Evidence for an intrarenal renin-angiotensin system in the rainbow trout, Oncorhynchus mykiss

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Brown, J. Anne, Richard K. Paley, Shehla Amer, and Stephen J. Aves. Evidence for an intrarenal renin-angiotensin system in the rainbow trout, Oncorhynchus mykiss. Am J Physiol Regulatory Integrative Comp Physiol 278: R1685–R1691, 2000.—Physiological and molecular approaches were used to investigate the existence of an intrarenal renin-angiotensin system (RAS) in rainbow trout. Inhibition of angiotensin-converting enzyme by captopril (5 × 10⁻⁴ M) rapidly decreased vascular resistance of the trunk of the trout, perfused at 19 mmHg, resulting in an increased perfusate flow rate and a decreased intrarenal dorsal aortic pressure. A profound diuresis occurred in the in situ perfused kidney and reflected both increased glomerular filtration rates and decreased water reabsorption (osmolyte reabsorption was unchanged). Renal and vascular parameters recovered once captopril treatment was stopped. Diuretic and vascular effects of captopril on the in situ trout kidney concur with an inhibition of known vasoconstrictor and antiuretic actions of angiotensin II. However, at a higher perfusion pressure (28 mmHg), captopril had no effect on intrarenal aortic pressure or perfusate and urine flow rates, suggesting that the trout intrarenal RAS is activated by low perfusion pressures/flows. Existence of the renal RAS in trout was further supported by evidence for angiotensinogen gene expression in kidney as well as liver.

perfused trout kidney; angiotensinogen mRNA expression; angiotensin-converting enzyme inhibition; captopril; teleost

THE RENIN-ANGIOTENSIN SYSTEM (RAS) was originally perceived as a classical circulating hormonal system, with angiotensinogen (synthesized by the liver) acted on by circulating renin (produced predominantly by the kidney), resulting in formation of ANG I. ANG I is cleaved as it circulates by angiotensin-converting enzyme (ACE) to form the octapeptide ANG II. It is now clear that in mammals at least, in addition to this circulating RAS, tissue-specific systems function alongside the systemic system and that in the kidney, the renal RAS has probable paracrine actions on renal function and renal vascular tone (13, 15, 34, 35). Not all features of the mammalian RAS are identical in nonmammalian vertebrates (16, 17), and it is not known whether an intrarenal RAS occurs and plays any role in regulation of renal function of lower vertebrates.

The present studies employed both physiological and molecular approaches to investigate the existence or otherwise of a functional renal RAS in the rainbow trout, Oncorhynchus mykiss. In teleost fishes, including the rainbow trout, renal renin is a central part of the classical systemic RAS (2, 25, 26, 29), and there is biochemical evidence for ACE activity in the rainbow trout kidney (19, 30). The synthesis of angiotensinogen, along with renin and ACE, would provide a complete renal RAS capable of acting independently from the systemic RAS. We employed a molecular approach to investigate angiotensinogen gene expression and thus the possible production of angiotensinogen by the trout kidney as previously demonstrated in mammals (13, 34, 35).

Elucidation of the actions of a renal RAS (as distinct from a circulating RAS) would be difficult in vivo, but the perfused trunk preparation with the kidney in situ (1, 4) offers the potential to demonstrate an active renal RAS in the absence of angiotensinogen synthesis by the liver or ACE conversion of ANG I by the gills. The ability to manipulate and control perfusion conditions enabled the effects of perfusion pressure (and the linked effects on flow) to be investigated. The vascular and renal effects of ACE inhibition of any intrarenal ANG II formation were investigated at two perfusion pressures.

MATERIALS AND METHODS

Animals. Rainbow trout (Oncorhynchus mykiss) of both sexes, weighing 200–400 g, were obtained from a local hatchery (Exmoor Trout, North Molton, UK), fed on trout pellets (Pauls Agricultural, Redstock, UK), and held in aerated Exeter tap water (in mM: 0.54 Na, 4.2 K, 1.2 Ca, 0.72 Mg, 0.36 Cl, 5.5 glucose, 2 CaCl₂, 5 NaHCO₃, and 10.2 HCO₃⁻) at 10°C under a natural photoperiod for at least 2 wk before experimental study.

