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LOCALIZATION OF ANGIOTENSIN II RESPONSES IN THE TROUT CARDIOVASCULAR SYSTEM

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Summary

The renin/angiotensin system (RAS) is a tonic anti-drop regulator of arterial blood pressure in many teleosts. In trout, angiotensin II (ANG II) has no direct constrictor effect on large arteries or veins and the identity of specific cardiovascular pressor effectors is unknown. Potential targets of angiotensin activation were examined in the present experiments using perfused organs and isolated tissues from the rainbow trout *Oncorhynchus mykiss*.

Perfused gill (arches 2 and 3), perfused skeletal muscle-kidney (*via* the dorsal aorta; PDA) and perfused splanchnic (*via* the celiacomesenteric; PCM) circulations vasoconstrict in response to salmonid ANG II in a dose-dependent manner. ANG II was significantly ($P \leqslant 0.05$) more potent in the PCM than in the PDA, and both preparations were more responsive than the gills: $pD_2=8.0\pm0.20$ (10) for PCM; $pD_2=7.5\pm0.07$ (13) for PDA; $pD_2=6.9\pm0.21$ (8) for gill arch 3; $pD_2=6.7\pm0.23$ (8) for gill arch 2; mean ± S.E.M. (*N*), respectively. Salmonid angiotensin I (ANG I) also produced a dose-dependent constriction of the PDA and PCM. Angiotensin converting enzyme (ACE) activated nearly 100 % of ANG I to ANG II in a single pass through the PDA, whereas PCM conversion was estimated to be less than 10 %. Inhibitors of adrenergic constriction partially prevented ANG II responses in the PDA but did not affect PCM responses.

ANG II did not affect paced rings of ventricular muscle in the presence of high or low $[Ca^{2+}]$ or epinephrine concentrations, nor did it have any inotropic or chronotropic effects in the *in situ* perfused heart. Red blood cell swelling was unaffected by ANG II. Similarly, the effects of ANG II on gut, urinary bladder and gall bladder smooth muscle were negligible or non-existent; thus, an increase in splanchnic resistance due to extravascular compression can be discounted.

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These results indicate that, in trout, the systemic microcirculation is the major cardiovascular effector of angiotensin-mediated pressor responses. In addition, the RAS has little direct effect on non-vascular smooth muscle or the heart. From an evolutionary perspective, the initial site of direct systemic RAS action appears to be the vascular microcirculation.

Introduction

The mammalian renin/angiotensin system (RAS) is a complex anti-drop effector of blood pressure and volume regulation. The biologically active end product of this system, angiotensin II (ANG II), as well as several truncated forms, both directly and indirectly stimulate the heart, blood vessels and fluid regulatory systems to prevent hypotension. The vascular effects of ANG II include direct vasoconstriction, stimulation of endothelial secretion of vasoactive contracting and/or relaxing factors and augmentation of vascular adrenergic neuronal tone (Phillips *et al.* 1993; Reid, 1992; Saxena, 1992; Timmermans *et al.* 1993). These mechanisms, however, are not equally distributed throughout the vasculature (Grega and Adamski, 1987; Minami and Toda, 1988). ANG II is inotropic in the heart and it may also affect the rhythm-generating and rhythm-conducting systems (Grinstead and Young, 1992; Saavedra *et al.* 1993). Evidence is accumulating that many tissues have their own complete RAS (Phillips *et al.* 1993) and thus that the physiological effects of ANG II may be expressed through both paracrine and endocrine pathways.

All components of the RAS have been identified in bony fish (Olson, 1992), and it has been suggested that the RAS evolved as a modulator of the sympathetic nervous system (Wilson, 1984*a*,*b*) or as an intrarenal hormone to control nephron function (Henderson *et al.* 1980). *In vivo* administration of angiotensin converting enzyme (ACE) inhibitors, which block endogenous ANG II formation, lowers blood pressure in many teleosts (Galardy *et al.* 1984; Madey *et al.* 1984; Lipke and Olson, 1990; Platzack *et al.* 1993). The onset of hypotension following ACE inhibition occurs too rapidly to be attributed to a renal effect and, in cod, ACE inhibition is a more potent hypotensive stimulus than α-adrenoceptor blockade (Platzack *et al.* 1993). These findings indicate that the RAS is an important tonic pressor system in many teleosts and that ANG II has direct effects on the cardiovascular system.

There is relatively little information on the site or mode of ANG II action in teleosts (reviewed in Olson, 1992). ANG-II-mediated constriction of large arteries and veins *in vitro* is either weak or non-existent (Carroll, 1981; Lipke and Olson, 1990; Conklin and Olson, 1994*a*,*b*) and ANG II may, in fact, relax vessels contracted by other agonists (Conklin and Olson, 1994*b*). It therefore seems unlikely that the actions of ANG II on large vessels contribute significantly to its pressor activity *in vivo*. Other potential systemic effectors of ANG II pressor action, such as the microcirculation, heart, gills, blood or extravascular sites, have not, to our knowledge, been examined and are the focus of the present study.

Materials and methods

Animals

Rainbow trout [*Oncorhynchus mykiss* (Walbaum), 150–500 g], purchased from a local

hatchery (Robinson Trout Farm, Grand Haven, MI) and maintained at the University of Notre Dame in aerated well water (12 °C), were used for all experiments except the *in situ* perfused heart. They were fed a maintenance diet of commercial trout pellets (Purina) up to 24 h prior to experimentation. Trout were exposed to a 12 h:12 h or 16 h:8 h light:dark (L:D) photoperiod appropriate for the season. Trout used in the *in situ* perfused heart preparation (500–800 g) were obtained from a local hatchery (West Creek Trout Farms, Aldergrove, BC) and held in aerated, dechlorinated water at 15 °C at Simon Fraser University. They were fed daily *ad libitum* with a commercial trout chow.

In vitro *procedures*

Trout were stunned by a blow to the head and 1 ml of heparin (1000 i.u. ml^{-1}) in phosphate-buffered saline (PBS) was injected *via* a mid-ventral incision into the bulbus arteriosus and allowed to circulate for 2–3 min. The head was then severed from the body and the tissues were removed or the appropriate vessels isolated.

Perfused trunk

The perfused trunk preparation has been described previously (Olson and Villa, 1991). With this method, a cannula is inserted into the dorsal aorta for perfusion of the skeletal muscle-kidney circulation, and a second cannula in the celiacomesenteric artery permits splanchnic perfusion. The two circuits were perfused with individual peristaltic pumps and flow was adjusted to produce an arterial pressure of between 10 and 20 mmHg.

The dorsal aorta and celiacomesenteric artery were perfused for 15–25 min with PBS. Cumulative angiotensin I (ANG I) and ANG II dose–response curves were obtained by step-wise tenfold increments of peptide concentration. The pD_2 was determined from individual dose–response curves and was calculated as the negative logarithm of the ANG II concentration that produced a half-maximal response (i.e. $-\log EC_{50}$). The efficacy of ANG I as a direct vasoconstrictor (or the extent of ANG I to ANG II conversion) was determined in separate experiments by perfusion of the dorsal aorta with the angiotensin converting enzyme inhibitor captopril (10^{-5} mol l^{-1}) for 20 min prior to 10^{-7} mol l^{-1} ANG I infusion. Tachyphylaxis to ANG II (see Fig. 1) was examined in separate experiments by four consecutive 15 min perfusions of 10^{-7} mol l^{-1} ANG II, each followed by a 20–30 min washout with PBS.

