The Journal of Experimental Biology 214, 3985-3991

doi:10.1242/jeb.065243

RESEARCH ARTICLE

Urotensin II and its receptor in the killifish gill: regulators of NaCl extrusion

David H. Evans[1,3,*], Kelly A. Hyndman[2,3], Emily Cornwell[3] and Patrick Buchanan[3]

[1]Department of Biology, University of Florida, Gainesville, FL 32611, USA, [2]Experimental Medicine Section, Department of Medicine, Georgia Health Sciences University, Augusta, GA 30912, USA and [3]Mount Desert Island Biological Laboratory, Salisbury Cove, ME 04672, USA

*Author for correspondence (devans@ufl.edu)

Accepted 12 September 2011

SUMMARY

The peptide urotensin II (UII) and its receptor (UT) mediate cardiovascular and renal effects in both mammals and fishes. In both groups, vasopressor and diuretic responses predominate, although, in mammals, some secondary vasodilatation is found, mediated by secondary release of nitric oxide or prostacyclin. In fishes, gill extrusion of NaCl is inhibited by UII, but a single study has determined that UT is expressed in gill vasculature, not on the epithelium that mediates the transport. To begin to clarify the pathways involved in UII inhibition of gill transport, we have cloned the cDNA encoding UII and UT from the euryhaline killifish (*Fundulus heteroclitus* L.) gill and spinal cord, quantified UT mRNA expression in various tissues and measured relative expression in gill tissue from fish acclimated to seawater (SW) *vs* fresh water (FW). We have also localized UT in the gill epithelium, and measured the effect of UII on ion transport across the opercular epithelium. We found that both UII and UT are synthesized in the gill of *F. heteroclitus* and that gill UT mRNA levels are ~80% higher in SW- *vs* FW-acclimated individuals. In addition, UII inhibits NaCl transport across the opercular epithelium in a concentration-dependent manner, and this inhibition is at least partially mediated by both nitric oxide and a prostanoid.

Supplementary material available online at http://jeb.biologists.org/cgi/content/full/214/23/3985/DC1

Key words: gill, transport, urotensin II.

INTRODUCTION

Urotensin II (UII) is a member of a somatostatin-like family of small peptide hormones that was first described and subsequently isolated from the caudal neurosecretory system (urophysis) of fishes (e.g. Bern et al., 1962; Pearson et al., 1980; Conlon et al., 1997; Tostivint et al., 2006; Bern, 2008; Conlon, 2008; Lu et al., 2008). Urotensin II has now been found in the mammalian brain and other tissues, and is emerging as an important component of various disease states, including: hypertension, atherosclerosis, heart failure, pulmonary hypertension, diabetes and renal failure (e.g. Ross et al., 2010). In mammals, UII has been shown to be a very potent vasoconstrictor (Douglas and Ohlstein, 2000; Maguire and Davenport, 2002), but endothelium-dependent dilatation has also been described, which is mediated by secondary release of nitric oxide (NO) and/or prostacyclin (Katano et al., 2000; Ishihata et al., 2005). UII also affects renal function in mammals by reducing glomerular filtration, urine flow and sodium excretion; but direct effects on tubular transport have not been described (Ashton, 2006), despite the finding of strong immunostaining for both UII and its receptor (UT) in the epithelial cells of renal tubules in rats and humans (e.g. thin limbs of loop of Henle in rats, proximal and distal convoluted tubules and collecting ducts in humans) (Shenouda et al., 2002; Maguire et al., 2004; Ashton, 2006; Abdel-Razik et al., 2008).

In fishes, early physiological studies demonstrated that UII was a general vasopressor and also contracted the urinary bladder, and oviductal and rectal smooth muscle of the eel (*Anguilla* sp.) (Lederis et al., 1971; Chan, 1975), and increased urine flow and urinary electrolyte concentrations in a dose-dependent manner in the eel in freshwater (FW) (Chan, 1975). Urophysial extracts also produced diuresis (but urinary Na^+ retention) and increased gill Na^+ uptake in the goldfish (*Carassius auratus*) (Maetz et al., 1964). Moreover, UII was found to inhibit the short-circuit current (I_{sc}) across the isolated jaw skin of the marine, long-jawed goby (*Gillichthys mirabilis*), tissue that, like the gill epithelium, contains numerous mitochondrion-rich cells (MRCs) and secretes Cl^- (Marshall and Bern, 1979). In the same species, UII stimulated active Na^+ uptake by both the urinary bladder in animals acclimated to seawater (SW) (Loretz and Bern, 1981) and the posterior intestine in animals acclimated to 5% SW (Loretz et al., 1985). In contrast, UII inhibited the I_{sc} across the posterior intestine of FW-acclimated *Anguilla anguilla* at concentrations of 10–100 nmol l^{-1}, but stimulated the I_{sc} at 500 nmol l^{-1} (Baldisserotto and Mimura, 1997). Given the strategies of hyper-osmoregulation (Evans and Claiborne, 2009), these responses to UII appear to be adaptive for teleosts in FW. Somewhat surprisingly, plasma levels of UII were reduced immediately after transfer of the euryhaline flounder (*Platichthys flesus*) from SW to FW, although plasma levels were similar in fish in the two salinities after acclimation (Bond et al., 2002; Lu et al., 2006). Urophysial levels of UII peptide were significantly higher in the FW-acclimated flounder, but UII mRNA in the central nervous system was the same in both salinities (Lu et al., 2006).

The receptor for UII, termed UT, was first discovered in rats by screening for genes that produce peptide-binding receptors, and was termed GPR14. This orphan receptor was transfected into HEK-293 cells and a calcium mobilization assay was used to screen potential ligands. The screening demonstrated that goby UII was the most potent ligand, indicating that GPR14 is actually the UII receptor (Liu et al., 1999; Nothacker et al., 1999). Flounder UT has

now been cloned, and high levels of expression (mRNA) are seen in the ovary, heart, spinal cord, caudal neurosecretory system and brain. UT expression is also seen in key osmoregulatory tissues, such as gill, kidney, bladder and gut. In both the kidney and gill, the protein can be immunolocalized to vascular elements (Lu et al., 2006). UT mRNA levels in both the kidney and gill of the flounder were lower in FW- than SW-acclimated individuals, and these differences were seen between 8 and 24 h after transfer from SW to FW (Lu et al., 2006).

