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Review

Nitric oxide control of lower vertebrate blood vessels by vasomotor nerves☆

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Abstract

In mammals, much is understood about the endothelial and neural NO control mechanisms in the vasculature. In contrast, NO control of blood vessels in lower vertebrates is poorly understood, with the majority of research focusing on the presence of an endothelial NO system; however, its presence remains controversial. This study examined the mechanisms by which NO regulates the large blood vessels of non-mammalian vertebrates. In all species examined, the arteries and veins contained a plexus of NOS-positive perivascular nerves that included nerve bundles and fine, varicose nerve terminals. However, in the large arteries and veins of various species of fishes and amphibians, no anatomical evidence was found for endothelial NOS using both NADPH-diaphorase and eNOS immunohistochemistry. In contrast, perinuclear NOS staining was readily apparent in blue-tongue lizard, pigeon and rat, which suggested that eNOS first appeared in reptiles. Physiological analysis of NO signalling in the vascular smooth muscle of short-finned eel and cane toad could not find any evidence for endothelial NO signalling. In contrast, it appears that activation of the nitrergic vasomotor nerves is responsible for NO control of the blood vessels.

Keywords: Nitric oxide; Nitric oxide synthase; Nitrergic nerves; Blood vessel; Vasodilation; Autonomic nervous system

Contents

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1. Introduction

1.1. Nitric oxide control of mammalian blood vessels

During the 1970s, it was discovered that nitroso compounds such as nitroprusside and nitroglycerin stimulate the production of the second messenger, guanosine 3′:5′-cyclic monophosphate (cGMP), by activating an intracellular soluble guanylyl cyclase (GC) receptor (see Murad et al., 1978). This led to the proposal that the conversion of nitroso compounds to NO might be responsible for the vasodilatory effects elicited by nitroprusside and nitroglycerin. Accordingly, NO was found to induce a potent vasodilation via cGMP (Gruetter et al., 1979), but it remained to be determined if NO was an endogenous signalling molecule. In 1980, Furchgott and Zawadzki discovered the presence of a labile, endothelium-derived substance that caused relaxation of isolated blood vessels of rabbit (Furchgott and Zawadzki, 1980). They demonstrated that the relaxation of rabbit aorta by acetylcholine (ACh) required the presence of an intact endothelium, and that ACh stimulated the release of a substance from the endothelium that was termed endothelium-derived relaxing factor (EDRF). However, it was not until 1987 that Palmer et al. demonstrated that the EDRF is pharmacologically identical to NO (Palmer et al., 1987). Over the last twenty years, NO has emerged as one of the most important signalling molecules in the integration of biological control systems.

In cells, NO is generated by the enzyme nitric oxide synthase (NOS) of which there are three isoforms: brain or neuronal NOS (nNOS or NOS I); inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III) (Alderton et al., 2001). Both eNOS and nNOS have been broadly classified as forms of constitutive NOS because they are continuously expressed (Lowenstein et al., 1994). In addition, they are both activated by an increase in intracellular calcium that is facilitated by ligands that activate the calcium second messenger system (Moncada et al., 1991). Conversely, iNOS is expressed under various conditions and is not dependent on intracellular calcium signalling for activation (Schmidt et al., 1993). The location of NOS in cells can be readily determined by the use of NADPH-diaphorase (NADPH-d) histochemistry, and immunohistochemistry (IHC) using antibodies that are specific to the three mammalian NOS isoforms or an antibody that recognizes the constitutive forms of NOS. In mammalian vascular endothelium, eNOS is located in the caveolae associated with the plasma membrane and within the Golgi apparatus, which accounts for the punctate, perinuclear staining observed following NADPH-d histochemistry or endothelial NOS IHC (Andries et al., 1998; Goligorsky et al., 2002; O'Brien et al., 1995). Neuronal NOS occurs primarily in neurons of the central and peripheral nervous systems, and those neurons that synthesise and release NO are termed nitrergic nerves. In mammals, nitrergic nerves mainly innervate blood vessels in the cerebral, gut and pelvic regions, and in combination with the endothelium, nitrergic nerves provide a second means of NO control of vascular tone in specific blood vessels (Toda and Okamura, 2003).

