Biochemical Pharmacology 83 (2012) 608–615

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Urocontrin, a novel UT receptor ligand with a unique pharmacological profile

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ARTICLE INFO

Article history:
Received 31 October 2011
Accepted 8 December 2011
Available online 16 December 2011

Keywords:
Urotensin II
Urotensin II-related peptide
Insurmountable antagonism
Urocontrin
Human UT receptors
Aortic ring bioassay

ABSTRACT

In recent years, several studies have demonstrated that urotensin II (UII) and urotensin II-related peptide (URP) can exhibit differential biological activity. So far, known antagonists of the urotensin II receptor (UT) are of limited usefulness for investigating the specific pathophysiological role of UII or URP. Therefore, identification of new compounds able to discriminate UII- and URP-associated biological activities is crucially needed. In the present study, we report preliminary data regarding the pharmacological properties of a novel UT ligand termed urocontrin, *i.e.* [Bip^4]URP, that is able to reduce the *ex vivo* efficacy of hUII- but not URP-induced vasoconstriction in rat aortic rings. *In vivo* studies support the pharmacological profile described above. Although urocontrin exert some residual agonist activity, this compound should be useful for the rational design of potent molecules that would allow discriminating specific biological action mediated by UII or URP.

1. Introduction

Numerous G protein-coupled receptor (GPCR) systems have been identified where multiple endogenous ligands can interact with a shared cognate receptor. These ligand clusters can be derived from different gene transcripts or from differential processing of a common transcript or precursor protein. The variety of pro-opiomelanocortin-derived peptides active at melanocortin receptors is an example of the latter [1], whereas the various families of chemokines may provide the most extensive example of the former [2].

Urotensin II (UII) is currently considered as the most potent endogenous human vasoconstrictor, being more than one order of magnitude more potent than endothelin-1 (ET-1) *in vitro* [3]. Following its initial isolation from the *Gillichthys mirabilis* urophysis, UII isoforms have been subsequently characterized or isolated in other species including human [4]. A comparison of UII primary sequences revealed a striking homology among species. In fact, from fish to man, evolution favored the conservation of residues 5–10, while the N-terminal segment varies in length and composition [4]. Recently, an octapeptidic paralog called UII-related peptide (URP) has been identified in rodents and humans [5]. URP shares with UII the fully conserved C-terminal cyclic hexapeptide CFWKYC core but differs in the physicochemical characteristics of the amino acid preceding the disulfide bridge (acidic *versus* hydrophobic) [6].

Pharmacologically, UII and URP are the endogenous ligands of the G protein-coupled receptor known as urotensin II receptor (UT). Both peptides are concomitantly expressed with the UII receptor in several human tissues including brain, lung, heart, pancreas and kidney as well as vasculature, and until recently were thought to exert redundant biological activities [4,5]. However, studies have reported a differential action for these two peptides on cell proliferation [7], and distinctive myocardial contractile activities [8]. Moreover, in isolated ischaemic heart experiments, both peptides were able to reduce myocardial injury through creatine kinase reduction but only UII was able to reduce atrial natriuretic peptide production [8]. As such, the specific role of each peptide in UTI-associated pathophysiological states, including atherosclerosis, heart failure, hypertension, pre-eclampsia, diabetes, renal disease and liver disease, must be clarified [4,9,10].

The structural homology between UII, URP and somatostatin has often raised the question regarding the cross reactivity of these peptides to explain divergent activities. As a matter of fact, UII is able to activate sst2 and sst5 receptors [11], and some somatostatin antagonists were found to antagonize UT-associated

Abbreviations: SDS, sodium dodecylsulfate; RP-HPLC, reversed-phase high performance liquid chromatography; MALDI-TOF-MS, matrix-assisted desorption ionization time-of-flight mass spectrometry; Bip, (*S*)-2-amino-3-(1,1′-bipheny-4-yl) propanoic acid.

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doi:10.1016/j.bcp.2011.12.009

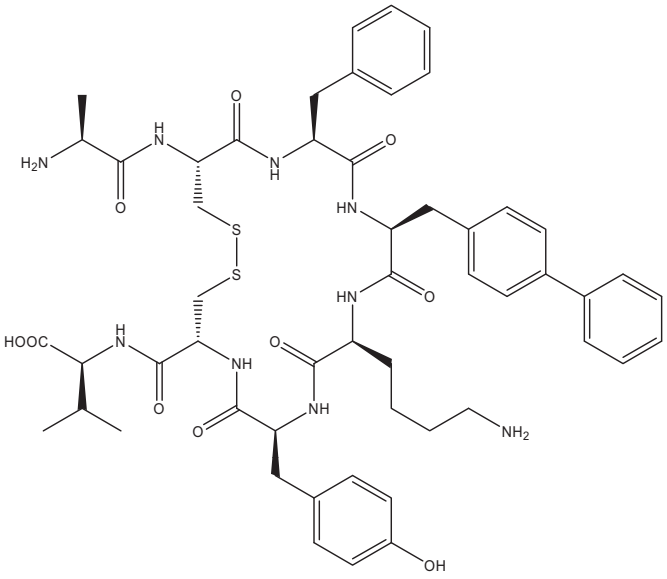

Fig. 1. Structure of the novel UT ligand urocontrin, *i.e.* [Bip[4]]URP.

activity [12]. Noteworthy, the somatostatinergic system, like the urotensinergic system, is composed of several peptides sharing high sequence homology and acting on the same receptors. For instance, cortistatin, a member of the somatostatin family, can bind and activate all somatostatin receptors and shares many biological activities but also possesses unique functional activities [13,14]. Interestingly, it has been found that the genes encoding these neuropeptides, *i.e.* UII, URP, somatostatin, and cortistatin, originate from a common ancestor [15].

