Responses of the trout cardiac natriuretic peptide system to manipulation of salt and water balance

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Johnson KR, Olson KR. Responses of the trout cardiac natriuretic peptide system to manipulation of salt and water balance. Am J Physiol Regul Integr Comp Physiol 296: R1170–R1179, 2009. First published January 28, 2009; doi:10.1152/ajpregu.90880.2008.—Natriuretic peptides (NPs) are evolutionarily conserved hormones that affect blood pressure and fluid volume through membrane-bound guanylate cyclase (GC)-linked natriuretic peptide receptors-A and -B (NPR-A and NPR-B, respectively) in a variety of vascular, renal, and other tissues. The principal physiological stimulus for cardiac NPs in fish is somewhat debated between two prominent theories: regulation of salt balance (osmoregulatory hypothesis) or prevention of volume expansion (cardioprotective hypothesis). In the present study, we examined atrial and ventricular expression of trout NPs, atrial (ANP), brain (BNP), and ventricular (VNP) using Northern (mRNA), Western (NP pro-hormone), and qPCR (GC-NPR-A and -B mRNA) blot analysis following independent manipulation of plasma salt and volume levels after chronic exposure to freshwater (FW, volume loaded, salt depleted), saltwater (SW; volume depleted, salt loaded), or freshwater trout fed a high-salt diet (FW-HSD; volume and salt loaded). We also measured NP transcriptional response to acute (2 h) volume expansion with dialyzed plasma (VE; 80% blood vol) or volume depletion by hemorrhage (VD, 20% blood vol every 30 min for 2 h) with real-time PCR. In essentially all instances, increased expression of the NP system was associated with FW-HSD or plasma expansion. There were no differences in NP expression between chronically adapted FW and SW fish, and hemorrhage decreased atrial ANP and VNP mRNA. These results indicate that rainbow trout cardiac NPs and cardiovascular GC-NPRs respond principally to volume, not salt overload, and this suggests that the primary function of trout cardiac NP system is to protect the heart.

Natriuretic peptide receptors; hypervolemia; hypervolemia; osmoregulation; cardioprotection

Natriuretic Peptides (NPs) and their membrane-bound receptors (NPRs) comprise a small family of evolutionarily conserved regulators of cardiovascular and body fluid homeostasis in numerous vertebrates (2, 8, 19, 20, 23, 35, 55, 62). Four NPs have been identified among vertebrate classes to date (24, 58, 2, 8, 34, 61). The physiological role of NPs has been examined in detail, and cardiac NPs are generally accepted as serving to protect the heart from blood volume expansion and detrimental cardiodilation by effectively reducing circulating blood volume through diuresis and transcapillary fluid efflux and by reducing blood pressure through potent vasodilatory effects (10, 40, 49).

The physiological actions of NPs are primarily targeted through two natriuretic peptide receptors, NPR-A and NPR-B, via the formation of cGMP. In mammals, NPR-A exhibits the greatest affinity for ANP and BNP, while NPR-B is the primary receptor for CNP (2, 8, 20, 32). A third receptor, NPR-C, is relatively nonspecific and is predominantly associated with clearing NPs from the circulation. An analogous profile is found in teleosts, with the addition of a non-guanylate cyclase (GC) receptor (NPR-D) in the Japanese eel (28, 55, 62).

The primary stimulus for cardiac NP synthesis and release in mammals has been well documented as cardiac stretch (2, 8, 34, 61). The physiological role of NPs has been examined in detail, and cardiac NPs are generally accepted as serving to protect the heart from blood volume expansion and detrimental cardiodilation by effectively reducing circulating blood volume through diuresis and transcapillary fluid efflux and by reducing blood pressure through potent vasodilatory effects (10, 40, 49).