The effects of the adrenergic antagonists phentolamine (10^{-5} mol l^{-1}) and bretylium (10^{-5} mol l^{-1}) on the ANG II response were examined by pre-perfusing the drug for 10 min, followed by infusion of 10^{-7} mol l^{-1} ANG II for 10–15 min, PBS for 15 min and a second dose of ANG II for 15 min. Antagonist exposure was maintained throughout the experiment. This protocol was designed to evaluate the adrenergic component during both the initial and tachyphylactic periods of ANG-II-induced vasoconstriction.

(Sar^1-Thr^8)-ANG II (10^{-7} mol l^{-1}), an angiotensin analog with putative antagonist activity, was examined using the protocol described above for adrenergic antagonists. Other angiotensin type 1 (AT_1) receptor inhibitors, dithiothreitol (10^{-5} mol l^{-1}; Chiu *et al.* 1989) and losartan (DuP 753; 10^{-6} mol l^{-1}; Timmermans *et al.* 1993), were infused for 15 min followed by infusion of inhibitor plus ANG II (10^{-6} mol l^{-1}) for an additional 10 min.

Interaction of ANG II and catecholamine-induced vasoconstriction was examined in preparations continuously perfused with epinephrine and the β-adrenoceptor antagonist propranolol (10^{-5} mol l^{-1}). The perfusion protocol was: propranolol (10 min); propranolol plus epinephrine (15 min); ANG II plus propranolol plus epinephrine (15–20 min). Two concentrations of epinephrine were used, 10^{-6} and 10^{-5} mol l^{-1} in the perfused dorsal aorta, and 10^{-7} and 10^{-6} mol l^{-1} in the celiacomesenteric artery. These concentrations have been found to produce borderline-threshold and near-maximal vasoconstrictory effects, respectively, in these preparations (Xu and Olson, 1993*a*).

Isolated perfused gill

The second and third pair of gill arches were isolated and the afferent branchial artery of one arch from each pair was cannulated with a 20 gauge stainless-steel cannula attached to polyethylene tubing (PE 90). Efferent branchial arteries were not cannulated to minimize trauma to the vessel and because this pathway accounts for over 90 % of the branchial resistance (Olson, 1984). The arches were placed in individual 500 ml tapwater baths and perfused with a peristaltic pump at 0.32 ml min^{-1}. A T in the cannula tubing, near the arch, was connected to a pressure transducer. Arches were perfused for 30 min with PBS then for 10 min each with increasing ANG II concentrations (10^{-9} to 10^{-5} mol l^{-1}) followed by 10 min perfusion with 10^{-5} mol l^{-1} acetylcholine (ACh). In several experiments, arches were perfused with PBS (30 min), 10^{-6} mol l^{-1} ACh (10 min) and 10^{-6} mol l^{-1} ANG II plus 10^{-6} mol l^{-1} ACh (10 min).

Ventricular rings

The heart was removed and placed in cold (4 °C) PBS. A single ring, approximately 2 mm wide, was cut from the middle of the ventricle, perpendicular to the base–apex axis. The ring was fastened to a 280 μm diameter steel hook and suspended from a Grass FT03C force–displacement transducer in a 35 ml water-jacketed (12 °C) muscle chamber and aerated with 100 % oxygen. Two coiled platinum electrodes were placed on either side of the heart and the heart was stimulated with 5 V of 40 ms duration at 0.33 Hz. Prior to experimentation, the ventricular rings were equilibrated for 30 min at 0.5–1 g of diastolic tension. The effects of angiotensin II were examined under three conditions; low [Ca^{2+}] (0.5 mmol l^{-1} Ca^{2+} PBS), high [Ca^{2+}] (2.0 mmol l^{-1} Ca^{2+} PBS) and low [Ca^{2+}]+EPI (low-Ca^{2+} PBS with 10^{-8} mol l^{-1} epinephrine). Ventricular rings were incubated in one of these solutions for 35–45 min and then angiotensin II was added in tenfold incremental doses from 10^{-9} to 10^{-6} mol l^{-1} at 10 min intervals. Only one incubation condition was used per ring.

In situ *perfused heart*

The *in situ* perfused heart preparation was performed as described previously (Keen *et al.* 1993). The sinus venosus was cannulated *via* a hepatic vein and an output cannula was inserted into the ventral aorta to a point confluent with the bulbus arteriosus. The ductus Cuvier were ligated, thereby crushing the cardiac branches of the vagus nerve; heart rate was maintained by the sino-atrial pacemaker rhythm. The pericardium was not disturbed and the heart received Tes-buffered saline (TBS) supplemented with

5×10^{-9} mol l^{-1} epinephrine throughout the experiment. Input and output pressures were measured using Micron pressure transducers (Narco Life Sciences, Houston, TX), while outflow (cardiac output) was monitored with an in-line electromagnetic flow probe and meter (Zepeda Instruments, Seattle, WA). Pressure and flow signals were amplified and displayed on a chart recorder (Gould, Cleveland, OH). Signals were also fed into a computer and stored for further analysis.

The following procedures were employed to determine whether ANG II affected intrinsic heart rate or shifted the Starling relationship to alter maximum cardiac output and/or maximum power output of the heart. In all instances, the heart was first allowed to stabilize for a 10 min period at basal mass-specific cardiac output of 18–20 ml min^{-1} kg^{-1} body mass, thereby approximating the *in vivo* resting condition at 15 °C. After stabilization, ANG II was added to the perfusate to produce a concentration of 10^{-10} mol l^{-1}. The response of the heart was then monitored for 5 min. After this, small increases in filling pressure (preload) were used to maximize cardiac output, thus setting the heart at the apex of the Starling curve. Myocardial power output was then examined by increasing the diastolic afterload from a resting level of 37 mmHg to a maximum of 57 mmHg. After completion of these trials, the basal condition was re-established and the heart was allowed to stabilize for 5 min. The ANG II concentration was then increased to 10^{-8} mol l^{-1} and the procedures were repeated. After completion of Starling and power trials with 10^{-8} mol l^{-1} ANG II, a final set of trials was performed at an ANG II concentration of 10^{-6} mol l^{-1}. Three Starling trials and three power trials were thus conducted on each heart. Control hearts were subjected to the same series of trials without the addition of ANG II to the perfusate.

Stroke volume and cardiac output were determined from flow and rate measurements. Mass-specific myocardial power output (in mW g^{-1} ventricular mass) was calculated as:

$$\text{Power} = [\dot{V}\text{b}(P_\text{o} - P_\text{i})k]\ (\text{g}^{-1}),$$

where $\dot{V}$b is cardiac output in ml min^{-1}, $P_\text{o}-P_\text{i}$ is the pressure differential between input and output pressure in mmHg, and k (2.19×10^{-3} mW min ml^{-1} $mmHg^{-1}$) is a conversion factor to mW.

Spontaneous periods of asystole may occur in the *in situ* heart preparation if perfusate epinephrine concentration is reduced below *in vivo* resting levels. If epinephrine is not restored, at least to this level, these episodes increase, eventually resulting in complete asystole (J. E. Keen, unpublished observation). The effects of RAS inhibition on heart rate and myocardial run-down were examined in four hearts in which spontaneous asystolic periods occurred. The *in situ* perfused heart was prepared as above and allowed to stabilize at the resting work level as previously described. The heart was then perfused with epinephrine-free TBS until arrhythmias, expressed as a progressively increasing frequency of asystolic intervals, developed. Captopril (10^{-5} mol l^{-1}) was then added to the perfusate and the heart was perfused for an additional 45 min.

