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Effects of chronic dietary salt loading on the renin angiotensin and adrenergic systems of rainbow trout (*Oncorhynchus mykiss*)

Steve F. Perry,[1] Kate Ellis,[1] Jordan Russell,[1] Nicholas J. Bernier,[2] and Colin Montpetit[1]

[1]*Department of Biology, University of Ottawa, Ottawa, Ontario; and* [2]*Department of Integrative Biology, University of Guelph, Guelph, Ontario, Canada*

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Perry SF, Ellis K, Russell J, Bernier NJ, Montpetit C. Effects of chronic dietary salt loading on the renin angiotensin and adrenergic systems of rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol Regul Integr Comp Physiol* 301: R811–R821, 2011. First published June 22, 2011; doi:10.1152/ajpregu.00244.2011.—Previous studies have demonstrated that chronic dietary salt loading causes hypertension and a decreased sensitivity of the systemic vasculature to α-adrenergic stimulation and other hypertensive stimuli (e.g. hypercapnia) in rainbow trout (*Oncorhynchus mykiss*). This reduced sensitivity to hypertensive stimuli is consistent with a possible blunting of homeostatic responses normally aimed at raising blood pressure. To test this idea, we examined the consequences of long-term salt feeding and the associated hypertension on the interactive capacities of the renin angiotensin system (RAS) and adrenergic systems to elevate blood pressure in trout. Secretion of catecholamines in response to a range of doses of homologous ANG II in vivo and in situ (using a perfused posterior cardinal vein preparation) was reduced in the salt-fed fish. The reduced sensitivity to ANG II could not be explained by alterations in stored catecholamine (adrenaline or noradrenaline) levels or the general responsiveness of the chromaffin cells to depolarizing stimuli (60 mmol/l KCl). Despite the decreased responsiveness of the chromaffin cells to ANG II, plasma catecholamines were increased to a greater extent in the salt-fed fish during acute hypoxia (a condition that activates the RAS). Interestingly, the pressor effects of ANG II in vivo were actually heightened in the salt-fed fish. The increased pressor response to exogenous ANG II was likely attributable to its direct interaction with vascular ANG II receptors because the effect persisted even after blockade of α-adrenergic receptors. Treating fish with the vascular smooth muscle relaxant papaverine caused similar reductions in blood pressure and increases in plasma ANG II levels regardless of diet. Similarly, inhibition of angiotensin converting enzyme with lisinopril reduced blood pressure equally in control and salt-fed fish. These results indicate that, while long-term dietary salt loading blunts the response of trout chromaffin cells to ANG II, the RAS itself appears to be unaffected. Indeed, the capacity of ANG II to elevate blood pressure is not compromised nor do fish exhibit a reduced capacity to mount an acute humoral adrenergic stress response during acute hypoxia.

high dietary salt; hypertension; catecholamines; chromaffin cells; angiotensin II; hypoxia; control of blood pressure

ALTHOUGH EPIDEMIOLOGICAL studies (21) have revealed a significant correlation between dietary salt intake and blood pressure in humans, the underlying mechanisms have not been clearly identified (for reviews see Refs. 6 and 30). In most individuals, the vascular volume expansion associated with moderately increased levels of ingested salt is restored rapidly by increased urine production, and thus the related increases in blood pressure typically are transient. Certain humans, however, exhibit heightened sensitivity to salt, including a significant proportion (~30%) of persons with essential hypertension (an elevation of blood pressure of unknown origin) (67). In addition to an impaired capacity of the kidney to excrete the added salt, other factors implicated in promoting essential hypertension include a salt-mediated increase in sympathoadrenal activity (41, 65), stimulation of the renin angiotensin system (RAS) (13, 20), and impairment of vascular endothelial function (7).

Typically, experimental studies of essential hypertension utilize animal models derived from genetically modified inbred strains of rodent (12) or exploit increased dietary salt supplements in animals experiencing compromised renal function (e.g., via unilateral nephrectomy). However, in freshwater (FW) rainbow trout (*Oncorhynchus mykiss*), dietary salt loading [e.g., (59)] is capable of inducing chronic hypertension in fish experiencing otherwise normal kidney function (10, 14, 39). The increased blood pressure in salt-fed trout reflects increased cardiac output (10) associated with increased extracellular and vascular fluid volumes (39) that persist despite increasing glomerular filtration rate and urine production (59). Although elevated systemic vascular resistance (of yet unknown origin) is the principal mediator of chronic essential hypertension in humans (6), it appears to be less significant in salt-mediated hypertension in FW trout (10).

Given that chronic hypertension can be readily facilitated by simple addition of dietary salt in otherwise normal fish, the FW trout is an attractive alternate vertebrate model to assess the long-term consequences of elevated blood pressure on physiological function. Using this approach, Chen et al. (10) demonstrated that rainbow trout experiencing sustained increases in blood pressure exhibited blunted pressor responses to exogenous catecholamines or hypercapnia. The latter condition is known to elevate blood pressure owing to the combined effects of increased circulating catecholamine levels and sympathetic nerve activity (47). Thus, one known consequence of chronically elevated blood pressure in trout is a reduced sensitivity of systemic α-adrenergic receptors to catecholamines of humoral or neural origin. This result is consistent with the notion that chronically raised blood pressure elicits responses aimed at minimizing further increases in pressure. While numerous neurohumoral systems contribute to blood pressure regulation in fish (22, 25, 26, 33, 37), two systems that are believed to play particularly significant roles are the RAS (42, 64) and the adrenergic systems [encompassing the net effects of catecholamines of humoral and neural origin (3, 52)]. In addition to the individual effects of each system on blood pressure, there is also marked interplay between the RAS and adrenergic systems. For example ANG II is a potent activator of catechol-

Address for reprint requests and other correspondence: S. F. Perry, Dept. of Biology, Univ. of Ottawa, 30 Marie Curie, Ottawa, Ontario, K1N 6N5, Canada (e-mail: sfperry@uottawa.ca).

amine secretion from piscine chromaffin tissue (4, 8, 40) and, if similar to the situation in mammals, sympathetic neurotransmission is likely enhanced by ANG II (29). Thus, the restoration of blood pressure associated with the recruitment of the RAS during hypotension is the result of the combined and interactive actions of ANG II and catecholamines (2) with the relative importance of each varying within those few species that have been examined (3). However, in certain models of hypertension, including spontaneously hypertensive rats (SHR), the RAS is inappropriately and permanently altered both centrally and peripherally to promote an elevation of blood pressure (13, 20, 30). For example, the elevation of blood pressure in SHR is caused, at least in part, by increased activity of the central (brain) RAS leading to increasing sympathetic neuronal activity and a blunting of the barostatic reflex (66). In rainbow trout, central administration of ANG II leads to an increase in blood pressure that, unlike when delivered peripherally, causes tachycardia (27) rather than reflex bradycardia (25).

With this background, the present study was designed to examine the consequences of long-term salt feeding and the associated hypertension on the interactive capacities of the RAS and adrenergic systems to elevate blood pressure in FW rainbow trout. The central hypothesis was that the capacity of the RAS to stimulate catecholamine secretion would be diminished in hypertensive trout, and when coupled with a presumed downregulation of the RAS and its downstream effector sites, this would result in a blunting of the pressor effects of exogenous ANG II and a reduced ability of trout to restore blood pressure following acute hypotension.