In the cerebral and pelvic regions of mammals, it is now well-established that neuronal NOS is located in parasympathetic vasodilator nerves. Using nNOS IHC and/or NADPH-d histochemistry, NOS containing nerve cell bodies has been located in most of the neurons in the cranial parasympathetic ganglia, which have been shown to be the origin of the nitrergic nerves that innervate the cerebral arteries (Young et al., 2000). Neuronal NOS has also been located in a small population of sympathetic vasodilator nerves that innervate the arteries supplying skeletal muscle, and in subpopulations of sensory nerves in vessels such as the mesenteric artery (Young et al., 2000). Electrical nerve stimulation of mammalian cerebral blood vessels induces non-adrenergic, non-cholinergic (NANC) vasodilation that was abolished by tetrodotoxin (TTX). Furthermore, in most species the vasodilation was abolished by NOS inhibitors and the soluble GC inhibitor, oxadiazole quinoxalin-1 (ODQ), but was unaffected by the removal of the endothelium (see Toda and Okamura, 2003). Therefore, it was proposed that nerve stimulation directly activated the release of NO from nitrergic nerves and, in fact, NO appears to be the dominant neurotransmitter responsible for the nerve-mediated, endothelium-independent vasodilation (Young et al., 2000; Toda and Okamura, 2003). In addition to electrical nerve stimulation, the nicotinic receptor agonist, nicotine and ACh can mediate a NO vasodilation by stimulating perivascular nitrergic nerves (Toda and Okamura, 2003). The cellular mechanisms underpinning the nicotine-mediated vasodilation are different from electrical nerve stimulation since it is generally TTX-resistant (see Toda and Okamura, 2003).

1.2. Nitric oxide control of non-mammalian blood vessels

The role of NO in the control of vascular tone in non-mammalian vertebrates has received less attention than mammals. Studies in birds (Hasegawa and Nishimura, 1991), reptiles (Knight and Burnstock, 1993), and amphibians (Rumbaut et al., 1995; Knight and Burnstock, 1996) provided evidence that an endothelial NO system was present in the vasculature. In fish, there is conflicting evidence for endothelial NO signaling that is correlated with the type of approach used. In perfused vascular beds of various species of fish, vascular resistance was affected by the NO precursor, L-arginine, and inhibition of NOS, and it was concluded that NO was being generated by an eNOS (Hylland and Nilsson, 1995; Nilsson and Söderström, 1997; Mustafa et al., 1997; Mustafa and Agnisola, 1998). However, studies using isolated, vascular rings have provided convincing evidence that the EDRF is, in fact, a prostaglandin rather than NO (Olson and Villa, 1991;

Kågström and Holmgren, 1997; Park et al., 2000). In non-mammalian vertebrates, there is little information on NO control of the vasculature by nitrergic nerves. This paper will discuss recent data from our laboratory that provides evidence that nitrergic nerves provide NO control of the large arteries and veins of lower vertebrates.

2. Materials and methods

2.1. Animals

Giant shovelnose rays, *Rhinobatus typus*, of either sex and with a body weight of 300–500 g, and epaulette sharks, *Hemiscyllium ocellatum*, with a body weight of 500–700 g were seine-netted on Heron Island Reef, Great Barrier Reef, Australia. Collared sea bream, *Gymnocranius audleyi*, of either sex and with a body mass of 300–400 g were obtained by line-fishing off Heron Island Reef. The fish were held in a seawater aquarium at 24 °C. All animals were caught under permit approval according to the Great Barrier Reef Marine Park Regulations (permit number G99/474) and the General Fisheries permit (permit number PRM02030C). Prior to experimentation, the animals were sacrificed by a sharp blow to the head, and pithed. Common pigeons, *Columba livia*, with a body weight of 300–400 g, were purchased from a commercial supplier in Geelong and used on arrival at Deakin University. Prior to experimentation the pigeons were sacrificed by an overdose of carbon dioxide. Blue-tongue lizards, *Tiliqua scincoides*, with a body mass of 250–300 g were purchased from a commercial supplier in Geelong and used on arrival. Prior to experimentation, the animals were sacrificed by an intramuscular injection of ketamine HCl (100 μL/100 g), and then decapitated and pithed. American bullfrogs, *Rana catesbeiana*, with a body mass of 150–250 g, were obtained from a commercial supplier in Tokyo (Japan) and used on arrival at the University of Tokyo. Prior to experimentation, the animals were sacrificed by decapitation, followed by the pithing of the spinal cord. The collection, maintenance and sacrifice of Australian short-finned eels, *Anguilla australis*, cane toads, *Bufo marinus*, and Sprague Dawley rats, *Rattus norvegicus*, have been previously described (Jennings et al., 2004; Broughton and Donald, 2002, 2005).

