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Deletion of the UT receptor gene results in the selective loss of urotensin-II contractile activity in aortae isolated from UT receptor knockout mice

*,[1]David J. Behm, [2]Stephen M. Harrison, [1]Zhaohui Ao, [3]Kristeen Maniscalco, [2,5]Susan J. Pickering, [2,6]Evelyn V. Grau, [4]Tina N. Woods, [4]Robert W. Coatney, [3]Christopher P.A. Doe, [3]Robert N. Willette, [1]Douglas G. Johns & [1]Stephen A. Douglas

[1]Department of Vascular Biology, Cardiovascular and Urogenital Diseases Center of Excellence for Drug Discovery, GlaxoSmithKline, King of Prussia, PA 19406-0939, U.S.A.; [2]Department of Comparative Genomics, GlaxoSmithKline, New Frontiers Science Park, Harlow CM19 5AW; [3]Department of Investigative Biology, Cardiovascular and Urogenital Diseases Center of Excellence for Drug Discovery, GlaxoSmithKline, King of Prussia, PA 19406-0939, U.S.A. and [4]Department of Laboratory Animal Sciences, GlaxoSmithKline, King of Prussia, PA 19406-0939, U.S.A.

1   Urotensin-II (U-II) is among the most potent mammalian vasoconstrictors identified and may play a role in the aetiology of essential hypertension. Currently, only one mouse U-II receptor (UT) gene has been cloned. It is postulated that this protein is solely responsible for mediating U-II-induced vasoconstriction.

2   This hypothesis has been investigated in the present study, which assessed basal haemodynamics and vascular reactivity to hU-II in wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice.

3   Basal left ventricular end-diastolic and end-systolic volumes/pressures, stroke volumes, mean arterial blood pressures, heart rates, cardiac outputs and ejection fractions in $UT^{(+/+)}$ mice and in $UT^{(-/-)}$ mice were similar.

4   Relative to $UT^{(+/+)}$ mouse isolated thoracic aorta, where hU-II was a potent spasmogen ($pEC_{50} = 8.26 \pm 0.08$) that evoked relatively little vasoconstriction ($17 \pm 2\%$ 60 mM KCl), vessels isolated from $UT^{(-/-)}$ mice did not respond to hU-II. However, in contrast, the superior mesenteric artery isolated from both the genotypes did not contract in the presence of hU-II. Reactivity to unrelated vasoconstrictors (phenylephrine, endothelin-1, KCl) and endothelium-dependent/independent vasodilator agents (carbachol, sodium nitroprusside) was similar in the aorta and superior mesenteric arteries isolated from both the genotypes.

5   The present study is the first to directly link hU-II-induced vasoconstriction with the UT receptor. Deletion of the UT receptor gene results in loss of hU-II contractile action with no 'nonspecific' alterations in vascular reactivity. However, as might be predicted based on the limited contractile efficacy recorded *in vitro*, the contribution that hU-II and its receptor make to basal systemic haemodynamics appears to be negligible in this species.
British Journal of Pharmacology (2003) **139,** 464 – 472. doi:10.1038/sj.bjp.0705254

*Author for correspondence at: Department of Vascular Biology (UW2510), Cardiovascular and Urogenital Diseases Center of Excellence for Drug Discovery, GlaxoSmithKline, PO Box 1539, 709 Swedeland Road, King of Prussia, PA 19406-0939, U.S.A.;
E-mail: David_J_Behm@gsk.com
[5]Current address: Centre for Preimplantation Genetic Diagnosis, Thomas Guy House, Guy's Hospital, London SE1 9RT, U.K.
[6]Current address: The Sanger Centre, Wellcome Trust Genome Campus, Hinxton, Cambs CB10 1SA, U.K.

Introduction

Urotensin-II (U-II) is a cyclic peptide (Pearson *et al.*, 1980) originally isolated from the goby urophysis (Bern & Lederis, 1969), a highly vascularized endocrine organ located at the caudal end of teleost fish spinal cords. Isoforms of this peptide are found not only in fish (Bern *et al.*, 1985; Conlon *et al.*, 1996), but also in amphibians (Conlon *et al.*, 1992) and mammals (Coulouarn *et al.*, 1998; 1999). In addition to

altering metabolic (Sheridan & Bern 1986; Kelsall & Balment 1998; Silvestre *et al.*, 2001), CNS (Gartlon *et al.*, 2001; Filipeanu *et al.*, 2002; Lu *et al.*, 2002; Pelletier *et al.*, 2002), osmoregulatory (Shenouda *et al.*, 2002) and pulmonary function (Hay *et al.*, 2000), U-II exhibits profound effects upon the cardiovascular system (Douglas & Ohlstein, 2000a; Maguire & Davenport, 2002). Indeed, human urotensin-II (hU-II) has been characterized as the most potent mammalian vasoconstrictor identified (Ames *et al.*, 1999; Douglas *et al.*, 2000). Recently, elevated U-II expression has been reported in both hypertensive (Matsushita *et al.*, 2001) and heart failure patients (Douglas *et al.*, 2002; Richards *et al.*, 2002) suggesting that U-II isopeptides may play a role in regulating mammalian cardiovascular homeostasis. Similarly, U-II levels are higher in patients on dialysis (Totsune *et al.*, 2001), with renal tubular abnormality (Matsushita *et al.*, 2001) and portal hypertension (Heller *et al.*, 2002), suggesting that U-II may also participate in the (patho)physiology of renal and liver disease.

The G-protein-coupled receptor originally termed GPR14/SENR (Marchese *et al.*, 1995; Tal *et al.*, 1995), and recently renamed the urotensin-II receptor (UT) by IUPHAR (see Douglas & Ohlstein, 2000b), has been identified as a receptor for U-II (Ames *et al.*, 1999; Liu *et al.*, 1999). Human, rat, mouse and monkey UT receptors have been cloned (Marchese *et al.*, 1995; Tal *et al.*, 1995; Ames *et al.*, 1999; Elshourbagy *et al.*, 2002). The amino-acid sequence identity of the monkey UT receptor is 97 and 77% identical to the human and rat sequence, while the mouse UT receptor is 76 and 93% identical to the human and rat sequence, respectively. However, it is unclear as to whether these sequences represent receptor homologues or sequence paralogues. Data to support the existence of UT receptor subtypes are ambiguous at best. Using multi-tissue Northern analysis, Liu *et al.* (1999) observed different UT receptor transcript sizes in human tissues. Functional data suggesting the existence of UT receptor subtypes have also been reported. Coy *et al.* (2000) demonstrated that although the somatostatin analogue (Cpa-c[D-Cys-Pal-D-Trp-Lys-Val-Cys]-Cpa-amide) was able to inhibit U-II-induced phasic contraction in the rat isolated aorta, the peptidic moiety was unable to block tonic contraction. Further, Camarda *et al.* (2002) demonstrated that [Orn^8]U-II acts as a full agonist at HEK293 cells expressing recombinant rat UT receptor, but is a competitive antagonist in rat isolated aortae. Although these observations could be interpreted as consistent with the existence of UT receptor subtypes, such evidence is far from definitive.

The UT receptor is clearly expressed in vascular tissue (Ames *et al.*, 1999; Maguire *et al.*, 2000), and it is possible therefore that U-II exerts its effects on vascular smooth muscle tone via an interaction with UT. However, a direct link between U-II, the UT receptor and vasoconstriction has yet to be established (Ames *et al.*, 1999; Douglas & Ohlstein, 2001a). In this study we have used homologous recombination in embryonic stem (ES) cells to generate mice lacking the UT receptor coding region to determine the effect(s) of this receptor on vascular reactivity both *in vivo* and *in vitro*.

Methods

All procedures were performed in accredited facilities in accordance with institutional guidelines (Animal Care and Use Committee, GlaxoSmithKline), the requirements of the United Kingdom Animals (Scientific Procedures) Act (1986) and the Guide for the Care and Use of Laboratory Animals (DHSS #NIH 85-23).