Thus, it is apparent that UII and its receptor UT may play various roles in fish osmoregulation, but the evidence for specific sites and pathways is sparse. Importantly, it has often been difficult to determine cardiovascular *vs* direct, epithelial transport effects. To begin the fill these gaps, we cloned UII and UT from the euryhaline killifish (*F. heteroclitus*) gill and spinal cord, quantified UT mRNA expression in killifish tissues and measured relative expression in gill tissue from FW- *vs* SW-acclimated individuals. We also localized UT protein in the gill epithelium, and measured the effect of UII on ion transport (short-circuit current, I_{sc}) across the opercular epithelium, a model for the gill epithelium. Our data suggest that both UII and UT are synthesized in the gill, and that UII inhibits active NaCl extrusion by the SW gill epithelium, *via* a signaling axis that involves both NO and a prostanoid.

MATERIALS AND METHODS

The research protocols in this study were approved by IACUC committees at the University of Florida and the Mount Desert Island Biological Laboratory. Adult killifish [*F. heteroclitus* (Linnaeus 1766); ~5 g, both sexes] were collected using minnow traps in North East Creek, Mount Desert Island, ME, USA, during the summers of 2006–2008 and maintained in running SW tanks (16–20°C) for at least 1 week. For the I_{sc} experiments, fish were shipped by air to Gainesville, FL, USA, and maintained for more than 1 week in 150 gallon (~570 l) stock tanks (Rubbermaid, Winchester, VA, USA) in recirculating, filtered SW (~32‰). When needed, fish were killed by cervical section and pithing before removal of tissue.

Salinity challenges

Hyndman and Evans previously described the killifish salinity challenge experimental design (Hyndman and Evans, 2007). In short, killifish were subjected to one of four treatments: (1) SW to FW; (2) SW sham (SW to SW); (3) FW to SW; or (4) FW sham (FW to FW). At 0, 3 and 24 h (acute acclimation) and 30 days (chronic acclimation) after transfer, six killifish were killed by decapitation, the gills from the right side were snap frozen for RNA analysis, and the second gill arch from the left side was preserved in 4% paraformaldehyde (in 10 mmol l^{-1} PBS) for immunohistochemical analyses (Hyndman and Evans, 2007).

Molecular analyses

For the molecular studies, the genes encoding UII and UT were initially cloned and sequenced from the killifish gill and spinal cord, respectively. Total RNA was isolated using TRI-Reagent (Sigma, St Louis, MO, USA). For UII, gill 3′ cDNA was produced by reverse transcribing gill RNA using Invitrogen's GeneRacer kit (Carlsbad, CA, USA) and was amplified using degenerate primers designed using Codehop (Rose et al., 2003) (supplementary material Table S1). Degenerate primers were designed against the fish UII protein sequences listed in supplementary material Table S2. The initial amplicon was predicted to be ~560 bp. Gill 3′ CDNA was used in a touchdown PCR reaction with 0.125 U of Ex Taq, hot start, DNA polymerase (Takara Bio, Madison, WI, USA), 10 pmol of degenerate primer, 30 pmol of 3′ RACE primer (Invitrogen), 0.25 mmol l^{-1} of each dNTP and 1× PCR buffer (Takara Bio). The touchdown PCR parameters were: 94°C for 2 min; 5 cycles of 94°C for 30 s, 72°C for 30 s; 5 cycles of 94°C for 30 s, 70°C for 30 s; 30 cycles of 94°C for 30 s, 50°C for 30 s, 72°C for 30 s; and a final cycle of 72°C for 5 min. PCR products were visualized using 1% ethidium bromide staining of 1.5% Tris–Borate–EDTA (TBE) agarose gels, ligated into pCR®4-TOPO vectors, and transformed into TOP10 chemically competent cells using a TOPO TA Cloning®Kit (Invitrogen) to enable sequencing. Plasmid DNA was then sequenced in both directions at the Marine DNA Sequencing Facility at the Mount Desert Island Biological Laboratory.

For the UT, spinal cord RNA was reverse transcribed using Invitrogen's Superscript III kit following manufacturer's instructions. This cDNA was amplified with degenerate UT primers designed against the fish UT protein sequences listed in supplementary material Table S2. The initial amplicon was predicted to be 603 bp. Spinal cord cDNA was used in a PCR reaction with 0.125 U of Ex Taq, hot start, DNA polymerase (Takara Bio), 10 pmol of degenerate primers, 0.25 mmol l^{-1} of each dNTP and 1× PCR buffer (Takara Bio). The PCR parameters were: 94°C for 2 min; 40 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 40 s; and a final cycle of 72°C for 7 min. PCR products were visualized using 1% ethidium bromide staining of 1.5% TBE agarose gels. This PCR resulted in a DNA smear, so the PCR product was nested with primer degenUTF2 (supplementary material Table S1), and run with the same PCR parameters as above for 25 cycles. This was followed by a final PCR with primer degenUTF3 and the aforementioned PCR parameters. This resulted in an amplicon of the predicted size of ~381 bp. This PCR product was cloned and sequenced as described above. To extend the killifish UT sequence, we performed a 3′ extension using the killifish specific primer FHUTF1 and the degenerate primer degenUTR1. This PCR was predicted to produce an amplicon of 496 bp. The PCR parameters were: 94°C for 2 min; 40 cycles of 94°C for 30 s, 45°C for 30 s, 72°C for 40 s; and a final cycle of 72°C for 7 min. Again, the products were visualized, cloned and sequenced as stated above.

To obtain the 5′ and 3′ ends of the killifish UT mRNA, 5′–3′ RACE was performed using Invitrogen's GeneRacer kit. The 5′ end was obtained by using 5′ spinal cord cDNA, the primers RACE5′UTR1A (supplementary material Table S1) and 5′ Race prime (Invitrogen), and the touchdown PCR parameters: 94°C for 2 min; 5 cycles of 94°C for 30 s, 72°C for 30 s; 5 cycles of 94°C for 30 s, 70°C for 30 s; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 30 s; and a final cycle of 72°C for 5 min. The predicted amplicon was ~700 bp and with the killifish we obtained a product of 676 bp. This product was cloned and sequenced. Finally, the 3′ end was obtained by using primers RACE3′UTF1, 3′ RACE primer (Invitrogen), and the touchdown PCR parameters: 94°C for 2 min; 5 cycles of 94°C for 30 s, 72°C for 90 s; 5 cycles of 94°C for 30 s, 70°C for 90 s; 30 cycles of 94°C for 30 s, 55°C for 30 s, 72°C for 90 s; and a final cycle of 72°C for 15 min. The predicted amplicon was 1160 bp, and this PCR product was cloned and sequenced. BLAST searches were performed on all cDNAs sequenced to confirm identity.