Isolated erythrocytes

Blood was drawn from the hemal arch into heparinized syringes, centrifuged, and the plasma and buffy coat were removed. The cells were washed four times in 4 volumes of

PBS. They were then resuspended in 2 volumes of PBS (hematocrit=30–35 %) and 0.32 ml samples were pipetted into 4 ml glass vials. In one protocol, the samples were incubated in tenfold increments of ANG II (10^{-10} to 10^{-5} mol l^{-1}) and hematocrits were determined, in duplicate, at 10, 45 and 120 min. In the second protocol, the cells were incubated for 60 min in increasing concentrations of norepinephrine (3×10^{-10} to 10^{-5} mol l^{-1}) either with or without 10^{-6} mol l^{-1} ANG II. All cells were incubated at 12 °C on an inclined (45 °) rotating (10 revs min^{-1}) mixer. Blood from 3–4 fish was pooled for each experiment, and each experiment was repeated three times using different blood donors.

Nonvascular smooth muscle

The effects of ANG II on nonvascular smooth muscle were examined *in vitro* in the smooth muscle chambers described above for vascular rings. Longitudinal and circular strips were removed from the esophagus, stomach, anterior and posterior intestine and urinary bladder; only longitudinal strips were taken from the gall bladder. The tissues were equilibrated at a resting tension of 1.0 g (500 mg for urinary bladder) for a minimum of 60 min, and then exposed either to tenfold increasing doses of ANG II (10^{-10} to 10^{-5} mol l^{-1}) or to a single 10^{-6} mol l^{-1} dose. In other preparations, the muscle was pre-contracted with 10^{-6} mol l^{-1} ACh prior to cumulative, or single, ANG II treatments.

In vivo *procedures*

Arterial pressure

Dorsal aortic blood pressure was measured in conscious, chronically cannulated trout as described previously (Galardy *et al.* 1984). Consecutive bolus ANG II injections (3×10^{-9} mol kg^{-1} body mass in 0.9 ml kg^{-1} body mass of 0.9 % NaCl) were administered at 25–35 min intervals.

Chemicals

Losartan (DuP 753; COZAAR) was a gift from DuPont Merck, Wilmington, DE. Salmonid angiotensin I [(Asn^1-Val^5-Asn^9)-angiotensin I], salmonid angiotensin II [(Asn^1-Val^5)-angiotensin II], (Sar^1-Thr^8)-angiotensin II, dithiothreitol, epinephrine, phentolamine and bretylium were purchased from Sigma (St Louis, MO). All other chemicals were reagent grade. PBS consisted of (in mmol l^{-1}): 126.0 NaCl; 4.1 KCl; 0.5 $CaCl_2$; 1.1 $MgSO_4$; 3.4 KH_2PO_4; 14.2 Na_2HPO_4; 5.0 D-glucose; pH was adjusted to 7.8 at 12 °C. TBS consisted of (in mmol l^{-1}): 124.1 NaCl; 3.1 KCl; 2.5 $CaCl_2$; 0.9 $MgSO_4$; 5.0 D-glucose; 11.8 Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid], sodium salt; 8.2 Tes, free acid. TBS perfusates were bubbled with 100 % O_2 and maintained at pH 7.8 and 15 °C (Keen *et al.* 1993). The osmolarity of the solutions was 280 mosmol l^{-1}.

Statistical analyses

Analysis of variance (ANOVA), with the Student–Newman–Keuls method for multiple comparisons, or paired *t*-tests were used for statistical comparisons. Results are expressed as means ± S.E.M.

Results

Perfused trunk

Both ANG I and ANG II produced dose-dependent increases in perfusion pressure in the perfused dorsal aorta and celiacomesenteric artery preparations (Figs 1A,B, 2). ANG I was nearly as potent as ANG II in the perfused dorsal aorta, whereas it was less effective in the perfused celiacomesenteric preparation. Maximum pressure produced by

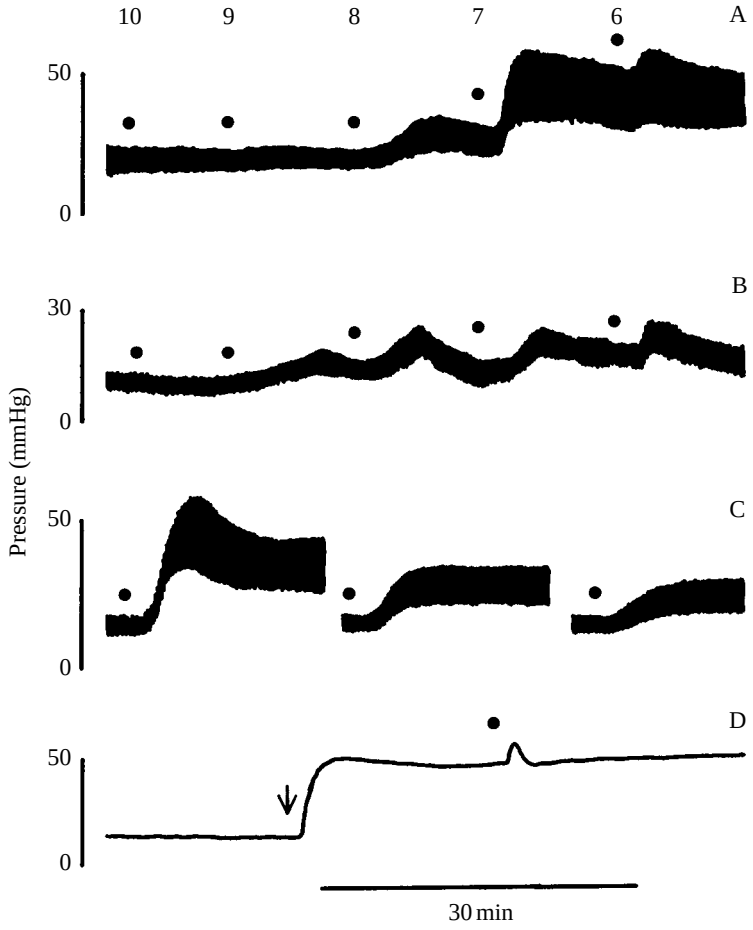

Fig. 1. Pressure tracings of the perfused trunk. (A,B) Cumulative ANG II dose–response characteristics of the perfused dorsal aorta (A) and celiacomesenteric artery (B). Dots indicate concentration of ANG II (−log molar concentration indicated above A) in the perfusate. (C) Tachyphylaxis of perfused dorsal aorta to repetitive treatment with 10^{-7} mol l^{-1} ANG II. After each ANG II treatment the preparation was perfused for 20–30 min with ANG-II-free phosphate-buffered saline. Left and center tracings are first and second exposures, respectively; the tracing on the right is the fourth ANG II treatment. (D) Effect of ANG II (10^{-7} mol l^{-1}; infusion initiated at dot) on perfused dorsal aorta previously treated with 10^{-5} mol l^{-1} propranolol and contracted with 10^{-5} mol l^{-1} epinephrine (arrow). Pressure pulsations were damped with an air bubble reservoir.

