain much higher levels (140–420 pg/ml) that are in the same range as the IC_{50} value (120 pmol/l i.e. 167 pg/ml) calculated for the insulinostatic activity of UII, suggesting that UII might constitute a diabetogenic factor.

In summary, in the rat pancreas, rat UII inhibits insulin secretion induced by secretagogues acting on the beta cell via different mechanisms, glucose, carbachol, GLP-1 and a calcium channel agonist (BAY K 8644). Concerning the two UII analogs tested, only [3-iodo-Tyr⁶]UII(4–11), reportedly possessing GPR14-mediated contractile activity (10), behaved as an insulinostatic agent. This finding would support the view that UII acts on the pancreatic beta cell through the GPR14 receptor, a point that deserves further investigation. The fact that relatively high concentrations of UII are present in pancreatic tissue indicates that UII may play a physiological function in the control of insulin secretion. The insulinostatic effect of UII brings under consideration its possible implication in the defective beta cell function associated with type 2 diabetes.

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