The tissue distribution of killifish UT was determined using duplexing, semi-quantitative reverse transcriptase PCR as previously described (Hyndman and Evans, 2007) (supplementary material Table S1). Killifish gill UT mRNA levels were quantified by real-time PCR following the methods of Hyndman and Evans (Hyndman and Evans, 2007) (supplementary material Table S1).

Phylogenetic and sequence analyses

Vertebrate UII protein sequences were mined from GenBank (Benson et al., 2008) and Ensembl (Flicek et al., 2010). These 17 sequences were aligned using CLUSTALX (Larkin et al., 2007). UT protein sequences were also mined from GenBank and Ensembl (supplementary material Table S2), and aligned with CLUSTALX. The phylogenetic relationship among these sequences was determined using maximum likelihood and PhyML (Guindon and Gascuel, 2003). The WAG empirical model of amino acid substitutions was used (Whelan and Goldman, 2001), and the gamma and proportion of invariant sites was optimized. Statistical significance of the branches was determined by non-parametric bootstrapping (100 replicates). Additionally, pairwise similarity comparisons between vertebrate UT protein sequences were made on a site-by-site basis using BLOSUM62 matrix.

Immunohistochemistry

UT protein expression was determined by immunohistochemistry following the methods of Hyndman and Evans (Hyndman and Evans, 2007). Killifish gills were probed with anti-UT antibody (8 ng of GRP14:E-18 antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA). This antibody is an affinity purified, goat polyclonal raised against the N-terminus of rat UT. It was previously used to immunolocalize UT in the flounder gill and kidney; its specificity to flounder gill and kidney UT, and to rat kidney UT was demonstrated by western blot and preabsorption with the antigenic peptide (Lu et al., 2006). In the killifish, however, western blots were negative (K.A.H., unpublished results). Immunoreactivity to mouse anti-chicken Na^+,K^+-ATPase was used to identify the MRCs of the gill (1/1000 dilution used). This antibody (α5) was developed by Dr Douglas Fambrough, and was obtained from the Developmental Studies Hybridoma Bank, which was developed under the auspices of the National Institute of Child Health and Human Development of the University of Iowa (Department of Biological Sciences, Iowa City, IA 52242, USA). Non-specificity of the secondary antibody was tested by omitting the primary antibody. No staining was observed.

Measurement of I_{sc}

For the physiological studies, preparation, mounting and measurement of I_{sc} produced by the opercular epithelium was performed as described previously (Evans et al., 2004), with the exception that current and voltage electrodes were purchased from World Precision Measurements (Naples, FL, USA). The I_{sc} across the killifish opercular epithelium is generated by the net secretion of Cl^- across this tissue (Karnaky et al., 1977). To maximize and normalize electrical parameters for each epithelium, the initial I_{sc} was stimulated by the addition of sufficient isoproterenol (β-adrenergic receptor agonist; dissolved in killifish Ringer solution) (Karnaky et al., 1977) to produce a concentration of 10^{-5} mol l^{-1} on the basolateral surface. An equivalent volume of killifish Ringer solution was added to the apical surface to maintain a matched volume on the two sides of the epithelium. In most cases, the epithelia reached an I_{sc} of at least 50 μA cm^{-2} and a resistance of >35 Ω cm^{-2} after stimulation. All subsequent changes in I_{sc} were calculated as a percentage of the initial isoproterenol stimulation to normalize each tissue response. During each experiment, tissue resistance was monitored by the automatic generation of a ±1 mV pulse every minute by the voltage clamp and recording the I_{sc} deflection. No systematic changes in tissue resistance were observed during the course of any experiments. Goby UII (American Peptide, Sunnyvale, CA, USA) was dissolved in 1% acetic acid, subdivided and lyophilized, and stored at –20°C until made up to the desired concentration in killifish Ringer solution. The nitric oxide synthase (NOS) inhibitor L-NAME (L-nitro-arginine methyl ester) and cyclo-oxygenase (COX) inhibitor indomethacin were dissolved and stored as described previously (Evans et al., 2004). To test the effect of these putative inhibitors, they were applied to the basolateral side at concentrations of 10^{-4} and 10^{-5} mol l^{-1}, respectively, to tissues (that had been stimulated with 10^{-5} mol l^{-1} isoproterenol), with the subsequent addition of 10^{-5} mol l^{-1} goby UII. The final volume of added agonist/inhibitor never exceeded 4% of the initial experimental volume of 2 ml.

All data are expressed as means ± s.e. Values for statistical differences were calculated by the appropriate, two-tailed, paired or unpaired Student's *t*-tests, and concentration dependence data were analyzed using repeated measures ANOVA and the appropriate post-tests. In the data analysis of the effect of UII on the isoproterenol-stimulated I_{sc}, each tissue served as its own control, but paired tissues (two operculae from the same fish) were used for the effects of either indomethacin or L-NAME on the UII-mediated inhibition of the I_{sc}. In all cases, $P<0.05$ was taken as significant, and specific statistical analyses were performed using Prism 5.0c (GraphPad Software, San Diego, CA, USA) on a MacBook Pro and are indicated in the text and figure legends. Quantitative real-time PCR values were tested using two-factor ANOVA (treatment and time), and when significance was found, specific differences between sham and treatment were determined using a Bonferroni *post hoc* test.