NPs also have direct, apparently cardioprotective, effects on the heart. NPR-A activation by ANP inhibits collagen synthesis by cardiac fibroblasts and atrial and ventricular cardiomyocytes, as well as extracellular matrix secretion of cardiac fibroblasts (47, 48). The NP/NPR-A pathway also inhibits hypertrophy in primary cultures of cardiac myocytes (3, 51). Under volume loading conditions, cardiac NPR-A and -B expression is significantly elevated (5, 30, 54) and upregulation of ANP and BNP production in the hypertrophied heart has led to the suggestion that autocrine/paracrine effects of the NP signal might serve as an endogenous defense against maladaptive cardiac hypertrophy that would otherwise result from chronic volume overload (38, 52). NPs also provide antimitogenic activity elsewhere in the cardiovascular system as ANP/NPR-A induces PKG inhibitory effects on proliferation and extracellular matrix production in vascular smooth muscle cells in vitro (9, 25).

In many aspects, physiological actions of the NP system in mammals are consistent with those found in fish. The effects of exogenous NP administration in fish in vivo include a reduction of whole body blood and extracellular fluid volume through their potent diuretic and natriuretic renal effects (12, 14). NPs also appear to be constitutively released in freshwater trout (11) and are well known for their potent vasodilatory effects (18, 42, 45, 53). In the saltwater (SW) eel, ANP has demonstrated potent antidiuretic effects, and both ANP and VNP are produced in the eel intestine and act locally to inhibit salt uptake in SW-adapted fish (37, 63).

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There are two somewhat different hypotheses regarding the physiological function of piscine NPs. The osmoregulatory hypothesis proposes that the NP family is primarily involved in the regulation of salt balance and functions to decrease salt load in saltwater teleosts (16, 36, 55, 57–59). This hypothesis is primarily based on studies in the eel showing plasma ANP is elevated following freshwater (FW) to SW transfer, local synthesis of VNP and NP inhibition of salt uptake in the eel intestine, and transient ANP elevation upon hypertonic saline injection. The cardioprotective hypothesis proposes that the primary action of NPs is to reduce blood volume and blood pressure and thereby protect the heart from excessive afterload or preload (19, 26). This hypothesis is supported by observations that NPs or NP-like immunoreactivity have been found in essentially all fishes examined to date, from primitive stenohaline hagfish to more derived euryhaline teleosts, including stenohaline FW species; release of cardiac NP or pro-NP fragments or plasma NPs are positively correlated with volume-loading conditions; and NPs reduce venous capacitance and branchial and systemic resistance in trout in vivo thereby effectively reducing both preload and afterload.

Euryhaline fish, such as trout, offer a unique opportunity to examine salt- and volume-dependent regulatory systems because they readily adapt to FW (volume loaded, salt depleted), SW (volume depleted, salt loaded), or FW while consuming a high-salt diet (FW-HSD; volume and salt loaded). Thus, salt and water balance can be manipulated independently and noninvasively. The cardiovascular implications of these chronic states have recently been delineated (4, 43); compared with FW, SW adaptation significantly reduces dorsal aortic pressure, central venous pressure, and blood volume, while FW-HSD significantly increases dorsal aortic pressure, ventral aortic pressure, central venous pressure, blood volume, and cardiac output (mainly by an increase in stroke volume as heart rate remains unchanged). Furthermore, gill morphology of FW-HSD trout resembles that of SW fish (46), thus providing additional evidence that both conditions produce a similar salt load with disparate effects on volume.

In the present study, we examined ANP, BNP, and VNP mRNA expression and pro-NP peptide levels in the atrium and ventricle of trout chronically adapted to FW, SW, and FW-HSD. Expression levels of these cardiac NPs were also analyzed following acute volume expansion with trout plasma and hemorrhage. Furthermore, the mRNA expression levels of NPR-A and -B in the heart and vasculature were quantified following chronic adaptation to FW, SW, and FW-HSD. We show that the NP system is preferentially activated by volume expansion and that this likely serves a cardioprotective role, presumably analogous to that found in mammals.

MATERIALS AND METHODS

All animal experiments were approved by the institutional animal care and use committee.

Animals. Adult rainbow trout (Oncorhynchus mykiss) were obtained from a local hatchery (Harietta Hills Trout Farm, Harrietta, MI). FW rainbow trout (525–877 g) were maintained in a 2,000-liter fiberglass tank with constant nonchlorinated 14°C well-water circulation under 12:12-h light-dark cycles. Fish were fed a maintenance diet of commercial trout pellets (Purina) for up to 48 h prior to experimentation.

