Vascular bed-specific endothelium-dependent vasomotor relaxation in the hagfish, *Myxine glutinosa*

Jun Feng,1,2* Kiichiro Yano,1,3* Rita Monahan-Earley,1,4 Ellen S. Morgan,1,4 Ann M. Dvorak,1,4 Frank W. Sellke,1,2 and William C. Aird1,3,5

1The Center for Vascular Biology Research, 2Department of Surgery, 3Division of Molecular and Vascular Medicine, and 4Department of Pathology, Beth Israel Deaconess Medical Center, Boston, Massachusetts; and 5Mount Desert Island Biological Laboratory, Salisbury Cove, Maine

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Feng J, Yano K, Monahan-Earley R, Morgan ES, Dvorak AM, Sellke FW, Aird WC. Vascular bed-specific endothelium-dependent vasomotor relaxation in the hagfish, *Myxine glutinosa*. Am J Physiol Regul Integr Comp Physiol 293: R894–R900, 2007. First published May 30, 2007; doi:10.1152/ajpregu.00080.2007.—The last common ancestor of hagfish and gnathostomes was also the last common ancestor of all extant vertebrates that lived some time more than 500 million years ago. Features that are shared between hagfish and gnathostomes can be inferred to have already been present in this ancestral vertebrate. We recently reported that hagfish endothelium displays phenotypic heterogeneity in ultrastructure, lectin binding, and mechanisms of leukocyte adhesion. Thus, phenotypic cell heterogeneity evolved as an early feature of the endothelium. In the present study, we wanted to extend these observations by determining whether hagfish endothelium plays a role in mediating vasomotor tone. Response of mesenteric and skeletal muscle arteries to a variety of mediators was assessed by videomicroscopy. Phenylephrine and acetylcholine induced vasoconstriction of mesenteric and skeletal muscle arteries. Bradykinin (BK) and ADP promoted vasorelaxation in precontracted mesenteric arteries but not those from skeletal muscle. BK- and ADP-mediated vasorelaxation of the mesenteric artery was abrogated by mechanical denudation of the endothelium but was unaffected by Nω-nitro-ω-arginine methyl ester. Indomethacin significantly inhibited the vasodilatory response to ADP but not BK. The nitric oxide donor sodium nitroprusside resulted in endothelium-independent relaxation of both mesenteric and skeletal muscle arteries. Together, these data suggest that site-specific endothelium-dependent vasorelaxation is an evolutionarily conserved property of this cell lineage.

endothelium; hagfish; vasomotor tone

ENDOTHELIAL CELLS PARTICIPATE in many physiological processes, including the control of hemostasis, leukocyte trafficking, and vasomotor tone. The endothelium displays remarkable heterogeneity in structure and function. Proximate causes of the endothelial cell heterogeneity include spatial and temporal differences in the net signal input as well as site-specific epigenetic modification of endothelial cell DNA (reviewed in Ref. 1). In addition to proximate mechanisms, every biological trait, including the phenomenon of endothelial cell heterogeneity, requires an evolutionary explanation that describes the phylogeny of the trait and the fitness advantage that the trait provides at the species level.

* J. Feng and K. Yano contributed equally to this work.

Address for reprint requests and other correspondence: W. C. Aird, Beth Israel Deaconess Medical Center, Molecular and Vascular Medicine; RW-663, 330 Brookline Ave., Boston MA 02215, (e-mail: waird@bidmc.harvard.edu).

Endothelium, as defined by an abluminal continuous mesoderm-derived layer of cells interconnected by specialized junctional complexes is present in all vertebrates yet absent in other phyla. Thus, the endothelium arose in a common ancestor to vertebrates following the divergence of tunicates some 500–540 million years ago (8). Since the last common ancestor of hagfish and gnathostomes was also the last common ancestor of all extant vertebrates, features of endothelial cells that are shared between hagfish and gnathostomes can be inferred to have already been present in this ancestral vertebrate.