Following the discovery of a human urotensin II isoform, several structure–activity relationships highlighted the importance of intracyclic residues in regard to binding affinity but also biological activity. For instance, it was observed that replacement of Phe, Lys, and Tyr residues modulated the binding affinity and/or the biological activity of UII [6]. However, little attention has been paid to the Trp residue in either UII or URP. Structure–activity relationships have suggested that the indole NH function may establish a hydrogen bond with some UT residue or that the electron rich indole system was involved in a cation-π interaction with the Lys[8] side chain [16]. Finally, docking models, in accordance with structure-activity relationship data, suggested that Trp[7] in UII interacts with the receptor *via* polar rather than hydrophobic interactions [17]. Recently, inversion of configuration of the Trp moiety in URP resulted in a partial agonist able to block hUII-associated aortic ring contraction [18]. We therefore hypothesized that replacement of the indole moiety in URP could lead to new ligands, and more specifically new antagonists, of the urotensinergic system. We evaluated the impact of various non-proteinogenic hydrophobic and/or polar amino acids on the biological activity of URP and identified urocontrin, *i.e.* [Bip[4]]URP (Fig. 1), as having the striking ability to reduce the efficacy of hUII- but not URP-induced vasoconstriction in a rat thoracic aorta assay. We report herein the *in vitro* and *in vivo* pharmacological profile of urocontrin and we believe this compound represent the first peptide analog of a new class of urotensinergic ligands.

2. Materials and methods

2.1. Materials

Fmoc-protected amino acids, Wang polystyrene resin and TBTU (2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate) reagent were purchased from Chem-Impex (Wood Dale, IL, USA). Solvents for solid phase peptide synthesis and purification were obtained from Fisher Scientific (Nepean, ON, Canada) whereas trifluoroacetic acid (TFA) was from PSIG (Montreal, QC, Canada). $Na^{125}I$ was purchased from Perkin Elmer (Montreal, QC, Canada). Other chemicals including D-glucose, Trizma® hydrochloride, Trizma® base, bovine serum albumin, manganese(II) chloride, chloramine-T, sodium bisulfite, sodium chloride, sodium bicarbonate, calcium chloride, magnesium sulfate, N,N-di-iso-propylethylamine (DIPEA), saline, isoflurane, potassium chloride, and potassium phosphate monobasic, were ordered from Sigma–Aldrich (Mississauga, ON, Canada).

2.2. Animals

Adult male Sprague-Dawley rats (Charles Rivers, St-Constant, QC, Canada) weighing 250–300 g or 350–400 g were housed in cages under controlled illumination (12:12 h light–dark cycle), humidity, and temperature (21–23 °C) and had free access to tap water and Purina rat chow. All experimental procedures were performed in accordance with regulations and ethical guidelines from the Canadian Council for the Care of Laboratory Animals and received approvals by the institutional animal care and use committee of the Institut National de la Recherche Scientifique-Institut Armand-Frappier and the animal ethics and research committee of the Montreal Heart Institute.

2.3. Peptide synthesis and characterization

All URP derivatives were synthesized manually using a standard solid phase peptide synthesis approach with Fmoc chemistry. Couplings of the protected amino acids were obtained with TBTU (1 eq.) and DIPEA (2 eq.) in DMF for 1 h and monitored with the qualitative ninhydrin test. A 3-eq. excess of the protected amino acids based on the original substitution of the Fmoc-Val-WANG resin (0.7 mmol g^{-1}) was used in most cases. Fmoc removal was achieved with 20% piperidine in DMF for 20 min. All peptides were cleaved from the resin support with simultaneous side chain deprotection by treatment with TFA containing 1,2-ethanedithiol (2.5%), water (2.5%) and tri-iso-propylsilane (1%) for 1.5 h at room temperature. The diethyl-ether-precipitated crude peptides were solubilized in 70% acetic acid (1 mg/mL) and then cyclized by the addition of iodine (10% solution in methanol) until appearance of a stable orange color [19]. Thirty minutes later, ascorbic acid was added to quench the excess of iodine. Crude cyclic lyophilized peptides were purified by preparative reverse-phase HPLC, using a linear gradient from eluent A to B with 1% B per 2 min increments (Eluent A = H_2O, 0.1% TFA, Eluent B = 60% CH_3CN/40% A, 0.1% TFA). Homogeneity of purified fractions was assessed by RP-HPLC and MALDI-TOF mass spectrometry in linear mode using α-cyanohydroxycinnamic acid as matrix. Pure fractions (>98%) containing the product were pooled and lyophilized. The synthesis of ET-1 and hUII were carried out as reported elsewhere [20,21].

2.4. Cell culture and binding experiments

Transfected CHO cells (from Drs H. Vaudry and C. Dubessy, Rouen, France) overexpressing the human urotensin receptor (hUT) [18] were maintained in Ham-F12 medium with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 UI/mL each of penicillin and streptomycin, 400 μg/mL G418. Synthetic hUII (10 μg) and URP (10 μg) were radiolabeled with 0.5 mCi $Na^{125}I$, using the chloramine-T technique, as previously described [22]. Iodinated ^{125}I-hUII or ^{125}I-URP were purified on a C_{18} cartridge, collected and stored at −20 °C until use. Binding assays were performed, as reported [18], using CHO cells stably transfected with hUT. Non-specific binding, determined in the presence of

1 μM unlabeled hUII, ranged between 10 and 15% of total binding. For dissociation studies, CHO–hUT cells were incubated for 2 h with ^{125}I-hUII or ^{125}I-URP (0.2 nM) [23], and dissociation of receptor-bound radioligand measured at different intervals, was initiated by the addition of a supra-maximal concentration of hUII (10^{-6} M) or URP (10^{-6} M) alone, or by the simultaneous addition of hUII (10^{-6} M) and urocontrin (10^{-6} M) or URP (10^{-6} M) and urocontrin (10^{-6} M). The cells were then washed, lysed, and the radioactivity counted on a γ-counter (1470 Automatic Gamma Counter, Perkin Elmer, Canada).