Chronic SW adaptation. A maximum of 10–15 FW trout were adapted to SW (1,000 mosM) at one time in a 500-liter Instant Ocean Culture System (model WM-500, Aquarium Systems, Eastlake, OH) using synthetic sea salts supplied by the manufacturer. The fish were initially adapted to 300 mosM SW for 3–5 days, and the osmolarity was gradually increased to full-strength SW over the next 2 wk. They were maintained at this salinity for a minimum of an additional 2–3 wk before experimentation. Temperature, pH, osmolarity, and total ammonia were measured daily and, with the exception of osmolarity, were not different from FW.

Chronic FW-HSD adaptation. A maximum of 10–15 FW rainbow trout were placed in a 750-liter flow-through tank in aerated well water (14°C) and fed a high-salt diet (HSD) containing 12% NaCl at 2% total body wt/day, for a minimum of 3 wk before experimentation (FW-HSD). The HSD was prepared by crushing commercial trout pellets and mixing the powder with 12% by weight non-iodinized Kosher salt. A limited amount of deionized water was added to this mixture, and the paste was then squeezed through a potato ricer and dried at room temperature overnight. This HSD diet feeding regimen has been shown to increase gill (chloride cell) numbers and Na⁺-K⁺-ATPase activity, dorsal aortic pressure, central venous pressure, and blood volume in FW rainbow trout (4, 43, 50).

Acute volume manipulations. Methods for cannulation of the dorsal aorta (DA) have been described in detail (42). Briefly, trout were anesthetized in benzocaine (ethyl-p-aminobenzoate; 1:12,000 wt:vol) prior to surgery. The DA was cannulated percutaneously through the roof of the buccal cavity with heat-tapered polyethylene tubing (PE-60); the gills were not irrigated during this brief procedure. The following day, trout were either volume expanded with dialyzed plasma infusion (40% of estimated total blood volume, 35 ml·kg⁻¹·h⁻¹, for 2 h; 80% total volume expansion), an 80% total blood volume (TBV) continuous hemorrhage (20% TBV every 30 min for 2 h), or untreated and used as a cannulated control. The dialyzed plasma was obtained from a donor fish and dialyzed overnight at 14°C in dialysis tubing with a molecular weight cutoff of 7 KDa (Pierce, Rockford, IL).

Antibody production. New Zealand White specific pathogen-free (SPF) juvenile rabbits were used for the production of homologous trout ANP (tANP) and trout VNP (tVNP) polyclonal antibodies. The rabbits were adapted to a temperature-stable environment with a 12:12-h light-dark cycle. The rabbits were injected subcutaneously with 50–500 μg of tANP coupled to BSA or 25 μg of tVNP coupled to keyhole limpet hemocyanin (21). The protein conjugates were mixed with complete Freund’s adjuvant (first injections) or incomplete Freund’s (subsequent injections). Rabbit antisera avidity and specificity to tANP or tVNP were confirmed via slot blotting throughout the immunization process with test bleeds from the marginal ear vein (21). For the production of homologous tBNP antibodies, rainbow trout mature NPs (ANP, BNP, VNP, and CNP) were aligned to complete ANP (first injections) or incomplete Freund’s (subsequent injections). Rabbit antisera avidity and specificity to tANP or tVNP were confirmed via slot blotting throughout the immunization process with test bleeds from the marginal ear vein (21). For the production of homologous tBNP antibodies, rainbow trout mature NPs (ANP, BNP, VNP, and CNP) were aligned to determine regions of lowest similarity. The most unique amino acid segment of tBNP was found in the carboxy-terminal 9 residues: VGKYNAKTR, this sequence was commercially synthesized and used for tBNP polyclonal antibody production in New Zealand White rabbit antibodies by Genescript (Piscataway, NJ).