Targeting the UT gene and generation of mutant mice

Gene targeting was performed in murine E14.1 ES cells, replacing the single coding exon of the UT receptor locus with a positive selection cassette containing the neomycin phosphotransferase gene (Neo) driven by the phosphoglycerate kinase I (PGK) promoter. 5′- and 3′-homology arms, both of ~4.0 kb, were cloned from a 129SVJ mouse genomic bacterial artificial chromosome (BAC) library and placed on either side of the positive selection cassette. Homologous recombination in neomycin-resistant ES cells was confirmed by Southern blot of *Bam*HI-digested genomic DNA using an ~800 bp *Bam*HI/*Sma*I restriction fragment as the 5′ external probe (which detects 6.5 and 6.0 kb bands at the wild-type and targeted locus, respectively). Approximately one in 80 G418-resistant clones had undergone homologous recombination. Homologous recombination at the 3′ end was confirmed in these ES cell clones by Southern blot of *Hind*III-digested genomic DNA using a ~700 bp *Xmn*I/*Hind*III restriction fragment as the 3′-external probe (which detects 5.5 and 5.0 kb bands at the wild-type and targeted locus, respectively). Three targeted clones were injected into C57B16/J-derived blastocysts. Male chimeras were crossed with C57B16/J females to give N1F0 offspring, which were subsequently intercrossed to generate N1F1 offspring. In addition, N1F0 offspring were successively backcrossed to C57B16/J females to generate N5F0 mice. These were intercrossed to create an N5F1 population.

Genotyping of study populations

N1F1 and N5F1 study populations were genotyped by polymerase chain reaction (PCR) and Southern blot of genomic DNA isolated from the hearts of animals used in the studies. Hearts of wild-type (UT$^{(+/+)}$) and UT receptor knockout (UT$^{(-/-)}$) mice were cut into small pieces (~1 mm) and placed into polypropylene tubes. Extraction buffer (2 ml; 10 mM Tris-HCl pH 8.0, 50 mM NaCl, 100 mM ethylenediaminetetraacetic acid (EDTA), 0.5% SDS, 20 μg ml^{-1} RNase, 100 μg ml^{-1} Proteinase K) was added and samples were incubated at 50–55°C for 4 h until completely lysed. The mixture was then extracted two times each with phenol, phenol/chloroform and chloroform (Maniatis *et al.*, 1989). Genomic DNA was precipitated by adding 2.5 volumes of cold ethanol, washed with 70% v v^{-1} ethanol (−20°C) and dissolved in 200 μl of (10 mM Tris-HCl, 1 mM EDTA TE, pH 8.0). Once dissolved, the purity and concentration of the DNA were measured by spectrophotometry (absorbency at 260 and 280 nm wavelength).

PCR amplification was performed in 50 μl aliquots (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 17 mM MgCl$_2$, 200 nM 2′-deoxynucleotide 5′-triphosphates (dNTPs), 10% v v^{-1} dimethylsulphoxide, 1.25 U *Taq* DNA polymerase; Perkin-Elmer, Norwalk, CT, U.S.A.) using 200 ng of genomic DNA as the template and PCR primers specific to the neomycin resistance gene present at the targeted locus (5′-TGA ACA AGA TGG ATT GCA CGC AGG TTC TCC GGC-3′ and 5′-GCC AAG CTC TTC AGC AAT ATC ACG GGT AGC-3′,

yielding an ~700 bp product) and mouse UT gene-specific primers (5′-CTG GCT GAC CTG CTG TAT CTG CT-3′ and 5′-CAG GGT CAC ACA AAG CAC TCT CA-3′, yielding an ~900 bp product). A 500 bp mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplicon was used as the internal control (5′-TGG CCA AGG TCA TCC ATG AC-3′ and 5′-GTC CAC CAC CCT GTT GCT GTA G-3′, yielding an ~500 bp product). Amplification was performed for 30 cycles at 60°C annealing for 30 s, 72°C extension for 90 s and 94°C denaturing for 30 s. Amplification of a 500 bp (GAPDH)/700 bp doublet alone corresponded to a UT$^{(-/-)}$ genotype and a 500 bp (GAPDH)/900 bp doublet alone corresponded to a UT$^{(+/+)}$ genotype.

Genomic DNA (20 μg) was digested with *Bam*HI and run on an agarose gel (1%). The agarose gel was then treated with denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 45 min, followed by neutralization with 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 30–40 min. The DNA was transferred to a nylon membrane (GeneScreen Plus, NEN Life Science Products, MA, U.S.A.) and probed with cDNA corresponding to the full-length mouse UT receptor open reading frame (ORF). cDNA fragments were labelled with [α-^{32}P] 2′-deoxycytidine 5′-triphosphate (dCTP) using standard random primed methods (T7 QuickPrime; Pharmacia Biotech, Piscataway, NJ, U.S.A.). Membranes were prehybridized for 2 h at 42°C and incubated overnight at 42°C with 1×10^9 cpm μg^{-1} denatured radiolabelled probe in standard buffer (50% deionized formamide, $6 \times$ sodium chloride, sodium citrate (SSC), $5 \times$ Denhardt's reagent, 0.5% sodium dodecyl sulphate (SDS), 100 μg ml^{-1} denatured, fragmented salmon sperm DNA). Membranes were washed under conditions of low stringency (three 15 min washes in $1 \times$ SSC, 0.1% SDS at 28°C) followed by a high stringency wash in $0.1 \times$ SSC/0.1% SDS for 30 min at 55°C. Hybridization signals were detected by conventional X-ray autoradiography (Hyper film, Amersham Life Science, U.K.) and phosphor imaging (Storm 860, Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Haemodynamics and echocardiography

Male wild-type (UT$^{(+/+)}$) and homozygous UT receptor knockout (UT$^{(-/-)}$) mice, anaesthetized with 1.5% isoflurane, underwent transthoracic echocardiographic determination of left ventricular end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), cardiac output (CO) and ejection fraction (EF). Further to this, mice were reanaesthetized the following day for haemodynamic evaluation where a fluid-filled catheter was inserted into the left carotid artery for the measurement of mean arterial blood pressure (MAP) and heart rate (HR). The catheter was then advanced into the left ventricle (LV) to obtain measurements of left ventricular end-systolic (LVESP) and end-diastolic pressure (LVEDP). At the end of the study, selected organs (right and left kidney, heart, right and left ventricle and lungs) were isolated and wet weights were measured.

Preparation and utilization of mouse isolated aortae and mesenteric arteries

Male (4 months; 27 g) wild-type (UT$^{(+/+)}$) and UT receptor knockout (UT$^{(-/-)}$) mice were anaesthetized with inhaled isoflurane (5% in O$_2$) and killed by cervical dislocation.

Proximal descending thoracic aortae were isolated and cleaned of adherent tissue. Vessels approximately 3 mm in length were suspended in 10 ml organ baths containing Krebs of the following composition (mM): NaCl, 112.0; KCl, 4.7; KH$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; CaCl$_2$, 2.5; NaHCO$_3$, 25.0; dextrose, 11.0. Krebs was maintained at 37 ± 1°C and aerated with 95% O$_2$: 5% CO$_2$ (pH 7.4). For contraction studies, vessels were denuded of endothelium by rubbing with a fine forceps and indomethacin (10 μM) was added to the buffer. Changes in isometric force were measured under 0.5 g optimal resting tension using FT03 force–displacement transducers (Grass Instruments, Quincy, MA, U.S.A.) coupled to Model 7D polygraphs.

The Halpern–Mulvany wire myograph (Model 610M; Danish Myo Technology, Denmark) was used for measurement of isometric force development of endothelium-intact superior mesenteric arteries (optimal resting tension of 0.5 g) and data were recorded using a Grass 7400 direct thermal recorder.