2.2. In vitro organ bath physiology

Physiological experiments were only performed on blood vessels from shovelnose ray, short-finned eel and cane toad. After sacrifice, large arteries and veins were dissected free and placed in an appropriate physiological saline for the species (shovelnose ray, Donald et al., 2004; eel, Jennings et al., 2004; toad, Broughton and Donald, 2002, 2005). Individual rings of approximately 4–5 mm in length were mounted horizontally between two hooks for the measurement of isometric force production, and placed in an organ bath. The rings were bathed in 15 mL of physiological saline that had appropriate temperature control and aeration for the species (shovelnose ray, Donald et al., 2004; eel, Jennings et al., 2004; toad, Broughton and Donald, 2002, 2005). The force transducer (Grass-FT03) was linked to a PowerLab™ data collection system and an IBM compatible computer, which recorded data for further analysis. An initial tension of 0.5 g was applied to the blood vessels, and they were allowed to equilibrate for 30 min. In some experiments, the endothelium was removed by rubbing with a toothpick and the extent of removal was determined by haematoxylin and eosin staining of fixed blood vessels. Prior to administering various vasodilatory substances, each vessel was pre-constricted with endothelin-1 (ET-1, Auspep), and vasoconstriction was allowed to reach its maximum. The extent of vasodilation was determined for each vasodilator by scoring the degree of relaxation as a ratio by assigning a relaxation to pre-constriction levels as 100%. For experiments, matched controls were used from the same animal for comparison of drug effects. Data are expressed as mean ± one standard error (SE) of five or more experiments, and statistical analysis was performed with independent *t*-tests using the SPSS (10.0) statistical package; a p value ≤ 0.05 was considered significant.

2.3. NADPH diaphorase histochemistry and immunohistochemistry

A range of different type of blood vessels from the species listed above was dissected free and immersed in phosphate buffered saline (PBS, 10 mM phosphate buffer and 150 mM NaCl; pH 7.4) at 4 °C, and fixed for 2 h in 4% formaldehyde (pH 7.4) at 4 °C, and washed in 10 mM PBS (3×10 min). Whole mounts and vessel sections were processed for NADPH diaphorase histochemistry using standard protocols (see Broughton and Donald, 2002). The vessels were observed under a light microscope (Olympus) and were photographed with a digital colour system (Spot 35 Camera System). For IHC, vessels were fixed in 4% paraformaldehyde as whole mounts, washed in 10 mM PBS (3×10 min), incubated in DMSO (3×10 min) and washed in 10 mM PBS (5×2 min). The blood vessels were then incubated in either a polyclonal antibody raised against mouse endothelial NOS (1:1000; O'Brien et al., 1995) or a polyclonal antibody raised against sheep neuronal NOS (1:4000; Anderson et al., 1995) for 24 h at room temperature in a humid box. The following day, the vessels were washed in 10 mM PBS (3×10 min) and incubated in either a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1:200, for eNOS) or FITC-conjugated goat anti-sheep IgG (1:200, for nNOS) (Zymed Laboratories, San Francisco) for 3–4 h at room temperature in a humid box. The blood vessels were washed in 10 mM PBS (3×10 min) and mounted in buffered glycerol, and observed under

Table 1
Summary of the findings on the presence of NOS in blood vessels of various vertebrate species

	Endothelial NOS	Neuronal NOS
Elasmobranchs: shovelnose ray, epaulette shark	Not detected	Perivascular nerves Arteries and veins
Teleosts: bream, eel	Not detected	Perivascular nerves Arteries and veins
Amphibians: toad, bullfrog	Not detected	Perivascular nerves Arteries and veins
Reptiles: blue-tongue lizard	Perinuclear staining in Golgi of endothelial cells	Perivascular nerves Arteries and veins
Birds: pigeon	Perinuclear staining in Golgi of endothelial cells	Perivascular nerves Arteries and veins
Mammals: rat	Perinuclear staining in Golgi of endothelial cells	Not determined

a fluorescence microscope (Zeiss) using a FITC filter and photographed as above.