Western blot analysis. For cardiac protein preparation, atria and ventricles were homogenized in PBS, pH 7.5 with 5 mM EDTA and 1× HALT protease inhibitor cocktail (Pierce, Rockford IL), centrifuged at 10,000 rpm for 15 min; supernatant protein concentrations from the crude protein extracts were determined using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). For SDS-PAGE separation, 50 μg of total protein were electrophoretically separated on a 12% polyacrylamide gel under denaturing conditions, then transferred to a nitrocellulose membrane (Bio-Rad Laboratories) using a wet transfer unit. The membranes were blocked in 5% TBS-Tween-milk for 1 h at room temperature. After blocking, incubations with the tANP, tBNP, or tVNP primary antibodies were conducted overnight at 4°C, while those for horseradish peroxidase-conjugated anti-rabbit...
IgG secondary antibody (Pierce, Rockford IL) were for 1 h. Blots were incubated with enhanced chemiluminescence Western blotting agent (Amersham, Arlington Heights, IL) for 1 min and exposed to X-ray film for 1–5 min. To account for deviation in the amount of protein loaded, the same membranes were stripped with four washes of TBS-Tween and subsequently rebotted for levels of b-tubulin to enable standardization (murine monoclonal anti b-tubulin antibody, Sigma-Aldrich, St. Louis MO). Western blots were quantified by densitometry using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Northern blot analysis. Total RNA was extracted from various tissues using TRIzol reagent, (Molecular Research Center, Cincinnati OH), and 15 μg of RNA were separated on a formaldehyde-agarose gel and blotted onto a nitrocellulose membrane (Bio-Rad Laboratories). The membrane was UV cross-linked using Spectrolinker XL-1500 (Spectronics, Westbury, NY) for 30 s. The membrane was prehybridized in 6× SSC containing 50% formamide, 3× Denhardt’s solution, 1% SDS, and calf thymus DNA at 42° for 12 h; then it was hybridized with tANP, tBNP, or tVNP cDNA probe products radiolabeled with α-32P dCTP (Amersham) in 6× SSC containing 50% formamide, 3× Denhardt’s solution and 1 SDS at 42° overnight. The primers used in DNA probe synthesis are listed in Table 1, and accession numbers according to the conditions described by the manufacturer. Forward and reverse primers for tANP, tBNP, tVNP, NPR-A, NPR-B, and GAPDH were generated using MacVector software (Table 1) and were subsequently validated for use with real-time PCR by determining the optimal amplification efficiency and primer concentrations as described by the system manufacturer (Applied Biosystems, Foster City, CA). Accession numbers for ANP, BNP, and VNP are the same as above, other sequences used in generating qPCR primers are NPR-A DQ174276, NPR-B DQ174277, and GAPDH AF027130. For real-time PCR, primers were added to 25 μl total reaction volume using reagents provided in the ABgene Absolute QPCR SYBR Green Mix (ABgene, Rochester, NY). Final concentrations of the sense and antisense primers were determined for each primer pair based on optimal amplification efficiency. Reactions were carried out on an ABI 7700 Thermocycler (Applied Biosystems). Conditions were set to the following parameters: 2 min at 94°C followed by 40 cycles each for 15 s at 95°C, 1 min at 60°C, and 1 min at 72°C. The Ct (defined as the cycle number at which the fluorescence exceeds a threshold level) was determined for each reaction, while quantification was accomplished using the ΔΔCt method (33). The target Ct was determined for each sample and then normalized to the GAPDH mRNA Ct from the same sample (GAPDH mRNA Ct subtracted from the target Ct yields the ΔCt). These values were then compared with control

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<th>Primer</th>
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<th>qPCR primers</th>
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<td>ANP SYBR 5’</td>
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<td>CTCCTTTTAGACTGAGGCTAAGGCGCTGAGGT</td>
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Fig. 1. Northern blot analysis of atrial natriuretic peptide (ANP; A), brain natriuretic peptide (BNP; B), and ventricular (VNP; C) mRNA expression following adaptation to freshwater (FW), saltwater (SW), or freshwater high-salt diet (FW-HSD). Expression levels for all peptides were significantly greater in FW-HSD trout than either SW or FW fish. There were no differences between FW and SW fish (P < 0.05).
levels using the 2-ΔΔCt method and expressed as fold difference compared with an appropriate control sample.