Physiological studies of the in situ perfused trout kidney. The technique for in vitro perfusion of the trunk of the trout with the kidney in situ has already been described in detail (1, 4). Briefly, fish were lightly anesthetized, killed with a blow to the head, heparinized through the caudal vein, weighed, and then decapitated directly behind the base of the pectoral fins. A tapered polyethylene cannula with flowing filtered (0.45 µm) physiological saline (in mM: 130 NaCl, 4.2 Cl, 1.2 MgSO₄, 2 CaCl₂, 5 NaHCO₃, and 5.5 glucose, continuously aerated with 0.03% CO₂ in air to give a pH 7.6) was inserted ~2 cm into the dorsal aorta to a position posterior to the bulk of the head kidney. Per fusate flow was measured throughout all experiments as the difference between saline delivery from perfusion pumps supplying the constant pressure head and measured overflow from the pressure head.
A fine catheter, threaded through the aortic catheter until its tip was positioned at the approximate transition between head and excretory kidney, enabled measurement of perfusate pressure within the dorsal aorta (RP 1500 pressure transducer and Narco Biosystems recorder).

The kidney was exposed by a ventral incision, all viscera except the kidney were removed, and minor arteries arising from the kidney were tied off. The bladder was cannulated for collection of timed urine samples into preweighed tubes, and urine flow was determined gravimetrically. Urine flow rates (V) were expressed in microliters per minute per kilogram body weight using the body weights obtained at the start of each experiment. All experiments were carried out in a controlled temperature room at 10 ± 1°C, with physiological saline delivered from a constant pressure head. Three experimental groups were run. Group 1 (n = 3 preparations), in which the pressure head was set at 28 mmHg (38 cmH2O) as in previous perfused kidney studies (1, 4). Groups 2 and 3 (n = 6 preparations in each case), in which perfusion was at a relatively low pressure head, 19 mmHg (25 cmH2O). In groups 2 and 3, inulin (0.25 g/l), Sigma) was added to the perfusate to determine inulin clearances as a measure of glomerular filtration rate, as in previous studies (1, 4). In group 3 studies, additional glucose was also included in the perfusate (final concentration 25 mM) to saturate tubular reabsorptive capacity and enable measurement of tubular transport maxima for glucose (TmG) as an indirect measure of the population of filtering glomeruli (9).

In all experiments, the perfused preparations were initially allowed to stabilize for a period of 1 h. The preparations were run for a further 2 h of perfusion; previous studies have demonstrated that renal and vascular function remains stable for at least 3 h perfusion (1, 4). In group 1 and 2 experiments, after stabilization, four 15-min "control" urine samples were collected before addition of the ACE inhibitor captopril (Sigma) at 5 × 10^{-4} M and collection of a further four 15-min urine samples. In group 3 experiments, after stabilization, four 10-min control urine samples were collected, then captopril (5 × 10^{-4} M) was added and a further four 10-min urine samples collected, after which captopril was removed, and a further four 10-min urine samples were collected during resumption of control perfusion.

Analyses and calculations. Inulin was analyzed in group 2 and 3 experiments, as previously described (1), using the resorcinol technique and glomerular filtration rates (GFR) were calculated as the product of urine flow and urinary-to-perfusate inulin concentration ratios ([U/P]inulin).

In group 2, in which urine volumes collected generally exceeded 100 µl, urinary osmolalities were determined by freezing point depression (Roebling Automatic Microosmometer), and osmolar clearance (C_{osm}) was calculated as the product of V and the urinary-to-perfusate osmolality ratio ([U_{osm}/P_{osm}]). It was then possible to calculate free-water clearance (C_{H2O}) as the difference between V and C_{osm} and to express both C_{H2O} and C_{osm} as a percentage of the filtered volume, GFR.