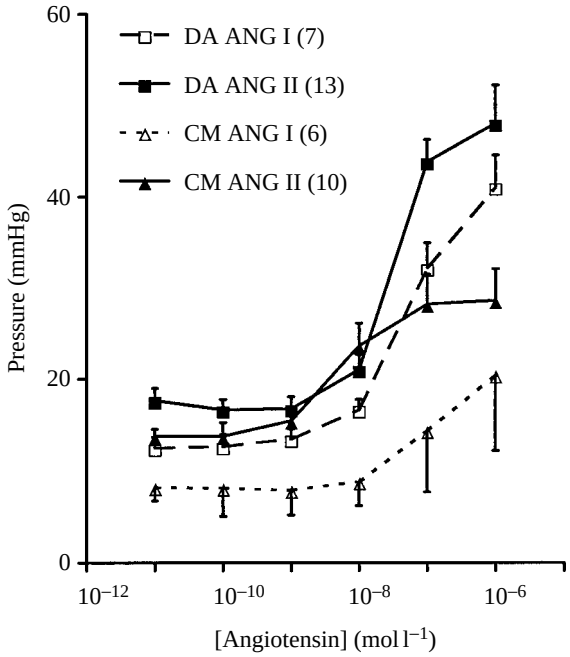

Fig. 2. Cumulative dose–response effects of angiotensin I (ANG I) or angiotensin II (ANG II) on perfusion pressure in the perfused dorsal aorta (DA) or celiacomesenteric artery (CM). Mean ± S.E.M.; *N* is indicated on the graph.

Table 1. *pD_2 values for angiotensin-II- or angiotensin-I-stimulated pressor responses in tissues perfused* via *the dorsal aorta (skeletal muscle-kidney), celiacomesenteric artery (splanchnic) and perfused gill arches 2 and 3*

	Dorsal aorta	Celiacomesenteric artery	Arch 2	Arch 3
Angiotensin II[a]	7.5±0.07 (13)	8.0±0.20[b] (10)	6.7±0.23 (8)	6.9±0.21 (8)
Angiotensin I	7.39±0.06[c] (7)	7.01±0.14[b,c] (6)		

pD_2 was calculated from individual dose–response curves as the negative logarithm of the angiotensin II concentration (in mol l^{-1}) producing a half-maximal response ($-\log EC_{50}$); values are mean ± S.E.M. (*N*).

[a]All angiotensin II pD_2 values are significantly different ($P \leqslant 0.05$) from each other except for values for arches 2 and 3.

[b,c]Significant difference between common symbols.

10^{-5} mol l^{-1} ANG II in both preparations during cumulative dose–response experiments was usually less than that produced by 10^{-7} or 10^{-8} mol l^{-1} ANG II (*N*=5 each, not shown). pD_2 values determined from individual dose–response curves for perfused

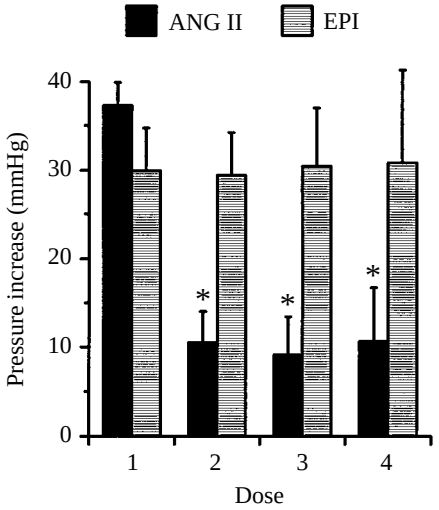

Fig. 3. Increase in dorsal aorta perfusion pressure produced by repetitive doses (1–4) of 10^{-7} mol l^{-1} angiotensin II (ANG II) or 10^{-5} mol l^{-1} epinephrine (EPI). Mean + S.E.M.; *N*=6, 6, 5 and 3 for ANG II doses 1–4 and 5, 5, 5 and 4, for EPI doses 1–4, respectively. Preparation perfused for 15 min with agonist, 30 min with PBS then agonist perfusion repeated; epinephrine-treated preparations were continuously perfused with 10^{-5} mol l^{-1} propranolol. *Significantly different from the control value ($P \leqslant 0.05$).

dorsal aorta and celiacomesenteric arteries are shown in Table 1. The perfused celiacomesenteric artery preparation was more sensitive to ANG II and less sensitive to ANG I than the perfused dorsal aorta. There was no significant difference in pD_2 in dorsal aorta perfused with ANG I or ANG II, whereas the perfused celiacomesenteric artery was significantly less sensitive to ANG I. Captopril (10^{-5} mol l^{-1}) virtually eliminated the vasoconstrictor response to 10^{-7} mol l^{-1} ANG I in the perfused dorsal aorta; pressure increase with captopril pre-perfusion was 1.3±0.4 mmHg (*N*=5), without captopril ANG I increased perfusion pressure by 14.0±0.9 mmHg (*N*=3), $P \leqslant 0.01$.

Both perfused dorsal aorta and celiacomesenteric artery preparations exhibited considerable tachyphylaxis at higher angiotensin concentrations. This was evident as a rapid increase, then a decrease, in perfusion pressure in cumulative dose–response measurements (Fig. 1A,B) or by a reduction in the response to repeated treatment of ANG II with an intervening rinse of ANG-II-free perfusate (Fig. 1C). In the latter experiments, perfusion pressure increased rapidly during the first exposure to ANG II, then fell almost as quickly to a steady value that was approximately half that of the initial response (Fig. 1C). Subsequent treatment with identical concentrations of ANG II failed to produce a rapid, maximal response (Fig. 1C), while the magnitude of the steady response, although lower than that produced by the first treatment, usually remained unchanged (Fig. 3). By comparison, pressor responses in intact conscious trout were not tachyphylactic to repeated ANG II injections. Five consecutive ANG II injections increased dorsal aortic pressure by 40.8±3.7, 39.8±2.9, 39.7±3.1, 40.5±2.9 and

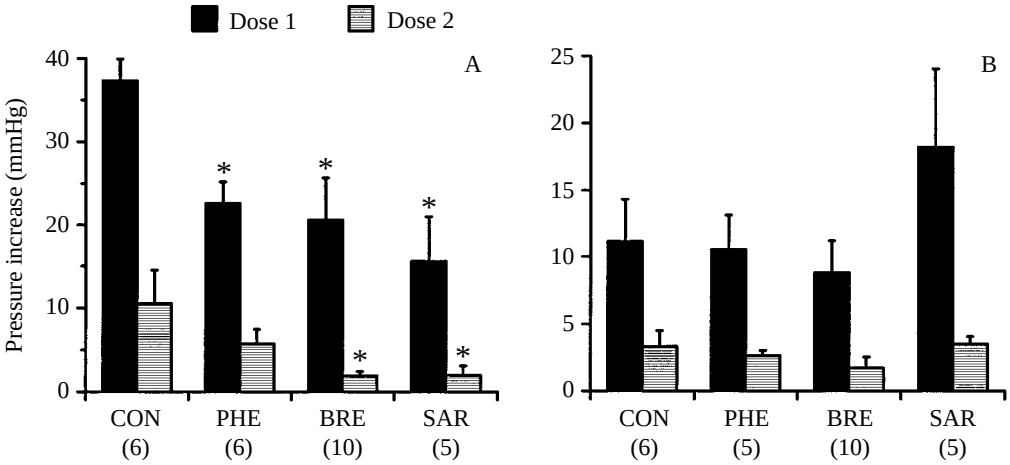

Fig. 4. Effects of antagonists on the pressure increase produced by two consecutive 10^{-7} mol l^{-1} angiotensin II infusions (doses 1 and 2) in the perfused dorsal aorta (A) or celiacomesenteric artery (B). CON, control; PHE, 10^{-5} mol l^{-1} phentolamine; BRE, 10^{-5} mol l^{-1} bretylium; SAR 10^{-7} mol l^{-1} (Sar^1-Thr^8)-angiotensin II. Antagonists were perfused prior to and during angiotensin exposure. Mean + S.E.M., *N* is indicated under the graph. *Significantly different from the respective control value ($P \leqslant 0.05$).