**Vessel isolation and vascular myography.** Trout were stunned by a blow to the head, and the EBAs were removed from the fish and placed in 4°C HEPES buffered saline (HBS) and used within 48 h. Vascular rings were cut into 3–4 mm lengths, mounted on 280-μm diameter stainless-steel hooks, and suspended in 5-ml water jacketed smooth muscle chambers (45) containing HBS at 14°C. Tension was measured on a Grass Model 7E or 7F polygraph (Grass Instruments). With this system, a change in tension equivalent to 5 mg could be detected. Baseline tensions were adjusted to 500 mg for a minimum of 1 h before experimentation. These vessels were pretreated with propranolol (10^{-5} M) for 10 min and then contracted with epinephrine (Epi; 10^{-5} M), washed four times with HBS and resting tension reestablished over the next 30 min. The vessels were then contracted a second time, as described above, and the effect of trout ANP (tANP used as agonist for NPR-A) or trout CNP (tCNP used as agonist for NPR-B) on active tonus was determined by step-wise addition of tANP or tCNP to Epi-precontracted vessels over the concentration range of 10^{-11}-10^{-6} M.

**Plasma sodium and chloride concentrations.** Plasma Na^+ and Cl^- concentrations were measured on a NOVA 5 ion analyzer. Blood samples were taken from the dorsal aortic cannula of FW, SW, and FW-HSD trout, and subsequently centrifuged for 15 min at 3,000 g. The plasma was then transferred to a separate sample tube and immediately analyzed using a NOVA 5 ion analyzer (Nova Biomedical, Waltham, MA).

**Chemicals.** Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO). HEPES-buffered trout saline was (in mmol/l): 145 NaCl, 3 KCl, 0.57 MgSO4·7H2O, 2CaCl2·2H2O, 5 glucose, 3 HEPES acid, and 7 HEPES Na^+ salt, pH adjusted to 7.8.

**Data analysis.** All experiments were repeated a minimum of three times, unless stated otherwise. Summarized levels of NP and NPR mRNA and pro-NPs were expressed as a fold-difference (means ± SE) vs. a designated reference treatment such as freshwater or DA-cannulated trout (the value for the reference treatment was arbitrarily set at 1). Data were analyzed by Student t-test or by one-way ANOVA followed by the Fisher protected least significant difference multiple range test. Concentration-response curves are presented for tANP and tCNP as the cumulative percent change of maximal effect (means ± SE). In nearly all instances, this resulted in the complete abolition of active tonus. The NP concentration producing half-maximal relaxation (EC_{50}) was determined from individual vessel concentration-response curves using curve-fitting software (Sigmaplot 2000), and EC_{50}s were analyzed by one-way ANOVA. Significance was assumed when \( P \leq 0.05 \).

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**Fig. 2.** Northern blot analysis of ventricular ANP (A), BNP (B), and VNP (C) levels following adaptation to FW, SW, or FW-HSD. Expression levels for all peptides were significantly greater in FW-HSD trout than either SW or FW fish. There were no differences in NP mRNA levels between FW-HSD and SW fish (n = 6, *P < 0.05).

**Fig. 3.** Western blot analysis of atrial pro-ANP (A), pro-BNP (B), and pro-VNP (C) levels following adaptation to FW, SW, or FW-HSD. Propeptide levels for all peptides were significantly greater in FW-HSD trout than either SW or FW fish. There were no differences in pro-NP levels between FW and SW fish (n = 5, *P < 0.05).
RESULTS

Effect of chronic adaptation on cardiac NP mRNA and prohormone. Atrial and ventricular ANP, BNP, and VNP mRNA was significantly greater in FW-HSD trout than in either FW or SW fish (Fig. 1, A–C, atrium and Fig. 2, A–C, ventricle). There were no significant differences in NP mRNA between FW and SW fish. Similarly, atrial and ventricular pro-ANP, pro-VNP, and pro-BNP peptide levels were significantly greater in FW-HSD trout than either FW or SW trout. There were no differences in pro-NP levels between FW and SW fish (n = 5, *P < 0.05).