3. Results and discussion

3.1. Location of NOS proteins in non-mammalian blood vessels

NADPH-d histochemistry showed that NOS-positive perivascular nerves were present in the large arteries and veins of each species that were examined (Table 1; Fig. 1). Furthermore, nNOS IHC revealed a pattern of nNOS-immunoreactive nerves that were very similar to that observed using NADPH-d histochemistry (Fig. 1). In all blood vessels examined, nNOS was observed in nerve bundles and in fine varicose nerve fibres (Table 1; Fig. 1). In addition, nNOS-positive ganglion cells were observed in arteries and veins of shovelnose ray (Fig. 1A) and epaulette shark. Thus, it is probable that both methods are revealing a population of perivascular nerves that contain nNOS, which have the potential to mediate NO neurotransmission to the vascular smooth muscle. Previously, the presence of NOS in perivascular nerves of non-mammalian vertebrates has been reported in fishes (epaulette shark, Renshaw and Dyson, 1999; goldfish, Brüning et al., 1996; trout, Esteban et al., 1998; Jiménez et al., 2001) and in crocodiles (Kågström et al., 1998; Axelsson et al., 2001) and birds (Cuthbertson et al., 1997).

In contrast to showing nNOS in perivascular nerves, the anatomical demonstration of eNOS in the vascular endothelium was dependent on the vertebrate class being examined. Both NADPH-d histochemistry and eNOS IHC could not reveal any punctuate, perinuclear NOS staining in the vascular endothelial cells of arteries and veins of the piscine and amphibian species that were examined (Table 1; Fig. 2A,C,F); it was assumed that the mammalian eNOS antibody would cross-react with fish and amphibian eNOS if it is

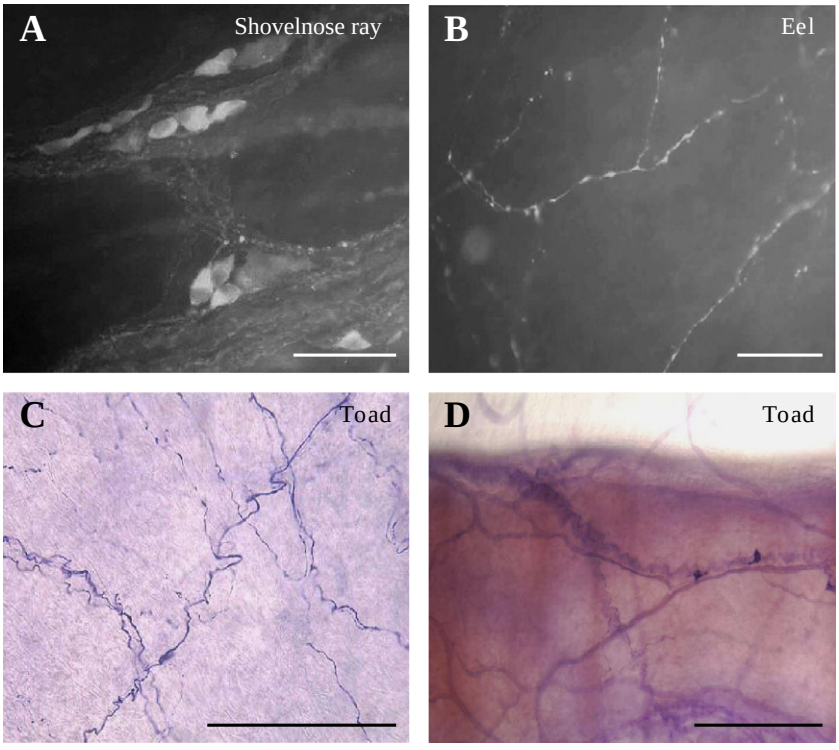

Fig. 1. Wholemount preparations of blood vessels prepared for nNOS IHC (A, B) and NADPH-d histochemistry (C, D). Perivascular, nitrergic nerves were present in the dorsal aorta of shovelnose ray (A), short-finned eel dorsal aorta (B), toad lateral aorta (C), and toad mesenteric arterioles (D). A, B bar = 40 μm; C, D bar = 100 μm.