Following a 60 min equilibration period, vessels were treated with standard concentrations of KCl (60 mM) and phenylephrine (1 μM) to which subsequent agonist-induced responses were normalized. Once the contractile response to phenylephrine had plateaued, carbachol (10 μM) was added to the vessels in order to evaluate endothelial integrity.

Cumulative concentration–response curves to phenylephrine (0.1 nM – 10 μM), angiotensin-II (0.1 nM – 10 μM), endothelin-1 (0.1 nM – 1 μM) and hU-II (0.01 nM – 3 μM) were obtained for each vessel by adding the spasmogen to the tissue bath at half-log increments. During relaxation studies, vessels were preconstricted with an EC$_{80}$ concentration of phenylephrine and contractile tone was reversed by adding cumulative amounts of carbachol (1 nM – 30 μM) or sodium nitroprusside (SNP; 0.1 nM – 1 μM). Each response was allowed to plateau before the addition of subsequent agonist concentrations. Vessels were allowed to recover for at least 30 min between subsequent agonist–response curves, and were not exposed to subsequent agonists after treatment with either endothelin-1 or hU-II.

Statistical and data analysis

All values are expressed as mean $\pm$ s.e.m. and n represents the total number of animals from which the vessels were isolated. Statistical comparisons were made using an unpaired, two-tailed *t*-test or Fisher's exact tests and differences were considered significant when $P \leqslant 0.05$. Concentration–response curves were fitted to a logistic equation as previously described (Douglas *et al.*, 1995):

$$R = \frac{R_{max} \cdot C^{n_H}}{EC_{50}^{n_H} + C^{n_H}}$$

where R is the contraction response; C, the concentration of agonist; EC$_{50}$, the concentration of agonist required to produce a half-maximal response; n_H, the Hill coefficient; R_{max}, the maximal contractile or relaxant response.

Drugs and reagents

hU-II was custom synthesized by California Peptide Research Inc. (Napa, CA, U.S.A.). Angiotensin-II, carbachol, endothelin-1, phenylephrine and SNP were from Sigma (St Louis, MO,

U.S.A.). All other reagents used were of analytical grade. All drug solutions were made fresh on the day of experimentation and stored in a light-tight container on ice.

Results

Targeting of the UT gene and generation of mutant mice

Targeted deletion of the mouse UT receptor gene was successfully achieved using standard homologous recombination in ES cells. Specifically, the single coding exon of the UT receptor was replaced with the positive selection cassette, thus removing all coding sequence (Figure 1a). Chimeric mice derived from the targeted 129Ola ES cell lines were crossed with C57B1/6J females to generate N1F0 heterozygotes, which were intercrossed giving rise to overtly healthy N1F1 homo-
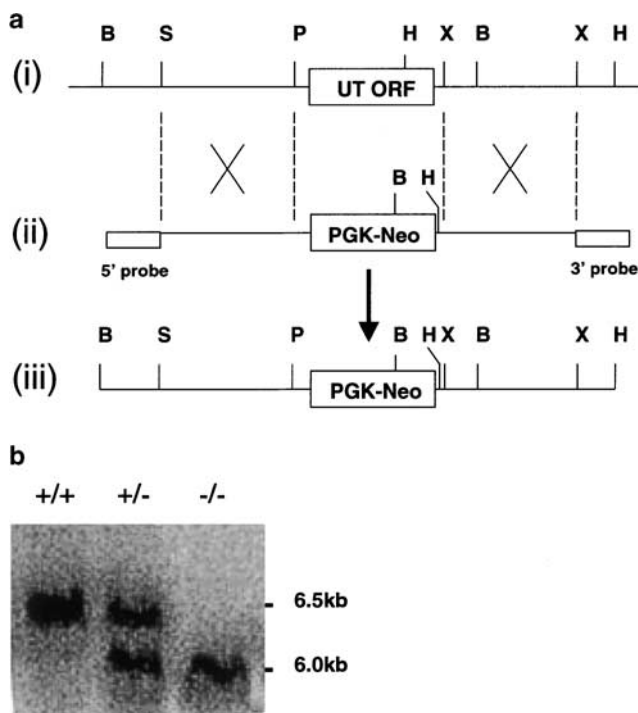
zygous mutant offspring in the expected Mendelian ratio. Successful targeting of the locus and germline transmission was confirmed by Southern analysis (Figure 1b). The N5F1 study population was generated by successive backcrossing of male heterozygotes to C57B1/6J females to generate N5F0 heterozygotes, which were then intercrossed.

PCR and Southern blot analysis of heart genomic DNA was used to identify wild-type mice and mice homozygous for the UT targeted mutation (Figure 2). Wild-type mice show the UT-specific PCR product of 900 bp and lack the 700 bp neomycin resistance gene product. Conversely, mice homozygous for the UT targeted mutation show the neomycin resistance gene product of 700 bp and lack the 900 bp UT-specific PCR product (Figure 2a). This PCR genotyping was confirmed by Southern blot analysis of *Bam*HI-digested genomic DNA probed with a UT gene-specific restriction fragment where, in contrast to UT$^{(+/+)}$ mice, the 6.5 kb *Bam*HI restriction fragment containing the UT receptor gene was absent in UT$^{(-/-)}$ mice (Figure 2b).

Basal haemodynamics, echocardiography and body weight

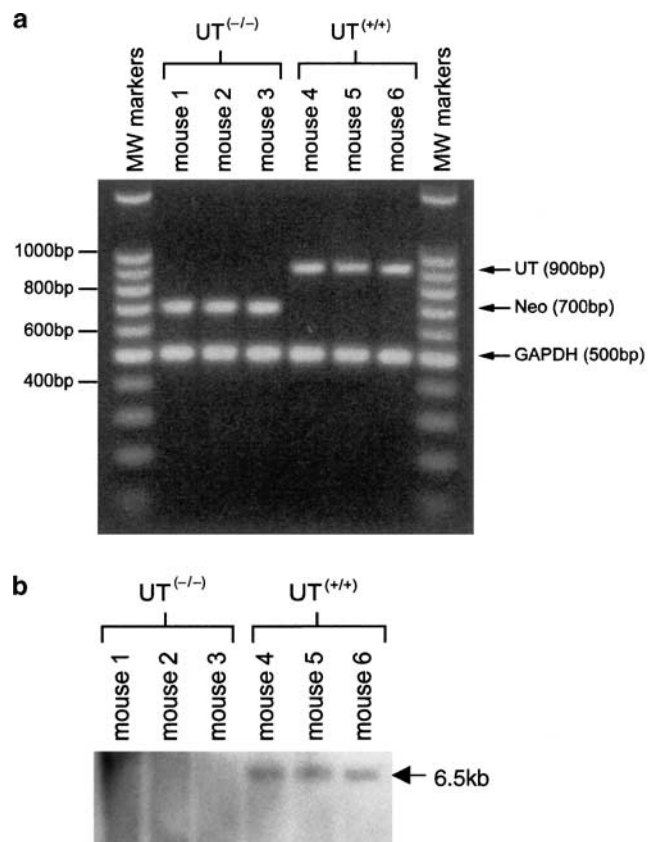Targeted deletion of the UT receptor did not alter basal systemic haemodynamics or major organ weights. Basal left

Figure 1 Disruption of the UT receptor gene by homologous recombination. (a) Targeting strategy for the UT receptor locus. (i) Structure of the UT receptor genomic locus. The single coding exon is indicated as urotensin-II receptor open reading frame ('UT ORF'). The position of the ~800 bp *Bam*HI/*Sma*I (B/S; see below) and *Xma*I/*Hind*III (X/H; see below) restriction fragment probes used to identify homologous recombination events at the 5′ and 3′ ends, respectively, are also indicated, (ii) Structure of the targeting construct. The 5′ and 3′ arms of homology are ~4 kb *Sma*I – *Pst*I (S-P; see below) and ~4 kb *Xmn*I (X, see below) restriction fragments, respectively. The *Pst*I site (P; see below) is 158 bp upstream of the UT initiation codon and the 5′ *Xmn*I site (X; see below) is 40 bp downstream of the termination codon. The PGK-Neo selection cassette is indicated. (iii) Structure of the targeted locus. Homologous recombination replaces the single coding exon with a PGK-Neo selection cassette. Restriction sites: B, *Bam*HII; S, *Sma*I; P, *Pst*I; H, *Hind*III; X, *Xmn*I. (b) Southern blot analysis of N1F1 offspring tail DNA (wild-type, UT$^{(+/+)}$; heterozygote, UT$^{(+/-)}$; homozygous UT receptor knockout, UT$^{(-/-)}$), digested with *Bam*HI and probed with the *Bam*HI/*Sma*I restriction fragment. The 6.5 kb wild-type locus specific band and 6.0 kb targeted locus specific band are indicated.