Effect of acute volume manipulation on atrial NP mRNA. Atrial expression of ANP, VNP, and BNP mRNA was significantly greater in FW-HSD trout than in either FW or SW fish (Fig. 1, A–C, atrium and Fig. 2, A–C, ventricle). There were no significant differences in NP mRNA between FW and SW fish. Similarly, atrial and ventricular pro-ANP, pro-VNP, and pro-BNP peptide levels were significantly greater in FW-HSD trout than either SW or FW, and there were no significant differences in propeptide levels between FW and SW trout (Fig. 3, A–C, atrium and 4, A–C, ventricle).

Effect of acute volume manipulation on atrial NP mRNA. Atrial expression of ANP, VNP, and BNP mRNA was significantly greater in trout subjected to an acute volume expansion with dialyzed trout plasma than in either control or hemorrhaged fish, and ANP and VNP mRNA was significantly lower in hemorrhaged fish than in controls (Fig. 5).

Cardiovascular distribution of NPR-A and NPR-B receptors. NPR-A and NPR-B mRNA was expressed in all tissues examined: atrium, ventricle, brain, EBA, CMA, VA, and ACV (Fig. 6, A and B). Quantification of NPR-A expression showed significantly elevated mRNA levels in the EBA and VA compared with the heart, brain, and other vasculature (Fig. 6A). NPR-B expression was significantly elevated in the EBA, VA, and ACV, while the lowest levels were found in the atrium (Fig. 6B).

Effect of chronic adaptation on cardiac GC-NPRs. NPR-A and NPR-B expression levels in the atrium and ventricle were analyzed following adaptations to FW, SW, or FW-HSD. As shown in Fig. 7, NPR-A and NPR-B mRNA expression in both the atrium and ventricle of FW-HSD trout was significantly greater than expression in the same tissue of either FW or SW (n = 6 all groups). With the exception of atrial NPR-B expression, NP receptor expression in cardiac tissue from SW trout was lower than receptor expression in FW trout (n = 6, all groups). Note the relative expression of atrial NPR-B in FW-HSD trout compared with FW trout, which was 5- to 20-fold greater than FW-HSD/FW expression in any other group.

Effects of chronic adaptation on vascular GC-NPRs and in vitro NP vasoactivity. Expression of NPR-A and -B, as well as sensitivity of effenter branchial arteries to NPs following FW, SW, or FW-HSD adaptation is shown in Fig. 8. There were no
significant differences in EBA NPR-A expression levels between FW, SW, or FW-HSD trout, whereas NPR-B mRNA expression was significantly lower in FW-HSD than FW adapted fish. Both ANP and CNP relaxed precontracted EBA; however, there was no significant effect of any environment on the respective EC50s (Table 2, Fig. 8).

Plasma Na+ and Cl− concentrations following chronic adaptations. Plasma Na+ and Cl− concentrations were determined in trout after 3 wk of adaptation to FW, SW, or FW-HSD. Plasma Na+ and Cl− concentrations were significantly greater in SW trout compared with FW trout (Table 3). No other differences were observed.

DISCUSSION

Chronic perturbation of salt and water balance by FW, SW, and FW-HSD adaptation. The effects of FW-SW adaptation on piscine salt balance have long been studied; however, only recently has there been a comprehensive analysis of the effects of FW, SW, and FW-HSD on fluid balance and cardiovascular function in fish (4, 43). Compared with FW trout, SW-adapted trout have a significantly lower blood volume, extracellular fluid volume, dorsal aortic, and central venous and mean circulatory filling pressure. Because of the decreased blood volume, SW trout also operate on the lower end of the vascular capacitance curve, which gives the artifactual appearance of an increase in vascular compliance. These responses are reversed when FW trout are fed a high-salt diet and the increased intravascular pressures, increased stressed blood volume, and increased cardiac output are the expected results of the associated volume expansion.