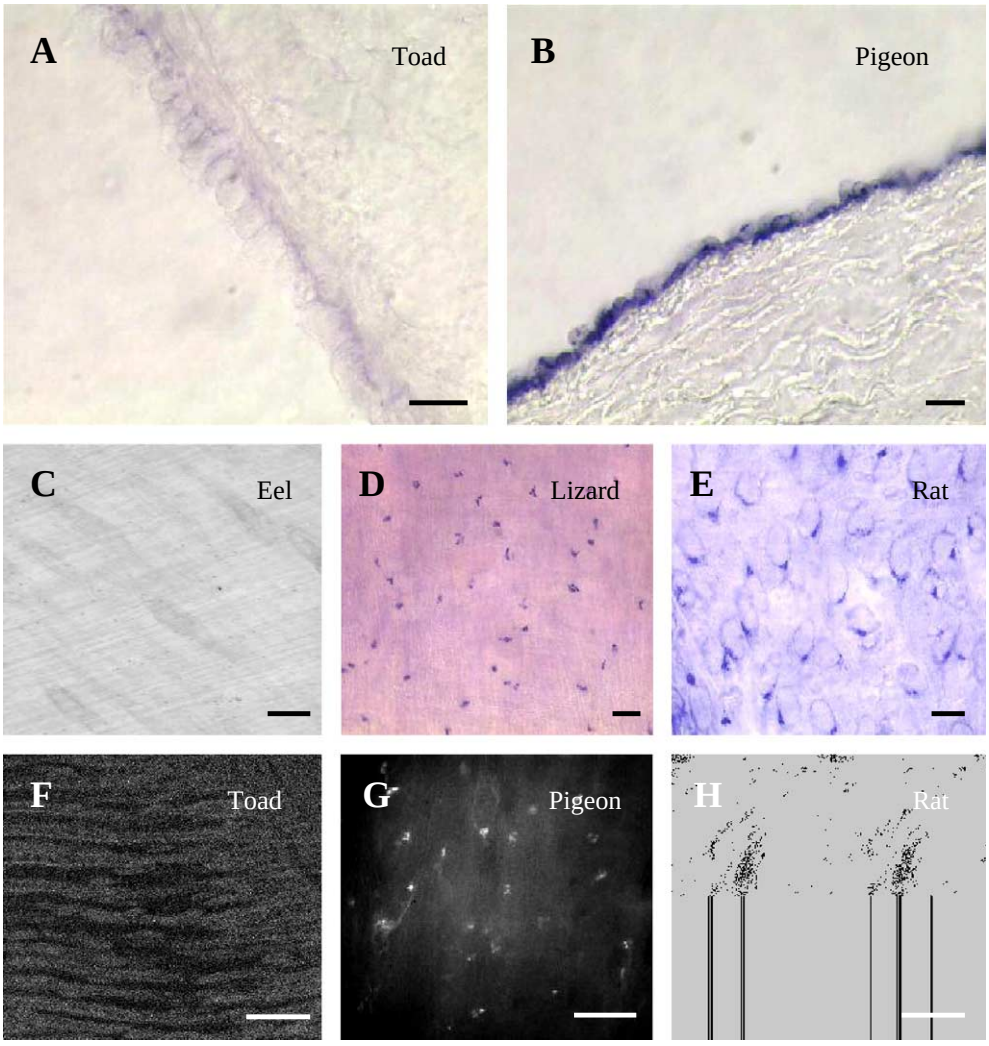

Fig. 2. Wholemount (C–H) and sectioned (A, B) preparations of blood vessels prepared for NADPH-d histochemistry (A–E) and eNOS IHC (F–H). Punctate, perinuclear staining typical of NOS was observed in the vascular endothelium of blue-tongue lizard (D), pigeon (B, G) and rat (E, H). In contrast, a similar pattern of NOS staining was not revealed in the vascular endothelium of toad (A, F) and short-finned eel (C). A, B bar=30 μm; C–H bar=10 μm.

present. In contrast to fish and amphibians, we found the typical NOS staining pattern in endothelial cells of blue-tongue lizard, pigeon and rat (Table 1; Fig. 2B,D,E,G,H). Therefore, it was concluded that eNOS may not be expressed in the vascular endothelial cells of fish and amphibians. This is in contrast to Fritsche et al. (2000) who showed eNOS immunoreactivity in the dorsal vein of the developing zebrafish. In addition, constitutive NOS immunoreactivity has been reported in the endocardial endothelium of the heart of the edible frog, *Rana esculenta* (Sys et al., 1997). Thus, the presence of eNOS in the cardiovascular system of fish and amphibians is equivocal and requires further research.