RESULTS

Killifish UII and UT

The 3′ end of the UII cDNA was cloned from the killifish gill (631 bp, GenBank HQ634243). An open reading frame (ORF) of 98 amino acids was found within this sequence from base pair positions 1–298 (GenBank HQ634243) and the active UII peptide is encoded from bp 162 to 298 (amino acids 87–98). Fig. 1 depicts the active UII killifish sequence, which shares a 58% amino acid identity with human UII (shaded amino acids). The goby UII peptide used in our physiological experiments also shares 58% amino acid identity with killifish UII (Fig. 1). However, the C-terminal hexapeptide receptor recognition site (Cys–Phe–Trp–Lys–Tyr–Cys) is 100% conserved among vertebrates (Vaudry et al., 2010), including the killifish and goby (Fig. 1).

UT cDNA was sequenced from the killifish spinal cord; 1832 bp were cloned and sequenced, with an ORF from position 216 to 1307 that translates into a 363 amino acid protein (GenBank HQ634242). This sequence was confirmed to be UT by BLAST search and phylogenetic analysis (Fig. 2). The killifish UT grouped with the other known fish UTs, and outside of the tetrapod UT group (Fig. 2). Pairwise similarity analysis was performed and revealed that human UT and killifish UT share 63% similarity at the amino acid level, while the other vertebrates share >69% similarity. The fish UTs are >90% similar to killifish UT (those shown in Fig. 2).

Fig. 1. Comparision of human (NP_068835), killifish (AEG64706) and goby (Pearson et al., 1980) urotensin II (UII) active peptide sequences. Goby urotensin II is used in the experiments reported in Fig. 6. Identical amino acids are shaded in gray.

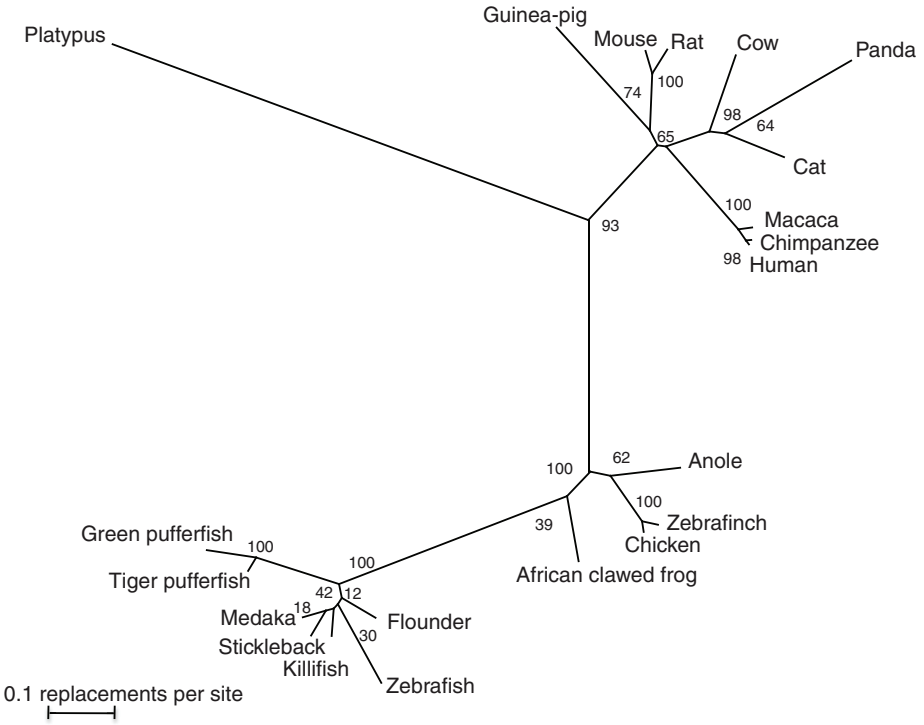

Fig. 2. An unrooted, maximum likelihood tree of the vertebrate urotensin II receptor (UT) protein. Numbers at nodes represent percentage bootstrap. Scale bar represents the number of replacements per site.

The distribution of the killifish UT mRNA was determined by duplexing reverse transcriptase PCR. As depicted in Fig. 3, the UT has a ubiquitous distribution, with relatively high expression in the gill, heart, brain, spinal cord and kidney.

Within the killifish gill, large, ovoid cells in the interlamellar region were immunoreactive to anti-UT (Fig. 4). These cells were also immunoreactive to anti-Na^+,K^+-ATPase, a common marker for the gill MRCs (Evans et al., 2005). As our western blot was negative, we cannot be certain about the specificity of the anti-UT antibody in killifish tissues, but the co-localization with Na^+,K^+-ATPase in the MRCs is consistent with our physiological data (see below).

UT mRNA expression during salinity acclimation

Chronic acclimation to FW (30 days) resulted in an ~80% reduction in UT mRNA expression in gill tissue compared with that in the SW killifish gill (Fig. 5A, P=0.01). Acute (24 h) acclimation to FW from SW had no effect on gill UT mRNA expression (Fig. 5B); however, acute acclimation to SW from FW resulted in a significant 4-fold increase in gill UT mRNA after 24 h (Fig. 5C) (salinity P=0.0487, time P=0.0370, interaction P=0.0397).

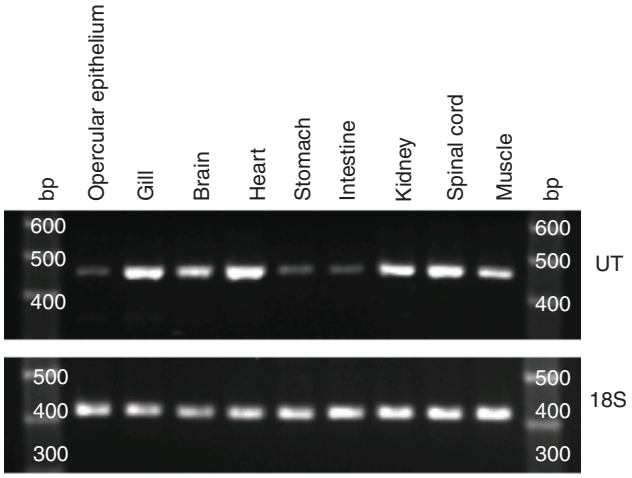

Fig. 3. mRNA expression of UT in the killifish. It is ubiquitously expressed with relatively high expression in the gill, heart and kidney. 18S represents the loading control.