2.5. Organ bath experiments

Male rats (Sprague-Dawley, 250–300 g) were killed by CO_2 asphyxiation. As previously described [21], the thoracic aorta was then cleared of surrounding tissues and excised from aortic arch to the diaphragm. From each vessel, conjunctive tissues were removed and the clean vessel was cut into 4 mm rings. The endothelium was removed by rubbing gently the vessel intimal surface. All preparations were placed in 5 mL organ baths filled with oxygenated normal Krebs-Henseleit solution. Contractile responses to 40 mM KCl were used as control at the beginning and the end of each experiment.

Agonistic activity was measured by increasing the concentration of each peptide in the organ chamber (3×10^{-11}–3×10^{-6} M). For antagonist behavior, thoracic aortic ring were first exposed to urocontrin for 15 min, and then cumulative concentration–response curves to hUII, URP or ET-1 (10^{-10}–10^{-6} M) were constructed. The amplitude of the contraction induced by each concentration of peptide was expressed as a percentage of the KCl-induced response.

Finally, the ability of urocontrin to reverse the hUII-induced contraction was also evaluated. Vessels were pre-contracted with 4 nM hUII (pre-determined EC_{80}) and once contraction had reached a plateau, contractile tone was reversed by adding increasing log unit concentrations of urocontrin (10^{-10}–10^{-6} M).

2.6. Hemodynamic assessment

Male Sprague-Dawley rats, weighing 350–400 g, were anesthetized by isoflurane inhalation delivered in 100% oxygen (1 L/min) from an Ohmeda Tec 4 anesthetic vaporizer (Somatechnology, Inc., Bloomfield, CT, USA). Anesthesia was maintained by mask inhalation of isoflurane vaporized at concentrations of up to 3% in the induction phase, at 1.5% during acute surgical procedures and at 0.8–1.0% during experimental observations. After anesthesia, a polyethylene catheter (PE 10) was inserted into the right jugular vein to inject saline (0.9%) or drugs. To measure arterial blood pressure, an incision was made at the common right carotid artery where a microtip pressure transducer catheter (model SPR-407, 2F, Millar Instruments, Houston, TX, USA) was inserted. A period of stabilization of 30 min was observed before the experimental protocols (described below) and arterial blood pressure was evaluated as previously described [24]. During isoflurane inhalation, no breathing complications or changes in blood pressure was observed. Mean values of mean arterial pressure (MAP) was 114 ± 3 mmHg at basal and was not modified by saline bolus injections.

2.7. Effect of urocontrin on a single intravenous injection of hUII or URP in anesthetized rats

In this set of experiments, five groups of rats (n = 5–7 rats) were used. Group 1 was employed to evaluate the intrinsic property of urocontrin at low (100 nmol/kg) and high dosage (1000 nmol/kg). Other groups were used to assess the hemodynamic profile of a single intravenous (*i.v.*) injection (200 μL over a 10 s period) of either hUII (10 nmol/kg) or URP (10 nmol/kg) in the absence (control) or in the presence (treated) of urocontrin. In treated groups, prior to hUII or URP administration (10 nmol/kg), rats were exposed to urocontrin (100 nmol/kg or 1000 nmol/kg) for a period of 30 min. Baseline hemodynamic parameters were assessed for 5 min before bolus injection of the drugs. Hemodynamic changes were continuously captured by the pressure probe for 1 h. Maximum rise and maximum drop in blood pressure were determined through an analysis using the PowerLab software (ADInstruments, Colorado Springs, CO, USA).

2.8. Effect of urocontrin on repeated intravenous injections of hUII

In the control group (n = 4 rats), rats received two doses of hUII (10 nmol/kg) with a 30 min interval between the two injections. As for the treated group (n = 4 rats), the same protocol was applied except that an additional dose of urocontrin (1000 nmol/kg) was given 30 min prior to the injection protocol performed in the control group.

2.9. Data analysis

Binding and functional experiments were performed at least in triplicate and data, expressed as mean ± S.E.M., were analyzed with the Prism software (Graph Pad Software, San Diego, CA, USA). In all experiments, n represents the total number of animals studied or individual assays performed. EC_{50}, pEC_{50}, pIC_{50} as well as E_{max} values were determined from corresponding concentration-response curves obtained by using a sigmoidal dose-response fit with variable slope. Non-competitive antagonist affinities (pK_b) were determined as previously described using the method of Gaddum where equiactive concentrations of agonist, in the absence or presence of urocontrin, were compared in a linear regression [25]. Statistical comparisons of binding affinities and contractile potencies of URP and its analogues were analyzed using unpaired Student's t-test and differences were considered significant when $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

3. Results

3.1. Radiolabeled hUII or URP binding to UT receptors is inhibited by urocontrin

As shown in Table 1, unlabeled hUII and URP inhibited ^{125}I-hUII binding to UT in CHO–UT cells with an IC_{50} of 13.2 nM and 12.4 nM, respectively. Similarly, unlabeled hUII and URP inhibited ^{125}I-URP binding with an IC_{50} of 32.5 nM and 31.8 nM, respectively (Table 1). Binding of ^{125}I-hUII and ^{125}I-URP to UT receptors was also inhibited by urocontrin with an apparent IC_{50} value of 386 nM and 339 nM, respectively (Table 1). Overall, this compound is able to consistently and completely displace both radioligands, *i.e.* ^{125}I-hUII and ^{125}I-URP.