3.2. Physiology of NO control of blood vessels

In the shovelnose ray, no evidence was found for a NO signalling system. The NO donor, sodium nitroprusside (SNP, 10^{-4} M), did not mediate vasodilation of aortic rings preconstricted with ACh (10^{-5} M), which is consistent with previous studies that could not demonstrate NO vasodilation in the vascular smooth muscle in the dogfish, *Squalus acanthias* (Evans and Gunderson, 1998; Evans, 2000). Since nitrergic nerves were found in the dorsal aorta of shovelnose ray, nicotine was used to determine if activation of the nitrergic nerves affected the vascular smooth muscle. Nicotine (3×10^{-4} M) caused an endothelial-dependent vasodilation that was not affected by inhibition of NOS with L-NNA (N^{ω}-Nitro-L-arginine, 10^{-4} M), and which was antagonised by indomethacin suggesting that nicotine was stimulating the cyclooxygenase pathway (Donald et al., 2004). It is very intriguing that the blood vessels of shovelnose ray (and other elasmobranchs) appear to lack a signalling pathway for NO-mediated vasodilation, despite the presence of nitrergic nerves.

In contrast to elasmobranchs, the NO donor SNP (10^{-4} M) caused a potent vasodilation of the preconstricted dorsal aorta and intestinal vein of short-finned eel that was blocked by ODQ (10^{-5} M), suggesting that NO stimulates the

production of cGMP via a soluble GC. This observation is consistent with previous studies that have shown that the vascular smooth muscle of most teleost fish contains a NO receptor that mediates vasodilation (see Jennings et al., 2004 for refs). In contrast, Pellegrino et al. (2002) reported a cGMP-mediated vasoconstrictor effect of NO in the branchial circulation of the European eel, *Anguilla anguilla*, using the NO donors, SNP and SIN-1, at a range of concentrations.

In teleost fish, the source of NO that mediates vascular regulation is controversial. In mammals, ACh has been used as a pharmacological tool to indirectly stimulate the production of NO via eNOS (Moncada et al., 1991); however, it is clear that ACh generally causes vasoconstriction in peripheral blood vessels of fish. In short-finned eel, applied ACh over a range of concentrations always caused vasoconstriction of the dorsal aorta and intestinal vein, even after the vessels had been pre-constricted with ET-1 (10^{-8} M). However, application of nicotine (3×10^{-4} M) induced an endothelium-independent vasodilation in both vessels (Fig. 3), which was reproducible in the same preparation and blocked by ODQ (10^{-5} M, Fig. 3) and L-NNA (10^{-4} M, Fig. 3). In addition, the nicotine-mediated vasodilation was significantly reduced by N^{ω}-Propyl-L-arginine (PLA, 10^{-5} M), a more specific inhibitor of nNOS. Hence, it is likely that nicotine is specifically activating nitrergic nerves as it does in many mammalian blood vessels (Toda and Okamura, 2003), since there was no evidence for the release of non-nitrergic

