

Vasoactivity of the Ventral Aorta of the American Eel (*Anguilla rostrata*), Atlantic Hagfish (*Myxine glutinosa*), and Sea Lamprey (*Petromyzon marinus*)

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ABSTRACT To determine if vascular smooth muscle from teleost and agnathan fishes expresses receptors for signaling agents that are important in vascular tension in other vertebrates, we exposed rings of aortic vascular smooth muscle from the eel (*Anguilla rostrata*), the hagfish (*Myxine glutinosa*), and the lamprey (*Petromyzon marinus*) to a suite of putative agonists, including: acetylcholine, endothelin, nitric oxide, natriuretic peptides, and prostanoids. Acetylcholine constricted aortic rings from the eel, but had no effect on the rings from lamprey. On the other hand, endothelin constricted rings from all three species. Use of receptor-specific ET agonists demonstrated that only ET_A receptors are expressed in the eel and lamprey aorta. The nitric oxide donor sodium nitroprusside or nitric oxide itself dilated rings from the eel, but both agonists constricted rings from the hagfish and NO produced a biphasic response (constriction followed by dilation) in the lamprey. Two natriuretic peptides, eel ANP and porcine CNP, produced marginally significant dilation in the eel aorta, human ANP dilated the hagfish rings, and pCNP and eANP dilated the lamprey rings. The prostanoids PGE₁ and PGE₂ both dilated the eel aortic rings, and PGE₁ and carbaprostacyclin (stable PGI₂ agonist) dilated the hagfish and lamprey rings. Our results suggest that receptors for a variety of vasoactive signaling agents are expressed in the aortic smooth muscle of the earliest vertebrates (lamprey and hagfish), as well as the more advanced teleosts (eel). *J. Exp. Zool.* 289:273–284, 2001. © 2001 Wiley-Liss, Inc.

It is becoming increasingly clear that both teleost and elasmobranch fishes express a variety of receptors for vasoactive signaling agents in their blood vessels, including both arteries and veins. The neurotransmitter acetylcholine constricted isolated vessels in the teleosts *Oncorhynchus mykiss* (Small et al., '90; Olson and Villa, '91; Miller and Vanhoutte, '92; Farrell and Johansen, '95) and *Salmo salar* (Sverdrup and Helle, '94; Sverdrup et al., '94), as well as the shark *Squalus acanthias* (Evans and Gunderson, '98a). The peptide endothelin also constricted isolated vessels in *O. mykiss* (Olson et al., '91; Wang et al., '99) and *S. salar* (Sverdrup et al., '94), another teleost, *Amiurus melas* (Poder et al., '91), and *S. acanthias* (Evans et al., '96; Evans, '2000). Vessels in *O. mykiss*, *S. salar*, *Gadus morhua*, *Opsanus beta*, (teleosts), and *S. acanthias* dilated in response to natriuretic peptides (Duff and Olson, '86; Price et al., '90; Evans, '91; Evans et al., '93a; Sverdrup and Helle, '94; Takei et al., '94; Tervonen et al., '98). In addition, infusion of either heterologous (human ANP) or homologous (eel ANP) into the

teleost *Anguilla japonica* lowered arterial blood pressure (Takei et al., '89), although decreases in cardiac output may have accounted for some of the hypotension observed (Oudit and Butler, '95). More recently, receptors for natriuretic peptides have been localized in eel gills, using radioligand (Mishina and Takei, '97) and Northern Blot analyses (Kashiwagi et al., '99).

The nitric oxide donor sodium nitroprusside (SNP) dilated vessels in *O. mykiss* (Small and Farrell, '90; Small et al., '90; Olson and Villa, '91) and *Anguilla anguilla* (Schwerte et al., '99). In addition, L-arginine (a NO precursor) dilated the in situ, coronary artery of *O. mykiss* (Mustafa et al., '97). Surprisingly, SNP and NO actually constricted the ventral aorta of *S. acanthias* (Evans

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and Gunderson, '98b), and our more recent data demonstrated that a small, conductance artery (anterior mesenteric) and even an abdominal vein (posterior intestinal) from this species constricted when SNP or NO was applied (Evans, 2001). The prostanoids PGE₁ and carbaprostacyclin (stable analogue of PGI₂) dilated the ventral aorta of *S. acanthias* (Evans and Gunderson, '98b), and the cyclooxygenase (mediates prostanoid synthesis) inhibitor indomethacin inhibited the endothelium-dependent dilation (in response to the Ca²⁺ ionophore A23187) in this tissue as well as in various vessels in *O. mykiss* (Olson and Villa, '91). Both of these groups suggested that a prostanoid, not NO, is the dominant endothelium-derived relaxing factor in fishes, contrary to the situation in mammals (e.g., Godecke et al., '98). Somewhat surprisingly, one study found that PGI₂ constricted a strip of ventral aortic tissue in the conger eel, *Conger conger*, but it did dilate an aortic strip from the shark, *Scyliorhinus stellaris* (Piomelli et al., '85).

In agnathan fishes, only a single, vasoactive signaling system has been functionally defined to date. We found that atrial natriuretic peptide dilated the ventral aorta of the hagfish, *Myxine glutinosa* (Evans et al., '93). However, a few morphological studies have localized nitric oxide synthase (Schober et al., '94) and receptors for natriuretic peptides (Kloas et al., '88; Toop et al., '95, '98) in a single species of hagfish and two lamprey species.

The present study was undertaken to broaden the database on vascular reactivity in fishes, especially agnatha, in order to provide information that might allow hypotheses on the evolution of these systems in the lower vertebrates. In particular, we were interested in whether nitric oxide is dilatory, as it is in mammals and apparently teleost fishes, or constrictory, as it appears to be in elasmobranchs.