MATERIALS AND METHODS

Experimental Animals

Two hundred rainbow trout (*O. mykiss*), including both sexes, were obtained from the Linwood Acres Trout Farm (Campellcroft, ON, Canada) and transported to the University of Ottawa Aquatic Care Facility. Upon arrival, the trout were divided into control and experimental groups of 100 fish each and housed separately in 1,300-liter fiberglass tanks. The tanks were supplied continuously with well-aerated, dechloraminated, City of Ottawa tap water at 13°C and were subjected to a constant 12:12-h light-dark photoperiod. Prior to beginning experimentation, 20 fish from each tank were chosen at random and weighed to provide an estimate of the average weight of the fish in each tank. The average weight of the control fish was 343 g, while the experimental fish weighed an average of 370 g. These estimates were later used to calculate the amount of food that would be provided to each group of fish through the duration of the experiment, with each fish receiving ~1% of its body weight at each feeding. Prior to beginning the different diets, all fish were fed commercial trout pellets five times per week. All experiments were approved by the University of Ottawa Animal Care Protocol Review Committee (protocol BL-226) and conformed to the guidelines established by the Canadian Council on Animal Care for the use of animals in research.

Normal and High-Salt Diets

The feeding regimen was based on a previous study (50) that in turn was adopted from a procedure described by Salman and Eddy (59). The control fish were fed 1% of their average body weight daily for a period of 4 wk with commercial trout pellets composed of 41% crude protein, 11% crude fat, 3.5% crude fiber, 1% calcium, 0.85% phosphorus, 0.45% sodium, 6,800 IU/kg vitamin A, 2,100 IU/kg vitamin D, 80 IU/kg vitamin E, and 200 IU/kg vitamin C. Similarly, the experimental fish were fed 1.1% of their average body weight per day for the same period with a reconstituted diet containing 11% (by weight) NaCl to induce hypertension (50). After 4 wk of daily feeding, the biomass in each tank had increased to such an extent that the oxygen content of the water could not be maintained at safe levels. Thus, the fish were fed on alternate days to avoid excessive growth while maintaining the hypertension that had been established during the previous month. Experimentation commenced once the initial 4 wk of daily feeding was complete, because this time frame was previously demonstrated to be effective at eliciting hypertension (50).

Surgical and Experimental Procedures

In vivo experiments. Surgical procedures were carried out according to Bernier and Perry (5). Fish were anesthetized by immersion in an oxygenated solution of 10 mg/ml benzocaine (ethyl-*p*-aminobenzoate; Sigma-Aldrich). Upon cessation of voluntary swimming movements (but before breathing had stopped), the trout were removed from anesthetic, weighed, and transferred to a surgical table where the gills were irrigated continuously with the same anesthetic solution for the duration of the surgery. To permit the measurement of dorsal aortic blood pressure (P_{DA}) and blood sampling, and to permit injections of drugs (in the event of venous cannula failure), an indwelling polyethylene cannula (PE-50; Clay-Adams) was implanted into the dorsal aorta via percutaneous puncture (61). The cannula was flushed with chilled heparinized (50 U/ml ammonium heparin) Cortland saline (69) and sealed with a needle.

The caudal vein was similarly cannulated with PE-50 tubing and served as an injection site throughout each experiment. To expose the vein, a lateral incision was made in the caudal peduncle 1 cm below the lateral line, and the underlying tissue was separated by using a cotton swab. The cannula was inserted directly into the vein in the anterograde direction, flushed with chilled heparinized Cortland saline, and sealed. The incision was closed using a running stitch, and the protruding cannula was anchored to the skin by using silk sutures.

The final surgical procedure involved the placement of a 3S ultrasonic flow probe (Transonic Systems) around the bulbus arteriosus to measure cardiac output (V_b). By using a ventral midline incision, the pericardial cavity was exposed, and the pericardium was gently dissected to expose the bulbus. The probe was coated with KY lubricating jelly (acting as an acoustic coupling agent) and secured around the bulbus. The incision was closed by using a running stitch, and the probe lead was anchored to the ventral surface of the trout. Fish were revived on the surgical table by switching to normal water; once opercular movements became frequent and regular, the fish were transferred into transparent holding chambers, which were themselves placed in individual opaque boxes. The fish were supplied with 13°C, flowing, aerated water and allowed to recover in darkness for 24 h prior to experimentation.

After the 24-h recovery period, the dorsal aortic cannula was flushed with heparinized saline and connected to a pressure transducer (model 1050BP; UFI, Morro Bay, CA), calibrated at the outset of each experiment against a static column of water, and used to measure P_{DA} (10). The flow probe was connected to a small animal blood flow meter (model T106; Transonic Systems) to measure cardiac output. The probes used in these experiments were precalibrated with human blood at 13°C prior to delivery. Dorsal aortic blood pressure and V_b analog signals were converted (50 samples/s) to digital data by using a data acquisition system (BioPac Systems, Santa Barbara, CA) and stored using AcqKnowledge data acquisition software (10). Systemic resistance (R_S) was calculated as mean P_{DA} [(systolic + diastolic pressure)/2] divided by V_b (i.e., $R_S = P_{DA}/V_b$), heart rate (f_H) was derived from the pulsatile blood flow trace and stroke volume (V_S) was calculated as V_b divided by f_H (i.e., $V_S = V_b/f_H$). The f_H and V_b data are not presented in this paper.

In situ experiments. The fish were killed by a sharp blow to the head, weighed, and placed on ice. A ventral incision was made from the anus to the pectoral girdle, and the tissues overlying the heart were removed by blunt dissection to expose the ventricle and the bulbus arteriosus. An inflow cannula (PE-160; Clay-Adams) was inserted into the posterior cardinal vein (PCV), and an outflow cannula (PE-160) was inserted into the ventricle through the bulbus arteriosus. Prior to beginning the experiments, the preparations were perfused for 20 min with modified aerated Cortland saline (46) (in mmol/l: 125 NaCl, 2.0 KCl, 2.0 $MgSO_4$, 5.0 $NaHCO_3$, 7.5 glucose, 2.0 $CaCl_2$, and 1.25 KH_2PO_4, final pH 7.8) to allow catecholamine levels to stabilize. Perfusion was accomplished using positive pressure differences between the surface of the saline and the outflow cannula, resulting in a relatively constant flow (~1.0 ml/min).

Experimental Protocols

Series 1: effects of ANG II on plasma catecholamines in vivo and catecholamine secretion in situ. In vivo experiments were conducted on fishes fitted only with dorsal aortic cannulae. After removal of a blood sample (0.3 ml) to assess basal plasma catecholamine levels, fish were given three successive bolus injections (0.3 ml) of 100, 300, and 500 pmol/kg of homologous [Asn^1-Val^5]-ANG II (11), each delivered over a period of 30 s; each injection was followed by 0.2 ml of saline to clear the cannula. Because a previous study demonstrated that peak catecholamine levels were achieved 2 min after injection (5), a single blood sample per dose of ANG II was withdrawn at 2 min postinjection. Each blood sample was replaced by an equivalent volume of saline, collected in a 1.5-ml microcentrifuge tube, and immediately centrifuged at 10,000 *g* for 15 s. The plasma was frozen in liquid nitrogen and stored at −80°C for later analysis of catecholamines. The injections were separated by 30 min, which is sufficient time for catecholamine levels and cardiovascular parameters to return to baseline (5).

For In situ experiments, following a 20-min stabilization period (see above), a single sample was collected in a preweighed microcentrifuge tube to assess basal catecholamine secretion rates prior to any experimental procedure. While perfusion was continued, the preparation was given two successive bolus injections (0.3 ml via a 3-way valve attached to the inflow cannula) of [Asn^1,$Va1^5$]-ANG II ranging from $l0^{-12}$ to 10^{-7} mol/kg body wt, allowing dose-response relationships to be established (4). To avoid receptor desensitization potentially resulting from multiple doses of ANG II being delivered to a single preparation, each preparation was injected with two doses only of ANG II (the higher of the 2 doses always was the final injection). Outflow samples were collected over 1-min intervals for a 5-min period following injection. Preliminary experiments and previous studies (4) demonstrated that maximal rates of catecholamine secretion are achieved within 3 min of injection. The preparations were allowed to recover for 20 min before the higher dose of ANG II was injected. All samples were frozen immediately in liquid nitrogen and kept frozen at −80°C until analysis of catecholamine content.