There is also evidence to support the hypotheses that both SW and FW-HSD adaptations are salt-loading conditions. SW-adapted trout experience salt loading through drinking seawater and a passive inward diffusion of ions. Although the effects of SW adaptation on plasma electrolytes are somewhat variable; that is, some studies show an increase in plasma ions in SW fish (i.e., this study), while others do not (briefly summarized in Ref. 43), the transition of the gill from a salt-absorbing (FW) to a salt-secreting (SW) organ has been well documented (17). Similar variability exists in plasma ions of FW vs. FW-HSD adapted fish, in which plasma ions may (43) or may not (Table 3, this study; Ref. 4) significantly increase relative to FW fish. However, as with SW fish, FW-HSD adaptation also transforms the gill of FW fish from a salt-absorbing to a salt-secreting epithelium that is both biochemically (50) and phenotypically (46) similar to SW fish.

Thus, it is clear that adapting trout to FW, SW, and FW-HSD is a unique tool with which to impose distinct and independent stresses on trout fluid and salt balance; FW fish are volume loaded and salt depleted, SW fish are volume depleted and salt loaded, and FW-HSD fish are volume and salt loaded. Because the increase in cardiac NP mRNA, prohormone and receptor levels were consistently increased in FW-HSD, but not in SW-adapted fish, we conclude that chronic volume, not salt, overload is the primary stimulus for the trout NP system.
Acute perturbation of blood volume. Acute volume expansion and hemorrhage of FW fish are also useful protocols to manipulate volume status in that there is little time for gill remodeling, and even a hemorrhage-induced increased drinking will not contribute to a salt load. Thus, the emphasis can be placed on the effects of blood volume manipulation on the NP system. In trout, volume expansion with trout plasma immediately increases both arterial and venous pressures (43), which results in a nearly instantaneous increase in the volume of the microcirculation and a slower shift of fluid from the vascular into the interstitial compartment (44) without a significant change in plasma osmolarity or [Na+] (13). Hemorrhage decreases both arterial and venous blood pressures (43) and mobilizes fluid from both intravascular and interstitial compartments (44). Trout capillaries are permeable to plasma protein, and trout can readily tolerate the addition or removal of large volumes of fluid (44). Because the half-time for fluid translocation either into or out of the interstitial compartment following plasma loading or hemorrhage, respectively, is on the order of 15 min (44), the protocols in the present experiments ensure that venous pressure will be more or less continuously perturbed for the duration of the experiment.

Similar to the chronic volume-loading experiments, acute volume loading was an effective stimulant of atrial NP mRNA production. There was clearly a difference in the magnitude of increase in NP mRNA, however, as volume expansion increased BNP mRNA nearly 30-fold, whereas the increase in VNP mRNA was ~7-fold, and ANP mRNA only increased by 50% (Fig. 5). A preference for increased BNP production is also the hallmark for chronic cardiodilation in the mammalian ventricle (61). Interestingly, in eels, a 28% volume expansion with hypertonic saline (that did not change plasma osmolarity) significantly elevated plasma ANP and VNP levels, while a single saline-dextran infusion (28% of blood volume) did not affect plasma ANP and VNP (27). Unlike our chronic experiments, however, acute volume depletion significantly reduced atrial NP mRNA levels. This suggests that there is tonic ANP production in trout. This has been suggested previously, as it has been reported that inhibition of the NP clearance receptor in unanesthetized, cannulated trout produces a hypotensive response similar to that produced by intra-arterial injection of ANP (11) and the observation that ANP is continuously secreted by the in situ perfused trout heart (6, 7). Furthermore, 10 min following a single 28% volume reduction, eel plasma ANP and VNP levels were significantly reduced (27).