MATERIALS AND METHODS

Freshwater (yellow) American eels (*Anguilla rostrata*; mixed sexes, 200–900 g) were purchased from a commercial supplier and maintained in running sea water tanks (ca. 16°C) at the Mt. Desert Island Biological Laboratory, Salsbury Cove, Maine for at least one week before use. They had been captured in the Union River (near Bangor, Maine) during the months of July and August, 1997 and 1998. Atlantic hagfish (*Myxine glutinosa*; mixed sexes, 50–100 g) were purchased from the Huntsman Marine Laboratory (St. Andrews, New Brunswick, Canada), delivered in

refrigerated tanks and maintained in running sea water tanks (ca. 16°C) at the MDIBL for at least 1 week before use. They had been trapped in the Bay of Fundy (near St. Andrews) in June, 1998. Landlocked sea lampreys (*Petromyzon marinus*; gravid females, 100–300 g) were trapped in various tributaries of Lakes Huron and Michigan (May, 1998) and maintained by the staff of the Hammond Bay Biological Station (Millersburg, MI). They were airfreighted to the MDIBL and maintained in filtered well water in a Living Stream aquarium (Frigid Units Inc., Toledo, OH) at 12–14°C, under license from the Maine Department of Inland Fisheries. Lamprey carcasses were incinerated at the end of the experiments by the Jackson Laboratory, Bar Harbor, Maine.

Eels were anesthetized in 0.1% MS-222 (Sigma Chemical) in sea water, hagfish in 0.2% 1-Phenoxy-2-Propanol (Pfaltz and Bauer) in sea water, and lampreys in 0.1% MS-222 in well water. Fish were double-pithed after cervical transection, and the ventral aorta was removed by dissection and placed into iced Ringer's solution, appropriate for a given species. Teleost and hagfish Ringer's solutions were formulated as described previously (Robertson, '66; Evans et al., '89; Evans, '91) and lamprey Ringer's (modified from Urist, '63) contained (mM/l): NaCl (130), KCl (5.1), CaCl₂ (2.74), MgSO₄ (1.2), NaH₂PO₄ (2.6), NaHCO₃ (15), glucose (5.6). The fish Ringer's maintained a pH of 7.6–7.8 when bubbled with 1% CO₂/99% oxygen.

Cross-sectional rings were mounted in 10 ml of the appropriate Ringer's solution in organ baths and maintained at 12°C (as described previously; Evans, '98; Gunderson '98b). Initial tensions were maintained at ca. 100 g (eel), ca. 150 mg (hagfish), or ca. 200 mg (lamprey) for 30–60 min, because preliminary experiments determined that the respective rings were most responsive at those tensions. Tension was recorded by either Gould-Statham or WPI strain transducers connected through a Biopac MP100WS data acquisition system (using AcqKnowledge III software) to a Macintosh Powerbook 140 computer. After the rings reached a stable tension, putative agonists were added cumulatively to the experimental bath in increments totaling <4% of the initial volume.

Solutions of acetylcholine (ACh, Sigma), human endothelin (ET-1, American Peptide), sarafotoxin S6c (SRX S6c; American Peptide), sodium nitroprusside (SNP, Sigma), porcine C-type natriuretic peptide (pCNP, Peninsula Laboratories), human atrial natriuretic peptide (hANP, Peninsula Laboratories), eel atrial natriuretic peptide (eANP;

Peninsula Laboratories), and carbaprostacyclin (CPR) and two E-type prostaglandins, PGE₁ or PGE₂ (Cayman Chemicals), were made as described previously (Evans et al., '96; Evans and Gunderson, '98b) and stored at -20°C until diluted. A saturated NO solution (in distilled water) was prepared as described previously (Evans and Gunderson, '98b). Initial experiments consisted of the sequential addition of some or all of the following: ACh (0.1 mM), ET-1 (0.1 μM), SNP (0.1 mM), NO (4.2 μM), pCNP (0.1 μM) or hANP (0.1 μM), CPR (1 μM), and PGE₁ (1 μM) to a given ring. In subsequent experiments, to differentiate between ET_A and ET_B receptors in eel and lamprey aortae, paired rings were exposed to 0.1 μM of either ET-1 or SRX S6c. This was followed by the addition of either 0.1 μM eANP or pCNP, to differentiate between NPR_A and NPR_B receptors. Specific concentrations of all agonists were chosen because our earlier studies had determined that they produced near maximal responses in shark vascular smooth muscle (e.g., Evans et al., '93; Evans et al., '96; Evans and Gunderson, '98b).

All data are expressed as mean ± s.e. (N). Tension changes (measured relative to the tension at the end of the previous experimental period) were compared to zero using Prism (GraphPad Software) and accepted as significant with a $P < 0.05$ (Student's t -test, two tailed, except where noted).

These protocols conformed to NIH Guidelines

and were approved by the IACUC at MDIBL and the University of Florida.

RESULTS

Eel

Addition of acetylcholine produced significant constriction of isolated aortic rings from *A. rostrata*; subsequent addition of either the NO donor SNP or NO itself, followed by either PGE₁ or PGE₂ produced significant dilation in all cases (Figs. 1, 3). On the other hand, if ET-1 is added after ACh, the rings constricted significantly (Figs. 2, 3). If paired rings are exposed to either ET-1 or the ET_B receptor-specific agonist SRX S6c, only the former produced constriction. (Fig. 4A,B). Both eANP ($P = 0.06$, one tailed) and pCNP ($P = 0.03$, one tailed) usually dilated the rings when added after the ET analogues (Fig. 4A,B).