To determine whether the responsiveness of the chromaffin cells to nonspecific depolarizing stimulation was altered by salt feeding, in situ preparations derived from control or salt-fed fish were administered bolus injections of 60 mmol/l KCl (55) to depolarize cell membranes. Outflowing perfusate samples were collected for the ensuing 3 min to determine the maximum rates of KCl-evoked catecholamine secretion. In a separate experiment, fish from either diet regimen were euthanized (overdose with benzocaine), the PCV was removed, divided into thirds (anterior, medial, and posterior PCV) and placed into preweighed 1.5 ml microcentrifuge tubes. Once the wet tissue weight was determined, 1 ml of 4% perchloric acid containing 2 mg/ml of EDTA, and 0.5 mg/ml of sodium bisulfate was added to each tube, and the samples were homogenized. The supernatants were then diluted 100 times in the same perchloric acid solution and stored at −80°C until subsequent analysis of stored catecholamine levels (see below).

Series 2: effects of acute hypoxia on plasma catecholamine and ANG II levels. Fish used in these experiments were fitted with a dorsal aortic cannula only. Acute hypoxia was achieved by replacing the air supplying a water/gas equilibration column with N_2. The desired water P_{O_2} (40–45 mmHg) was preset and established by adjusting the rate of water and/or N_2 flow through the column. This level of hypoxia was chosen on the basis of previous studies (24, 49) that demonstrated significant catecholamine release and ANG II mobilization in rainbow trout by using this protocol. Blood samples (0.3 ml) were withdrawn during the normoxic period and 10 min after reaching the targeted 40–45 mmHg P_{O_2}. Blood samples were immediately transferred to microcentrifuge tubes containing 10 μl of EDTA and centrifuged (10,000 *g* for 1 min); the plasma layer was removed and stored at −80°C (24). The water P_{O_2} within the experimental box was monitored continuously by using a peristaltic pump (flow rate 0.6 ml/min) that withdrew water from each individual trout box and passed it across a P_{O_2} electrode (Cameron Instruments) connected to an O_2 meter (Cameron Instruments). Generally, the desired P_{O_2} in the experimental box was reached within 10 min and thereafter never varied more than ± 5 mmHg. After experimentation, water was restored to normoxic levels and the fish were allowed to recover.

Series 3: effects of ANG II with and without prior α-adrenergic receptor blockade on cardiovascular function in vivo. After calibrating the pressure transducer and cardiac flow probe and verifying the patency of each cannula, the fish were left to stabilize for at least 30 min or until stable baseline recordings of P_{DA}, V_b, f_H, and R_S were obtained. After recording baseline cardiovascular variables, a blood sample (0.3 ml) was taken for analysis of basal plasma ANG II levels and treated and stored as in *series 2*. Following the restabilization of cardiovascular parameters (if the fish was disturbed during blood sampling), saline was injected (1 ml/kg) over a period of 30 s to assess the effects of injection, itself on cardiovascular variables. Consecutive injections (1 ml/kg) of ANG II were administered over 30 s at doses of 100 and 500 pmol/kg. These doses of ANG II were reported by Bernier et al. (3) to elicit dose-dependent increases in the circulating levels of adrenaline in rainbow trout. The net responses to ANG II injections in trout are therefore assumed to result from the combined vasopressor effects of ANG II and catecholamines of humoral (5) and neural origin (3, 34, 36). The fish were allowed to recover (30–60 min) from the transient increases in blood pressure accompanying each injection of ANG II before experimentation was continued. Upon recovery from the second injection of ANG II, prazosin hydrochloride (1 ml/kg, 1 mg/kg, each) was injected over a period of 10 min (3) to block α-adrenergic receptors, and after 60 min the 500 pmol/kg dose of ANG II was repeated. All injections were administered via the caudal vein cannula, except when the patency of the cannula could not be confirmed by blood withdrawal. In those few instances, the dorsal aortic cannula was used as the site of injection, which meant a temporary loss of blood pressure data.

Series 4: effects of acute hypotension or angiotensin converting enzyme (ACE) inhibition on cardiovascular function in vivo. Once the stability of blood pressure had been established during an initial period of monitoring, control baseline P_{DA} was recorded for 10 min, and the control or salt-fed fish were administered an intravenous injection of the smooth muscle relaxant papaverine hydrochloride (0.4 ml/kg; RBI) at 10 mg/kg over a 10-min period (3). Each injection was followed by 0.3 ml of saline to clear the caudal vein cannula, and the effects on P_{DA} were monitored continuously over the following 30 min.

In a separate group of fish, control baseline cardiovascular parameters were recorded for 10 min, after which trout were administered an intravenous injection of the ACE inhibitor lisinopril (1 ml/kg; Sigma-Aldrich) at 10^{-4} mol/kg over a 2-min period (2). Each injection was followed by 0.3 ml of saline to clear the cannula, and P_{DA} was monitored over the following 30 min.

Analytical Techniques

ANG II RIA. Plasma ANG II concentrations were determined by RIA according to the methods of Bernier et al. (2) with modifications. Briefly, the incubation mixture for the standard curve consisted of 0.1 ml standard [Asn^1,Val^5]-ANG II ligand (Sigma), 0.1 ml antiserum raised against [Asp^1,Ile^5]-ANG II (1:7000 dilution; cat. no. T-4005; Bachem, Torrance, CA), and 0.1 ml normal rabbit serum (1:250 dilution; cat. no. 869019; Calbiochem, Gibbstown, NJ). After 20 h at 4°C, 0.05 ml ^{125}I-labeled [Asp^1,Ile^5]-ANG II (~8,000 CPM; specific activity = 2,200 Ci/mmol; PerkinElmer, Woodbridge, ON, Canada) was added to the mixture and incubated for another 24 h at 4°C. Bound antigen was precipitated with 0.1 ml pansorbin cells (0.25%; Calbiochem) for 5 h at 4°C and thereafter centrifuged at 3,200 rpm for 1 h at 5°C. Supernatants were removed, and precipitates were counted on a WIZARD2 gamma counter (Perkin Elmer). In the incubation mixture of unknown samples, the 0.1 ml of standard ligand was replaced with extracted trout plasma. All measurements were made in triplicate. The dilution curve of immunoreactive ANG II in extracted trout plasma was parallel to the standard curve of [Asn^1,Val^5]-ANG II. All samples were measured in a single assay and the intra-assay coefficient of variation was 7.2% (n = 5).

HPLC determination of plasma catecholamines. Plasma samples were alumina-extracted for catecholamines as previously described (70). Then 200 μl of extracted sample was injected via an HPLC autosampler; the HPLC consisted of a Varian ProStar 410 solvent delivery system (Varian Chromatograhy Systems, Walnut Creek, CA) connected to a Decade II electrochemical detector containing a VT-03 electrochemical flow cell (both Antec Leyden, Zoeterwoude, The Netherlands). Adrenaline and noradrenaline concentrations (in nmol/l) were calculated relative to standards of known concentration and 3,4-dihydroxybenzalamine hydrobromide was incorporated in all standards and unknowns as an internal standard.

Statistical Analysis

Data are presented as means ± 1 SE throughout this study. Time series data or experiments involving multiple injections were analyzed (SigmaStat 3.5; SPSS) by two-way, repeated-measures ANOVA (Figs. 1, 3–8) or unpaired Student's t-test (Fig. 2). The data in Table 2 did not exhibit a normal distribution and were analyzed by Mann-Whitney's rank sum test. When significant differences were identified by ANOVA, post hoc multiple comparison tests (Bonferroni t-test) were used to identify the specific data points exhibiting statistically significant differences from a single control value; in all cases the fiducial limit of significance was set at 5%.