I_{sc}

Goby UII inhibited the isoproterenol-stimulated I_{sc} generated by the isolated killifish opercular epithelium in a concentration-dependent manner, reaching significance at 10^{-7} mol l^{-1} and a 60% reduction at a UII concentration of 10^{-5} mol l^{-1} (Fig. 6A). The apparent IC_{50} is 1.7×10^{-6} mol l^{-1}. The inhibition of the isoproterenol-stimulated I_{sc} by 10^{-5} mol l^{-1} UII was partially, but significantly, blunted by pretreatment with either 10^{-4} mol l^{-1} L-NAME (Fig. 6B, P=0.03) or 10^{-5} mol l^{-1} indomethacin (Fig. 6C, P=0.04). Neither L-NAME nor indomethacin had a significant effect on the I_{sc} when applied alone (data not shown).

DISCUSSION

The translated UII sequence was 58–77% identical to a variety of vertebrates, while the hexapeptide receptor recognition site was 100% identical among all vertebrates examined in the phylogenetic analysis of this study. This degree of identity is similar to that of endothelin, another paracrine/autocrine expressed in the fish gill (Hyndman and Evans, 2007). Likewise, we found the killifish UT to have a high degree of homology with the vertebrate UTs (>63% homology) (Vaudry et al., 2010). Thus, both the UII peptide and UT receptor have been well conserved during vertebrate evolution.

The nearly ubiquitous expression of mRNA for UT in tissues ranging from brain and spinal cord to osmoregulatory tissues like operculum, gill, intestine and kidney (Fig. 3) is similar to that described for the euryhaline flounder (Lu et al., 2006). The flounder, however, appears to express relatively less UT in the gill (compared with other tissues in that species) and more in the intestine than the killifish. Nevertheless, it is clear that the mRNA for UII and UT is expressed in the relevant osmoregulatory tissues in the killifish, as in the flounder. The immunohistochemistry demonstrates that the

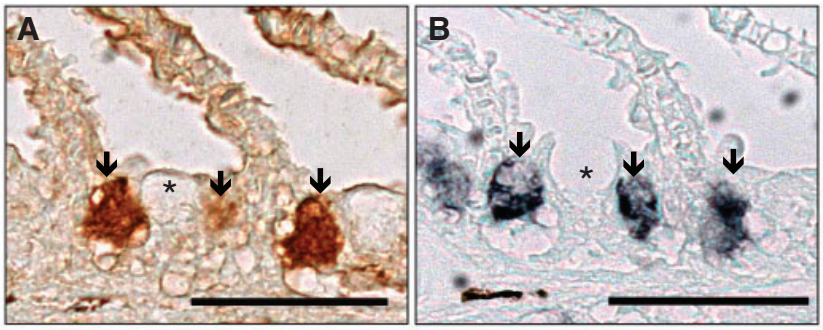

Fig. 4. Representative adjacent, serial sections of killifish gill, illustrating immunoreactivity to anti-UT (brown, A) and anti-Na^+,K^+-ATPase (blue, B). The UT is expressed in the mitochondrion-rich cells (MRCs) of the killifish gill. Arrows point to the same cells that are immunoreactive to both antibodies. Asterisk indicates mucous cell. Scale bars, 50 μm.

UT protein is also expressed in the gill epithelium in the killifish; specifically, within cells that also express the ubiquitous transport enzyme Na^+,K^+-ATPase (Fig. 4). As staining for relatively high concentrations of NKA characterizes the ion-transporting cells of the gill epithelium (termed mitochondrion-rich cells or MRCs) (e.g. Evans et al., 2004), our data provide the first evidence that the UII receptor can be localized to the gill MRCs. In the flounder, however, UT was localized to vascular elements in the gill, not to transport cells in the epithelium (Lu et al., 2006), suggesting that species differences may exist. Although the flounder and killifish are both euryhaline species that can tolerate direct SW to FW transfers and *vice versa*, in the wild these fishes have different habitat usages. Killifish are a resident, intertidal species (Gibson, 1999) and the killifish used in this study were caught at the mouth of the tidal Northeast Creek on Mount Desert Island. These fish experience daily fluctuations in environmental salinity (Marshall, 2003). In contrast, the flounder is a migratory fish that is nocturnal, burrows and frequently enters FW (Kottelat and Freyhof, 2007). It is plausible, therefore, that given the different habit usage, and resident *vs* migratory behaviors, these fishes may have different intrinsic distributions of UT. Comparative studies using a variety of euryhaline (catadromous, andromous, migratory and resident, etc.) fishes would help elucidate these differences.

The expression of UT mRNA in the gill tissue of the killifish is directly related to salinity, at least after 30 days of acclimation. Specifically, the expression is reduced by 80% when the fish are acclimated to FW. Similar studies using the flounder gill produced nearly equivalent results (Lu et al., 2006). Interestingly, the expression of UT mRNA did not change 24 h after transfer of SW-acclimated killifish to FW, but did increase in FW-acclimated killifish 24 h after transfer to SW. In flounder, the opposite has been described: no change 24 h after transfer from FW to SW, but a significant decline 24 h after transfer from SW to FW. Whether this discrepancy is species or protocol specific remains to be determined; however, it is clear that both species display salinity-dependent differences in the expression of UT mRNA from gill tissue, with highest expression in SW. We were unable to measure plasma levels of UII, but it has been demonstrated that the plasma levels are the same in FW- *vs* SW-acclimated flounder (Bond et al., 2002; Lu et al., 2006), but fall within 8 h of acute transfer of the flounder from SW to FW (Lu et al., 2006). Conversely, urophysial content of UII is significantly higher in flounder 24 h after transfer from SW to FW (Lu et al., 2006), suggesting inhibition of secretion, but not synthesis in the urophysis, by hypo-osmotic stress. Thus, the reduction in both the expression of gill receptor mRNA and plasma peptide levels suggests a down-regulation of the UII–UT axis in