Hagfish

ET-1 produced significant constriction of the hagfish aortic ring, as did subsequent addition of SNP and NO (Fig. 5A,B). When hANP was added next, the rings dilated, and they dilated even further when CPR (PGI₂ agonist) and PGE₁ were added subsequently (Figs. 5A,B).

Lamprey

ACh did not alter the tension in the lamprey aortic ring (data not shown), but ET-1 produced

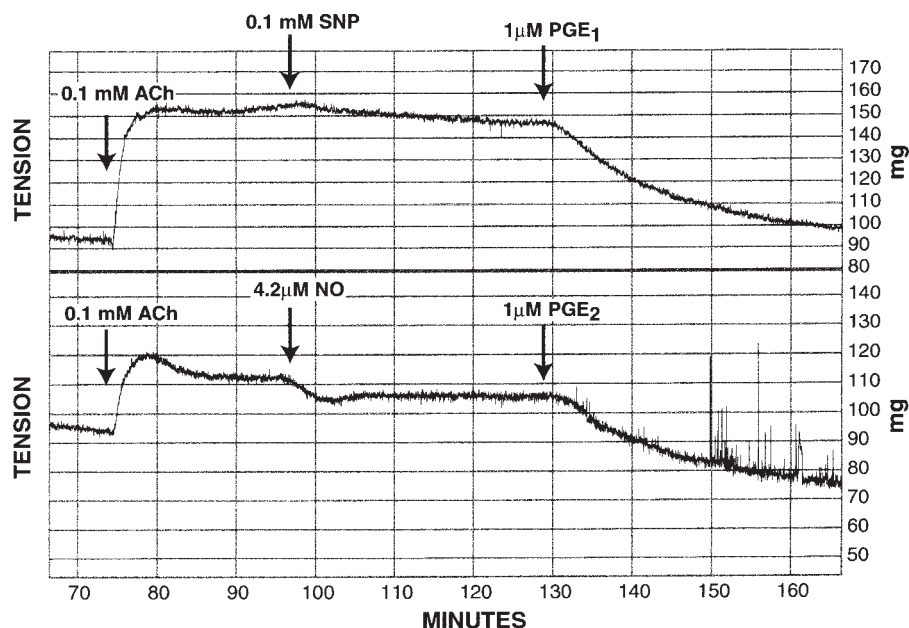


Fig. 1. Representative plots of the effect of ACh followed by SNP or NO and then either PGE₁ or PGE₂ on aortic rings from two eels, *Anguilla rostrata*. See text for details.

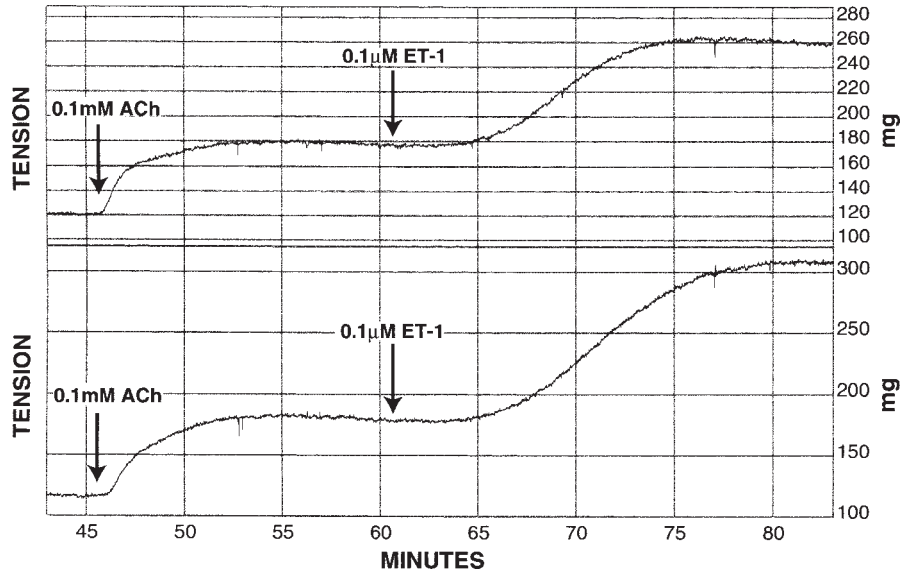


Fig. 2. Representative plot of the effect of ACh followed by ET on aortic rings from two eels. See text for details.

significant constriction (Figs. 6, 8). Subsequent addition of SNP did not affect the ring, but addition of NO produced a biphasic response: slight initial constriction followed by a much more sig-

nificant dilation (Fig. 7). Porcine CNP produced significant dilation when added next, as did both carbaprostacyclin and PGE₁ (Figs. 6, 8). In paired rings, ET-1, but not SRX S6c constricted the rings,