3.2. Effects of urocontrin on hUII- and URP-induced rat aortic ring contraction

In the rat isolated aorta bioassay, hUII and URP evoked a dose-dependent contraction (Table 2) with a pEC_{50} of 8.98 ± 0.21 (E_{max} of 124 ± 8% response to 40 mM KCl; n = 10) and 8.09 ± 0.18 (E_{max} = 100 ± 6%; n = 10), respectively. Exposure to increasing concentrations of urocontrin up to 3 μM induced only a weak vasoconstriction (E_{max} = 9% at $10^{-5.5}$ M). This compound was later tested for its ability to antagonize hUII- or URP-mediated contraction of isolated rat aortic rings. As shown in Fig. 2A and Table 3, various concentrations of urocontrin produced a significant rightward shift of the hUII concentration-response curve and the maximal hUII

Table 1
Physicochemical properties and binding affinities of hUII, URP, and urocontrin to recombinant human UT.

Cpd name	Sequence	Purity[a]	MS[b] calc	MS[b] found	Binding (^{125}I-hUII)			Binding (^{125}I-URP)		
					IC_{50} (nM)[c]	pIC_{50}	n	IC_{50} (nM)[c]	pIC_{50}	n
hUII	H-Glu–Thr–Pro–Asp–Cys–Phe–Trp–Lys–Tyr–Cys–Val-OH	≥98%	1387.6	1388.1	13.2	7.88 ± 0.07	3	32.5	7.49 ± 0.11	3
URP	H-Ala–Cys–Phe–Trp–Lys–Tyr–Cys–Val-OH	≥98%	1016.4	1016.4	12.4	7.90 ± 0.11	4	31.8	7.50 ± 0.11	4
Urocontrin	H-Ala–Cys–Phe–Bip–Lys–Tyr–Cys–Val-OH	≥98%	1053.4	1053.4	386.0	6.41 ± 0.12	3	338.8	6.47 ± 0.12	4

[a] Percentage of purity determined by HPLC using buffer system: A = H_2O (0.1% TFA) and B = 60% CH_3CN/40% A with a gradient slope of 1% B/min, at flow rate of 1 mL/min on a Vydac C_{18} column. Detection at 214 nm.
[b] MALDI mass spectral analysis (m/z). The observed m/z of the monoisotope compared with the calculated $[M+H]^+$ monoisotopic mass.
[c] IC_{50} represent the concentration giving 50% of binding inhibition.

response was not attainable. For instance, pre-treatment with urocontrin at 10^{-6} M, produced a significant suppression (E_{max} = 61 ± 7%) of the maximum contractile response to hUII with a significant shift in the concentration-response curve (pEC_{50} = 7.60 ± 0.29) (Table 3). These results were further analyzed as recently reported for a non-peptidic UII antagonist [25]. Consistent with non-competitive antagonism, the slope of the double reciprocal plot of equiactive concentrations of agonist in the presence and absence of 1 μM urocontrin was linear with a slope of 15.00 ± 0.62, equating to a pK_b of 7.15 (Fig. 2C). Interestingly, a slight but non-significant rightward shift (pEC_{50} = 7.74 ± 0.12) with a non-significant reduction of efficacy (E_{max} = 102 ± 5%) was observed with URP (Fig. 2B). Finally, urocontrin potently (pIC_{50} = 7.72 ± 0.15; n = 6) and efficaciously (92% suppression) reversed contractile tone established in the rat isolated aorta with an EC_{80} dose of hUII (Fig. 2D).

3.3. Effects of urocontrin on endothelin-1-induced contraction of rat aortic ring

To further characterize the properties of urocontrin, its specificity as a ligand of the urotensinergic system was assessed by examining the effect of urocontrin on the contractile response to endothelin-1. Indeed, it was demonstrated that somatostatin ligands (agonists and antagonists) are able to potentiate the ET-1-induced effect in the rat aorta [26]. Mean EC_{50} values with and without urocontrin (10^{-6} M), respectively, were as follows: ET-1 pEC_{50} = 7.85 ± 0.25 (E_{max} = 127.3 ± 10.3%; n = 8) and 8.06 ± 0.15 (E_{max} = 118.5 ± 5.7%; n = 6). Thus, this compound is not able to alter the concentration-response curves of ET-1-mediated contraction. Consequently, it is unlikely that urocontrin exerts its action through the activation of somatostatin receptors.

3.4. Influence of urocontrin on the dissociation rate of hUII or URP

To determine whether the insurmountable antagonism of hUII by urocontrin observed in the functional assay could be due to the compound binding at a site different from the agonist binding domain, CHO–UT cells were incubated with either ^{125}I-hUII or ^{125}I-URP until binding equilibrium was reached. Dissociation of the bound radioligand was then initiated by high concentration of the corresponding unlabeled peptide in the absence or presence of an excess of urocontrin. The supramaximal concentration of unlabeled hUII or URP causes dissociation of the bound radioligand from its receptor site. If the addition of the ligand alters the