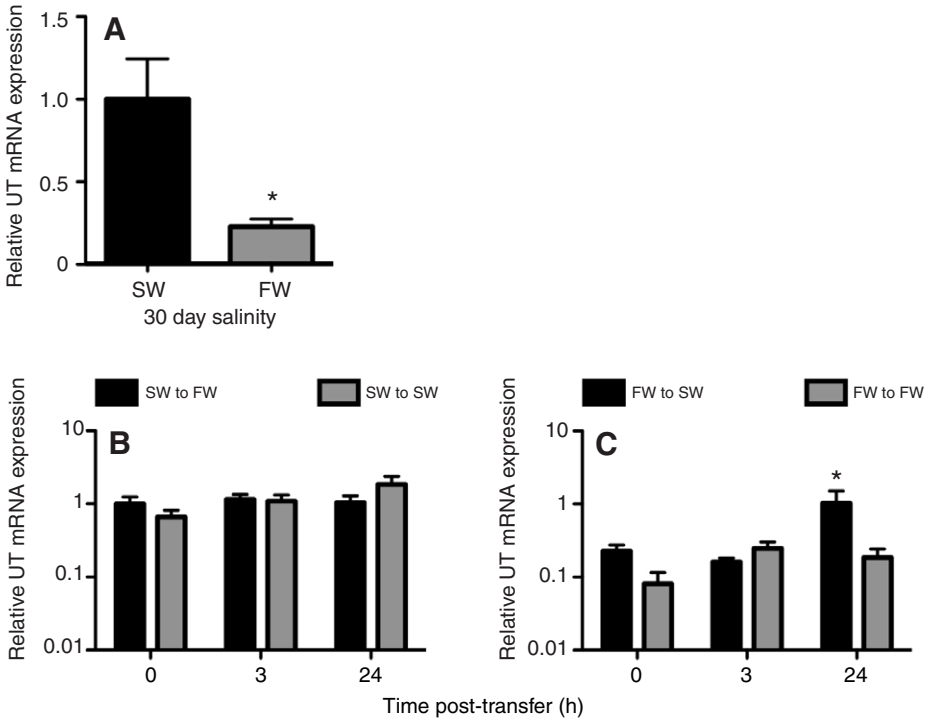

Fig. 5. Relative killifish gill UT mRNA expression. (A) Gill UT is significantly lower in the chronically acclimated, freshwater (FW) killifish gill (P=0.014, N=6). (B) UT gill mRNA expression is not altered with acute FW acclimation to FW (*P>0.05, N=5–6). (C) UT gill mRNA expression increases over time during acclimation to seawater (SW) (N=5–6). All values are relative to chronic SW acclimation (A). *30 day SW *vs* FW, P=0.03 (A); *0 *vs* 24 h, P<0.01 (C). Note, B and C are logarithmic scales.

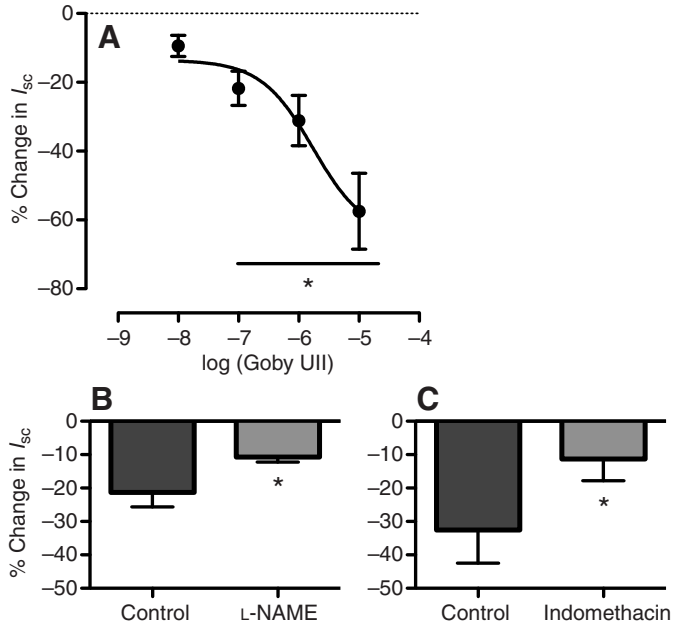

Fig. 6. Goby UII inhibits the short circuit current (I_{sc}) stimulated by isoproterenol (log of mol l^{-1} values) in the killifish opercular epithelium. (A) Dose–response curve. (B) Pretreatment with 10^{-4} L-NAME results in a significant reduction in the inhibition of the I_{sc} in response to 10^{-5} mol l^{-1} goby UII. (C) pretreatment with 10^{-5} mol l^{-1} indomethacin also results in a significant reduction in the inhibition of the I_{sc} in response to 10^{-5} mol l^{-1} goby UII. *P<0.05. N=6–8.

low salinities in both the killifish and flounder. This could be hemodynamically adaptive because UII is a known vasoconstrictor in fishes (e.g. Le Mevel et al., 2008), and vasoconstriction would not be adaptive in lower salinities, where the fish is already facing an expanded extracellular space because of osmotic uptake of water (e.g. Evans and Claiborne, 2009). Our clear signal for UT mRNA in the kidney (Fig. 3) confirms the data in the flounder, where it was shown that the receptor localizes to the glomerular arterioles and vessels surrounding the collecting duct (Lu et al., 2006). Similar to the gill, the renal tissue expressed lower UT mRNA in FW-acclimated flounders and the changes occurred 8–24 h after acute transfer (Lu et al., 2006).

The mRNA for the UT receptor is also expressed in the killifish intestine (Fig. 3), but at lower levels than in the gill, contrary to what has been described for the flounder, where intestinal expression was apparently higher than gill expression (Lu et al., 2006). It has been shown previously that UII stimulates intestinal NaCl uptake by the posterior intestine of the euryhaline goby (*G. mirabilis*) acclimated to 5% SW (Loretz et al., 1985).