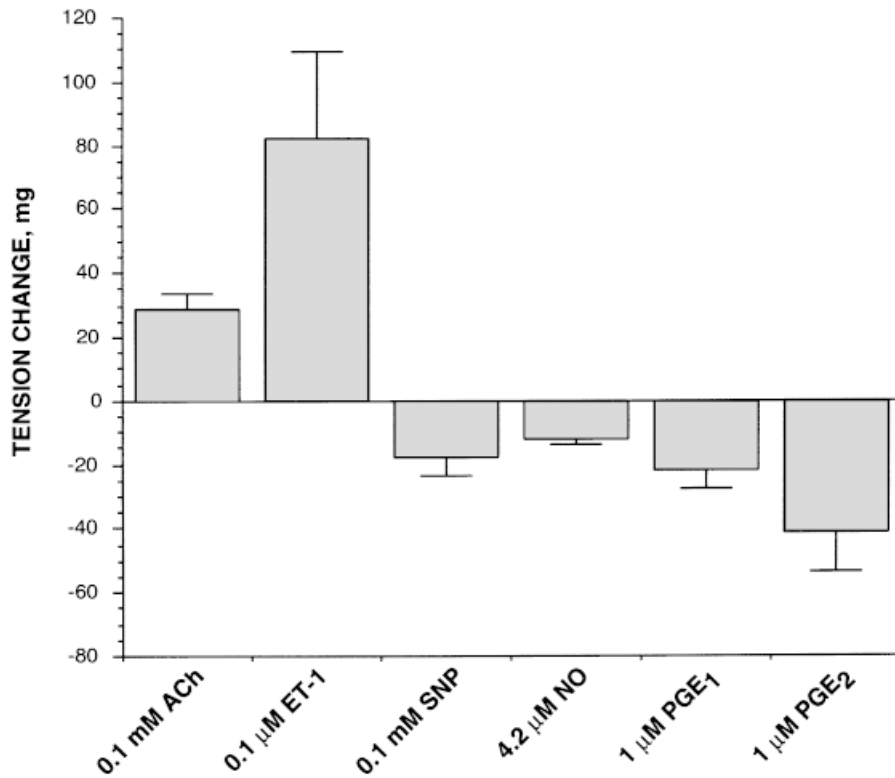


Fig. 3. Summary histogram of the effect of agonists on eel aortic rings. N = 5-17; all responses were significantly different from zero ($P > 0.05$).

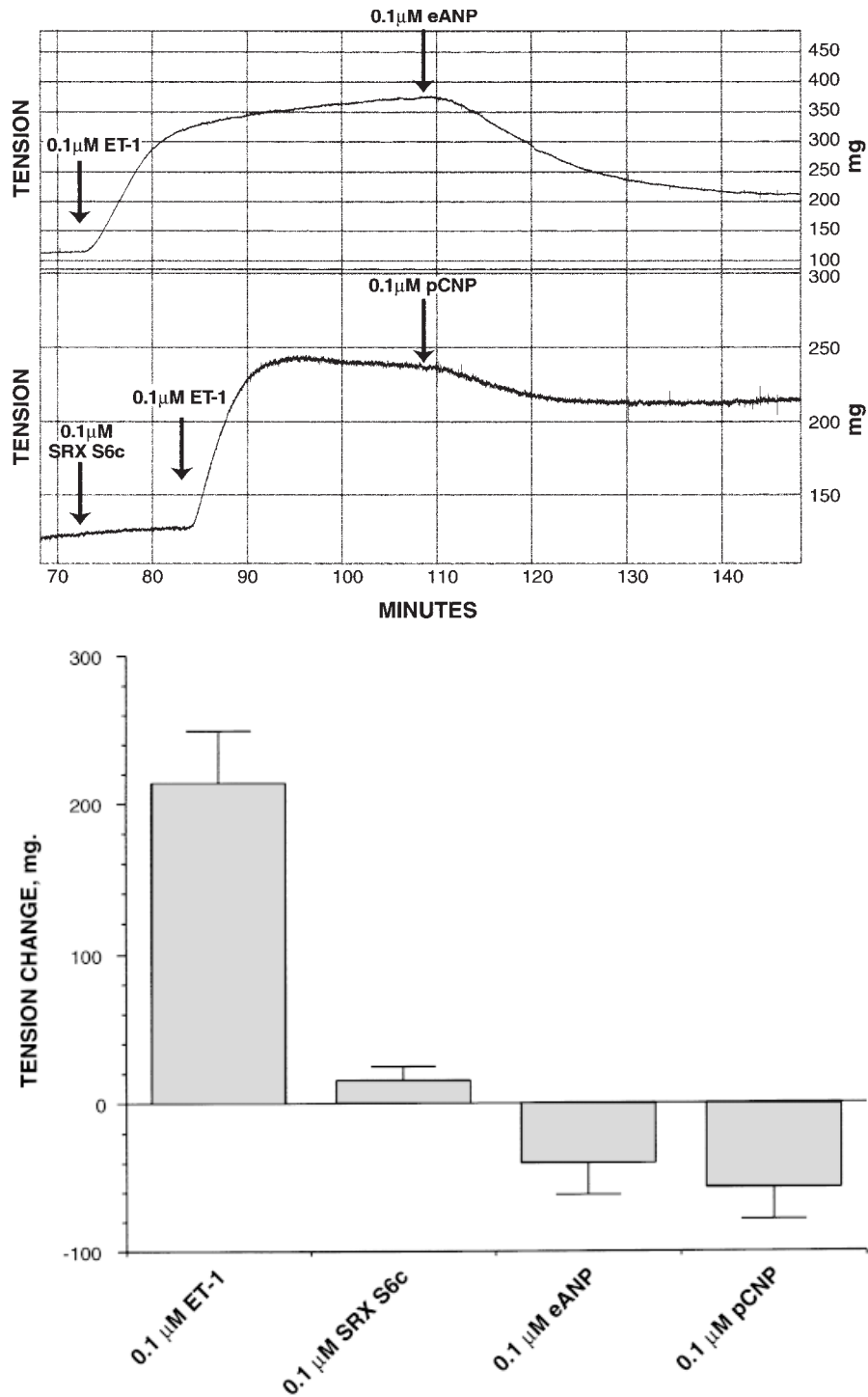


Fig. 4. (A) Representative plot of the comparative effects of ET-1 vs. SRX S6c and eANP vs. pCNP on paired aortic rings from a single eel. Note that in the bottom tracing SRX S6c produced no response, but the ring responded to ET-1 when it was added subsequently. See text for details. (B) Sum-

mary histogram for 6–9 aortae. The response to SRX S6c was not significantly different from zero. The responses to the NPs was only marginally significant using a one-tailed test. See text for details.