RESULTS

Series 1: Effects of ANG II on Plasma Catecholamines in Vivo and Catecholamine Secretion in Situ

The levels of circulating catecholamines under resting conditions were in keeping with values reported previously in the literature (e.g., see Ref. 3) and did not differ between the control and salt-fed fish for either adrenaline or (2.9–5.6 nmol/l) or noradrenaline (1.2–2.9 nmol/l; Fig. 1). The injection of homologous ANG II in vivo caused significant increases in plasma noradrenaline and adrenaline levels at the doses of 300 and 500 pmol/kg (Fig. 1). However, the increases in plasma adrenaline levels were roughly 10-fold higher than the corresponding noradrenaline levels. The levels of circulating catecholamines achieved after ANG II injection were markedly lower in the fish fed a high-salt diet (Fig. 1). Similarly, the secretion of catecholamines from chromaffin cells in situ (Fig. 2) was also significantly attenuated in the PCV preparations derived from the fish previously fed a high-salt diet. As in the

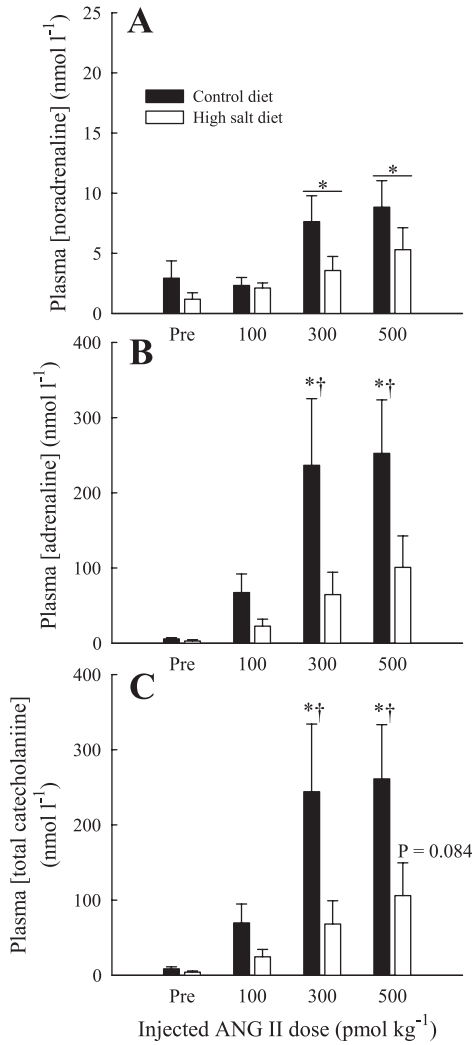

Fig. 1. In vivo effects of injections of homologous [Asn^1,Va1^5]-ANG II (100–500 pmol/kg) on the plasma concentrations of noradrenaline (*A*), adrenaline (*B*), and total catecholamines (*C*; sum of noradrenaline and adrenaline) in rainbow trout (*Oncorhynchus mykiss*) fed a control (n = 6) or a high-salt (n = 7) diet. *Significant differences ($P < 0.05$; Bonferroni multiple-comparison t-test) from the preinjection baseline values (Pre). Horizontal lines beneath an asterisk indicate a global effect of treatment (ANG II injection) with no interaction of diet (P = 0.02); two-way repeated-measures ANOVA. †Significant differences between the control and high-salt diet groups ($P < 0.05$; Bonferroni multiple-comparison t-test).

in vivo experiment, adrenaline was the predominant catecholamine secreted in response to ANG II accounting for ~80% of total catecholamines (noradrenaline plus adrenaline) released. The reduced sensitivity to ANG II exhibited by the salt-fed fish could not be explained by alterations in stored catecholamine levels within the PCV because there was no effect of diet on levels within any of three regions analyzed (Fig. 3, *A* and *B*). Similarly, the diminished capacity of the salt-fed fish to secrete catecholamines in response to ANG II was unrelated to any change in the general responsiveness of the chromaffin cells to depolarizing stimuli (60 mmol/l KCl) (Fig. 3, *C* and *D*).

Series 2: Effects of Acute Hypoxia on Plasma Catecholamine and ANG II Levels

Plasma catecholamine levels were increased in response to acute hypoxia (Fig. 4). Even though circulating ANG II con-

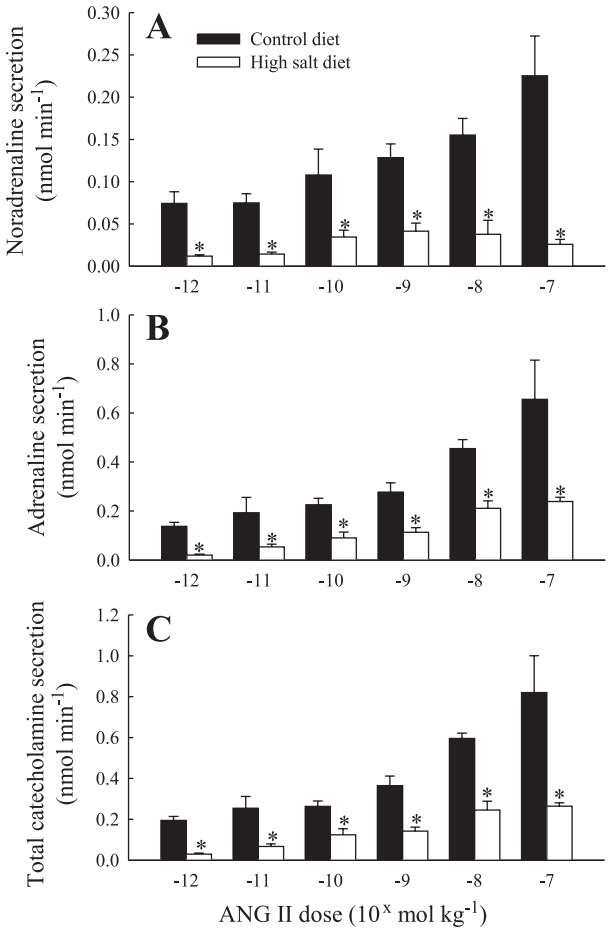

Fig. 2. Effects of bolus injections of increasing doses of homologous [Asn^1,Va1^5]-ANG II on noradrenaline (*A*), adrenaline (*B*), and total catecholamine (*C*; the sum of noradrenaline and adrenaline) secretion from a perfused posterior cardinal vein (PCV) preparation of rainbow trout (*Oncorhynchus mykiss*). The PCV preparations were derived from fish fed either a control or a high-salt diet. Each preparation was administered only 2 doses of ANGI II to achieve $n = 6$ for each dose, a total of 18 fish were used for each diet. *Significant difference in the rate of secretion between the control and salt-fed fish ($P < 0.05$; unpaired Student's *t*-test).

centrations were increased to a similar extent during hypoxia in both groups of fish (Table 1), and thus would have been expected to contribute less to catecholamine secretion in the fish fed high salt (Figs. 1–2), plasma catecholamine levels in the hypoxic fish nevertheless were markedly higher in the fish fed the high-salt diet (Fig. 4).

Series 3: Effects of ANG II With and Without Prior α-Adrenergic Receptor Blockade on Cardiovascular Function In Vivo

Baseline cardiovascular variables for untreated and prazosin-treated control and salt-fed fish are summarized in Table 2. The only variable affected by diet was P_{DA}, which was significantly increased by ~20% in the salt-fed fish. Although there was an obvious trend for prazosin to lower P_{DA} and R_S, the changes were not statistically significant when the entire data set (control plus salt-fed fish) was analyzed by two-way ANOVA. However, a simple unpaired one-tailed, *t*-test comparison of the P_{DA} data revealed a significant lowering of P_{DA} after prazosin treatment in the fish fed the control ($P < 0.001$) and salt-fed ($P = 0.032$) fish (Table 2).