We recently reported that hagfish endothelium is heterogeneous in ultrastructure and lectin binding (41). In addition, histamine-mediated leukocyte adhesion in dermal microvessels occurred primarily in venules and capillaries (41). Together, these data suggest that phenotypic heterogeneity evolved as an early feature of the endothelium.

In mammals, endothelial cells synthesize many vasoconstricting and vasodilatory molecules. Indeed, the balance of endothelial-derived vasomotor mediators is an important determinant of vasomotor tone (2, 19, 40). There is less information concerning the role for endothelium in mediating vasoconstriction or vasodilation in nonmammalian vertebrates. Several endothelial-derived vasomotor substances found in mammals have been implicated in fish, including prostaglandins, nonprostanoid endothelium-dependent relaxing factors, and vasoconstrictors (12, 13, 20, 21, 26, 30, 37). In the current study, we asked whether the endothelium plays a role in mediating vasomotor tone in hagfish, and if so, whether this function is differentially regulated across the vascular tree.

MATERIALS AND METHODS

Experimental animals. Atlantic hagfish, *Myxine glutinosa* were purchased from a commercial supplier who caught the specimens in the Bay of Fundy, Canada. Hagfish were maintained in the dark in circular tanks with running sea water at 10 ± 2°C. The animals were anesthetized in sea water containing tricaine methanesulfonate (MS 222; 1:2,500 wt/vol). Vessel segments were dissected out as described in *In vitro assessment of vasomotor tone*. The protocol was approved by the Animal Care and Use Committees at the Mount Desert Island Biological Laboratory and the Beth Israel Deaconess Medical Center.

Light and electron microscopy. For light microscopy, tissues were removed and either snap-frozen in liquid nitrogen or fixed in 10% formaldehyde, dehydrated in alcohol, and embedded in paraffin. For electron microscopy (EM), standard fixation, dehydration, and embedding were carried out as previously described (10). One-microme-
ter plastic sections were stained with alkaline Geimsa stain for viewing by light microscopy and 70- to 80-nm sections were stained with lead citrate and examined by EM in Philips 400 and CM10 electron microscopes by three different microscopists.

In vitro assessment of vasomotor tone. Microvessel studies were performed by in vitro organ bath videomicroscopy as described previously (3). Arteries were dissected from the mesentery (150–250 µm internal diameters) and parietal muscle (100–180 µm internal diameters) under a ×10–60 magnification microscope (Olympus Optical, Tokyo, Japan). Microvessels were placed in a microvessel chamber, cannulated with dual glass micropipettes measuring 40–80 µm in diameter, and secured with 10-0 nylon monofilament sutures (Ethicon, Somerville, NJ). Modified hagfish HEPES-buffered saline (HHBS) (pH 7.8) (474 mM NaCl, 8.05 KCl, 3.04 mM MgSO4 × 7 H2O, 5.1 mM CaCl2 × 2 H2O, 9 mM MgCl2 × 6 H2O, 5.55 mM glucose, 3.1 mM HEPES acid, and 7 mM HEPES Na+ salt) was cooled to 15°C with a Brinkmann RM6 Refrigerator-Circulating Bath (Brinkmann Instruments, Lauda, Germany) and continuously circulated through the microvessel chamber with a Masterflex roller pump (Cole Parmer Instrument, Chicago, IL). The vessels were pressurized to 3 mmHg in a no-flow state by using a burette manometer filled with Parmer Instrument, Chicago, IL). The vessels were pressurized to 3 mmHg in a no-flow state by using a burette manometer filled with HEPES buffer. With the use of an inverted microscope (×40–200 magnification, Olympus CK2; Olympus Optical) connected to a video camera, the vessel image was projected onto a television monitor. An electronic dimension analyzer (Living System Instrumentation, Burlington, VT) was used to measure the internal lumen diameter and wall thickness. Vessels were allowed to bathe in the organ chamber for at least 1 h prior to a pharmacologic intervention.