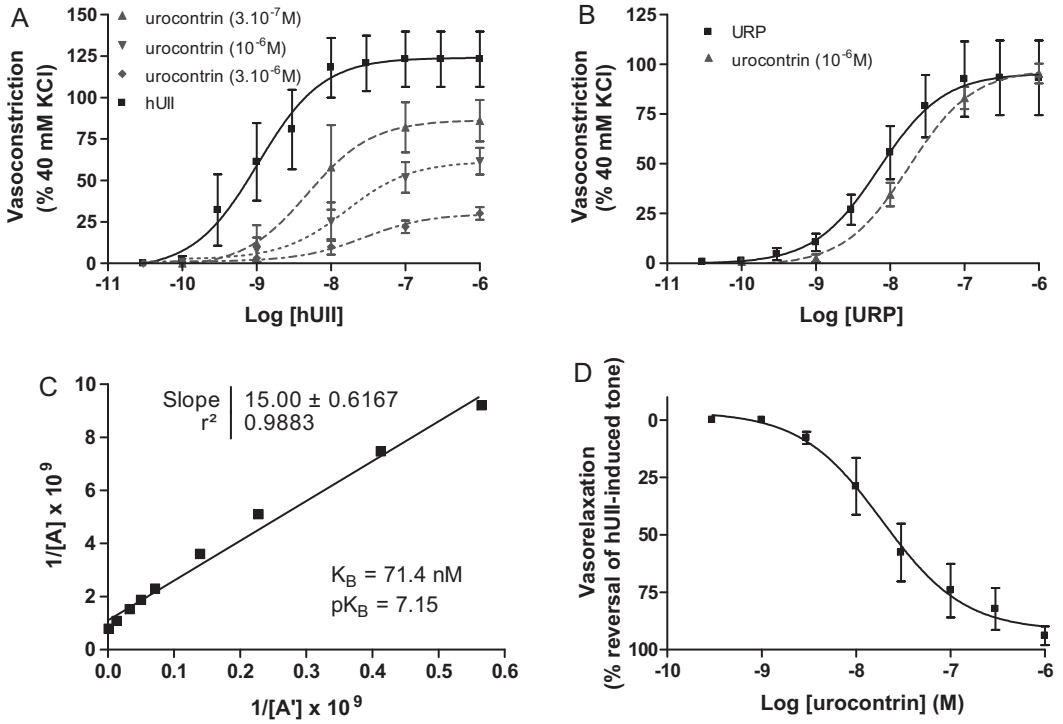

Fig. 2. Effects of urocontrin on (A) hUII- and (B) URP-induced contraction of rat aortic rings. Urocontrin suppressed the maximum contractile response to hUII in a concentration-dependent manner but not URP. (C) Double reciprocal plot of equiactive concentrations of hUII in the absence (A) and presence (A′) of 1 μM urocontrin is linear (consistent with non-competitive antagonism) with a slope of 15.0 ± 0.6, indicating a pK_b of 7.15. (D). Concentration-dependent relaxation response to urocontrin expressed as percentage of the original tone reversal established in endothelium denuded aortae with 4 nM hUII. Data represent the mean ± S.E.M. and n = 3–7 animals.

Table 2
Effect of urocontrin on the rat thoracic aorta.

Cpd name	Aortic ring contraction			*n*
	EC_{50} (nM)[a]	pEC_{50}	E_{max} (%)[b]	
hUII	0.8 (0.2–2)	8.98 ± 0.21	124 ± 8	10
URP	8 (3–18)	8.09 ± 0.18	100 ± 6	10
Urocontrin	$>10^{-6}$	–	9[c]	7

[a] Concentration producing 50% of the maximum effect. Values in parentheses are 95% confidence limits.
[b] The maximum efficacy is expressed as a percentage of the amplitude of the contraction induced by KCl (40 mM).
[c] Maximum efficacy at $10^{-5.5}$ M.

Table 3
Concentration-dependent inhibition of hUII-induced contraction of rat isolated aorta by urocontrin.

Urocontrin (μM)	hUII E_{max} (%KCl)	hUII pEC_{50}	*n*
Vehicle	124 ± 8	8.98 ± 0.21	10
0.3	86 ± 11	8.31 ± 0.37	3
1	61 ± 7**	7.60 ± 0.29*	5
3	30 ± 3***	7.55 ± 0.26*	6

All values are expressed as mean ± SEM. Statistical comparisons for both pEC_{50} and E_{max} values were performed using the unpaired Student's *t*-test analysis where $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ *versus* vehicle control values.

Table 4
Dissociation kinetics of ^{125}I-hUII or ^{125}I-URP bound to recombinant human UT receptors in CHO cells in the absence or presence of urocontrin.

	^{125}I-hUII			
	hUII	*n*	hUII + urocontrin	*n*
Half-life ($t_{1/2}$, min)	13.84 (11.11–18.36)	8	4.52 (3.60–6.07)**	7
Dissociation constant (k_{off}, min^{-1})	0.050 ± 0.006		0.153 ± 0.019**	
	^{125}I-URP			
	URP	*n*	URP + urocontrin	*n*
Half-life ($t_{1/2}$, min)	13.13 (10.52–17.44)	4	12.06 (9.12–17.47)	6
Dissociation constant (k_{off}, min^{-1})	0.052 ± 0.006		0.054 ± 0.006	

Statistical comparisons were performed using unpaired *t*-test analysis ($^{**}P < 0.01$ *versus* control values). Values in parentheses are 95% confidence limits.

dissociation rate of the radioligand, it must do so by interacting at a different «allosteric» site [27]. Fig. 3 and Table 4 show that the half-life ($t_{1/2}$ = 13.84 min) and dissociation constant (k_{off} = 0.05 min^{-1}) of ^{125}I-hUII was significantly different ($P < 0.01$) in the presence of urocontrin ($t_{1/2}$ = 4.52 min and k_{off} = 0.15 min^{-1}), indicating that it probably binds to a different binding site compared to UII. Surprisingly, no change in the dissociation rate of ^{125}I-URP (Table 4) was observed suggesting that urocontrin is able to bind the same site than URP, which seems to be different than that of UII.

3.5. Effect of urocontrin on hUII and URP hemodynamic action in anesthetized rats

The hypotensive action of UII and URP in anesthetized rats was previously investigated [28,29]. It was then observed that hUII and URP at a dose of 0.01 μmol/kg were able to significantly alter blood pressure. A dose-response curve, reflecting the effect of hUII on the change in blood pressure, was performed and a similar significant drop in blood pressure was observed at 0.01 μmol/kg (data not shown). However, at 0.03 μmol/kg, a subsequent reduction of the mean arterial pressure was obtained suggesting that the maximal efficacy was not attained. Consistent with this observation, it was previously demonstrated that UII (1.5–150 nmol/kg) dose-dependently reduced blood pressure in anesthetized rats [30]. However, the dose of 0.01 μmol/kg was used to further characterize the *in vivo* pharmacological effect of systemic injection of urocontrin.