The pressor effects of exogenous ANG II were significantly larger in the fish receiving the high-salt diet (Fig. 5). At the low dose used (100 pmol/kg), a significant rise in P_{DA} was observed only in the salt-fed fish (Fig. 5*A*), while at the higher dose (500 pmol/kg), the increases in P_{DA} and R_S were considerably greater in the salt-fed group (Fig. 5, *B* and *D*); while not statistically significant, there was an obvious trend for increasing R_S at the higher dose of ANG II in the control fish (Fig. 5*D*). At the higher dose of 500 pmol/kg, the maximal increases in P_{DA} elicited by ANG II were 8.1 ± 1.5 (mean ± SE) and 16.7 ± 4.2 mmHg in the control and salt-fed fish, respectively. The ANG II-mediated maximal increases in R_S were 0.16 ± 0.04 and 0.44 ± 0.11 mmHg·ml^{-1}·min^{-1}·kg^{-1} in the control and salt-fed fish, respectively. Neither dose of ANG II caused any significant differences in V_b, f_H, or V_S (data not shown).

After pretreating fish with prazosin (Fig. 6), the pressor responses to exogenous ANG II either were abolished (control diet) or attenuated (high-salt diet). In the salt-fed fish, prazosin treatment decreased ($P = 0.039$) the magnitude of the maximal increase in P_{DA} caused by ANG II injection from 16.7 ± 4.2 to 8.1 ± 1.5 mmHg. Similarly, the magnitude of the maximal increase in R_S was reduced ($P = 0.044$) from 0.44 ± 0.11 to 0.17 ± 0.04 mmHg·ml^{-1}·min^{-1}·kg^{-1}.

Series 4: Effects of Acute Hypotension or ACE Inhibition on Cardiovascular Function in Vivo

Treatment of fish with the smooth muscle relaxant papaverine caused similar transient decreases in P_{DA} and increased

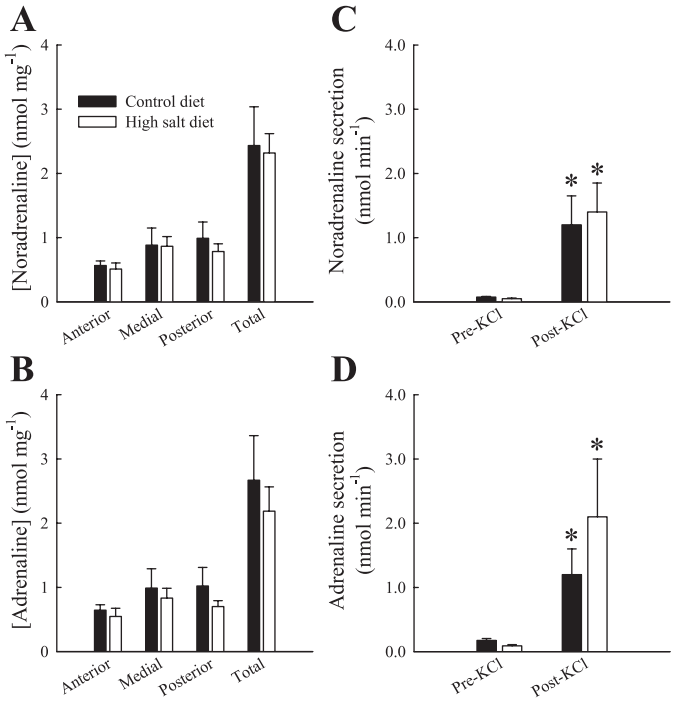

Fig. 3. *A* and *B*: storage levels of noradrenaline and adrenaline in distinct regions of PCV in rainbow trout (*Oncorhynchus mykiss*) fed a control ($n = 13$) or a high-salt diet ($n = 8$). *C* and *D*: effects of depolarizing levels of KCl (60 mmol/l) on noradrenaline and adrenaline secretion from a perfused PCV preparation of rainbow trout (*Oncorhynchus mykiss*). PCV preparations were derived from fish fed either a control ($n = 6$) or a high-salt ($n = 6$) diet. *Significant difference from the pre-KCl values ($P < 0.05$; Bonferroni *t*-test).

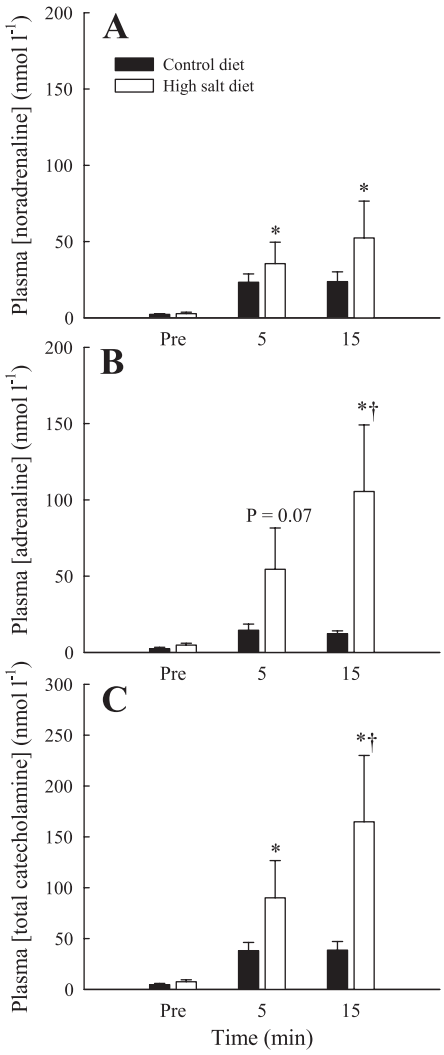

Fig. 4. Effects of acute hypoxia (water P_{O_2} = 40–45 mmHg) on plasma concentrations of noradrenaline (*A*), adrenaline (*B*), and total catecholamines (*C*; sum of noradrenaline and adrenaline) in rainbow trout (*Oncorhynchus mykiss*) fed a control (n = 8) or a high-salt (n = 6) diet. *Significant differences ($P < 0.05$; Bonferroni multiple comparison t-test) from the preinjection baseline values (Pre); †significant differences between the control and high-salt diet groups ($P < 0.05$; Bonferroni multiple-comparison t-test).

plasma ANG II levels in fish regardless of diet (Fig. 7). The R_S data were highly variable, however, and thus any statistically significant changes were obscured (Fig. 7*B*). There were no significant effects of papaverine on V_b or f_H (data not shown).

Table 1. *Effects of acute hypoxia (water P_{O_2} = 40–45 mmHg) on plasma concentrations of ANG II in rainbow trout (Oncorhynchus mykiss) fed a control or a high-salt diet*

	[Angiotensin II]	
	Control diet, n = 8	High-salt diet, n = 6
Prehypoxia	166.1 ± 51	163.5 ± 59.2
5-min hypoxia*	294.2 ± 86.7	462.7 ± 83.3
15-min hypoxia*	395.5 ± 119.9	593.9 ± 129.9

Values are means ± SE (pmol/l). Asterisks indicate a global effect of treatment (hypoxia) with no interaction of diet ($P < 0.001$); two-way repeated-measures ANOVA.