Experimental protocols. After equilibration in the organ chamber, hagfish arteries were incubated with either ACh (10^{-10}–10^{-5} M) or phenylephrine (Phe) (10^{-5} M) and washed three times with cold HHBS for 15–20 min (bubbled with room air). In other experiments, vessels were pretreated with the anticholinergic inhibitor atropine (10^{-5} M) or the α-adrenergic blocking agent prazosin (10^{-5} M) prior to perfusion with ACh or Phe, respectively. The small arteries did not develop significant tone spontaneously. Thus, in vasorelaxation studies, vessels were precontracted to a specified degree of 30–50% with ACh (10^{-7} M). Once steady-state tone was reached, vessels were exposed to varying doses of ADP (10^{-9} to 10^{-4} M), bradykinin (BK) (10^{-12} to 10^{-7} M), or sodium nitroprusside (SNP) (10^{-9} to 10^{-4} M). One to four interventions were performed on each vessel. The vessels were washed three times with cold HHBS 15–20 min and allowed to equilibrate in drug-free buffer for at least 30 min between interventions. In some cases, vessels were exposed to N\(^{G}\) nitro-L-arginine methyl ester (l-NAME) (10^{-5} or 10^{-4} M) or indomethacin (10^{-6} M) for 20–30 min prior to perfusion with ADP and BK. Endothelial denudation was carried out by advancing a human hair (~60 µm in diameter) into the lumen and gently abrading the luminal surface. Removal of endothelium was verified by transmission EM.

![Fig. 1. Morphology of the hagfish mesenteric arteries.](image-url)

**A:** whole mount image of the aorta, kidney, intestine, and skeletal muscle of the hagfish. The kidneys run along the length of the aorta (caudal end of the fish is to the right). The intestine has been lifted and “rolled” off the aorta. **B:** higher-magnification image of boxed area in A shows aorta, kidney, mesenteric arteries (used for vasomotor studies), and mesenteric vein. **C:** alkaline Geimsa-stained 1-µm plastic section of the mesenteric artery. The lumen is filled with nucleated red blood cells. **D:** electron microscopy (EM) of mesenteric artery shows endothelium (top) and vascular smooth muscle cells (bottom). Endothelial and smooth muscle cell layers are separated by a thick layer of extracellular matrix. **E:** higher-magnification EM of mesenteric artery reveals highly vesicular endothelial cells with caveolae and many cytoplasmic tubules. The well-developed junctional complexes along the lateral endothelial cell borders are seen on the right. **F:** EM of denuded mesenteric artery shows loss of endothelial surface and preservation of extracellular matrix. Underling smooth muscle cell demonstrates vacuolization.
Drugs. ACh, Phe, atropine, prazosin, ADP, BK, SNP, L-NAME, and indomethacin were obtained from Sigma (St. Louis, MO). All chemicals were dissolved in ultrapure distilled water. All stock solutions were stored at \( -20^\circ C \), and all dilutions were prepared daily.

Data analysis. Values are shown as means \( \pm \) SE. Repeated-measures ANOVA and Student’s \( t \)-test were used to compare the variables between vessels. The treatment effects were statistically examined by paired or independent two-tailed Student’s \( t \)-test. Statistical significance was taken at a probability value of \( < 0.05 \). The concentration effects (EC\(_{50}\)) were made using nonlinear regression (sigmoidal dose response).

RESULTS

Morphology of mesenteric and skeletal muscle arteries. A pair of arteries originate from the abdominal aorta at each myotomal segment, with each artery supplying a corresponding side of the intestinal wall (38). After penetrating the intestinal wall, these segmental arteries encircle the intestine and ultimately branch toward the lumen of the gut. Terminal arterioles give rise to a capillary network in the mucosa, which is responsible for absorption of nutrients. We isolated the artery originating from the aorta (Figs. 1, A and B). Light microscopy of alkaline Geimsa-stained 1-\( \mu \)m plastic sections revealed an endothelial lining and a 1- to 2-layer thick smooth muscle cell coat (Fig. 1C). Ultrastructurally, the endothelium (Fig. 1D) contained many tubular structures and caveolae (Fig. 1E) and well-developed junctional complexes (Fig. 1E).