Systemic injection of urocontrin at a dose of 0.1 μmol/kg caused no detectable effect on blood pressure. However, at a dose of 1 μmol/kg, it produced a hypotension comparable with that of hUII at 0.01 μmol/kg (Fig. 4). Since the maximum effect of urocontrin or hUII on the mean arterial pressure was not investigated, it is therefore impossible to clearly define urocontrin as a weak agonist or a partial agonist. Bolus *i.v.* injection of hUII (0.01 μmol/kg) or URP (0.01 μmol/kg) produced a biphasic hemodynamic response characterized by a rapid and transient pressor phase with a peak ($^{*}P < 0.05$) occurring after ~1 min, followed by a long lasting hypotension phase reaching a nadir ($^{*}P < 0.05$) at ~6 min (Fig. 5A and B). Even though similar effects were observed with a bolus injection of URP, the hypotension effect was significantly less pronounced as compared to the same dose of hUII ($^{¶}P < 0.05$) (Fig. 5B). Bolus injection of urocontrin (1 μmol/kg) significantly reduced ($^{§}P < 0.05$) hUII hypotensive action while preserving its pressor effect (Fig. 5A). At a lower dose, *i.e.* 0.1 μmol/kg, urocontrin had no effect on the hUII hemodynamic profile (data not shown). Interestingly, urocontrin (1 μmol/kg) had no effect on the biphasic response induced by URP (10 nmol/kg) (Fig. 5B).

3.6. Effect of urocontrin on repeated intravenous hUII injection

A recent report demonstrated a desensitization of the urotensinergic system following repeated injection of hUII in eight weeks old SHR rats [24]. However, no experiment was achieved on normotensive rats. As shown in Fig. 6A, injections of hUII 30 min apart produced equivalent biphasic responses that do

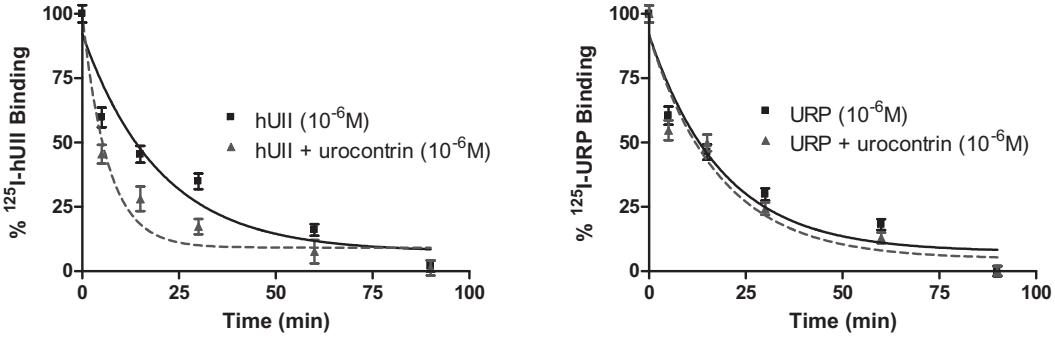

Fig. 3. Influence of urocontrin on the dissociation rate of hUII or URP. Dissociation time-courses of bound ^{125}I-hUII or ^{125}I-URP were assessed on living CHO cells over-expressing the human urotensin II receptor (n = 4–8).

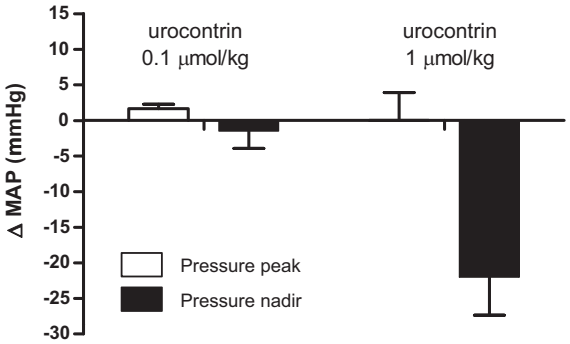

Fig. 4. Intrinsic agonistic property of urocontrin at low (100 nmol/kg) and high dosage (1000 nmol/kg). Data represent the mean ± S.E.M. and $n = 5–7$ animals.

not suggest a tolerance effect. The same protocol was then applied except that an additional dose of urocontrin (1 μmol/kg) was given 30 min prior to the injection protocol performed in the control group. As shown, in Fig. 6B, and in accordance with the previously described action on hUII hemodynamism (Fig. 5A), the first injection of hUII, 30 min after the initial injection of urocontrin, is characterized by a significantly reduced ($^{*}P < 0.05$) hypotensive action while preserving its pressor effect (Fig. 6B). Worthy of notice is the complete absence of response to a second injection of hUII, 30 min apart from the first injection of hUII, when rats were treated 1 h before with urocontrin (1 μmol/kg) (Fig. 6B).

4. Discussion

Urotensin II and UII-related peptide are paralog peptides sharing multiple biological actions as well as structural features. Recent studies have suggested that both peptides might regulate common but also divergent physiological [7,8] and pathophysiological actions [31]. Although sharing the same intracyclic core sequence, previous structure–activity relationship studies have highlighted differences in their recognition and activation processes. Indeed, inversion of configuration of the Trp residue in UII or URP demonstrated a differential participative effect of this residue in these structures. Though it led to a full and potent agonist when introduced in UII [21,32], the same modification in URP produced only a partial agonist able to completely abolish the UII-induced aortic ring contraction [18,33]. Altogether, these observations have raised the question about the possibility to design an analog that could block selectively UII- or URP-associated effects. Based on the unusual pharmacological profile observed following the inversion of configuration of the Trp residue in UII or URP, we have initiated a random structure–activity relationship study centered at position 4 of URP and identified [Bip^{4}]URP as a potential candidate for the conception of ligands aimed at discriminating UII- and URP-mediated biological activity.