39.7±3.1 mmHg (*N*=7), respectively. Similarly, tachyphylaxis was not observed after repetitive treatment with high concentrations (10^{-5} mol l^{-1}) of epinephrine in propranolol-blocked perfused trunks (Fig. 3).

The vasoconstrictory effect of ANG II in the perfused dorsal aorta (Fig. 4A) was inhibited by approximately 40 % by both the α-adrenoceptor antagonist phentolamine (10^{-5} mol l^{-1}) and the adrenergic nerve toxin bretylium (10^{-5} mol l^{-1}). (Sar^1-Thr^8)-ANG II (10^{-7} mol l^{-1}) reduced the pressor response of the perfused dorsal aorta by approximately 50 % (Fig. 4A). In contrast, phentolamine, bretylium and (Sar^1-Thr^8)-ANG II did not affect the ANG II response of the perfused celiacomesenteric artery (Fig. 4B). In fact, (Sar^1-Thr^8)-ANG II appeared to act as a partial agonist. The response of perfused dorsal aorta or celiacomesenteric artery to 10^{-7} mol l^{-1} ANG II was unaffected by pre-treatment with either 10^{-5} mol l^{-1} dithiothreitol (*N*=5) or 10^{-6} mol l^{-1} DuP 753 (*N*=2).

Tissues perfused with epinephrine at a concentration that was just at the vasoactive threshold (10^{-6} mol l^{-1}, dorsal aorta; 10^{-7} mol l^{-1}, celiacomesenteric artery) produced ANG-II-mediated constrictions that were similar to those of tissues not exposed to epinephrine (*N*=4 for each preparation, data not shown). ANG II had minor and inconsistent effects on perfusion pressure of dorsal aorta or celiacomesenteric artery preparations treated with near-maximal vasoconstrictor concentrations (10^{-5} and 10^{-6} mol l^{-1}, respectively) of the catecholamine. In some instances, perfusion pressure was slightly increased by ANG II (Fig. 1D), in other experiments it was slightly decreased, and in others there was a biphasic constrictor–dilator response. In all instances ANG II effects were transient.

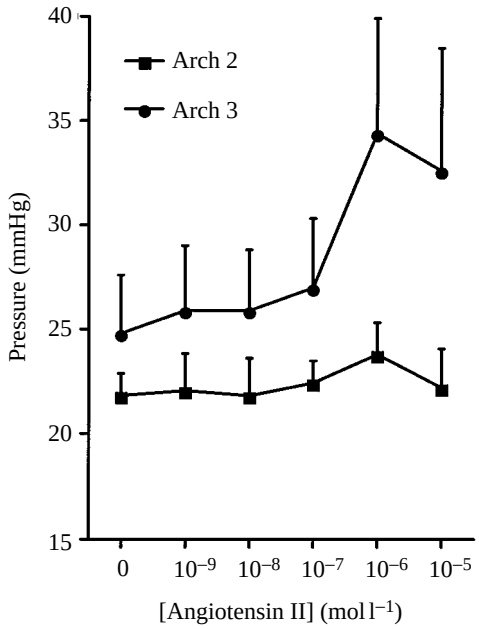

Fig. 5. Cumulative dose–response effects of angiotensin II (ANG II) on perfusion pressure of the second (*N*=9) or third (*N*=8) gill arch. Mean ± S.E.M.

Isolated perfused gill

Angiotensin increased perfusion pressure in both second and third gill arches, although the pressor effects were more pronounced in the latter (Fig. 5). The net increase in perfusion pressure (maximum pressure minus control) produced by ANG II infusion into the second arch (2.4±0.8 mmHg) was less ($P \leqslant 0.05$) than that produced in the third arch (11.1±3.3 mmHg). The ANG-II-mediated net increase in perfusion pressure was only 12.4 % (arch 2) and 33.0 % (arch 3) as great as the net increase in perfusion pressure produced by 10^{-5} mol l^{-1} ACh (20.6±2.7 mmHg, *N*=8, for gill arch 2 and 31.9±3.6 mmHg, *N*=7, for gill arch 3). In three out of nine second gill arches and six out of eight third arches, 10^{-9} mol l^{-1} ANG II produced an obvious 0.5–2 mmHg increase in perfusion pressure that was not augmented by 10^{-8} mol l^{-1} ANG II. All ANG-II-mediated vasoconstrictions exhibited tachyphylaxis, with maximum pressure responses usually lasting less than 2–3 min. Perfusion pressure during 10^{-5} mol l^{-1} ANG II exposure rarely increased; often it fell below the pressure produced by 10^{-6} mol l^{-1} ANG II (Fig. 5). There was no significant difference in pD_2 values between the second and third arches, although both arches were significantly less sensitive to ANG II than the perfused systemic tissues (Table 1). ANG II (10^{-6} mol l^{-1}) produced a further increase in arches contracted with 10^{-6} mol l^{-1} ACh (*N*=2, arch 3; *N*=1, arch 2); ANG-II-mediated dilation was not observed in any preconstricted gill.

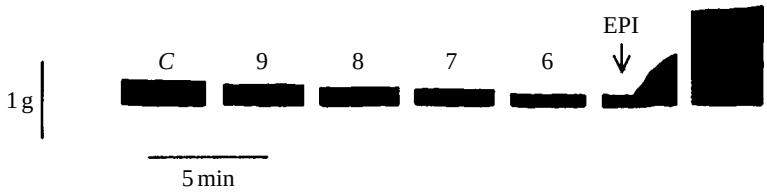

Fig. 6. Tension developed by an isolated ventricular ring incubated consecutively with PBS (*C*), graded angiotensin II (9-6; −log molar concentration) or 10^{-5} mol l^{-1} epinephrine (EPI). At this chart speed, individual contractions are fused; spaces between treatments are 10 min.

Ventricular rings

The tension produced by electrically paced ventricular rings incubated in PBS declined slowly over the course of 2 h by approximately 80–90 % (*N*=4, not shown). Tension could be restored at any time during this period by addition of epinephrine ($\geqslant 10^{-8}$ mol l^{-1}) or Ca^{2+}. The decline in tension was retarded or inhibited by continuous incubation with 10^{-8} mol l^{-1} epinephrine (*N*=4). Switching from low- to high-Ca^{2+} PBS increased tension two- to fourfold (*N*=6). Angiotensin II (10^{-10} to 10^{-6} mol l^{-1}) had no effect on tension in rings incubated in PBS (*N*=6; Fig. 6), high-Ca^{2+} PBS (*N*=6) or PBS containing 10^{-8} mol l^{-1} epinephrine (*N*=4). Furthermore, angiotensin II did not affect the inotropic effect of high (10^{-6} to 10^{-5} mol l^{-1}) epinephrine concentrations (*N*=4) or elevated [Ca^{2+}] (*N*=3).

In situ *perfused heart*

ANG II (10^{-10} to 10^{-6} mol l^{-1}) had no effect on intrinsic heart rate, stroke volume or cardiac output in the *in situ* perfused heart (Table 2). Similarly, ANG II did not affect power output at any afterload (Fig. 7). There was no significant change in any variable during the three control periods.