Underlying the endothelium was a thick band of extracellular matrix (Figs. 1, D and E) followed by a layer of smooth muscle cells (Fig. 1D). Gentle scraping of the inside of the blood vessel with a hair shaft resulted in denudation of most of the endothelial surface as determined by histochemistry (data not shown) and EM (Fig. 1F). Of note, the denudation procedure also altered the morphology of the smooth muscle cells, as evidenced by increased vacuolization (Fig. 1F).

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from the dorsal or ventral aorta (16). The artery penetrates the muscle mass, eventually branching into side branches and capillaries. We isolated the artery originating from the dorsal aorta (Fig. 2A). Light microscopy and EM revealed similar morphology to that of the mesenteric artery (Figs. 2, B–D).

**DISCUSSION**

Our finding that ACh induces vasoconstriction of hagfish mesenteric and skeletal muscle arteries (Fig. 1, A and B) is consistent with previous studies in hagfish, elasmobranchs, and teleosts. For example, ACh was shown to induce contraction of isolated ventral aortas from M. glutinosa (32); anterior mesenteric artery and posterior intestinal vein rings from the dogfish shark (Squalus acanthias) (11); cross-sectional aortic rings from American eel (Anguilla rostrata); unstimulated and precontracted isolated ventral aortas, celiacomesenteric arteries, and cardinal veins from the steelhead trout (Salmo gairdneri) (30); ventral aortas and celiacomesenteric arteries from the Atlantic salmon (Salmo salar) (36); and coronary arteries from the rainbow trout (Oncorhynchus mykiss) (35). An interesting exception is the Atlantic cod (Gadus morhua) in which ACh failed to induce vasoconstriction of the ventral or dorsal aortas (36). A previous study in O. mykiss demonstrated that ACh activates nicotinic cholinergic receptors on chromaffin cells, leading to the release of catecholamines (27). Collectively, these data suggest that in most fishes ACh does not interact with endothelial cells to induce release of endothelium-derived relaxing factors, but rather interacts with other cell types to promote cholinergic constriction.
Previous studies in teleosts have shown that NO or NO donors result in vasorelaxation (14, 20, 30, 35). For example, the NO and/or the NO donor SNP dilated vessels in *O. mykiss*, European eel (*Anguilla anguilla*) (34), American eel (*A. rostrata*) (14), Australian eel (*Anguilla australis*) (9, 20), and rainbow trout (*S. Gairdneri*) (30). In contrast to these results, studies in *A. anguilla* have demonstrated a soluble GC-dependent vasoconstricting effect of NO or NO donors in the branchial circulation (31). Moreover, NO and/or NO donors resulted in vasoconstriction of the ventral aorta, anterior mesenteric arteries, the posterior intestinal vein of *S. acanthias* (11, 13), and constriction of aortic rings from the hagfish and lamprey (14). The mechanism underlying NO-induced vasoconstriction in these species is unknown. Interestingly, Evans and Harrie (14) reported that 50% of the NO-mediated vasocostriction of shark aortic rings was inhibited by pretreatment with the superoxide dismutase mimetic Tempol, raising the possibility that NO interacts with superoxide to form the highly reactive peroxynitrite. In contrast to the results of the latter study, we have shown that SNP reproducibly induces endothelium-independent vasorelaxation of both mesenteric and skeletal muscle arteries in hagfish (Fig. 5, A and B), suggesting that the NO target enzyme guanylate cyclase is present in both artery types. The discordance in results between the two studies may relate to differences in study design. For example, in the previous report, the effects of SNP were examined in an isolated ring preparation of the aorta following precontraction with endothelin-1 (13), whereas our study employed organ bath videomicroscopy of nonprecontracted mesenteric and skeletal muscle arteries.