In radioligand competition assays, this compound, named urocontrin, was able to efficiently displace, in a similar manner, ^{125}I-hUII and ^{125}I-URP from hUT. In the rat aorta bioassay, this compound was almost devoid of any contractile activity and only a small residual agonist activity was observed at 3 μM. Surprisingly, urocontrin presented a dual pharmacological profile in this assay when used as an antagonist. Indeed, urocontrin seems to be a low-efficacy partial agonist that is able, at least at 10^{-6} M, to selectively and significantly reduce the hUII-induced contraction without altering URP-mediated vasoconstriction. GSK248451, SB-710411 and urantide, three putative UT receptor competitive antagonists that are classified as 'low-efficacy partial agonists' [34], were also able to block hUII-associated vasoconstriction. However, to the best of our knowledge, the ability of a UT peptide ligand to differentially alter hUII and URP biological activity has never been reported. Due to the high structural homology between UII, URP, and somatostatin, questions regarding the selectivity of this new ligand were raised. However, based on our results, it is unlikely that urocontrin may also act as a somatostatin receptor ligand, since it does not potentiate ET-1-induced effects in the rat aorta (a phenomenon thought to be closely connected to somatostatin receptor affinity). Insurmountable blockade may occur if the antagonist binds to an allosteric site, close to but not at the agonist binding site, and induces a conformational change in the receptor that compromises the ability of the agonist–receptor complex to elicit a response [35]. In accordance with our *ex vivo* experiments, urocontrin increased the dissociation rate of ^{125}I-hUII but not ^{125}I-URP from UT suggesting the existence of a slightly different binding pocket for hUII and URP within UT. Worthy of mention is the ability of urocontrin to fully displace both radioligands in a similar manner at the concentration used to initiate dissociation. Therefore the observed differences in kinetics cannot be attributed to the inability of urocontrin to efficiently displace ^{125}I-URP. Since hUII and URP differ only by the length and composition of their N-terminal domain [4], it appears that this region is probably responsible for this differential binding mode. A similar

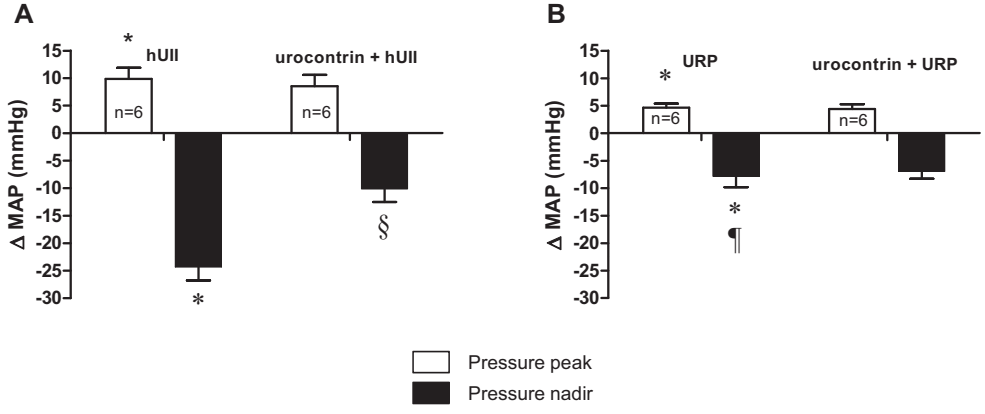

Fig. 5. Effect of urocontrin (0.01 μmol/kg) on (A) hUII and (B) URP hemodynamic actions in anesthetized rats. Data represent the mean ± S.E.M. and $n = 6$ animals. Significant differences between hUII- or URP-associated hemodynamic effects ($^{*}P < 0.05$) were determined by unpaired Student t-test. Significant differences between hUII-associated hemodynamic in the presence or absence of urocontrin pre-treatment were determined by unpaired Student t-test ($^{§}P < 0.05$). Significant differences between hUII- and URP-associated hemodynamic effects following the first injection were determined by unpaired Student t-test ($^{¶}P < 0.05$).