In group 3, perfusate ([P_{j}]) and urinary ([U_{j}]) glucose concentrations were determined using a glucose oxidase-peroxidase assay (Boehringer Mannheim), and TmG was determined as the difference between filtered glucose (GFR × [P_{j}]) and excreted glucose (V × [U_{j}]).

Statistical analyses of renal and vascular data. Data are presented as means ± SE of untransformed data. Inulin concentration ratios and relative clearances were arcsin transformed before statistical analyses. Renal and vascular parameters during collection of the last urine sample before experimental manipulation were statistically compared (using paired t-tests) with values during the last urine sample collected during administration of captopril. In recovery experiments (group 3), renal and vascular parameters during the last treatment period were also statistically compared with those in the final collection of the 40-min recovery period. Significance was accepted at P < 0.05 after application of the Bonferroni sequential rejection test to exclude type I errors when necessary (33).

Reverse Northern blots for investigation of angiotensinogen mRNA expression. To date, as far as we are aware, only mammalian angiotensinogen genes have been cloned. Our attempts to clone a fragment of the trout angiotensinogen cDNA by PCR using several different sets of degenerate primers derived from sequence data of the cloned human, mouse, rat, and sheep angiotensinogen cDNAs have so far failed. Expression of angiotensinogen mRNA was therefore investigated using a heterologous cDNA probe [rat angiotensinogen mRNA, a kind gift from K. R. Lynch (21)]. Before any use of the rat angiotensinogen cDNA probe in expression studies, it was essential to demonstrate reproducible cross-hybridization to specific trout genomic DNA fragments (by Southern blotting). For this, 30 µg trout genomic DNA obtained from liver was restricted with BamHI, EcoRI, Hind III and Pst I, fractionated (0.8% agarose gel), transferred to a nylon membrane (Hybond N, Amersham International), and probed with [α-32P]dCTP-rat angiotensinogen cDNA (~500 kBu/µg) prepared and purified using a Sequenase Random Primed DNA labeling kit (United States Biochemical) and NucTrap probe purification column (Stratagene). Blots were washed for 2 × 5 min at room temperature in 2× sodium chloride-sodium phosphate-EDTA (SSPE; 0.36 M NaCl, 20 mM NaH2PO4, pH 7.7, 2 mM EDTA) containing 0.1% SDS and 0.1% sodium pyrophosphate; then 2 × 5 min at room temperature in 0.2× SSPE, 0.1% SDS, 0.1% sodium pyrophosphate; then 2 × 15 min at 45°C in 0.2× SSPE, 0.1% SDS, 0.1% sodium pyrophosphate. Blots were exposed to intensified X-ray film for 6 days.

With the use of Northern blots of 20 µg total RNA or 5 µg mRNA from trout liver and kidney probed with the rat angiotensinogen cDNA probe and washed at low stringency, we failed to detect any expression of angiotensinogen in the trout. As an alternative and to enhance the potential signal in expression studies, the rat angiotensinogen cDNA was employed in "reverse Northern blots" (17, 22). For reverse Northern blotting, the cloned heterologous cDNA was restricted, size-separated, and bound to two separate membranes. One membrane was probed with cDNA derived from liver mRNAs; the second membrane was probed with cDNA derived from caulud kidney mRNAs. To prepare these mRNAs, trout were killed, liver and caudal kidney (posterior to the corpuscles of Stannius) were removed rapidly, and total RNA was extracted immediately (10). Poly(A)⁺ RNA was purified using an Oligotex mRNA kit (Qiagen) and quantified by ultraviolet spectrophotometry. To prepare the blots, plasmid DNA containing rat angiotensinogen cDNA (300 ng) was restricted with EcoRI and Hind III to remove the insert and fractionated (0.8% agarose) alongside various negative controls [plasmids 300 ng: pON163 containing Schizosaccharomyces pombe DNA, pMS10 containing Aspergillus nidulans DNA; 30 µg fission yeast (S. pombe) genomic DNA; 30 µg Escherichia coli genomic DNA] and a positive control (30 µg trout genomic DNA). Genomic DNAs were restricted with EcoRI. These DNAs were transferred to nylon membranes (Hybond N, Amersham International). The two blots prepared were prehybridized and probed with [α-32P]dCTP-cDNA (~750 kBu/µg) prepared from 1 µg trout liver mRNA or 1 µg trout kidney mRNA, respectively. Blots were washed (as...
described above for Southern blots) and exposed to X-ray film for 24 h.