While many investigations, including our own, suggest that NO is capable of mediating vasodilation of systemic vessels in fish, the source of NO is unclear. Currently, there is strong evidence that endothelium of amphibia, reptiles, birds, and...
mammals is capable of generating endothelial-derived NO (18, 22, 33). However, the question of whether fish endothelium generates NO is controversial. Vascular bed perfusion models provide support for the existence of endothelial NO. For example, l-arginine dilated intact coronary vasculature and isolated coronary arteries of O. mykiss (28, 29). Adenosine-mediated vasorelaxation of perfused trout hearts was partially inhibited by N\textsuperscript{G}-nitro-l-arginine (l-NA) (28). Moreover, perfusion of the intact coronary system with adenosine or l-Arg caused increased release of nitrite into the perfusate, an effect that was inhibited by pretreatment with l-NA (28). Chemical disruption of the endothelium resulted in attenuation of the vasodilatory effect of l-Arg and adenosine (28). Immunohistochemical approaches have yielded conflicting results with some studies supporting the presence of endothelial NO synthase (eNOS) in the circulatory system (17, 24, 39) and other studies failing to identify such activity (9, 20). In contrast to the perfusion model and the histochemical analyses, studies of isolated blood vessels from fish have consistently failed to identify an endothelial source of NO. NOS has been localized in the central and peripheral nervous system of fish (14) and some studies supporting the presence of endothelial NO synthesis (9). NOS isolated from the gill tissue of S. acanthias (15). BK had no effect on the coronary arteries, ventral aorta, or celiacomesenteric of rainbow trout (30, 35). Intra-arterial injection of BK in S. gairdneri failed to elicit a depressor effect (23). In vivo injection of BK into the eel or trout results in increased blood pressure (5, 23). In the current study, BK induced vasorelaxation of the mesenteric arterioles (Fig. 4B). The latter effect was dependent on the presence of endothelium (Fig. 4D) but was not inhibited by l-NAME or indomethacin (Fig. 5D). Although the use of human BK to test vasomotor response in hagfish arteries may not precisely reflect the extent of BK-mediated vasodilation, the current data suggest the presence of a nonprostanoid endothelium-dependent relaxing factor(s) in the mesenteric microcirculation of hagfish.

Importantly, hagfish arteries demonstrated vascular bed-specific vasomotor responses. For example, BK and ADP resulted in endothelium-dependent vasorelaxation of mesenteric but not skeletal muscle arteries (Fig. 4). These findings may be explained by the differential expression of BK and ADP receptors and/or site-specific differences in downstream signaling pathways. The gill afferent arteries were conspicuously insensitive to all agonists tested (data not shown). Site-specific vasomotor reactivity has been reported in other fish. For example, ET-1 has a more profound vasoconstricting effect on the posterior intestinal vein compared with the mesenteric artery of S. acanthias, whereas ACh produces more vasoconstriction of the artery (11). In O. mykiss, the vasoconstricting effect of angiotensin was greater in isolated unstimulated coronary arteries compared with celiacomesenteric artery, gill arch epibranchial artery, ventral aorta, or anterior cardinal vein (6). In another study in O. mykiss, angiotensin had a greater vasoconstricting effect than epinephrine on posterior cardinal vein, whereas the opposite was true with the anterior cardinal vein (7).

Our data are the first to unequivocally demonstrate vessel-specific diversity in endothelium dependent vasomotor tone in hagfish. The findings point to a hitherto unappreciated complexity in vasomotor regulation in these animals and suggest that similar functional heterogeneity of the endothelium was present in the common ancestral vertebrate.

GRANTS

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REFERENCES

ENDOTHELIUM-DEPENDENT VASOMOTOR TONE IN HAGFISH