Cardiovascular NPR responses to salt and volume manipulations. Availability of partial cDNA sequence information for rainbow trout NPR-A and NPR-B (39) has provided the opportunity to examine the distribution and potential regulation of these biological effectors of circulating NPs. In the FW trout, there were no significant differences in GC-NPR expression between the atrium and ventricle, although NPR-A mRNA expression was markedly elevated in the brain, EBA, and VA compared with the atrium (Fig. 6A). Similarly for NPR-B, the vascular tissues exhibited highest levels of mRNA with no differences between cardiac tissues or brain (Fig. 6B).
The effects of chronic adaptation on NP receptors are consistent with a volume overload, as it is evident that cardiac NPR-A and NPR-B transcriptional levels were primarily responsive to elevated blood volume as well. However, while cardiac NP transcription and propeptide levels were not different between FW and SW fish, both NPR-A and NPR-B were lower in volume-depleted SW trout. This additional decrease in SW cardiac NP receptors suggests a further decrement in volume-sensitivity, which is perhaps consistent with an initial attempt at myocardial remodeling in the low-volume SW fish. While comparable studies examining the GC-NPR expression response to volume expansion are not currently available in any other piscine model, elevation of cardiac NPR-A and NPR-B transcription levels in response to volume expansion in mammals is well documented (5, 15, 31, 54). In rats, elevated cardiac expression of GC-NPRs has been found in surgically induced cardiac hypertrophy (54). Furthermore, in rats, left ventricular NPR-A is markedly elevated following pressure overload-cardiac hypertrophy (5), and NPR-B knockout mice display progressive blood pressure-independent cardiac hypertrophy and elevated heart rate (30). The reduced transcription of cardiac GC-NPRs when trout are adapted from FW to SW suggests that even in FW fish, there is some tonic antihypertrophic stimulus. Similar to our study, eel NPR-B expression is reduced in the atrium and ventricle (along with numerous other tissues) following SW adaptation (29); hence, the cardiac response of GC-NPRs appears to be volume responsive and tonically regulated in eels as well as in trout.

Vascular expression of NPRs in FW fish was generally greater than cardiac expression (Fig. 6). This was especially noticeable in the most elastic vessels, the ventral aorta, and efferent branchial artery, where mean and pulse pressures are the greatest. Perhaps these receptors decrease vascular tone and thereby increase their compliance. As described above, NPRs appear to be tonically secreted into the blood of FW trout (11), and this may help buffer systolic pressure under resting conditions.

The vascular response of NPRs to chronic manipulation of water and salt balance is different from the cardiac response (Figs. 7 and 8). Where FW-HSD consistently increased cardiac NPRs, it had no effect on EBA NPR-A and decreased expression of NPR-B. This suggests that the vascular receptors may be more responsive to circulating NPRs, that is, if anything they are slightly downregulated by a predicted increase in circulating NPRs in FW-HSD fish and less affected by wall stress. This is somewhat similar to the mammalian response, in which chronic volume loading may decrease, or not change, vascular synthesis of GC-NPRs (31, 41). Vascular sensitivity to NPRs in mammals also appears to be reduced in chronic hypertension as a result of homologous GC-NPR desensitization by elevated circulating NPRs (20, 52). On the other hand, an upregulation of vascular GC-NPRs or increased vascular sensitivity for circulating NPRs would create a vicious volume-accommodating vasorelaxant cycle.

The physiological role of NPRs: the osmoregulatory and cardioprotective hypotheses. The present studies provide a variety of evidence that increased activation of the trout NP system is strongly correlated with volume expansion and not with salt balance. This supports the cardioprotective hypothesis and suggests that in the absence of volume loading, salt load is not an effective NP secretagogue. Volume-related effects have recently been observed in other fish as well. In zebrafish larvae, ANP transcriptional response was upregulated in larvae raised in dilute freshwater medium but downregulated in larvae raised in concentrated medium (22). In genetic mutations of the zebrafish atrium, weak-atrium fish exhibited marked upregulation of ANP expression in both the atrium and ventricle, a molecular response shared with mammalian cardiomyopathies (1). Thus, the collective diuretic, natriuretic, and vasodilatory effects of NPRs all serve to decrease afterload and preload. Furthermore, the increased volume-induced expression of cardiac NPR may provide additional protection.

Perspectives and Significance

Although the molecular progression from compensated cardiac hypertrophy to decompensated heart failure in mammals is poorly understood, one commonality is activation of the cardiac NP system. Results from the present study also suggest that trout cardiac NPRs are primarily responsive to volume perturbation and thus cardioprotection appears to be one of the primum functions of NPs. This role may be even of greater homeostatic value in chronically volume-loaded vertebrates such as freshwater fish, and we propose that these vertebrates will provide valuable insight into the basic mechanisms of cardiac NPR and GC-NPRs in cardiomyopathies.

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**REGULATION OF TROUT NATRIURETIC PEPTIDES**

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