Perfusion with epinephrine-free TBS produced increasing periods of asystole (not shown). Addition of captopril to four of these hearts resulted in the resumption of rhythmic contractions in all of them after 10–20 min. Captopril had no effect in epinephrine-replete hearts, nor did ANG II, up to 10^{-6} mol l^{-1}, appear to potentiate arrhythmias in any preparation.

Isolated erythrocytes

ANG II (10^{-10} to 10^{-5} mol l^{-1}) had no effect on hematocrit after 10, 45 and 120 min of incubation (not shown). Norepinephrine produced a dose-dependent increase in hematocrit independent of the presence of 10^{-6} mol l^{-1} ANG II (pD_2 without ANG II was 7.4±0.2 and pD_2 with ANG was 7.6±0.1; *N*=3 in both cases; Fig. 8).

Non-vascular smooth muscle

ANG II was generally ineffective, rarely a weak agonist and, in one instance, a weak antagonist in the tissues examined. ANG II produced a transient contraction in an

Table 2. *Cardiovascular variables determined at the apex of the Starling curve in the* in situ *perfused heart preparation during control (*N*=4) or angiotensin II (*N*=5) perfusion*

	Control 1	Control 2	Control 3	Angiotensin II (10^{-10} mol l^{-1})	Angiotensin II (10^{-8} mol l^{-1})	Angiotensin II (10^{-6} mol l^{-1})
Heart rate (beats min^{-1})	63.3±1.9	64.8±3.2	64.5±3.9	64.1±1.6	65.0±2.9	65.2±0.8
Preload (mmHg)	0.3±0.1	0.3±0.2	0.2±0.3	0.3±0.1	0.1±0.1	0.2±0.1
Afterload (mmHg)	39.2±0.4	39.2±0.5	39.2±0.5	39.9±0.2	40.0±0.2	40.0±0.3
Stroke volume (ml kg^{-1} body mass)	0.76±0.04	0.74±0.03	0.72±0.02	0.70±0.02	0.70±0.01	0.68±0.01
Cardiac output (ml min^{-1} kg^{-1} body mass)	48.0±3.1	47.8±3.2	46.4±3.5	46.4±0.8	47.3±0.8	46.7±0.7

Values are mean ± S.E.M.

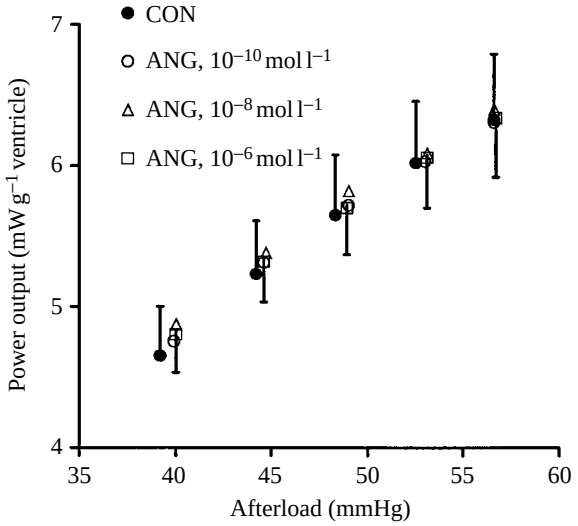

Fig. 7. Effect of afterload on power output in *in situ* trout hearts perfused without (CON; *N*=4) or with 10^{-10}, 10^{-8} or 10^{-6} mol l^{-1} angiotensin II (ANG; *N*=5). There was no difference in power output during the three control periods; only the first period is shown. Standard errors, shown only for CON and 10^{-6} mol l^{-1} ANG, were similar in all four groups.

otherwise unstimulated longitudinal stomach strip (475 mg; *N*=1), and transient and weak (50–75 mg) constrictions in one out of five, and one out of two strips of ACh-contracted longitudinal anterior and posterior intestine, respectively. In contracted urinary bladders,

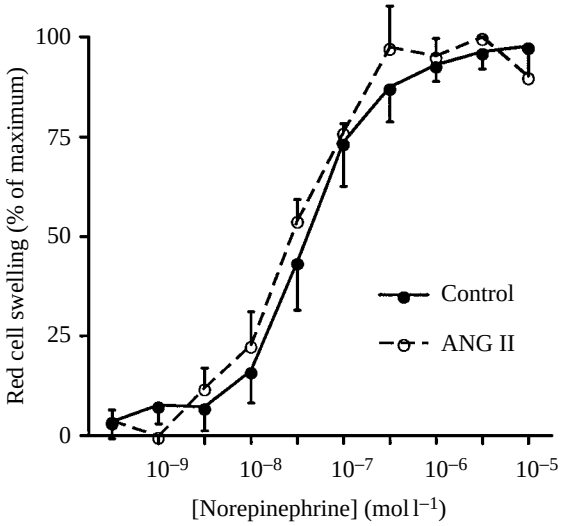

Fig. 8. Effects of graded norepinephrine concentrations on red cell swelling in the absence (Control) or presence of 10^{-6} mol l^{-1} angiotensin II (ANG II). Values are expressed as a percentage of the maximal response; mean ± S.E.M.; *N*=3 for each curve.

a weak (50 mg) relaxation was observed in one out of two circular strips and a 100 mg contraction in one out of four longitudinal strips. Uncontracted urinary bladders did not respond to ANG II. Unstimulated or ACh-contracted circular strips from esophagus, stomach, anterior and posterior intestine, and longitudinal esophageal and posterior intestinal strips did not respond to any dose of ANG II (*N*=2–6, for each tissue). ANG II was likewise ineffective in longitudinal strips of pre-contracted stomach (*N*=2), unstimulated anterior intestine (*N*=2) and gall bladder (*N*=2).

Discussion

The pattern of ANG-II-mediated vasoconstriction in trout varies as a function of the vascular segment (i.e. along the artery–microcirculation–vein) and the target organ. These variations appear to be neither random nor non-specific and they provide important information regarding the significance of ANG II action on vascular resistance. Other components, such as the degree of involvement of sympathetic activation, the action of ANG II antagonists and the intraorgan conversion of ANG I to ANG II are also often location-specific and point to the mechanisms through which ANG II affects overall regulation of systemic blood pressure in fish. Comparison of these patterns between trout and mammals can also provide an interesting insight into the evolution of this anti-drop effector system.

Localization of ANG II response

The importance of the trout microcirculation in mediating ANG-II-induced increased vascular resistance becomes apparent by comparing ANG II responses in perfused tissues

with those produced in large isolated vessels. Previous studies on trout (Lipke and Olson, 1990; Conklin and Olson, 1994*a*,*b*) have shown that otherwise unstimulated large arteries and most large veins are refractory to ANG II, whereas ANG II stimulation of pre-contracted vessels produces an endothelium-dependent vasodilation. Thus, large vessel responses appear to be generally incapable of increasing vascular resistance. This implies that the vasoconstrictory responses of perfused tissues to ANG II, as observed in the present study, occur in the microcirculation, i.e. the arteriole–capillary–venule segment. Because only the microcirculatory responses are consistent with the *in vivo* pressor effects of ANG II, the microcirculation appears to be the focal point of ANG-II-mediated increases in systemic vascular resistance. Furthermore, since ANG II does not directly affect cardiac performance or blood viscosity (i.e. red cell swelling), it is quite likely that the major, if not sole, systemic effector of angiotensin-mediated blood pressure regulation is the microcirculation.