Figure 2 UT receptor gene analysis of wild-type (UT$^{(+/+)}$) and UT receptor knockout (UT$^{(-/-)}$) mice as determined by (a) PCR and (b) Southern blot. PCR analysis indicates that the 900 bp UT receptor gene is replaced with the neomycin resistance gene product (Neo) of 700 bp in UT$^{(-/-)}$. The lack of UT receptor gene in UT$^{(-/-)}$ is confirmed by Southern blot analysis of *Bam*HI-digested heart genomic DNA probed with probed radiolabelled with cDNA corresponding to the full-length mouse UT receptor ORF.

ventricular EDV, ESV, SV, CO and EF determined by transthoracic echocardiography in the wild-type ($UT^{(+/+)}$) mice were similar to those values determined in UT receptor knockout ($UT^{(-/-)}$) mice (Table 1). Inaddition, mean arterial blood pressure, heart rate and left ventricular systolic and end-diastolic pressure were similar in both the genotypes (Table 1). In accord with these haemodynamic data, body weights and organ-to-body-weight ratios were similar in $UT^{(+/+)}$ and $UT^{(-/-)}$ mice (Table 2).

Effects of vasoactive agents in mouse-isolated arteries

Relative to the responses in $UT^{(+/+)}$ mice, where hU-II was a potent (EC_{50} of $\sim 5\,nM$) and low-efficacy ($R_{max} < 20\%$ response to 60 mM KCl) vasoconstrictor, aortae isolated from $UT^{(-/-)}$ mice did not respond to hU-II (Table 3, Figures 3 and 4). However, this observation was not accompanied by any general aberration in vascular reactivity, since responses to unrelated vasoactive agents were unaltered. Thoracic aortae

Table 1 Basal echocardiographic and haemodynamic measurements in wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice

Genotype	MBP (mmHg)	Heart rate (min^{-1})	LVESP (mmHg)	LVEDP (mmHg)	EDV (ml)	ESV (ml)	SV (ml)	CO (ml min^{-1})	EF (%)
$UT^{(+/+)}$	78 ± 3	383 ± 22	93 ± 7	2.5 ± 0.8	0.091 ± 0.002	0.057 ± 0.004	0.034 ± 0.018	14.0 ± 0.9	38 ± 2
n	7	7	5	5	15	15	15	15	15
$UT^{(-/-)}$	79 ± 3	369 ± 27	87 ± 4	1.0 ± 0.3	0.086 ± 0.003	0.052 ± 0.002	0.035 ± 0.002	14.2 ± 1.0	40 ± 2
n	8	8	8	8	16	16	16	16	16

All values are expressed as mean $\pm$ s.e.m. Statistical comparisons were made using unpaired two-tailed *t*-tests. No statistical differences were identified between the $UT^{(+/+)}$ and $UT^{(-/-)}$ mice.

Table 2 Organ-to-body-weight ratios in wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice

Genotype	Body weight (g)	Left kidney (10^{-3})	Right kidney (10^{-3})	Heart (10^{-3})	Left ventricle (10^{-3})	Right ventricle (10^{-3})	Lung (10^{-3})
$UT^{(+/+)}$ (n=8)	27.5 ± 0.9	6.1 ± 0.2	6.5 ± 0.2	4.2 ± 0.2	3.2 ± 0.1	0.8 ± 0.1	4.9 ± 0.2
$UT^{(-/-)}$ (n=8)	27.1 ± 0.6	6.2 ± 0.1	6.3 ± 0.1	4.3 ± 0.1	3.3 ± 0.1	0.8 ± 0.1	5.0 ± 0.1

All values are expressed as mean $\pm$ s.e.m. Kidney:, heart: and lung: to-body-weight ratios are expressed as mg g^{-1}. Statistical comparisons were made using unpaired two-tailed *t*-tests. No statistical differences were identified between the $UT^{(+/+)}$ and $UT^{(-/-)}$ mice.

Table 3 Responses to various vasoactive compounds in thoracic aortae and superior mesenteric artery isolated from wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice

Thoracic aorta	$-log[EC_{50}]$		R_{max} (% normalized)		Responders/n	
Vasoactive agent	$UT^{(+/+)}$	$UT^{(-/-)}$	$UT^{(+/+)}$	$UT^{(-/-)}$	$UT^{(+/+)}$	$UT^{(-/-)}$
Urotensin-II	8.35 ± 0.07	–	17 ± 2	$0*$	5/7	0/8*
Phenylephrine	7.33 ± 0.07	7.39 ± 0.04	115 ± 6	117 ± 8	6/6	6/6
Endothelin-1	8.51 ± 0.06	8.39 ± 0.19	15 ± 3	16 ± 2	9/9	8/10
Angiotensin-II	–	–	0	0	0/4	0/4
KCl	1.64 ± 0.03	1.58 ± 0.03	132 ± 8	136 ± 7	6/6	6/6
Carbachol	6.33 ± 0.17	6.08 ± 0.25	65 ± 5	67 ± 6	6/6	5/6
Sodium nitroprusside	8.87 ± 0.09	8.85 ± 0.10	100 ± 0	100 ± 0	6/6	6/6

Mesenteric artery	$-log[EC_{50}]$		R_{max} (% normalized)		Responders/n	
Vasoactive agent	$UT^{(+/+)}$	$UT^{(-/-)}$	$UT^{(+/+)}$	$UT^{(-/-)}$	$UT^{(+/+)}$	$UT^{(-/-)}$
Urotensin-II	–	–	0	0	0/6	0/6
Phenylephrine	5.83 ± 0.10	5.68 ± 0.13	148 ± 8	134 ± 14	6/6	6/6
Endothelin-1	8.66 ± 0.32	8.84 ± 0.21	10 ± 4	10 ± 3	4/4	5/5
Angiotensin-II	–	–	0	0	0/4	0/4
KCl	1.42 ± 0.04	1.42 ± 0.04	244 ± 44	384 ± 145	5/5	5/5
Carbachol	6.69 ± 0.16	6.41 ± 0.13	100 ± 0	100 ± 0	6/6	6/6
Sodium nitroprusside	8.66 ± 0.32	8.84 ± 0.21	100 ± 0	100 ± 0	6/6	6/6