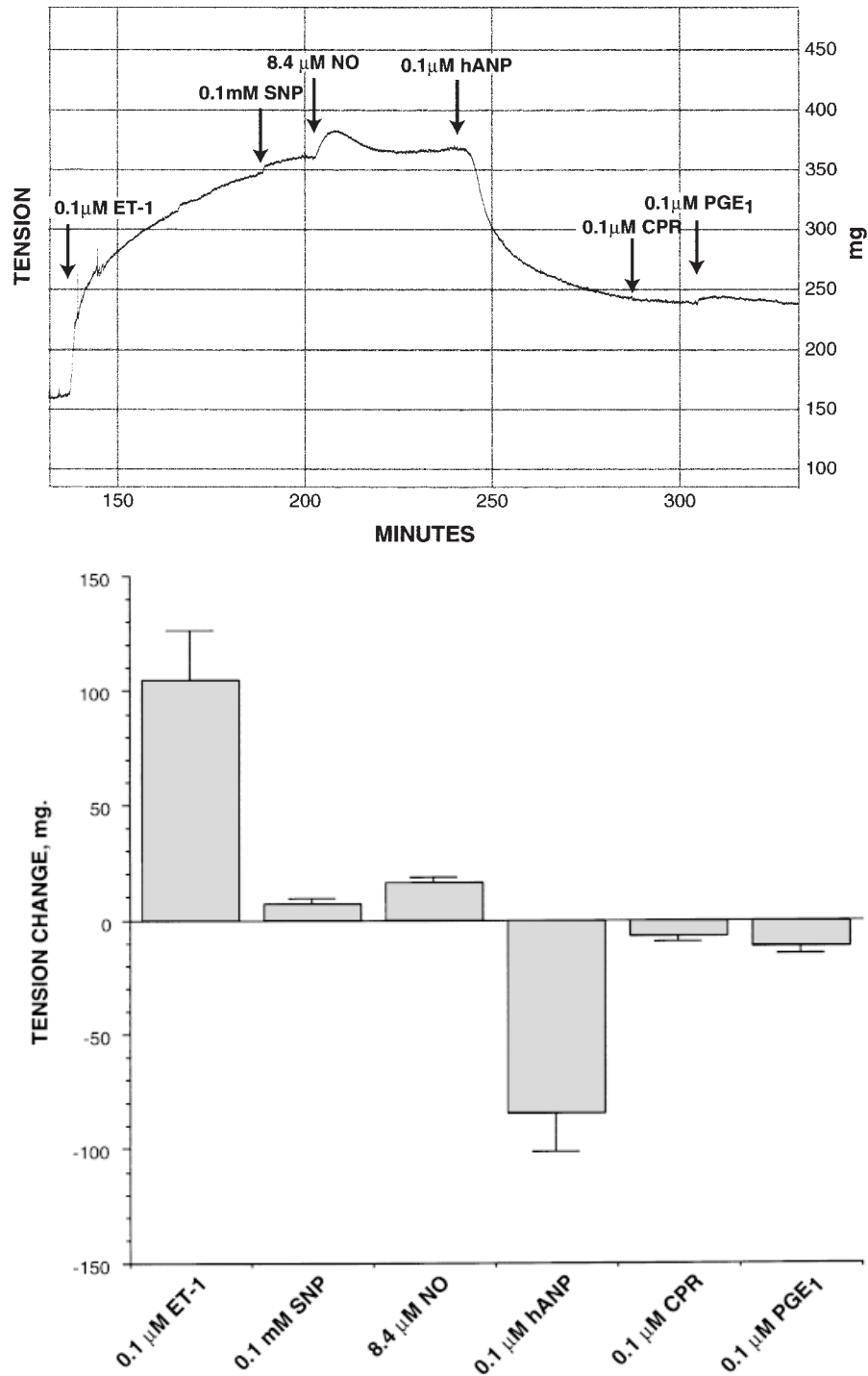


Fig. 5. (A) Representative plot of the effect of cumulative addition of ET-1, SNP, NO, hANP, CPR, and PGE₁ on an aortic ring from a single hagfish, *Myxine glutinosa*. See text for

details. (B) Summary histogram of the effects of agonists on 5 hagfish aortae. All responses were significantly different from zero ($P < 0.05$).

and subsequent addition of either eANP or pCNP dilated the rings (Fig. 9). The apparent difference between the responses to the two natriuretic peptides was not significant ($P = 0.10$).