There is an interesting dichotomy in trout in the order of potency of vasoconstrictor hormones in large *versus* small vessels. While angiotensin vasoconstrictor activity seems to be confined to the microcirculation, large vessels are considerably more sensitive than perfused tissues to catecholamines. Isolated rings of celiacomesenteric and efferent branchial arteries and anterior cardinal veins exhibit 10- to 100-fold greater sensitivity to either epinephrine or norepinephrine than either the perfused dorsal aorta or perfused celiacomesenteric artery preparation (Xu and Olson, 1993*a*). These differences may be related to the primary pathway (neuronal or humoral) of vascular activation. ANG II, a humoral vasoconstrictor, stimulates the microcirculation directly, whereas much of the adrenergic regulation of trout systemic vascular resistance has a neuronal origin (Bushnell *et al.* 1982). Under resting conditions, circulating catecholamines may support neuronal tone (Xu and Olson, 1993*a*) and may selectively affect large vessel tone without significantly altering total (i.e. microcirculatory) resistance.

Intravascular (large artery–small vessel–large vein) differences in the potency of various vasoactive hormones have also been observed in mammals (Grega and Adamski, 1987; Minami and Toda, 1988; Vicaut and Hou, 1993). For instance, the primary site of ANG II effects in the dog is the microcirculation, as it is in trout (present study). However, norepinephrine preferentially constricts both small vessels and veins but is less effective in large arteries in the dog (Grega and Adamski, 1987), a response virtually opposite to that of trout (Xu and Olson, 1993*a*). The physiological significance of these differences remains to be determined, although an interesting possibility is that the differences reflect, on an evolutionary time scale, the relative degree of direct and/or indirect dependence on the RAS and sympathetic nervous systems in blood pressure regulation.

Fish appear to place greater dependence on the RAS for tonic control of blood pressure than do mammals. ACE inhibitors readily lower dorsal aortic pressure in most fish, yet they seem to have little, if any, depressor effect on salt-replete mammals. It is well recognized that an important component of ANG-II-mediated vasoconstriction in fish is achieved through activation of the peripheral sympathetic nervous system (Nishimura, 1985; Olson, 1992; Wilson, 1984*a*,*b*). In fact, Wilson (1984*b*) concluded that ‘it is unlikely that ANG II exerts an important, direct vasoconstrictor action in fish’. The

results of the present experiments indicate that ANG II can exert a direct vasoconstrictor action and that the extent of direct *versus* indirect stimulation of the vasculature is organ-specific. Adrenoceptor inhibition reduced the ANG II vasoconstrictor response in the perfused dorsal aorta preparation by around 35 %, whereas the response of the perfused celiacomesenteric circuit to ANG II was unaffected (Fig. 4). Interestingly, the perfused celiacomesenteric artery preparation is more sensitive than the perfused dorsal aorta to the direct vasoconstrictory actions of both epinephrine and norepinephrine (Xu and Olson, 1993*a*). The relative importance of vasoconstriction mediated by direct ANG II stimulation of vascular smooth muscle *versus* indirect (*via* catecholaminergic nerves) stimulation on blood pressure regulation is not clear. Both adrenoceptor blockade (Xu and Olson, 1993*b*) and ACE inhibitors (Lipke and Olson, 1990) produce nearly the same degree of hypotension, supporting the conclusions of Wilson (1984*b*) that nearly all of the tonic pressor action of ANG II *in vivo* is mediated through sympathetic neural involvement. However, these findings also suggest that adrenergic control of vascular resistance is ineffective without a background of ANG II. Additional *in vivo* studies with combinations of adrenoceptor and RAS blockade, as well as measurements of plasma ANG II concentrations in trout, will be needed to resolve this issue.

Tissue specificity

Branchial resistance is a determinant of systemic arterial pressure in fish because branchial and systemic circulations are in series. Thus, systemic arterial pressure may be increased by increased systemic resistance, by decreased branchial resistance or by a combination of the two. Because ANG II increases vascular resistance in the perfused gill (Fig. 5) as well as in the trunk (Fig. 2), it is apparent that RAS regulation of dorsal aortic pressure is mediated through systemic vasoconstriction and does not have a branchial component. Furthermore, because the gill vasculature is less sensitive than the systemic circulation to the vasoconstrictory effects of ANG II, it is unlikely that branchial constriction interferes with the systemic pressor effects of the peptide. The slight vasoconstriction frequently observed in gills perfused with 10^{-9} mol l^{-1} ANG II may be indicative of a minor component of the branchial circulation that is more sensitive to the peptide. Whether this can be localized to a specific branchial vascular pathway, such as the non-respiratory circulation, remains to be determined.

Angiotensin converting enzyme

Similarities in the ANG I and ANG II pressure responses and pD_2 values when these peptides are perfused into the dorsal aorta indicate that there is virtually a stoichiometric conversion of the decapeptide into an active metabolite during a single transit through the vasculature. Because ANG II only increases resistance in the microcirculation, the activation process must occur either in the larger (proximal) arteries or directly at the site of vasoconstriction. Local ACE in the rat cremaster microcirculation can account for nearly 100 % conversion of ANG I to ANG II (Vicaut and Hou, 1993) and it is probably equally effective in the trout. It is not known whether the active metabolite produced in trout is ANG II or one of several truncated forms, i.e. ANG III or ANG-(1-7), also known to be vasoactive in various vertebrates (Khosla *et al.* 1983; Osei *et al.* 1993). However,

the ability of captopril to inhibit over 90 % of the ANG I response indicates that ACE is an essential step in the activation process and suggests, but does not prove, that ANG II, or one of its products, is the active metabolite produced. This also suggests that other enzymatic pathways for generating ANG II from ANG I, such as those catalyzed by neutral endopeptidase 24.11, prolyl endopeptidase 21.26 (Welches *et al.* 1993) or chymase (Urata *et al.* 1993), have little role in ANG II formation in the peripheral vasculature of trout.

The perfused dorsal aorta preparation does not permit separation of renal and skeletal muscle vascular resistances, nor is it possible to estimate the relative contribution of each to the total. However, it is doubtful if resistance in one of these parallel pathways is sufficiently below that in the other to account for all of the pressure changes observed. Therefore, conversion of ANG I to ANG II must occur simultaneously in both renal and skeletal muscle vessels. We have previously shown that ACE is present in renal tissue, but this enzyme was not detected in skeletal muscle homogenates (Lipke and Olson, 1988), perhaps because of the sparse vascularization of the latter (Duff *et al.* 1987). The present experiments suggest that ACE activity in skeletal muscle vessels is also an efficient process. Angiotensin activation is not as efficient in the perfused splanchnic circulation; in fact, the pD_2 values for ANG I and ANG II in this preparation suggest that the conversion efficiency is only around 10 %. Whether these differences are due to differences in local (tissue) RAS or reflect variations in remote (systemic) RAS control processes remains to be determined.

Tachyphylaxis

Tachyphylaxis to ANG II was evident in cumulative ANG II dose–response curves and following repeated exposure to a single ANG II concentration in the perfused trunk (Fig. 1). This tachyphylaxis probably somewhat biased the ANG II (and ANG I) dose–response curves to the left by reducing the magnitude of the response at the highest ANG II concentrations. However, this was unavoidable because the long duration of the tachyphylactic period (20–120 min) precluded washout of all ANG II effects between doses. Because epinephrine was not tachyphylactic, is is unlikely that ANG II tachyphylaxis was due to an inability of the contractile mechanism to sustain tension.