All values are expressed as mean $\pm$ s.e.m. Statistical comparisons were made using unpaired two-tailed *t*-tests or Fisher's exact tests: *$P < 0.01$ compared to values obtained in $UT^{(+/+)}$ mice. Responses to KCl are normalized to 1 μM phenylephrine and responses to phenylephrine, endothelin-1, angiotensin-II and urotensin-II are normalized to 60 mM KCl. Responses to carbachol and sodium nitroprusside are expressed as per cent reversal of 1 μM phenylephrine-induced tone.
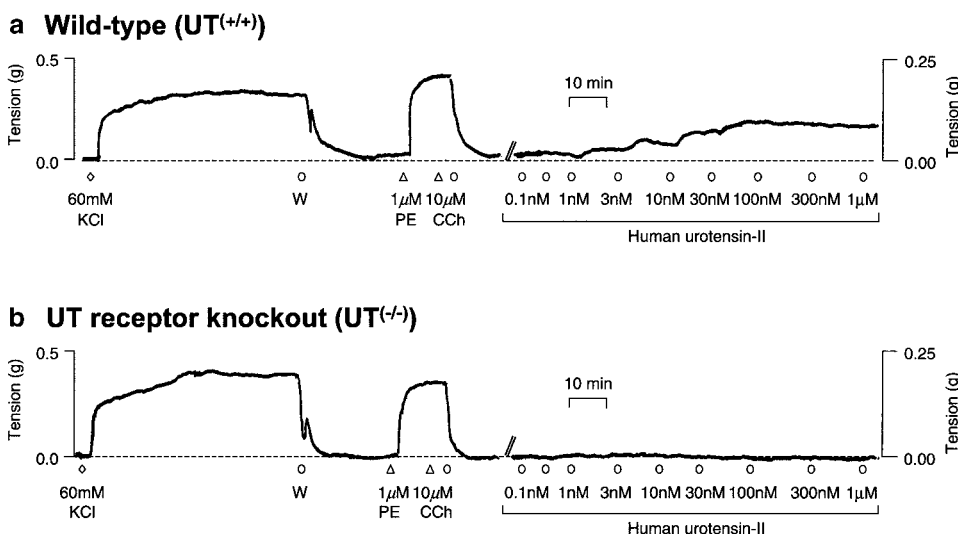
a Wild-type (UT$^{(+/+)}$)

b UT receptor knockout (UT$^{(-/-)}$)

Figure 3 Representative experimental traces illustrating the contractile responses to hU-II in the aortae isolated from wild-type (UT$^{(+/+)}$) and UT receptor knockout (UT$^{(-/-)}$) mice. In contrast to (a) UT$^{(+/+)}$ mice (where hU-II is a potent spasmogen evoking relatively little vasoconstriction), (b) aortae isolated from UT$^{(-/-)}$ do not respond to hU-II. Vessels were precontracted with 60 mM KCl. Following washing (W), endothelial cell integrity was evaluated by adding 1 μM phenylephrine (PE) and observing the response to 10 μM carbachol (CCh). The vertical and horizontal scale bars represent tension (0.5 and 0.25g) and time (10 min), respectively.
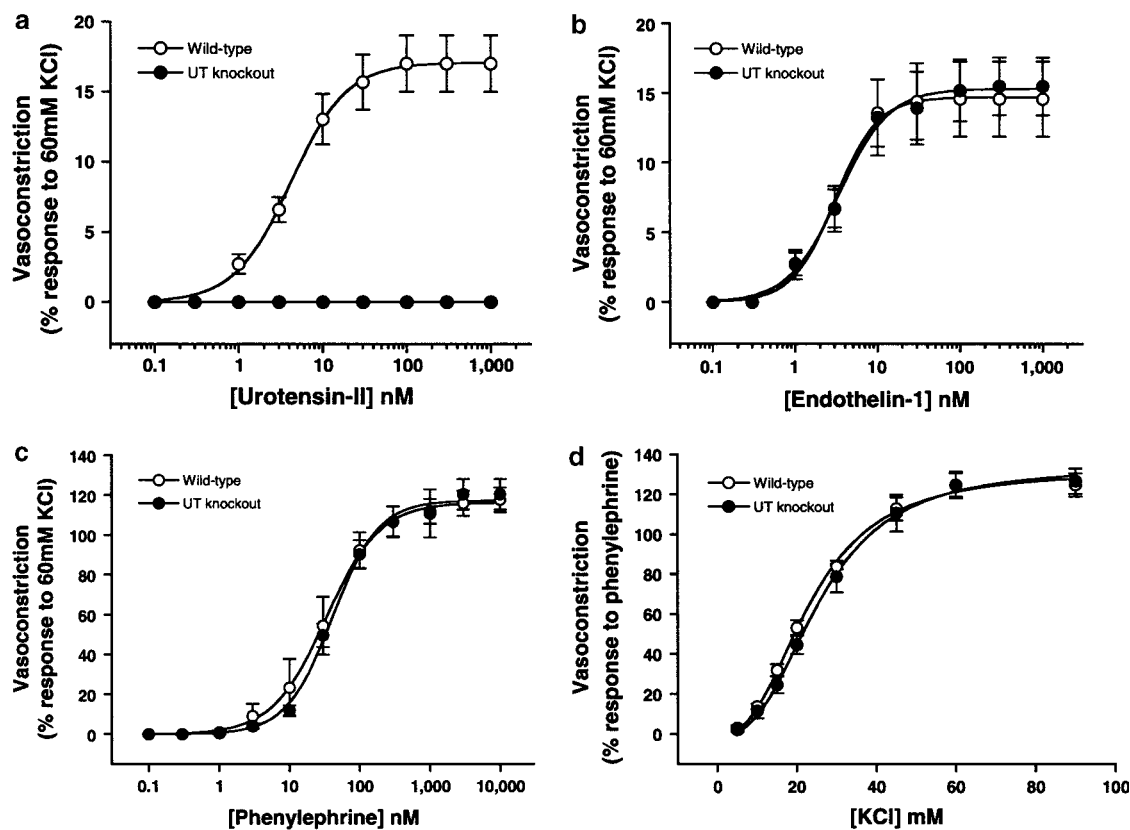

Figure 4 Loss of the UT receptor does not alter the responses to vasoconstrictors other than hU-II in the mouse isolated thoracic aorta. Log concentration-response curves to (a) hU-II, (b) endothelin-1, (c) phenylephrine and (d) KCl in aortae isolated from wild-type (UT$^{(-/+)}$) and UT receptor knockout (UT$^{(-/-)}$) mice. Values are mean and vertical bars represent the s.e.m. Curves were derived by fitting experimental data to a logistic equation (Douglas *et al.*, 1995).

isolated from UT$^{(+/+)}$ and UT$^{(-/-)}$ mice responded to cumulative concentrations of KCl, phenylephrine and endothelin-1 with similar potencies and efficacies (Table 3, Figure 4). Similarly, loss of the UT receptor did not alter the responses to

the endothelium-dependent and -independent vasodilators, carbachol and SNP (Table 3, Figure 5).

As in the aorta, responses to endothelin-1, phenylephrine, angiotensin-II, KCl, carbachol and SNP were similar in the
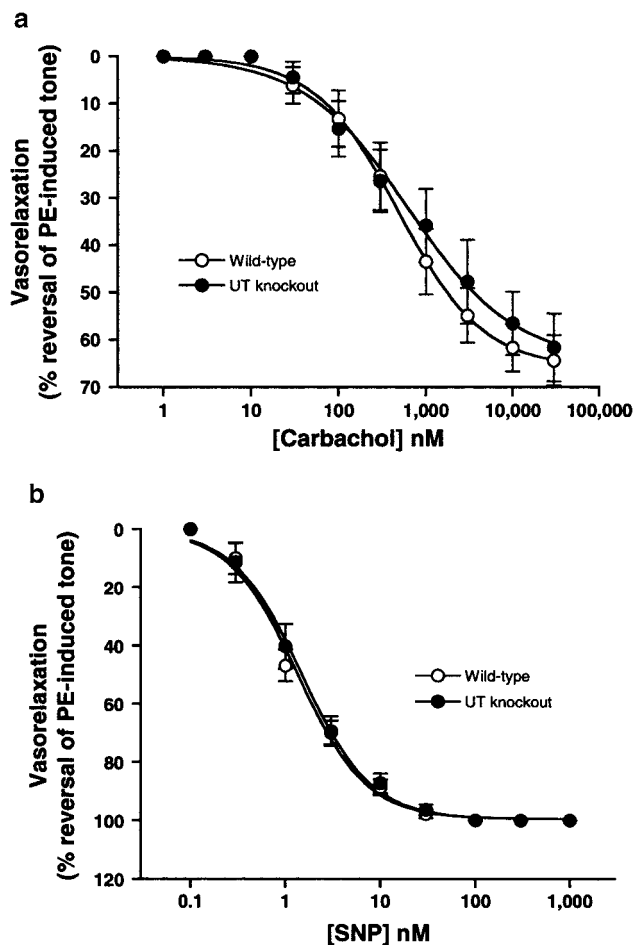
a