DISCUSSION

These data demonstrate that the ventral aortae of two agnathan species and a teleost express receptors for a variety of vasoactive signaling

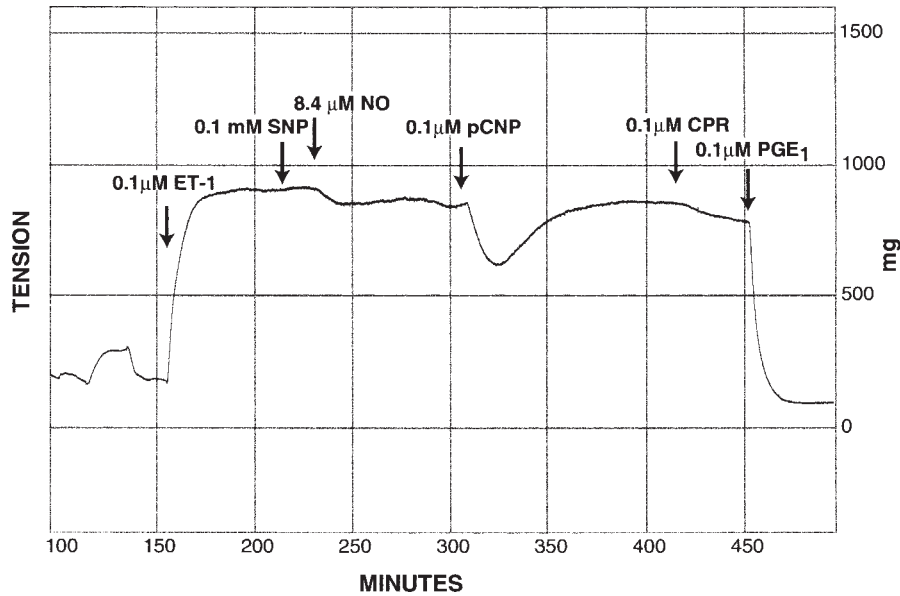


Fig. 6. Representative plot of the effect of cumulative addition of ET-1, SNP, NO, pCNP, CPR, and PGE₁ on an aortic

ring from a single lamprey, *Petromyzon marinus*. See text for details.

agents. The neurotransmitter acetylcholine constricted the vascular smooth muscle in the eel (Figs. 1, 2, 4), corroborating data from two other species of teleosts (both salmonids), as well as the spiny dogfish (see Introduction). Somewhat surprisingly, ACh did not affect the tension in the aortic rings from the lamprey, suggesting that cho-

linergic receptors are missing in the aortic vasculature of this species, and possibly this clade of agnathan evolution. We did test the effect of ACh on hagfish aortic rings and usually found constriction; however, the data were too variable to produce statistical significance. The current data do not allow us to characterize the subtype of cho-

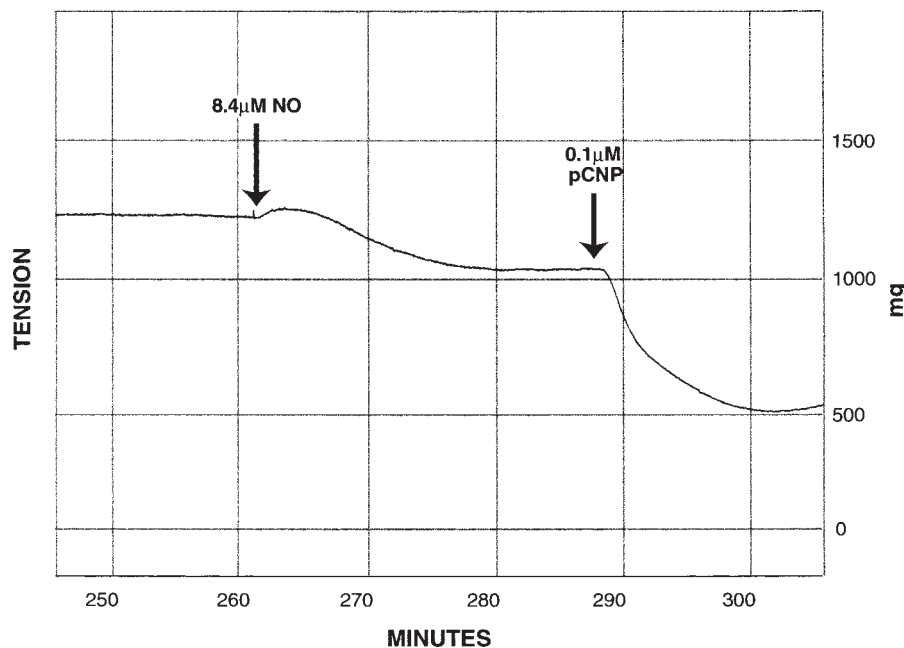


Fig. 7. Representative plot showing the biphasic response to NO applied to a single lamprey aortic ring.

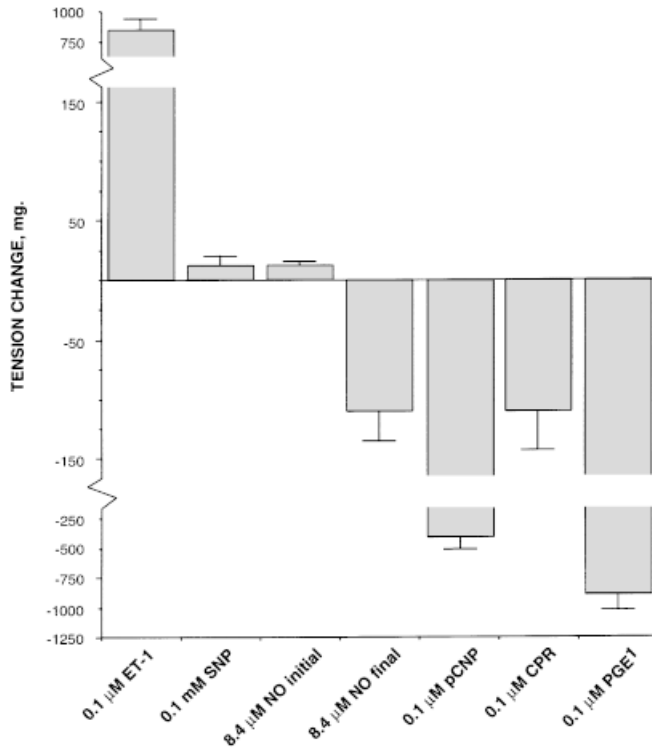


Fig. 8. Summary histogram of the effects of agonists on 6–20 lamprey aortae. All responses were significantly different from zero ($P < 0.05$), except the response to SNP.