Increased release of peptide and expression of receptor in high salinities suggests that the UII–UT axis may play some role in osmotic regulation in SW. Our results, and those published previously, suggest that this is the case, but as an inhibitor of gill salt extrusion, rather than as a stimulant. Early studies demonstrated that UII is a potent inhibitor of transport across the isolated chin skin of the marine goby *G. mirabilis* (Marshall and Bern, 1979), which secretes NaCl *via* MRCs in a manner similar to that described for the marine teleost gill and the opercular epithelium of the killifish, *F. heteroclitus* (e.g. Evans and Claiborne, 2009). We have now confirmed these finding for the killifish opercular epithelium, using a range of concentrations from 0.01 to 10 μmol l^{-1} of goby UII (Fig. 6A). This inhibition produced by UII can be reduced by pre-incubation of the tissue with either the NOS inhibitor L-NAME (Fig. 6B) or the COX inhibitor indomethacin (Fig. 6C). This finding suggests that, like the endothelin inhibition of NaCl transport across the same tissue (Evans et al., 2004; Hyndman and Evans, 2007), the UII inhibition of ion transport is mediated through both the NO and prostanoid paracrine signaling pathways, confirming what has been proposed by the vascular action of UII in mammalian blood vessels (e.g. Zhang et al., 2003; Ishihata et al., 2005; Ong et al., 2005). The fact that pretreatment with either L-NAME or indomethacin did not affect the I_{sc} after the application of isoproterenol suggests that secretion of either NO or prostaglandins is not important in isoproterenol-stimulated opercular epithelia until the subsequent application of UII. In a previous study (Evans et al., 2004), we found that pretreatment of unstimulated opercular epithelia with L-NAME stimulated the I_{sc} slightly, so it is clear that the putative role of NO secretion in modulating unstimulated or isoproterenol-stimulated opercular epithelia should be investigated further.

In conclusion, the UII–UT axis is found throughout the vertebrate taxa, and this study determined that it regulates ion transport in the gill of the euryhaline killifish. Much like endothelin, urotensin signals through a NO/prostanoid pathway to inhibit ion transport. When the killifish is in SW, this signaling pathway is thought to act as a brake on salt extrusion, helping the fish maintain ion homeostasis (Choe et al., 2006; Hyndman and Evans, 2007). It is unclear, however, whether there is cross-talk between UII and endothelin and these signaling pathways. The fish gill functions much like the mammalian renal epithelium, which is the principal site of UII production in humans (Nothacker et al., 1999). UII signaling has been proposed to be up-regulated in rodent models of hypertension (Abdel-Razik et al., 2008), where it functions to reduce glomerular filtration rate and acts directly on the tubules to promote sodium retention. This is in contrast to normotensive rodent models (WKY and Sprague–Dawley rats), where UII acts to inhibit sodium reabsorption (Abdel-Razik et al., 2008). Thus, UII signaling, either at the fish gill epithelium or mammalian tubular epithelium, is a regulator of ion transport.

ACKNOWLEDGEMENTS

This work is dedicated to Prof. Howard A. Bern, now in his 10th decade – the discoverer of urotensin II, and an important mentor in D.H.E.'s graduate career.

FUNDING

This work was supported by the National Science Foundation [IOB-0519579 to D.H.E.].

REFERENCES

Abdel-Razik, A. E., Forty, E. J., Balment, R. J. and Ashton, N. (2008). Renal haemodynamic and tubular actions of urotensin II in the rat. *J. Endocrinol.* **198**, 617-624.
Ashton, N. (2006). Renal and vascular actions of urotensin II. *Kidney Int.* **70**, 624-629.
Baldisserotto, B. and Mimura, O. (1997). Changes in the electrophysiological parameters of the posterior intestine of Anguilla anguilla (Pisces) induced by oxytocin, urotensin II and aldosterone. *Braz. J. Med. Biol. Res.* **30**, 35-39.
Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J. and Wheeler, D. L. (2008). GenBank. *Nucleic Acids Res.* **36**, D25-30.
Bern, H. A. (2008). From fish tail to human brain. Preface. *Peptides* **29**, 649-650.
Bern, H. A., Yagi, K. and Nishioka, R. S. (1962). The caudal neurosecretory system of fishes. *Gen. Comp. Endocrinol.* **2**, 96-110.
Bond, H., Winter, M. J., Warne, J. M., McCrohan, C. R. and Balment, R. J. (2002). Plasma concentrations of arginine vasotocin and urotensin II are reduced following transfer of the euryhaline flounder (*Platichthys flesus*) from seawater to fresh water. *Gen. Comp. Endocrinol.* **125**, 113-120.
Chan, D. K. O. (1975). Cardiovascular and renal effects of urotensins I and II in the eel, *Anguilla rostrata*. *Gen. Comp. Endocrinol.* **27**, 52-61.
Choe, K. P., Havird, J., Rose, R., Hyndman, K., Piermarini, P. and Evans, D. H. (2006). COX2 in a euryhaline teleost, *Fundulus heteroclitus*: primary sequence,