Initial exposure of the perfused dorsal aorta preparation to 10^{-7} mol l^{-1} ANG II produced a strong transient vasoconstriction followed by a sustained constriction of lesser intensity (Fig. 1). The transient constriction was not observed during subsequent ANG II treatments, whereas the sustained constriction remained, albeit with lower intensity, throughout four or more consecutive treatments. The biphasic response could be due to (1) rapid vasoconstriction and a delayed vasodilation superimposed on the constriction, (2) summation of direct ANG-II-mediated vasoconstriction with ANG-II-stimulated release of an exhaustible supply of endogenous vasoconstrictor(s) from the microcirculation, or (3) distinct ANG II constrictor receptors or second-messenger systems, one of which rapidly develops tachyphylaxis. The first two possibilities seem less likely, although they cannot be discounted at present. First, the two most likely endogenous vasoconstrictor candidates, catecholamines and endothelins, do not appear to be involved; neither phentolamine nor bretylium prevented the transient response (not

shown), even though they reduced the magnitude of the response (Fig. 4). Endothelin-mediated contractions in trout vessels typically have a much slower rate of onset (Olson and Villa, 1991). Second, although constriction and endothelium-dependent dilation may be concomitant events in mammalian arteries (Scheuer and Perrone, 1993), the perfused dorsal aorta preparation does not exhibit significant dilatory responses to ANG II when precontracted with other agonists (Fig. 1D). It is more likely that the biphasic response involves two populations of ANG II receptors or multiple intracellular second-messenger signals with different activation and processing rates, as reported in mammals (Griendling *et al.* 1988). The present experiments do not permit differentiation between the latter two possibilities, although ANG-II-mediated contraction/relaxation of precontracted trout arteries does appear to involve separate ANG II receptor populations (Conklin and Olson, 1994*b*). The fact that tachyphylaxis was not observed *in vivo* suggests that only one of these populations (or mechanisms of action) is predominant in the intact fish.

ANG II receptors

The ineffectiveness of classic mammalian peptide-substituted angiotensin receptor antagonists in fish has been known for some 15 years (Nishimura *et al.* 1978; summarized by Olson, 1992). It has recently been found that the latest generation of non-peptide angiotensin antagonists, those that have been shown to be highly selective against angiotensin receptor subtypes in mammals (Timmermans *et al.* 1993), are also unable to block ANG-II-induced relaxation of large arteries and veins in trout (Conklin and Olson, 1994*b*). The results of the present study support the contention that there are significant differences between most, if not all, angiotensin receptors in these two classes of vertebrates. This is somewhat surprising, because agonist potency of the angiotensins themselves is remarkably well conserved across species (Khosla *et al.* 1983). Thus, evolutionary modifications in the angiotensin receptor may have occurred at the inhibitory, but not the activator, site.

Cardiac effects

The refractory nature of the trout heart to ANG II is quite surprising in view of the number and variety of effects and effectors of ANG II action in the mammalian myocardium. Angiotensin has a number of effects on the mammalian heart, including positive inotropism and chronotropism, facilitation of cardiac sympathetic nerve activity, stimulation of myocardial growth and coronary vasoconstriction (Dostal and Baker, 1993; Grinstead and Young, 1992; Timmermans *et al.* 1993). Cardiac activation in mammals may occur through local and systemic RAS activity (Dostal and Baker, 1993; Phillips *et al.* 1993). Clearly, ANG II does not affect trout myocardial contractility. The lack of ANG II responses in the *in situ* perfused heart (Table 2) suggests that it probably has little direct effect on chronotropic mechanisms as well.

The mammalian RAS has also been implicated in the development of reperfusion arrhythmias, and inhibition of intracardiac ANG II formation reduces the duration of reperfusion arrhythmias regardless of any change in coronary resistance (Fleetwood *et al.* 1991). The ability of captopril to restore cardiac rhythm in trout hearts perfused with low

levels of epinephrine is difficult to explain in view of the general lack of any other chronotropic effects in this tissue. However, it has been suggested that captopril scavenges free radicals in ischemic dog hearts (Westlin and Mullane, 1988), and it is possible that it performs a similar function on the trout.

Erythrocytes

The present study shows that ANG II does not directly affect red cell volume and thus that it probably does not have short-term effects on blood viscosity. An indirect ANG II effect on blood viscosity, through a cortisol-stimulated increase in erythrocyte surface β-adrenoceptors (Reid and Perry, 1991), is also unlikely because (1) ANG II appears to play only a minor role in maintaining adrenocortical function in freshwater fish (Kenyon *et al.* 1985); and (2) cortisol effects on red cell swelling are only evident during hypoxia (Reid and Perry, 1991). Whether ANG II contributes to other slow-onset, stress-related effects on the erythrocyte remains to be determined.

Non-vascular smooth muscle

The general ineffectiveness of ANG II on trout gut is consistent with the reported absence of an ANG II effect in the gastrointestinal tract of dogfish shark *Squalus acanthias* (Opdyke and Holcombe, 1978) and carp *Cyprinus carpio* (Kitazawa *et al.* 1990). However, unlike the response in carp, where no ANG II responses were observed (Kitazawa *et al.* 1990), the presence of an occasional ANG-II-stimulated contraction in trout suggests that ANG II receptors are present. However, they probably exert little, if any, direct physiological control in trout. The lack of any significant ANG II effect in fish gut smooth muscle is strikingly different from observations on the mammalian gut, where ANG II, acting predominantly through AT_1-type receptors, is both directly and indirectly a potent stimulant of smooth muscle contractions (Schinke *et al.* 1991; Yang *et al.* 1993). It remains to be determined whether the lack of ANG II potency in fish gut smooth muscle is due to the relative unimportance of the RAS, or whether other key variables or cofactors were not present under the experimental conditions employed. A background of intermediary factors, such as prostaglandins or catecholamines, might be necessary, as has been shown to be the case for catecholamines during the sympathetic-nerve-mediated vasoconstrictor response in the perfused splanchnic circulation of trout (Xu and Olson, 1993*a*). Alternatively, RAS stimulation may only be required to accommodate drinking responses in saltwater-adapted fish, a possibility that needs further investigation. Similar arguments can be made regarding the effects of ANG II on urinary and gall bladders. However, until additional information is provided to the contrary, it must be assumed that the ANG II has virtually no direct effect on trout gut, gall bladder or urinary bladder smooth muscle.

Previous studies (Carroll, 1981; Opdyke and Holcombe, 1978; Opdyke *et al.* 1982) have shown that ANG II does not have a direct action on vascular or non-vascular smooth muscle in the elasmobranch *Squalus acanthias*. The present study shows that, whereas ANG II has potent effects on specific vascular smooth muscles in trout, cardiac and non-vascular smooth muscle are not directly influenced by this peptide. From an evolutionary perspective, and assuming there has been some phylogenetic progression from fish to

mammals, it appears that the initial role of the RAS as a direct stimulant of systemic non-skeletal muscle effectors was focused on the vasculature. Specifically, the primary function of the RAS was as an anti-drop regulator of blood pressure and this was achieved through the constrictory actions of ANG II on the microcirculation. Only through later evolutionary embellishment was this system integrated into the variety of cardiac and non-vascular smooth muscle control systems found in present-day mammals. Both the structure of the primary peptide signal and cross-species ANG II/effector interactions have been well preserved from teleosts to mammals (Nishimura, 1985; Olson, 1992; Wilson, 1984*b*). However, the functional attributes of the RAS have been greatly elaborated over time, while the inhibitory sites of the mammalian ANG II receptors appear to have little similarity to their antecedents.

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