RESULTS

Renal and vascular actions of captopril. In the three preparations perfused at a pressure head of 28 mmHg (group 1), captopril had no significant effect on perfusate flow rates, renal aortic pressures, or Vs (Table 1).

Captopril treatment of both groups 2 and 3 preparations perfused at a pressure head of 19 mmHg significantly reduced the aortic pressure and significantly increased perfusate flow rate. Data for group 3 preparations are shown in Fig. 1. In group 2, aortic pressure declined from 19.5 ± 0.3 to 15.9 ± 0.2 mmHg (P < 0.001), whereas perfusate flow rate increased from 8.7 ± 0.1 to 10.1 ± 0.2 ml/min (P < 0.01). Both parameters stabilized after 30 min treatment (see Fig. 1). Once captopril was removed (group 3 experiments), aortic pressure and perfusate flow fully recovered (Fig. 1).

Captopril administration at 19 mmHg perfusion pressure was associated with significant changes in a number of renal parameters. In all cases, once captopril was removed, a full recovery occurred and there were no statistical differences between values recorded in the clearance period before captopril treatment and those in the final “recovery” collection (30–40 min posttreatment). Captopril treatment of kidneys perfused at a 19-mmHg pressure head resulted in an increase in Vs (P < 0.001: Figs. 2 and 3). This partially reflected a significant rise in GFRs (P < 0.05, group 2 experiments; P < 0.001, group 3 experiments). The GFR increase of 42% (mean values) in group 3 studies (Fig. 3) was paralleled by a 42% rise in the tubular transport maxima for glucose (P < 0.001), indicating an elevated functional tubular mass as a result of a rise in the population of filtering glomeruli (Fig. 3).

Alongside the rise in GFR during captopril treatment, a decline in tubular water reabsorption also contributed to the renal diuresis. Despite the increased population of filtering nephrons, i.e., functional tubular mass, the total volume of tubular fluid reabsorbed (GFR minus V) was relatively stable during captopril treatment. As a proportion of GFR, the volume reabsorbed by group 3 kidneys declined from 41% GFR pretreatment to 28% GFR during captopril treatment. Comparable events occurred in group 2 kidneys with comparable values in pretreatment, i.e., after 45–60 min captopril inclusion in the perfusate.

Table 1. Captopril treatment

<table>
<thead>
<tr>
<th>Pretreatment</th>
<th>45–60 min Captopril Treatment</th>
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<tr>
<td>V</td>
<td>106.1 ± 3.2</td>
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<tr>
<td>Perfusion flow</td>
<td>8.81 ± 0.23</td>
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<tr>
<td>Aortic pressure</td>
<td>20.18 ± 0.43</td>
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Values are means ± SE. Effects of captopril treatment of perfused trout trunks with the kidney in situ, perfused at a constant pressure head of 28 mmHg (group 1, n = 3), on urine flow rate (V; µl·min⁻¹·kg body wt⁻¹), perfusate flow rate (ml/min), and aortic pressure (mmHg). Values for 15 min immediately before captopril treatment are compared with values in the final 15 min treatment, i.e., after 45–60 min captopril inclusion in the perfusate.