distribution, localization, and potential function in gills during salinity acclimation. *J. Exp. Biol.* **209**, 1696-1708.
Conlon, J. M. (2008). Liberation of urotensin II from the teleost urophysis: an historical overview. *Peptides* **29**, 651-657.
Conlon, J. M., Tostivint, H. and Vaudry, H. (1997). Somatostatin- and urotensin II-related peptides: molecular diversity and evolutionary perspectives. *Regul. Pept.* **69**, 95-103.
Douglas, S. and Ohlstein, E. (2000). 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.
Evans, D. H. and Claiborne, J. B. (2009). Osmotic and ionic regulation in fishes. In *Osmotic and Ionic Regulation: Cells and Animals* (ed. D. H. Evans), pp. 295-366. Boca Raton: CRC Press.
Evans, D. H., Rose, R. E., Roeser, J. M. and Stidham, J. D. (2004). NaCl transport across the opercular epithelium of *Fundulus heteroclitus* is inhibited by an endothelin to NO, superoxide, and prostanoid signaling axis. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **286**, R560-R568.
Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: Dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.
Flicek, P., Aken, B. L., Ballester, B., Beal, K., Bragin, E., Brent, S., Chen, Y., Clapham, P., Coates, G., Fairley, S. et al. (2010). Ensembl's 10th year. *Nucleic Acids Res.* **38**, D557-D562.
Gibson, R. N. 1999 Movement and homing in intertidal fishes. In *Intertidal Fishes. Life in Two Worlds* (ed. M. H. Horn, K. L. M. Martin and M. A. Chotkowski), pp. 97-125. San Diego: Academic Press.
Guindon, S. and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* **52**, 696-704.
Hyndman, K. A. and Evans, D. H. (2007). Endothelin and endothelin converting enzyme-1 in the fish gill: evolutionary and physiological perspectives. *J. Exp. Biol.* **210**, 4286-4297.
Ishihata, A., Ogaki, T., Aita, T. and Katano, Y. (2005). Role of prostaglandins in urotensin II-induced vasodilatation in the coronary arteries of aged rats. *Eur. J. Pharmacol.* **523**, 119-126.
Karnaky, K. L., Jr, Degnan, K. J. and Zadunaisky, J. A. (1977). Chloride transport across isolated opercular epithelium of killifish: a membrane rich in chloride cells. *Science* **195**, 203-205.
Katano, Y., Ishihata, A., Aita, T., Ogaki, T. and Horie, T. (2000). Vasodilator effect of urotensin II, one of the most potent vasoconstricting factors, on rat coronary arteries. *Eur. J. Pharmacol.* **402**, R5-R7.
Kottelat, M. and Freyhof, J. (2007). *Handbook of European Freshwater Fishes,* 646 pp. Cornol, Switzerland: Publications Kottelat.
Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R. et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**, 2947-2948.
Le Mevel, J. C., Lancien, F., Mimassi, N., Leprince, J., Conlon, J. M. and Vaudry, H. (2008). Central and peripheral cardiovascular, ventilatory, and motor effects of trout urotensin-II in the trout. *Peptides* **29**, 830-837.
Lederis, K., Bern, H. A., Nishioka, R. S. and Geschwind, I. I. (1971). Some observations on biological activity, chemical nature and subcellular organization of urophysial active principles. *Mem. Soc. Endocrinol.* **19**, 391-412.
Liu, Q., Pong, S. S., Zeng, Z., Zhang, Q., Howard, A. D., Williams, D. L., Jr, Davidoff, M., Wang, R., Austin, C. P., McDonald, T. P. et al. (1999). Identification of urotensin II as the endogenous ligand for the orphan G-protein-coupled receptor GPR14. *Biochem. Biophys. Res. Commun.* **266**, 174-178.
Loretz, C. A. and Bern, H. A. (1981). Stimulation of sodium transport across the teleost urinary bladder by urotensin II. *Gen. Comp. Endocrinol.* **43**, 325-330.
Loretz, C. A., Howard, M. E. and Siegel, A. J. (1985). Ion transport in goby intestine: cellular mechanism of urotensin II stimulation. *Am. J. Physiol.* **249**, G284-G293.
Lu, W., Greenwood, M., Dow, L., Yuill, J., Worthington, J., Brierley, M. J., McCrohan, C. R., Riccardi, D. and Balment, R. J. (2006). Molecular characterization and expression of urotensin II and its receptor in the flounder (*Platichthys flesus*): a hormone system supporting body fluid homeostasis in euryhaline fish. *Endocrinology* **147**, 3692-3708.
Lu, W., Abdel-Razik, A. E., Ashton, N. and Balment, R. J. (2008). Urotensin II: lessons from comparative studies for general endocrinology. *Gen. Comp. Endocrinol.* **157**, 14-20.
Maetz, J., Bourguet, J. and Lahlouh, B. (1964). Urophyse et osmoregulation chez *Carassius auratus*. *Gen. Comp. Endocrinol.* **4**, 401-414.
Maguire, J. J. and Davenport, A. P. (2002). Is urotensin-II the new endothelin? *Br. J. Pharmacol.* **137**, 579-588.
Maguire, J. J., Kuc, R. E., Wiley, K. E., Kleinz, M. J. and Davenport, A. P. (2004). Cellular distribution of immunoreactive urotensin-II in human tissues with evidence of increased expression in atherosclerosis and a greater constrictor response of small compared to large coronary arteries. *Peptides* **25**, 1767-1774.
Marshall, W. S. (2003). Rapid regulation of NaCl secretion by estuarine teleost fish: coping strategies for short-duration freshwater exposures. *Biochim. Biophys. Acta* **1618**, 95-105.
Marshall, W. S. and Bern, H. A. (1979). Teleostean urophysis: urotensin II and ion transport across the isolated skin of a marine teleost. *Science* **204**, 519-521.
Nothacker, H. P., Wang, Z., McNeill, A. M., Saito, Y., Merten, S., O'Dowd, B., Duckles, S. P. and Civelli, O. (1999). Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nat. Cell Biol.* **1**, 383-385.
Ong, K., Lam, K. and Cheung, B. (2005). Urotensin II: its function in health and its role in disease. *Cardiovasc. Drugs Ther.* **19**, 65-75.
Pearson, D., Shively, J. E., Clark, B. R., Geschwind, I. I., Barkley, M., Nishioka, R. S. and Bern, H. A. (1980). Urotensin II: a somatostatin-like peptide in the caudal neurosecretory system of fishes. *Proc. Natl. Acad. Sci. USA* **77**, 5021-5024.
Rose, T. M., Henikoff, J. G. and Henikoff, S. (2003). CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. *Nucleic Acids Res.* **31**, 3763-3766.
Ross, B., McKendy, K. and Giaid, A. (2010). Role of urotensin II in health and disease. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **298**, R1156-R1172.
Shenouda, A., Douglas, S. A., Ohlstein, E. H. and Giaid, A. (2002). Localization of urotensin-II immunoreactivity in normal human kidneys and renal carcinoma. *J. Histochem. Cytochem.* **50**, 885-889.
Tostivint, H., Joly, L., Lihrmann, I., Parmentier, C., Lebon, A., Morisson, M., Calas, A., Ekker, M. and Vaudry, H. (2006). Comparative genomics provides evidence for close evolutionary relationships between the urotensin II and somatostatin gene families. *Proc. Natl. Acad. Sci. USA* **103**, 2237-2242.
Vaudry, H., do Rego, J. C., Le Mevel, J. C., Chatenet, D., Tostivint, H., Fournier, A., Tonon, M. C., Pelletier, G., Conlon, J. M. and Leprince, J. (2010). Urotensin II, from fish to human. *Annu. NY Acad. Sci.* **1200**, 53-66.
Whelan, S. and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* **18**, 691-699.
Zhang, A. Y., Chen, Y.-F., Zhang, D. X., Yi, F.-X., Qi, J., Andrade-Gordon, P., de Garavilla, L., Li, P.-L. and Zou, A.-P. (2003). Urotensin II is a nitric oxide-dependent vasodilator and natriuretic peptide in the rat kidney. *Am. J. Physiol. Renal. Physiol.* **285**, F792-F798.