Table 2. *Effects of a salt-enriched diet and the α-adrenergic receptor antagonist prazosin on baseline cardiovascular variables in rainbow trout (O. mykiss)*

	P_{DA}	R_S
Control diet	22.2 ± 0.6 (34)	0.61 ± 0.03 (27)
High-salt diet	26.0 ± 0.9 (41)*	0.71 ± 0.06 (27)
Control diet, prazosin	15.6 ± 2.7 (5)	0.28 ± 0.03 (5)
High-salt diet, prazosin	20.2 ± 2.9 (5)	0.47 ± 0.06 (5)

Data are means ±1 SE; number per group are indicated in parentheses. P_{DA}, dorsal aortic blood pressure (mmHg); R_S, systemic vascular resistance ($mmHg \cdot ml^{-1} \cdot min^{-1} \cdot kg^{-1}$). *Significant differences from the corresponding values in the control fish (Mann-Whitney's rank sum test; $P < 0.05$).

Injection of the ACE inhibitor lisinopril caused similar decreases in P_{DA} and R_S in the control and salt-fed fish (Fig. 8) with no recovery observed during the 30-min period of monitoring. There were no significant effects of lisinopril on V_b or f_H (data not shown).

DISCUSSION

As in previous studies (10, 14, 39), feeding rainbow trout a diet supplemented with high salt (11% NaCl) caused a significant elevation of arterial blood pressure in otherwise healthy and untreated fish. Thus, salt-fed FW trout are emerging as a viable alternate model to study hypertension, especially compared with mammalian models that rely on imposed impairment of kidney function (62) or genetic modifications (60) to elicit secondary or primary hypertension, respectively. The increase in dorsal aortic blood pressure reported in the present study (17%) was intermediate between the salt-induced increases in P_{DA} reported in previous studies using similar protocols: 12% (39), 25% (14), and 37% (10). Thus, while not necessarily large, the increases in blood pressure associated with a high-salt diet are significant and repeatable. The increases in P_{DA} have been attributed to increases in V_b (10) related to increasing blood volume (39). In turn, the increasing blood volume is thought to reflect an augmentation of water entry across the gill that cannot be fully compensated, despite increased urine output (59). By analogy to the SHR, it is also possible that the hypertension in the salt-fed fish reflects an increased activity of the central RAS, which in trout, is known to increase blood pressure by increasing systemic resistance and causing tachycardia, while potentially contributing to a blunting of the barostatic reflex (25).

A distinction between secondary and primary hypertension is particularly important when attempting to evaluate the chronic consequences of high blood pressure on physiological systems. Because the origins of primary or essential hypertension remain poorly understood (13, 30), there is an inherent difficulty associated with trying to distinguish the consequences, from the causes, of the elevated blood pressure. For example, with respect to the secretion of catecholamines, it can be problematic to separate the consequences of chronic hypertension from the contribution of altered catecholamine secretion to causing the primary hypertension. Thus, the significance of the salt-fed FW trout model of hypertension is that it can be used to readily assess the impact of chronically high blood pressure in the absence of impaired kidney function or genetic modifications. Using this approach, the novel findings of the present study are that a long-term elevation of blood pressure

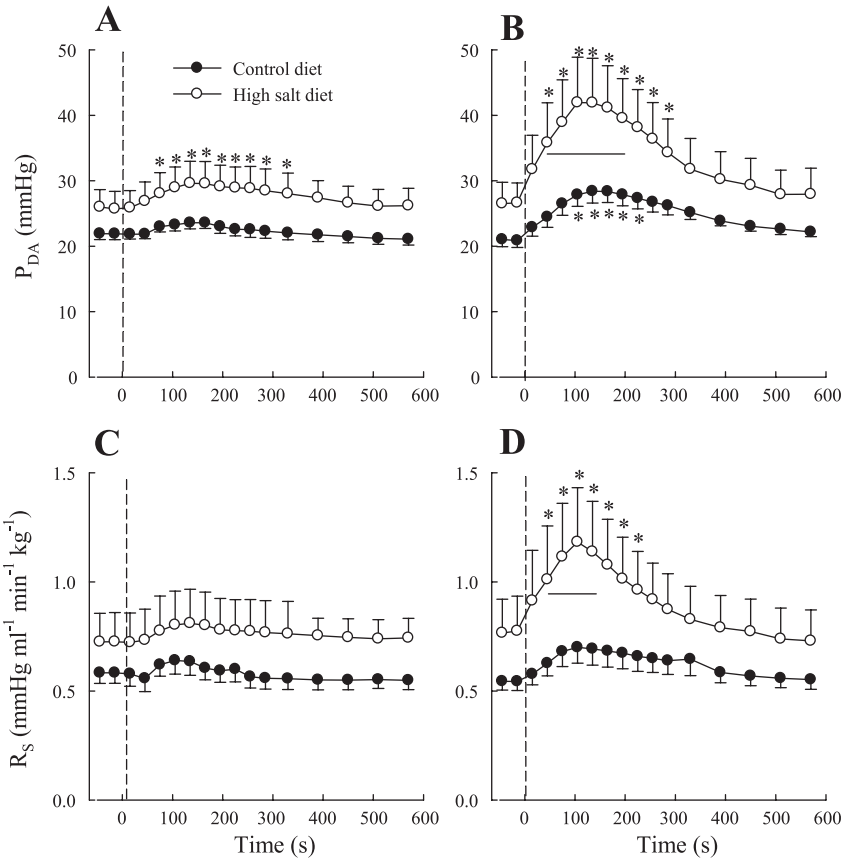

Fig. 5. In vivo effects of consecutive injections of low (100 pmol/kg) (*A* and *C*) and high (500 pmol/kg) (*B* and *D*) doses of homologous [Asn^1,Va1^5]-ANG II on dorsal aortic blood pressure (P_{DA}) (*A* and *B*) and systemic vascular resistance (R_S) (*C* and *D*) in rainbow trout (*Oncorhynchus mykiss*) fed a control (n = 7) or a high-salt (n = 9) diet. *Significant differences from the final preinjection value (−15 s); horizontal lines indicate significant differences between the control and salt-fed fish ($P < 0.05$; Bonferroni *t*-test).

induced by dietary salt in rainbow trout is associated with *1*) a significant blunting of the responsiveness of chromaffin cells to ANG II, yet an enhancement of catecholamine release into the circulation during acute hypoxia stress; and *2*) an apparently paradoxical increase in the vasopressor responses to ANG II.

Catecholamine Secretion in Hypertensive Fish

Catecholamine secretion in fish, as in other vertebrates, is largely mediated by activation of preganglionic sympathetic nerve fibers and subsequent interaction of the neurotransmitter acetylcholine with chromaffin cell cholinergic receptors (32, 44, 54). Additionally, however, a growing number of noncholinergic pathways and secretagogues are being identified in the stimulation or modulation of catecholamine secretion from piscine chromaffin tissue (45). Of particular relevance to this study is ANG II, one of the bioactive products of the RAS, which is mobilized in fish during periods of hypotension (1–3, 19, 35, 64). ANG II plays a critical role in raising blood pressure during periods of hypotension by its integrated effects on cardiovascular and renal function (12, 19). The predominant cardiovascular response mediated by ANG II is an increase in peripheral systemic resistance resulting from its direct effects on vascular smooth muscle contraction (18, 63) and indirectly by increasing circulating catecholamine levels following the stimulation of adrenaline (and to a lesser extent noradrenaline) secretion from chromaffin cells (2, 4). The relative importance of each of these pathways to increasing blood pressure varies among the species that have been examined. For example, in rainbow trout (3, 5) and, to a lesser extent, dogfish (1, 63) the mobilization of the RAS causes blood pressure to increase via peripheral vasoconstriction from the combined direct effects of ANG II and elevated circulating catecholamines (an α-adrenergic receptor-mediated effect). In eels (*Anguilla anguilla* or *A. rostrata*); however, the pressor effects of RAS mobilization result solely from the direct interaction of ANG II with vascular receptors (3, 64).