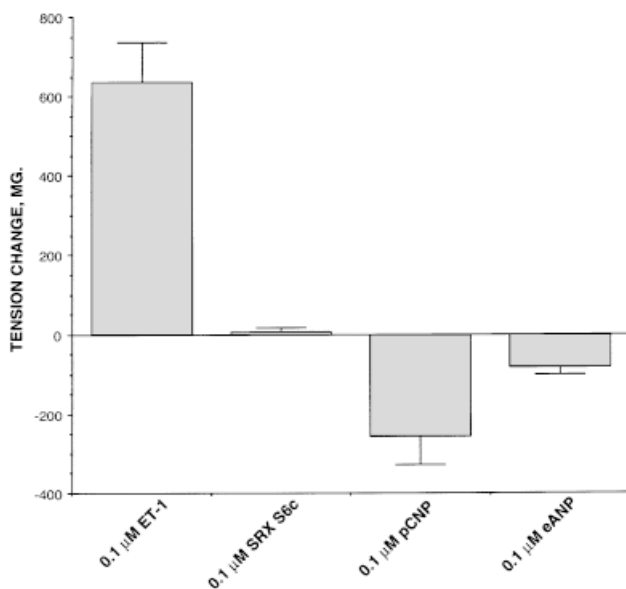


Fig. 9. Summary histogram of the comparative effects of ET-1 vs. SRX S6c and pCNP vs. eANP on paired aortic rings from the lamprey. $N = 4$, and all responses are significantly different from zero ($P < 0.05$) except for SRX S6c.

linergic receptor expressed in the eel (or hagfish), but our earlier data have demonstrated that an M_3 -type muscarinic receptor is expressed in the ventral aorta of *S. acanthias* (Evans and Gunderson, '98a). The site of secretion of acetylcholine is unclear, however, since cholinergic nerves have not been described in the ventral aorta of any fish species, but they may be present in the gills of teleosts. There is no evidence for cholinergic innervation of the systemic vasculature of any fish (e.g., Donald, '98). Thus, the actual function of cholinergic receptors in non-gill vasculature in most groups of fishes remains a mystery.

The vascular response to endothelin (Figs. 2–9) appears to be universal in all the fish groups examined so far (i.e., teleosts, elasmobranchs, agnatha; See Introduction). Indeed, ET appears to be the most effective vasoconstrictive agent in the lower vertebrates, just as it is in mammals (e.g., Miyauchi and Masaki, '99). In mammals, the dominant receptor appears to be the ET_A -type in arterial vessels (e.g., Levin, '96), but the ET_B receptor mediates constriction in veins and also some arterial preparations (e.g., Moreland et al., '92; Seo et al., '94; Teerlink et al., '94; White et al., '94). The ventral aorta (and gill) of the spiny dogfish expresses an ET_B -type receptor (Evans et al., '96; Evans and Gunderson, '99), but the lack of response to the ET_B -specific agonist SRX S6c in either the eel or lamprey aortic rings suggests that an ET_A -type receptor may predominate in these receptors in fish aortae is species-specific or clade-specific (i.e., elasmobranchs vs. teleosts and species. One might wonder if expression of ET_B vs. ET_A agnathans). Further data may answer that question. Presumably, in vivo, fish ET is secreted from endothelial cells, as it is in mammals, although it has now been shown to be secreted by a variety of tissues including the central nervous system, macrophages, and parathyroid in humans (e.g., Stjernquist, '98). However, ET is considered to act as a paracrine in most cases, signaling only nearby cells. Immunoreactive ET (antibodies raised against the mammalian peptide) has been localized in both teleost and elasmobranch gills (Zaccone et al., '96), as well as the central nervous system of an agnathan *Lampetra japonica* and a teleost *Oryzias latipes* (Kasuya et al., '91). In fact, ET-IR has been described in molluscs, insects, and the protochordate *Ciona intestinalis*, suggesting a very ancient origin of this polypeptide family (Kasuya et al., '91).

Our data confirm earlier studies (see Introduction) that the NO donor sodium nitroprusside and

NO itself are dilatatory when applied to a teleost (in this case, eel) ventral aortic ring (Figs. 1, 3). Unfortunately, only two congeneric eel species and a single salmonid (rainbow trout) have been tested to date, but it seems likely that teleosts in general express the nitric oxide-based, endothelium-derived relaxing system. However, the role of endothelium-derived NO in vascular hemodynamics in fishes has been questioned recently because many of the classical hallmarks (e.g., endothelium-dependent dilation produced by ACh) are missing; indeed, some authors have suggested that prostaglandins are the dominant EDRF in fishes (Olson and Villa, '91; Farrell and Johansen, '95; Evans and Gunderson, '98b). Our data also corroborate our earlier finding that NO produced constriction in two arteries and a vein of *S. acanthias* (Evans and Gunderson, '98b; Evans, 2001). In the present case, both the hagfish and lamprey aortic rings respond to SNP or NO by constricting, but the lamprey vessel subsequently dilated (Figs. 7, 8). Whether or not this constrictory response to NO is characteristic of vertebrates that have evolved before the teleosts remains to be determined by studies of other species, but it is clear that this response is not an anomaly. We have no ready explanation for the mechanism of NO-induced constriction in these vessels, but it is possible that it is mediated by NO interaction with superoxide to produce the very reactive peroxynitrite ion (e.g., Beckman and Koppenol, '96). In fact, our unpublished studies have demonstrated that 50% of the NO-induced constriction of the shark aortic ring can be inhibited by pretreatment with the superoxide dismutase mimetic Tempol, which would reduce the intracellular concentrations of superoxide (and thereby peroxynitrite; (e.g., Nilsson et al., '89). Nitric oxide synthase (which mediates the production of NO from L-arginine) has been localized (via histochemical and immunohistochemical techniques) in the central and peripheral nervous system of a variety of teleost, elasmobranch, and even agnathan fishes (e.g., Schober et al., '94; Bruning et al., '96; Funakoshi et al., '97; Holmqvist and Ekstrom, '97; Karila et al., '97; Cioni et al., '98), and we have recently cloned a 336-bp fragment of a NOS from gill tissue from *S. acanthias* (Evans, Farmerie, Holland, unpublished; GenBank Accession No. AF232227), which is >90% identical with human and rat neuronal NOS.