b

Figure 5 Loss of the UT receptor does not alter the responses to vasodilators in the mouse isolated aorta. Log concentration-response curves to (a) carbachol and (b) SNP in aortae isolated from wild-type ($UT^{(+/+)}$) and UT receptor knockout ($UT^{(-/-)}$) mice. Vessels were preconstricted with an EC_{80} concentration of phenylephrine and contractile tone was reversed by adding cumulative amounts of carbachol or SNP. Values are mean and vertical bars represent the s.e.m. Curves were derived by fitting experimental data to a logistic equation (Douglas *et al.*, 1995).

superior mesenteric arteries isolated from both the genotypes. However, in contrast to the response to hU-II observed in the aorta (vasoconstriction in $UT^{(+/+)}$ mice), the superior mesenteric artery isolated from both the $UT^{(-/-)}$ and $UT^{(+/+)}$ mice did not contract in the presence of hU-II (Table 3).

Discussion

U-II, among the most potent peptidic mammalian vasoconstrictors identified, is purported to elicit a contractile response via an interaction with its G-protein-coupled receptor, UT (Douglas & Ohlstein, 2000a, b; Maguire & Davenport, 2002). Interestingly, and in contrast to the majority of knockout studies investigating vasoactive peptide systems (where receptor subtypes exist typically), the hU-II/UT system is somewhat atypical inasmuch as no receptor subtypes have been identified to date. As in other mammalian species, one mouse UT receptor gene has been cloned. Therefore, it is assumed that this specific protein mediates U-II-induced vasoconstriction.

However, since this has not been previously investigated, the present study represents the first to provide a direct link between the UT receptor and vasoactivity, an association achieved via the generation of UT-receptor-deficient (knockout) mice.

In order to determine the effects of the UT receptor on cardiovascular homeostasis, basal haemodynamic measurements were made in wild-type mice ($UT^{(+/+)}$) and UT receptor knockout mice ($UT^{(-/-)}$). Basal echocardiographic and haemodynamic measurements in $UT^{(-/-)}$ mice were similar to those determined in $UT^{(+/+)}$ mice, suggesting that loss of the mouse UT receptor does not uncover an overt cardiovascular phenotype *in vivo*. This observation is not surprising since systemic administration of hU-II does not effect basal haemodynamics in this species (R. Willette, unpublished observation).

hU-II induces potent vasoconstriction in blood vessels isolated from the rat, rabbit, dog, pig, monkey (Douglas *et al.*, 2000; Saetrum Opgaard *et al.*, 2000; Camarda *et al.*, 2002) and man (MacLean *et al.*, 2000; Maguire *et al.*, 2000; Paysant *et al.*, 2001; Russell *et al.*, 2001; Camarda *et al.*, 2002). Vasoconstriction appears to be dependent on the species and anatomical origin of the vessel, that is, hU-II-induced contraction is restricted to isolated thoracic aortae in the rat and the spasmogen is a universal arterial vasoconstrictor in the cynomolgus monkey (Douglas *et al.*, 2000). Previous studies have demonstrated that vertebrate U-II isopeptides (fish, amphibian and mammalian) exhibit similar functional potencies across species (Gardiner *et al.*, 2001 [haemodynamic responses in the rat]; Russell *et al.*, 2001 [contractile activity in isolated trabeculae]). Indeed, competition binding studies reveal that both mouse and human U-II isopeptides exhibit similarly high affinities (2.5 and 4.0 nM K_is, respectively) for mouse UT receptor (Elshourbagy *et al.*, 2002). Together, these data demonstrate that hU-II is an agonist at the mouse UT receptor and that the relatively poor contractile activity described herein is not likely to reflect the use of a nonmurine (i.e. human) isoform of U-II in mouse isolated tissue. Although Douglas *et al.* (2000) reported no contractile response to hU-II in the mouse-isolated aorta, the ability of hU-II to contract various arteries isolated from wild-type ($UT^{(+/+)}$) mice was evaluated. As in the rat and dog, where vasoconstriction has only been observed in vessels proximal to the aortic arch (Douglas *et al.*, 2000), the present study reported that hU-II contracted $UT^{(+/+)}$ mouse isolated aortae but failed to elicit a contractile response in the superior mesenteric arteries. However, in contrast to the response previously observed in the rat aorta, hU-II induced relatively little vasoconstriction in the $UT^{(+/+)}$ mouse aorta (R_{max} was only $17\pm2\%$ response to 60 mM KCl). The fact that the present investigation was able to elicit a contractile response in wild-type $UT^{(+/+)}$ mice, albeit small in size, contrasts a previous report (Douglas *et al.*, 2000) where hU-II was purported to lack contractile activity in mouse aortae. This apparent disparity likely reflects difficulties encountered when trying to record such small responses (i.e. $<20\%$ response to 60 mM KCl, likely because of low UT receptor expression/spare receptor reserve in this tissue; Douglas, 2003).

In addition to being a potent vasoconstrictor in vessels isolated from several species, hU-II has been reported to induce vasodilation in rat (Bottrill *et al.*, 2000) and human (Stirrat *et al.*, 2001) isolated resistance arteries. Consistent with

the effects in rat-isolated resistance arteries, Gibson *et al.* (1986), Gardiner *et al.* (2001) and Lin *et al.* (2003) have all reported vasodilation to be the predominant cardiovascular response when hU-II is given systemically as a bolus dose in the rat (infusions, however, are associated with pressor responses in the rat; Bennett *et al.*, 2002). Although i.v. administration of hU-II does not induce a systemic hypotensive response in the mouse as it does in the rat (R. Willette, unpublished observation), it remains to be determined if a vasodilator response can be elicited with hU-II in mouse isolated arteries (and, if so, whether such a phenotype is lost as a function of UT deletion $UT^{(-/-)}$ mouse).

Once the contractile responses to hU-II were observed in tissues isolated from $UT^{(+/+)}$ mice, they were compared to those in $UT^{(-/-)}$ mice. Relative to $UT^{(+/+)}$ mouse isolated thoracic aorta where hU-II was a potent spasmogen, vessels isolated from $UT^{(-/-)}$ mice did not respond to hU-II. Thus, loss of the UT receptor results in the loss of U-II-induced contractile action. However, loss of the UT receptor gene did not alter the contractile responses of KCl, phenylephrine, endothelin-1 or angiotensin-II in the isolated aorta. Similarly, responses to the endothelium-dependent vasodilator carbachol or SNP, an endothelium-independent vasodilator, were not altered by the deletion of the UT receptor. Together, these data suggest that loss of the UT receptor results in the selective loss of hU-II contractile activity, that is, the lack of response to hU-II in $UT^{(-/-)}$ mice was not accompanied by any alteration in reactivity to unrelated spasmogens. However, even though the contractile efficacy of hU-II was low in mouse isolated aorta, it is pertinent to note that neither endothelin-1 nor angiotensin-II was able to elicit contractile responses equivalent to those seen with KCl or phenylephrine in this vessel. Although the aorta is a conduit vessel, since both angiotensin-II and endothelin-1 play a significant (patho)physiological role in the control of haemodynamic function/regional vascular resistance in man, one must recognize the potential shortcomings of using this species for studying the effects of vasoactive neurohormones.