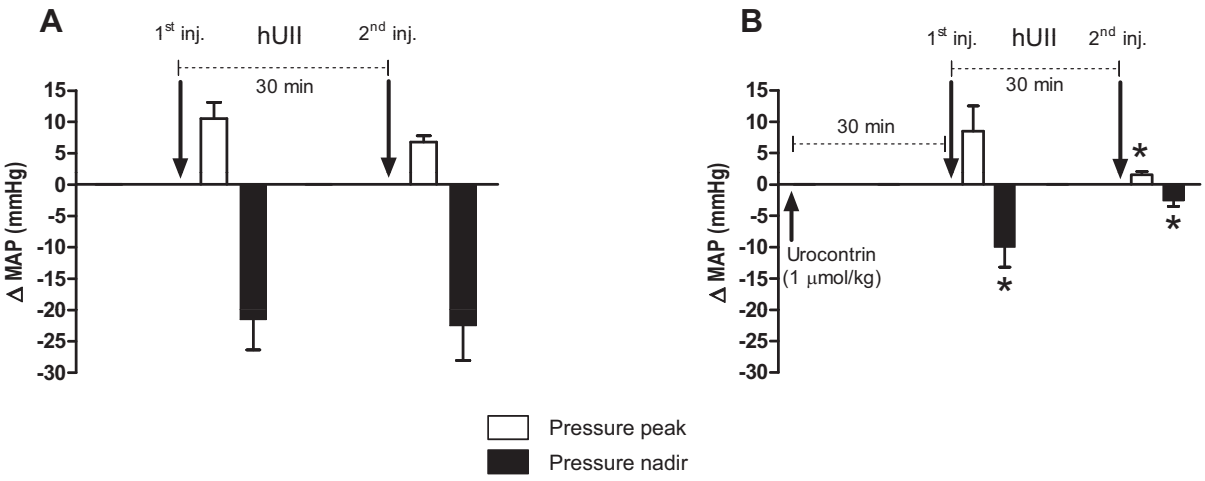

Fig. 6. (A) Effect of repeated hUII injections on hemodynamism in anesthetized rats. (B) Effect of urocontrin (0.01 μmol/kg) on repeated *i.v.* hUII injections in anesthetized rats. Significant differences between hUII injections following urocontrin pre-treatment or not were determined by unpaired Student *t*-test ($^*P < 0.05$). Data represent the mean ± S.E.M. and $n = 4$ animals.

assumption was also made to explain the divergent action of UII and URP on ischemic heart [8]. Studies are actually ongoing to demonstrate this hypothesis.

In anesthetized rats, hUII and URP exert a rapid and transient pressor phase with the maximal effect occurring almost 1 min after the administration. This first phase is followed by a hypotensive effect reaching its maximum 5 min after injection and with rather slow offsets as previously demonstrated in anesthetized WKY and SHR rats [24]. Interestingly, at the given concentration, the hUII-associated hypotension is significantly different from that observed with URP (Fig. 5A and B). In accordance with our results, intravenous bolus hUII injection was found to decrease mean arterial blood pressure in various anesthetized rat strain [24,28,36]. However, no biphasic hemodynamic profile was observed [24,28,36]. The mild initial and transient pressor effect may be easily overlooked and could be variable between rat strains as well as dependent on other experimental conditions such as the age of animals and choice of anesthetic agents. Further studies are ongoing to assess this specific phenomenon. The unique specificity of action of urocontrin was also observed *in vivo* where only the hUII-associated hemodynamic action, and more particularly the hypotensive effect, is modulated. Interestingly, urantide, a well known peptidic UII antagonist but also low-efficacy agonist was unable to reduce the hypotensive action of hUII *in vivo* under similar conditions [24] but was yet able to attenuate monocrotaline-induced cardiac hypertrophy [37] and plasma extravasation [34]. Once again, treatment with urocontrin induces a marked reduction of the hUII-associated hypotensive action after a first injection and a complete inhibition of UII-hemodynamic effect after the second injection, suggesting a slow dissociation rate of urocontrin from the receptor. Although the longevity of the antagonist-receptor complex is quoted in many studies to explain insurmountable antagonism, slowly interconverting receptor conformations, allosteric binding sites, and receptor internalization have been evoked as alternative explanations [38]. Further *in vitro* experiments are currently ongoing to assess those questions. However, desensitization of the urotensinergic system is unlikely to be responsible for the effect observed with hUII following urocontrin administration. Indeed, this hypothesis is supported by the absence of effect observed with URP (Fig. 5B) as well as the absence of desensitization of the system following repeated injection of hUII (Fig. 6A). Recently, insurmountable non-peptide antagonists were reported with GSK1562590 being able to reduce the maximum efficacy of UII-associated vasoconstriction in different aortic tissues but its efficacy against URP-mediated action was not assessed [25]. Interestingly, this compound also comprises a biphenyl group as in urocontrin and therefore a similar receptor interaction could be hypothesized. To the best of our knowledge, such modulation of the urotensinergic system was never observed before and therefore urocontrin represents the first compound of a completely new class of bitopic ligands that might be able to discriminate the physiological and pathophysiological activities of UII and URP.

With this study, we demonstrated the possibility to create UII specific antagonists that do not modulate *in vitro* and *in vivo* URP-associated action. All the reported antagonists, peptidic and non-peptidic, of the urotensinergic system were able to potently inhibit the UII-associated action *in vitro*. Nevertheless, their effects on the URP/UT system are unknown and might contribute to their failure once used for a specific therapeutic indication that is not associated with UII. For instance, distinct pathophysiological roles for UII and URP in hypertension have been suggested [31]. In this study, the mRNA expression of both UII and URP were up-regulated in the atrium of SHR rats when compared with age-matched WKY rats. However, the specific up-regulation of URP but not UII mRNA in aorta and kidney of SHR rats may suggest that UII and URP have distinct pathophysiological roles in hypertension [31]. Diabetic nephropathy is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. Consequently, and based on the previous report, such pathology could be associated with URP and not UII up-regulation. It is thus important to reassess the pharmacological profile of previously developed UII agonists and antagonists and assign specific physiological and pathophysiological roles for each peptide.

In summary, we demonstrated the unique pharmacological profile of urocontrin both *in vitro* and *in vivo*. Indeed, this compound blocking both *in vivo* and *in vitro* hUII-associated action but not URP-mediated effects represents the first analog of a new class of urotensinergic ligands. Such derivative would enable a better understanding of the pathophysiological role of the urotensinergic system and would allow discriminating *in vitro* and *in vivo* specific biological actions mediated by UII and/or URP. As such, urocontrin should prove to be useful as a chemical template for the rational design of novel UT receptor ligands, as well as a pharmacological tool for *in vitro* and particularly *in vivo* studies aimed at clarifying the role(s) played by the UII/URP/UT receptor system in physiology and pathology.

Acknowledgments

This work was supported by the Canadian Institutes of Health Research. JD is a National Researcher from the Fonds de la Recherche en Santé du Québec. The authors wish to thank Drs. Hubert Vaudry and Christophe Dubessy (Université de Rouen, France) for the CHO–UT transfected cell line.

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