Fig. 1. Effects of captopril on the time course of changes in aortic pressure (mmHg) and perfusate flow (ml/min) to the trout trunk perfused at 19 mmHg with the kidney in situ (group 3). Captopril significantly affected both parameters (P < 0.001), and there was a full recovery after its removal (final clearance values not significantly different from pretreatment values).

52% of GFR reabsorbed pretreatment declining to 40% GFR reabsorbed during captopril treatment. Hence, water reabsorption per filtering nephron declined. This was reflected by a significant decrease in urine-to-plasma inulin concentration ratios within the treatment period (P < 0.001 in both groups 2 and 3; Figs. 2 and 3), whereas in group 2 studies in which free-water clearances could be calculated, both free-water clearances and relative free-water clearances significantly increased (P < 0.001; Fig. 2). In group 2 experiments in which urine osmolality was measured, captopril treatment slightly increased the relative clearance of osmoles (31.3 ± 0.62% to 35.7 ± 0.96% GFR; P < 0.02), but this effect was very small compared with the fourfold rise in relative free-water clearance.

Angiotensinogen expression in trout kidney and liver. Radiolabeled rat angiotensinogen cDNA consistently and reproducibly cross-hybridized to specific trout genomic DNA fragments in Southern blots (Fig. 4). Progressive washing at higher temperatures resulted in complete removal of all cross-hybridizing species of DNA at 52°C, consistent with the use of a heterologous...
There were three detectable species (5.0, 2.0, and 1.4 kb) after restriction of trout DNA with BamHI and two detectable species (6 and 4.8 kb) with HindIII. Although salmonids have a tetraploid genome, they are in the process of rediploidization and loss of duplicated genes, so hybridization of the rat angiotensinogen cDNA to a single species (6 kb) after restriction with PstI or EcoRI is not unreasonable. The cross-hybridization to restricted trout DNA was reproducible in all cases and therefore justified the use of the heterologous probe for investigations of angiotensinogen expression in the trout.

DISCUSSION

There is evidence that in mammals, alongside a circulating RAS, tissue-specific RASs occur with the potential for paracrine actions (13, 15, 34, 35), but whether paracrine RASs occur in nonmammalian vertebrates is not yet known. The focus of the present studies was specifically on the kidney, where renin has been demonstrated histologically in most teleostean species examined to date, including the trout (29), and
Release of renin from perfused trout kidneys has been clearly demonstrated (2). There is also biochemical evidence for the occurrence of ACE in the kidneys of many lower vertebrates including the rainbow trout (19, 30), although the gill, the functional homolog of the mammalian lung, contains much higher levels and has been presumed to play the major role in activation of circulating ANG I and its conversion to ANG II (19, 20, 30, 32). So, two components of the RAS are synthesized by the trout kidney, but a complete intrarenal RAS requires the substrate angiotensinogen. The present studies suggested angiotensinogen gene expression in the trout kidney and therefore provide evidence for a complete intrarenal RAS in the trout, capable of functioning independently of the systemic RAS. To our knowledge, this is the first molecular evidence for a tissue-specific RAS in any fish species. Elucidation of the activation and physiological role of this system was explored in vitro, in the absence of the systemic RAS, using the perfused trunk preparation.

Captopril inhibition of ACE conversion of ANG I to ANG II in trout trunks perfused at 28 mmHg had no effect on perfusate flow rates, renal aortic pressure, or urine output. However, when perfusion pressure was lowered, captopril treatment resulted in significant vasodilation, increasing perfusate flow, and a renal diuresis occurred. Such effects are in agreement with the vasoconstrictor and antidiuretic actions of ANG II previously reported. ANG II induced vasoconstriction of the microcirculation of trout trunks perfused via the dorsal aorta (31). In whole trout, ANG II has a renal antidiuretic action (8, 14), although this effect may be compromised by pressor actions of intravenously administered ANG II (14, 28). The renal antidiuretic action of ANG II in the trout has, however, been confirmed in vitro using the perfused trout trunk and in situ kidney (9, 12).