Regardless of its mode of action in fish (direct or indirect via catecholamines), the end products of the RAS including ANG II (36) and ANG III (31) are hypertensive, and thus it was hypothesized that chronically elevated blood pressure induced by a high-salt diet would lead to a blunting of the physiological responses linked to ANG II, including a reduced responsiveness of the catecholamine-secreting chromaffin cells. Indeed, the capacity of the chromaffin cells to release catecholamines (largely adrenaline) in response to homologous ANG II in vivo or in situ was markedly diminished in the chronically hypertensive fish. While we did not evaluate whether the responsiveness of the chromaffin cells to other specific stimuli (secretagogues) also was altered by the high-salt diet, the secretory response to the nonspecific depolarizing agent KCl was unaffected. Thus, it is plausible that the decreased secretion of catecholamines in the hypertensive fish was a result of a change in the distribution of ANG II receptors. Because the dose of ANG II eliciting a half-maximal response (ED_{50}) for total catecholamine secretion was unchanged by diet (6.46×10^{-10} mol/kg in control fish vs. 1.27×10^{-10} mol/kg) in the salt-fed fish, it suggests that the ligand binding affinity of the chromaffin cell ANG II receptor was not modified but that a more likely explanation for the result was a reduced number of cell surface ANG II receptors in the hypertensive fish. Al-

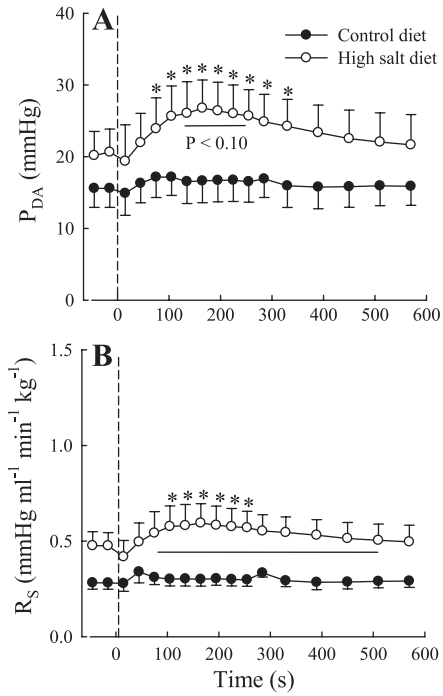

Fig. 6. Effects of α-adrenergic receptor blockade (using prazosin) on the in vivo effects of injecting 500 pmol/kg of homologous [Asn^1,Va1^5]-ANG II on dorsal aortic blood pressure (P_{DA}) (*A*) and systemic vascular resistance (R_S) (*B*) in rainbow trout (*Oncorhynchus mykiss*) fed a control ($n = 5$) or a high-salt ($n = 5$) diet. *Statistically significant differences from the final preinjection value (−15 s); horizontal lines indicate significant differences between the control and salt-fed fish ($P < 0.10$; Bonferroni *t*-test).

though the AT1 receptor has been implicated in mediating catecholamine secretion from mammalian chromaffin cells (9), the specific ANG II receptor subtype has not yet been identified in any fish species, although there is evidence that the piscine receptor is AT1-like (15, 57). Once the specific chromaffin cell ANG II receptor is identified in rainbow trout, future studies should be directed at quantifying their expression levels in response to dietary salt loading.

The blunted responsiveness of the chromaffin cells during salt-induced hypertension in rainbow trout differs markedly from results obtained from mammalian models of essential (primary) hypertension, which is associated with hyperactivity of the sympathoadrenal axis (23). The increased sympathoadrenal activity in essential hypertension is manifested by increased basal levels of circulating catecholamines (17, 43, 60), as well as increased responsiveness of the adrenal medulla (28) or isolated chromaffin cells (31) to acetylcholine or elevated (70 mM) KCl. Few, if any studies however, have examined the consequences of acquired (secondary) hypertension on mammalian chromaffin cells' responsiveness. While there is some evidence to suggest that elevated levels of stored chromaffin cell catecholamines may be a common feature of both essential and acquired hypertension (62), there is no obvious consequence of acquired hypertension on plasma catecholamine levels (16). We are unaware of any studies that have evaluated the effects of acquired hypertension on the responsiveness of mammalian chromaffin cells. The apparent absence of such studies is not surprising because chronic acquired hypertension is not easily achieved in mammals in the absence of disease (16), impaired kidney function, or the combination of increased exogenous salt and experimentally raised ANG II levels (41). The combined hypertensive effect of combining salt and low levels of ANG II (too low to otherwise affect blood pressure) is a particularly interesting situation because, except in salt-sensitive individuals, the addition of low levels of salt alone does not lead to an increase in pressure (20). Thus, it is reasoned that suppression of the RAS with increasing dietary salt is critical to prevent hypertension from developing (20) (see below for a discussion of modulators vs. nonmodulators).

While the responsiveness of the chromaffin cells to ANG II clearly was blunted in the hypertensive fish, the elevation of circulating catecholamines in these fish exposed to hypoxia was markedly greater than in the controls. The increase in circulating catecholamines in hypoxic trout can normally be

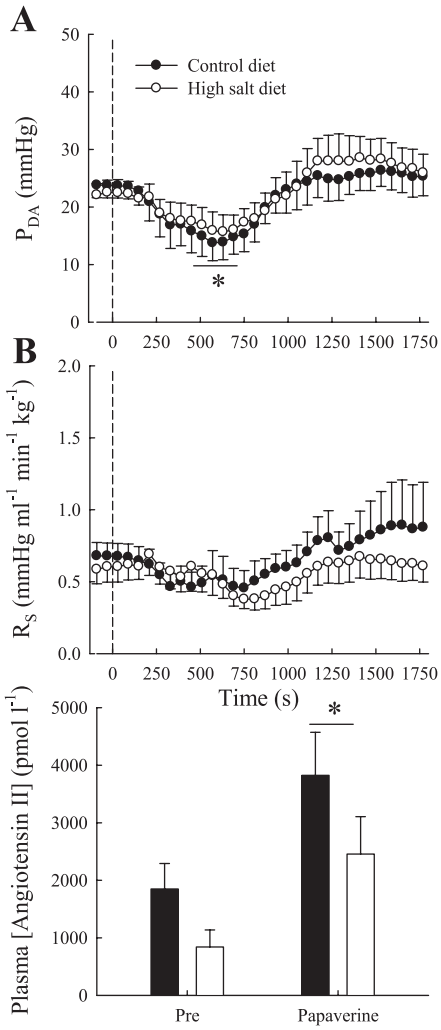

Fig. 7. Effects of the smooth muscle relaxant papaverine on dorsal aortic blood pressure (P_{DA}) (*A*) and systemic vascular resistance (R_S) (*B*) in rainbow trout (*Oncorhynchus mykiss*) fed a control ($n = 6$) or a high-salt ($n = 6$) diet. Horizontal lines associated with an asterisk indicate a global effect of treatment (papaverine injection) compared with the final preinjection values (−30 s) with no interaction of diet ($P < 0.001$); two-way repeated-measures ANOVA. *C*: effects of papaverine on plasma ANG II levels from samples withdrawn from fish fed a control (black bars; $n = 6$) or high-salt (white bars; $n = 8$) diet. The horizontal lines associated with an asterisk indicate a global effect of treatment (papaverine injection) with no interaction of diet ($P < 0.001$); two-way repeated-measures ANOVA.