In summary, the present study includes the first description of the generation of UT receptor knockout mice and constitutes the first direct link between the UT receptor and U-II-induced vasoconstriction. Deletion of the UT gene results in a loss of U-II contractile action within the mouse vasculature (without causing any 'nonspecific' alterations in vascular reactivity to other spasmogens). However, the contribution that U-II and its receptor make to the regulation/maintenance of basal systemic haemodynamics appears to be negligible in this species. Although loss of the UT receptor does not alter basal haemodynamics in the mouse, further experiments are required to assess the role of UT in the mouse under conditions of stress/pathology (e.g. salt-sensitive hypertension in bradykinin B_2 receptor knockout mice; Alfie *et al.*, 1996). Nevertheless, these mice may represent a useful tool for delineating other (patho)physiological roles for U-II outside the vasculature such as regulating endocrine/metabolic, CNS and pulmonary function. For example, U-II has been implicated in diabetes/obesity (U-II inhibits insulin release in the perfused rat pancreas; Silvestre *et al.*, 2001) and atherosclerosis (increased U-II binding in coronary artery atherosclerotic lesions; Maguire & Davenport, 2002). Since nonpeptide UT receptor antagonists are not currently commercially available, breeding UT receptor knockout mice onto a genetic background of diabetes, obesity and/or atherosclerosis (db/db, ob/ob and ApoE/LDLR deficient mice, etc.) may be an alternative/supplemental method for determining the influence of U-II and its receptor on the aetiology of cardiorenal disease.

References

ALFIE, M.E., YANG, X.P., HESS, F. & CARRETERO, O.A. (1996). Salt-sensitive hypertension in bradykinin B2 receptor knockout mice. *Biochem. Biophys. Res. Commun.,* **224,** 625 – 630.

AMES, R.S., SARAU, H.M., CHAMBERS, J.K., WILLETTE, R.N., AIYAR, N.V., ROMANIC, A.M., LOUDEN, C.S., FOLEY, J.J., SAUERMELCH, C.F., COATNEY, R.W., AO, Z., DISA, J., HOLMES, S.D., STADEL, J.M., MARTIN, J.D., LIU, W.S., GLOVER, G.I., WILSON, S., McNULTY, D.E., ELLIS, C.E., ELSHOURBAGY, N.A., SHABON, U., TRILL, J.J., HAY, D.W.P., OHLSTEIN, E.H., BERGSMA, D.J. & DOUGLAS, S.A. (1999). Human urotensin-II is a potent vasoconstrictor and agonist for the orphan receptor GPR14. *Nature,* **401,** 282 – 286.

BENNETT, T., MARCH, J.E., KEMP, P.A. & GARDINER, S.M. (2002). Regional haemodynamic responses to infusion of human urotensin II (hUII) in conscious Sprague – Dawley rats. *Br. J. Pharmacol.,* **135,** 200.

BERN, H.A., PEARSON, D., LARSON, B.A. & NISHIOKA, R.S. (1985). Neurohormones from fish tails: the caudal neurosecretory system. I. 'Urophysiology' and the caudal neurosecretory system of fishes. *Rec. Prog. Horm. Res.,* **41,** 533 – 552.

BOTTRILL, F.E., DOUGLAS, S.A., HILEY, C.R. & WHITE, R. (2000). Human urotensin-II is an endothelium-dependent vasodilator in rat small arteries. *Br. J. Pharmacol.,* **130,** 1865 – 1870.

CAMARDA, V., RIZZI, A., CALO, G., GENDRON, G., PERRON, S.I., KOSTENIS, E., ZAMBONI, P., MASCOLI, F. & REGOLI, D. (2002). Effects of human urotensin II in isolated vessels of various species; comparison with other vasoactive agents. *Naunyn-Schmiedeberg's Arch. Pharmacol.,* **365,** 141 – 149.

CONLON, J.M., O'HARTE, F., SMITH, D.D., TONON, M.C. & VAUDRY, H. (1992). Isolation and primary structure of urotensin II from the brain of a tetrapod, the frog *Rana ridibunda. Biochem. Biophys. Res. Commun.,* **188,** 578 – 583.

CONLON, J.M., YANO, K., WAUGH, D. & HAZON, N. (1996). Distribution and molecular forms of urotensin II and its role in cardiovascular regulation in vertebrates. *J. Expl. Zool.,* **275,** 226 – 238.

COULOUARN, Y., JEGOU, S., TOSTIVINT, H., VAUDRY, H. & LIHRMANN, I. (1999). Cloning, sequence analysis and tissue distribution of the mouse and rat urotensin II precursors. *FEBS Lett.,* **457,** 28 – 32.

COULOUARN, Y., LIHRMANN, I., JEGOU, S., ANOUAR, Y., TOSTIVINT, H., BEAUVILLAIN, J.C., CONLON, J.M., BERN, H.A. & VAUDRY, H. (1998). Cloning of the cDNA encoding the urotensin II precursor in frog and human reveals intense expression of the urotensin II gene in motoneurons of the spinal cord. *Proc. Natl. Acad. Sci. U.S.A.,* **95,** 15803 – 15808.

COY, D.H., ROSSOWSKI, W.J., CHENG, B.L., HOCART, S.J. & TAYLOR, J.E. (2000). Novel urotensin-II (UII) antagonists point to multiple receptor involvement in UII bioactivity. *Reg. Pep.,* **94,** 48.

DOUGLAS, S.A. (2003). Human urotensin-II as a novel cardiovascular target: 'heart' of the matter or simply a fishy 'tail'. *Curr. Opin. Pharmacol.* (In press).

DOUGLAS, S.A., BECK, G.R., ELLIOTT, J.D. & OHLSTEIN, E.H. (1995). Pharmacological evidence for the presence of three distinct functional endothelin receptor subtypes in the rabbit lateral saphenous vein. *Br. J. Pharmacol.,* **114,** 1529 – 1540.

DOUGLAS, S.A. & OHLSTEIN, E.H. (2000a). Human urotensin-II, the most potent mammalian vasoconstrictor identified to date, as a therapeutic target for the management of cardiovascular disease. *Trends Cardiovasc. Med.,* **10,** 229 – 237.

DOUGLAS, S.A. & OHLSTEIN, E.H. (2000b). Urotensin receptors. In: *The IUPHAR Compendium of Receptor Characterization and Classification*. ed. Girdlestone, D. pp. 365 – 372. London: IUPHAR Media.

DOUGLAS, S.A., SULPIZIO, A.C., PIERCY, V., SARAU, H.M., AMES, R.S., AIYAR, N.V., OHLSTEIN, E.H. & WILLETTE, R.N. (2000). Differential vasoconstrictor activity of human urotensin-II in vascular tissue isolated from the rat, mouse, dog, pig, marmoset and cynomolgus monkey. *Br. J. Pharmacol.,* **131,** 1262 – 1274.

DOUGLAS, S.A., TAYARA, L., OHLSTEIN, E.H., HALAWA, N. & GIAID, A. (2002). Congestive heart failure and expression of myocardial urotensin II. *Lancet,* **359,** 1990 – 1997.

ELSHOURBAGY, N.A., DOUGLAS, S.A., SHABON, U., HARRISON, S., DUDDY, G., SECHLER, J.L., AO, Z., MALEEFF, B.E., NASELSKY, D., DISA, J. & AIYAR, N.V. (2002). Molecular and pharmacological characterization of genes encoding urotensin-II peptides and their cognate G-protein-coupled receptors from the mouse and monkey. *Br. J. Pharmacol.,* **136,** 9 – 22.