The significant effects of captopril treatment of trout kidneys perfused in situ at 19 mmHg on the tubular reabsorption of water are also in line with an antago-
nism of tubular antidiuretic effect of ANG II that has previously been reported (8), although not always apparent (14). The small increase in relative osmolyte clearance induced by captopril treatment suggests that the intrarenal RAS may have some effect on ion handling processes in the renal tubules, but that the effects on water permeability extend beyond those predicted from the small changes in ion transport. After removal of captopril, the restoration of tubular water reabsorption appeared to be somewhat slower than the recovery of vascular tone and glomerular function. This may reflect more complex events likely to be associated with the changes in tubular water reabsorption and/or the involvement of secondary paracrine substances such as the renal prostanoïds (6, 7). Previous studies on the effects of ANG II in trout have predominantly focused on glomerular and vascular actions. More detailed study of tubular actions is justified both on the basis of the present studies and in view of previous studies demonstrating the occurrence of renal tubular receptors for angiotensin in teleosts including the trout (11, 23, 24).

The changes in vascular, glomerular, and tubular function during captopril treatment of preparations perfused at low pressures and recovery of all parameters once captopril was removed (with no effect in preparations perfused at higher pressures/flows) argues for a significant physiological activation of the renal RAS at low pressures/flows. Low blood volume (after hemorrhage) and associated low blood pressures have already been suggested to have a major impact on the release of renin from the fish kidneys (2, 25–27), though at that time these were only perceived to be part of a systemic RAS acting through the vasoconstrictor action of circulating angiotensins. More recently, treatment of trout head kidneys perfused via the posterior cardinal vein with captopril suggested that a head kidney RAS may exist (3) as catecholamine release induced by angiotensin was reduced by captopril treatment. The catecholamine release aids the “antidrop” effects of the systemic RAS (30, 31). The present studies imply that the renal actions of renally formed ANG II could aid in the “emergency response” to low blood volume/pressure. The present studies also lead us to speculate that in vivo, the renal RAS could play a subtle role in local control of nephron function, perhaps regulating discrete areas of vascular and glomerular perfusion. In whole animals, the existence of discrete areas of perfused glomeruli and nonperfused glomeruli is apparent (8, 9). Intermittent glomerular function is a central tenet of current ideas as to how teleostean kidneys operate, particularly within euryhaline species (5, 8, 9) with <40% of nephrons usually filtering at any one time (8). The present studies support the hypothesis that the renal RAS is involved in regulation of these patterns of perfusion and may also regulate tubular function amongst the population of nephrons.

In summary, our studies have provided evidence for angiotensinogen expression within the kidney, which along with biochemical evidence for renal ACE and renin, strongly supports the existence of an intrarenal RAS. The studies of the in situ perfused kidney suggest activation of this renal RAS by low perfusion pressure and/or the resultant low perfusate flow rate, generating renal ANG II with vascular and renal actions. In vivo, this activation could operate at a very subtle level regulating the operation and performance of discrete groups of the population of nephrons.

Perspectives

To date, renal studies of the actions of angiotensins have centered on the actions of circulating peptides. This study provides a new perspective and shows that the euryhaline trout possesses a functional renal RAS. We would expect that this renal RAS operates independently of the well-established systemic RAS. This has important functional implications in understanding the regulation of body fluid homeostasis in fish, and further studies will be essential to elucidate the modes of activation and the interactions between the operation of systemic and renal RASs. Measurement of renal levels of angiotensins needs to be explored in parallel with investigation of their circulating levels. We hypothesize that local renal vascular dynamics will act as one regulator of the renal RAS, perhaps achieving regulation of glomerular perfusion and intermittent filtration with insignificant impact on circulating angiotensins. Similarly, control of renal tubular function could be achieved through the renal RAS; studies on isolated renal tubules would help to distinguish direct actions of angiotensins from indirect, hemodynamically induced effects.

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