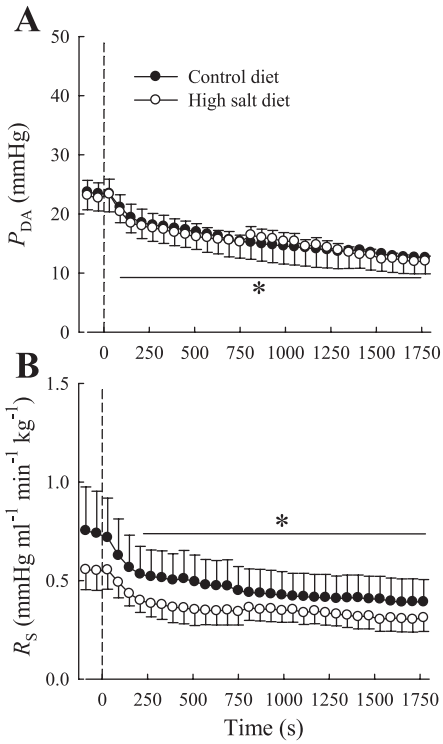

Fig. 8. Effects of the angiotensin converting enzyme (ACE) inhibitor lisinopril on dorsal aortic blood pressure (P_{DA}) (*A*) and systemic vascular resistance (R_S) (*B*) in rainbow trout (*Oncorhynchus mykiss*) fed a control ($n = 6$) or a high-salt ($n = 6$) diet. Horizontal lines associated with an asterisk indicate a global effect of treatment (lisinopril injection) compared with the final preinjection values (−30 s) with no interaction of diet ($P < 0.001$); two-way repeated-measures ANOVA.

attributed a variety of factors (for reviews, see Refs. 32, 44, 53, 54), including increasing neuronal stimulation following activation of O_2 chemoreceptors (56), local production of H_2S (48), and an elevation in plasma ANG II levels (24) (Table 1). Given that the sensitivity of the chromaffin cells to ANG II was blunted in the salt-fed fish and that plasma ANG II levels were elevated to a similar extent in both groups of fish, the greater secretion of catecholamines in the salt-fed hypoxic fish presumably reflected an increased contribution of one or more of the other stimulatory pathways, independently of RAS activation. The absolute baseline levels of ANG II (~200 pmol/l) and the magnitude of its increase during hypoxia (roughly 3-fold) were similar to the previous findings of Lapner and Perry (24).

Cardiovascular Effects of ANG II Are Enhanced in Salt-Fed Hypertensive Trout

Given that secondary hypertension is expected to initiate physiological responses aimed at lowering blood pressure and, in light of the dampened catecholamine secretion response to ANG II in the salt-fed fish (see above), the augmented cardiovascular responses to ANG II in the hypertensive fish were unexpected. As in previous studies (5), the rise in P_{DA} following the administration of ANG II was attributed to increasing systemic resistance (note, however, that while there was no statistical increase in R_S in the control group when the data were analyzed by 2-way repeated-measures ANOVA, the increases were statistically significant when analyzed by 1-way repeated-measures ANOVA). While the pressor responses to ANG II in rainbow trout reflect the combined direct constrictory effects of ANG II and the indirect effects of elevated circulating catecholamine levels (5), the significant attenuation of the catecholamine release response to ANG II in the salt-fed fish suggested that the greater pressor effects of ANG II in these fish were unrelated to circulating catecholamine levels. Indeed, this was verified by demonstrating that the increased responsiveness of the salt-fed fish to ANG II was retained even after the vasopressor effects of circulating (and neuronal) catecholamines was prevented by pretreatment of fish with the α-adrenergic receptor antagonist prazosin. Thus, the likeliest explanation for these results is that the high-salt diet, while leading to a decreased sensitivity or number of the chromaffin cell ANG II receptors, caused an increased affinity or number of vascular ANG II receptors associated with resistance vessels. The heightened effects of ANG II on cardiovascular responses in the fish experiencing chronic hypertension, while counterintuitive and in apparent conflict with the diminished responsiveness of the chromaffin cells, may reflect the fact that salt-induced hypertension in trout is accompanied by a decreased responsiveness of the systemic vasculature to circulating catecholamines (10). While speculative, it is possible that the blunted response of the vasculature to catecholamines, coupled with the diminished release of catecholamines from the chromaffin tissue, necessitates a heightened response of the vasculature to ANG II to maintain a normal capacity to regulate blood pressure during hypotension (see below).

In most humans, increased consumption of dietary salt and the ensuing increases in blood pressure are associated with reduced levels of ANG II levels that, in turn, contribute to a lowering of blood pressure (13); these individuals are termed modulators. However, in ~50% of patients (termed nonmodulators) with essential hypertension, plasma ANG II levels either are not lowered or the renal and cardiovascular responses to ANG II are increased inappropriately (13, 68). Thus in this respect, the cardiovascular responses of salt-fed hypertensive rainbow trout resemble those of some patients with essential hypertension.

Cardiovascular Responses During Induced Hypotension and Effects of RAS Blockade

The hypotensive responses of trout to the vascular relaxant papaverine were similar to those in previous reports (2, 3); P_{DA} declined rapidly but was fully recovered within 30 min. Unlike a previous study (2), the changes in R_S were not statistically significant owing to a high degree of variability in the V_b data. Clearly, however, on the basis of theory and previous results (2, 3) the initial fall in blood pressure accompanying papaverine is being driven largely by a decreased systemic resistance. While mechanisms vary among species (e.g., Ref. 3), the restoration of P_{DA} in rainbow trout after papaverine treatment largely reflects the combined vasoconstrictory effects of increased sympathetic nerve activity, elevated levels of circulating catecholamines, and increasing concentrations of ANG II (2). The increasing levels of ANG II, while contributing directly to vasoconstriction, also serve to stimulate catecholamine secretion from chromaffin tissue and activate sympathetic nerve fibers (38, 51).

In contrast to our prediction, the chronically elevated blood pressure associated with salt feeding did not impede the capacity of trout to restore blood pressure following the acute hypotension induced by papaverine. While we had hypothesized that the capacity to activate the RAS would be suppressed in the chronically hypertensive fish, clearly this was not the case. Indeed, the increases in plasma ANG II concentrations achieved after papaverine treatment were identical in the control and salt-fed fish. Surprisingly, however, the baseline levels of ANG II were exceptionally high (by ~10-fold) in both groups of fish compared with previous studies on rainbow trout (2, 3). One possible explanation for the unusually high resting levels of ANG II is the extensive surgery (cardiac flow probe, venous and arterial cannulae) compared with the previous studies [single arterial cannula only (e.g., Ref. 3)]. Regardless of the unusual baseline values, there were obvious further similar increases in plasma ANG II levels following the acute hypotension that accompanied papaverine injection.

Further evidence that the intrinsic activity of the RAS was unaffected by salt feeding was provided by results obtained using the ACE inhibitor lisinopril. As previously demonstrated (2, 3), lisinopril caused a marked and apparently unregulated reduction in P_{DA} owing to peripheral vasodilation (decreased R_S). The reduction in P_{DA} reflects the loss of existing vascular tone normally provided by the end products of the RAS. The identical fall in P_{DA} and R_S in the salt-fed and control fish suggests that the tonic activity of the RAS was unaffected by the chronic hypertension, a result that also did not support the original hypothesis of this study.

Concluding Remarks and Perspectives

The results of the present study suggest a paradoxical decrease and increase in ANG II receptor signaling at the level of the chromaffin cells and vasculature, respectively, in response to dietary salt loading. These findings may reflect tissue-specific regulation of ANG II receptor gene expression and/or tissue-specific downstream signaling. This is a broad avenue for future investigation. Despite the reduced capacity of the chromaffin tissue to respond to ANG II, the salt-fed fish were characterized by higher circulating levels of catecholamines in response to hypoxia. These results reinforce the importance of hypoxia as a proximal cue for catecholamine secretion in fish and the overall physiological importance of catecholamines in the response to a hypoxic stressor. The results also suggest the presence of redundant stimulatory pathways that ensure the secretion of catecholamines in response to hypoxia despite the hypertensive state of the animal, thereby ensuring a margin of safety in the hypoxia-elicited secretion of catecholamines.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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