FILIPEANU, C.M., BRAILOIU, E., LE DUN, S. & DUN, N.J. (2002). Urotensin-II regulates intracellular calcium in dissociated rat spinal cord neurons. *J. Neurochem.,* **83,** 879 – 884.

GARDINER, S.M., MARCH, J.E., KEMP, P.A., DAVENPORT, A.P. & BENNETT, T. (2001). Depressor and regionally-selective vasodilator effects of human and rat urotensin II in conscious rats. *Br. J. Pharmacol.,* **132,** 1625 – 1629.

GARTLON, J., PARKER, F., HARRISON, D.C., DOUGLAS, S.A., ASHMEADE, T.E., RILEY, G.J., HUGHES, Z.A., TAYLOR, S.G., MUNTON, R.P., HAGAN, J.J., HUNTER, J.A. & JONES, D.N. (2001). Central effects of urotensin-II following ICV administration in rats. *Psychopharmacology,* **155,** 426 – 433.

GIBSON, A., WALLACE, P. & BERN, H.A. (1986). Cardiovascular effects of urotensin II in anesthetized and pithed rats. *Gen. Comp. Endocrinol.,* **64,** 435 – 439.

HAY, D.W., LUTTMANN, M.A. & DOUGLAS, S.A. (2000). Human urotensin-II is a potent spasmogen of primate airway smooth muscle. *Br. J. Pharmacol.,* **131,** 10 – 12.

HELLER, J., SCHEPKE, M., NEEF, M., WOITAS, R., RABE, C. & SAUERBRUCH, T. (2002). Increases urotensin II plasma levels in patients with cirrhosis and portal hypertension. *J. Hepatol.,* **37,** 767 – 772.

KELSALL, C.J. & BALMENT, R.J. (1998). Native urotensins influence cortisol secretion and plasma cortisol concentration in the euryhaline flounder, *Platichthys flesus. Gen. Comp Endocrinol.,* **112,** 210 – 219.

LIN, Y., TSUCHIHASHI, T., MATSUMURA, K., ABE, I. & IIDA M. (2003). Central cardiovascular action of urotensin II in conscious rats. *J. Hypertens.,* **21,** 159 – 165.

LIU, Q., PONG, S.S., ZENG, Z., ZHANG, Q., HOWARD, A.D., WILLIAMS, D.L., DAVIDOFF, M., WANG, R., AUSTIN, C.P., MCDONALD, T.P., BAI, C., GEORGE, S.R., EVENS, J.F. & CASKEY, C.T. (1999). Identification of urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. *Biochem. Biophys. Res. Commun.,* **266,** 174 – 178.

LU, Y., ZOU, C, HUANG, D. & TANG, C. (2002). Cardiovascular effects of urotensin II in different brain areas. *Peptides,* **23,** 1631.

MacLEAN, M.R., ALEXANDER, D., STIRRAT, A., GALLAGHER, M., DOUGLAS, S.A., OHLSTEIN, E.H., MORECROFT, I. & POLLAND, K. (2000). Contractile responses to human urotensin-II in rat and human pulmonary arteries: effect of endothelial factors and chronic hypoxia in the rat. *Br. J. Pharmacol.,* **130,** 201 – 204.

MAGUIRE, J.J. & DAVENPORT, A.P. (2002). Is urotensin-II the new endothelin? *Br. J. Pharmacol.,* **137,** 579 – 588.

MAGUIRE, J.J., KUC, R.E. & DAVENPORT, A.P. (2000). Orphan-receptor ligand human urotensin-II: receptor localization in human tissues and comparison of vasoconstrictor responses with endothelin-1. *Br. J. Pharmacol.,* **131,** 441 – 446.

MANIATIS, T., FRITSCH, E.F. & SAMBROOK, J. (1989). *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

MARCHESE, A., HEIBER, M., NGUYEN, T., HENG, H.H., SALDIVIA, V.R., CHENG, R., MURPHY, P.M., TSUI, L.C., SHI, X., GREGOR, P., GEORGE, S.R., O'DOWD, B.F. & DOCHERTY, J.M. (1995). Cloning and chromosomal mapping of three novel genes, GPR9, GPR10, and GPR14, encoding receptors related to interleukin 8, neuropeptide Y, and somatostatin receptors. *Genomics,* **29,** 335 – 344.

MATSUSHITA, M., SHICHIRI, M., IMAI, T., IWASHINA, M., TANAKA, H., TAKASU, N. & HIRATA, Y. (2001). Co-expression of urotensin II and its receptor (GPR14) in human cardiovascular and renal tissues. *J. Hypertens.,* **19,** 2185 – 2190.

PAYSANT, J., RUPIN, A., SIMONET, S., FABIANI, J.N. & VERBEUREN, T.J. (2001). Comparison of the contractile responses of human coronary bypass grafts and monkey arteries to human urotensin-II. *Fundam. Clin. Pharmacol.,* **15,** 227 – 231.

PEARSON, D., SHIVELY, J.E., CLARK, B.R., GESCHWIND, I.I., BARKLEY, M., NISHIOKA, R.S. & BERN, H.A. (1980). Urotensin II; a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc. Natl. Acad. Sci. U.S.A.,* **77,** 5021 – 5024.

PELLETIER, G., LIHRMANN, I. & VAUDRY, H. (2002). Role of androgens in the regulation of urotensin II precursor mRNA expression in the rat brainstem and spinal cord. *Neuroscience,* **115,** 525 – 532.

RICHARDS, A.M., NICHOLLS, M.G., LAINCHBURY, J.G., FISHER, S. & YANDLE, T.G. (2002). Plasma urotensin II in heart failure. *Lancet,* **360,** 545 – 546.

RUSSELL, F.D., MOLENAAR, P. & O'BRIEN, D.M. (2001). Cardiostimulant effects of urotensin-II in human heart *in vitro. Br. J. Pharmacol.,* **132,** 5 – 9.

SAETRUM OPGAARD, O., NOTHACKER, H., EHLERT, F.J. & KRAUSE, D.N. (2000). Human urotensin II mediates vasoconstriction via an increase in inositol phosphates. *Eur. J. Pharmacol.,* **406,** 265 – 271.

SHENOUDA, A., DOUGLAS, S.A., OHLSTEIN, E.H. & GIAID, A. (2002). Localization of urotensin-II immunoreactivity in normal human kidneys and renal carcinoma. *J. Histochem. Cytochem.,* **50,** 885 – 889.

SHERIDAN, M.A. & BERN, H.A. (1986). Both somatostatin and the caudal neuropeptide, urotensin II, stimulate lipid mobilization from coho salmon liver incubated *in vitro. Reg. Peptides,* **14,** 333 – 344.

SILVESTRE, R.A., RODRIQUES-GALLARDO, J., EGIDO, E.M. & MARCO, J. (2001). Inhibition of insulin release by urotensin II – a study on the perfused rat pancreas. *Horm. Metab. Res.,* **33,** 379 – 381.

STIRRAT, A., GALLAGHER, M., DOUGLAS, S.A., OHLSTEIN, E.H., BERRY, C., KIRK, A., RICHARDSON, M. & MACLEAN, M.R. (2001) Potent vasodilator responses to human urotensin-II in human pulmonary and abdominal resistance arteries. *Am. J. Physiol. Heart Circ. Physiol.,* **280,** H925 – H928.

TAL, M., AMMAR, D.A., KARPUJ, M., KRIZHANOVSKY, V., NAIM, M. & THOMPSON, D.A. (1995). A novel putative neuropeptide receptor expressed in neural tissue, including sensory epithelia. *Biochem. Biophys. Res. Commun.,* **209,** 752 – 759.

TOTSUNE, K., TAKAHASHI, K., ARIHARA, Z., SONE, M., SATOH, F., ITO, S., KIMURA, Y., SASANO, H. & MURAKAMI, O. (2001). Role of urotensin II in patients on dialysis. *Lancet,* **358,** 774 – 775.