In the current study, responses of eel aortic rings to natriuretic peptides (pCNP and eANP; applied after ET-1 induced constriction) were measurable,

but marginally statistically significant. Other physiological and molecular studies suggest that natriuretic peptide receptors are present in the vascular smooth muscle of this genus, as well as other teleost genera (see Introduction). Importantly, our data (Fig. 3) suggest that both NPR-A and NPR-B are present since ANP has the highest affinity for NPR-A receptors, while CNP is the best agonist for NPR-B receptors (e.g., Takei, 2000). Our earlier studies had determined that both ANP and CNP could dilate the aortic ring of *S. acanthias*, but that CNP was effective at much lower doses, suggesting a predominance of NPR-B in elasmobranchs (Evans et al., '93a). Our finding that hANP dilated the hagfish aortic rings confirms our earlier studies, using rat ANP (Evans, '91). Our earlier work demonstrated that the hagfish aortic rings also responded to shark, killifish, and porcine CNP (Evans et al., '93b); thus, it appears that both NP receptors are present in hagfish vascular smooth muscle. This seems to be the case in the lamprey also, because its aortic ring responded to both agonists (Fig. 9). Thus, it appears that some form of NP receptor (possibly both NPR-A and NPR-B) is expressed in the vascular smooth muscle of fishes in general. In fact, it appears that gill tissue from *O. mykiss*, *O. beta*, *S. acanthias*, and *M. glutinosa* and *Geotria australis* (pouched lamprey) also expresses the so-called clearance receptor (NPR-C; not linked to guanylyl cyclase) for natriuretic peptides (Olson and Duff, '93; Donald et al., '94, '97, '99; Toop et al., '95, '98). Natriuretic peptides have been isolated from hearts and brain of the teleosts *A. japonica*, *Fundulus heteroclitus*, and *O. mykiss* as well as the elasmobranchs *S. acanthias*, *Triakis scyllia*, and *Scyliorhinus canicula* (Takei et al., '89; Price et al., '90; Takei et al., '90; Schofield et al., '91; Suzuki et al., '91; Takei et al., '91; Suzuki et al., '92; Takei et al., '94), and various immunological studies have localized NPs in brain and heart of *O. beta*, *S. acanthias*, and *M. glutinosa* (Donald and Evans, '92; Donald et al., '92). So it is clear that the natriuretic peptide system is expressed and important in all the major fish clades. A putative role for NPs in fish cardiovascular and osmoregulatory physiology is logical (Evans and Takei, '92; Evans, '95), but it has recently been suggested that the major role may be in protecting the fish heart from the effects of increased venous return (preload) and concomitant afterload, if aortic and gill resistance is not decreased (Farrell and Olson, 2000).

Prostaglandins (PGE₁, PGE₂, and the stable PGI₂

agonist CPR) dilated the aortic rings of all three species (Figs. 1, 4–7, 9), as they did in two elasmobranchs, *S. acanthias* (Evans and Gunderson, '98b) and *S. stellaris* (Piomelli et al., '85). Interestingly, Piomelli et al. (1985) described constriction of aortic rings from *C. conger* when PGE₂ was applied, but it is clear that this prostaglandin dilates the ventral aorta from the teleost, *A. rostrata* (Fig. 1), as well as the coronary artery of another, *O. mykiss* (Farrell and Johansen, '95). The basis for this discrepancy is unknown; nevertheless, it is apparent that PG receptors are present in blood vessels from all three fish clades. Characterization of the specific receptor involved in these responses is difficult from these experiments, and indeed, difficult in most cases because of the substantial cross-reactivity of both PGEs and PGI₂ on their respective receptors, termed IP and EP (e.g., Narumiya et al., '99). Prostaglandins are synthesized in the blood, heart, gills, kidney, and urinary bladder of a variety of teleosts and elasmobranchs (Herman et al., '84; Srivastava and Mustafa, '84; Herman, '90; Brown et al., '91; Mustafa and Jensen, '92; Knight et al., '95). We have already referred to the suggestion that PGs, not NO, are the dominant EDRF in fishes (see above).

In summary, these data confirm and extend the hypothesis that many of the vasoactive signaling systems that are expressed in mammals are present in the piscine vertebrates and therefore evolved nearly 500 million years ago. Specifically, it is now clear that vascular receptors for acetylcholine, endothelin, nitric oxide, natriuretic peptides, and prostaglandins are present in the earliest vertebrates, the Agnatha. However, delineation of specific receptor subtypes in various species is only possible if comparative concentration-response curves, radioreceptor analyses, or cloning protocols are undertaken for a given tissue. The specific roles of these putative receptors in fish cardiovascular regulation and osmoregulation are unclear at present, but should be a focus of future research.

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