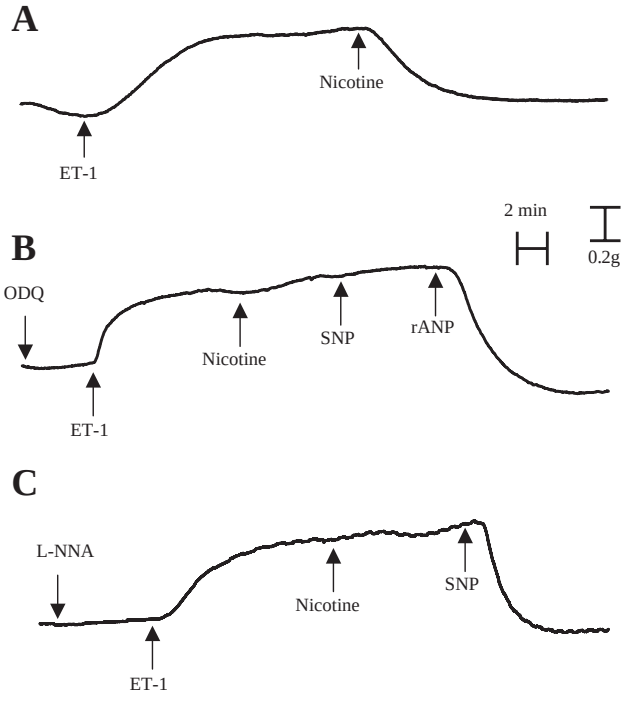

Fig. 3. Tension recordings from the intestinal vein of *A. australis* showing the vasodilatory effect of nicotine (A) and its effect in the presence of the soluble guanylyl cyclase (GC) inhibitor, ODQ (B) and the NOS inhibitor, L-NNA (C). Vessels were pre-incubated with ODQ (10^{-5} M) or L-NNA (10^{-4} M) for approximately 10 min prior to being constricted with endothelin-1 (ET-1; 10^{-8} M). No response was observed following the addition of nicotine (3×10^{-4} M) or SNP (10^{-4} M) to vessels incubated with the soluble guanylyl cyclase inhibitor, ODQ. In contrast, the vessel dilated following the addition of rat atrial natriuretic peptide (rANP 10^{-8} M), which mediates its effect through a particulate GC. Following maximal constriction in the vessel preincubated with L-NNA, nicotine (3×10^{-4} M) was administered, but, no vasodilatory effect was observed. Following this, the NOS independent NO donor, SNP (10^{-4} M), was added to the vessels, resulting in a marked vasodilation. Similar results were observed in the dorsal aorta ($N=5$).

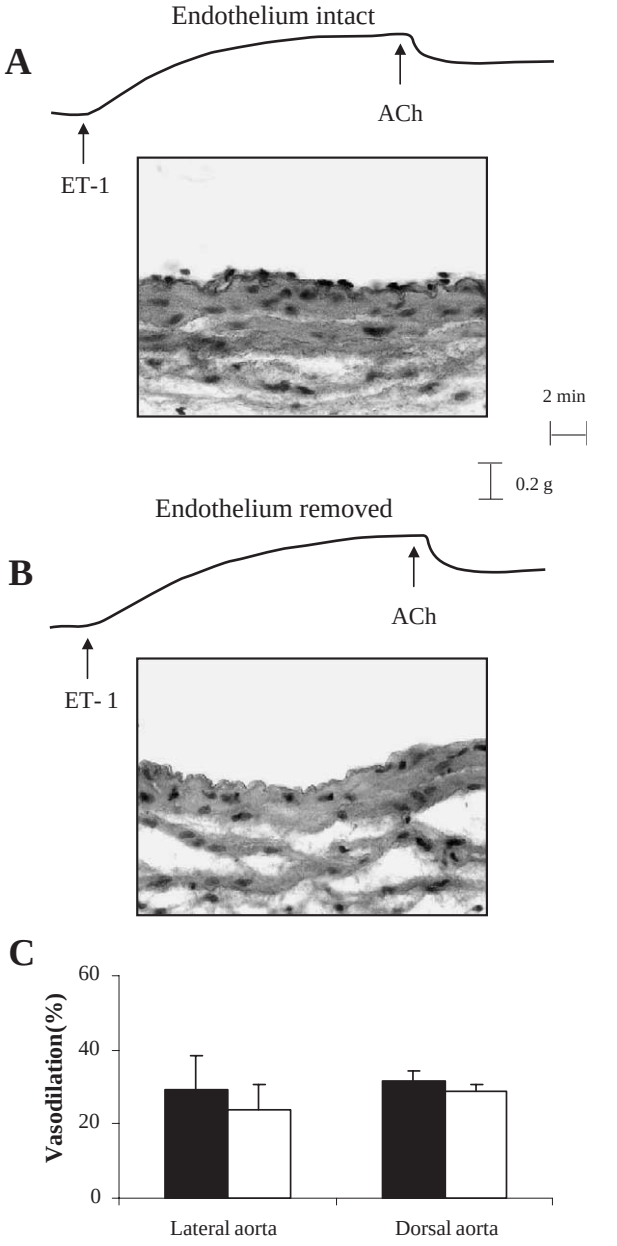

Fig. 4. Tension recordings showing the vasodilatory effect of ACh on the toad dorsal aorta with (A) or without (B) the endothelium. Haematoxylin and eosin staining was used to verify that the endothelium was present or removed (see inset). The preparations were exposed to ET-1 (10^{-8} M) until a maximum constriction was achieved and then ACh (10^{-5} M) was added. (C) Mean response of ACh on pre-constricted lateral aortae and dorsal aorta with (■) or without (□) the endothelium intact. Note that there is no significant difference in the ACh-mediated dilation (lateral aortae $P=0.26$, dorsal aorta $P=0.35$, $N=5$).

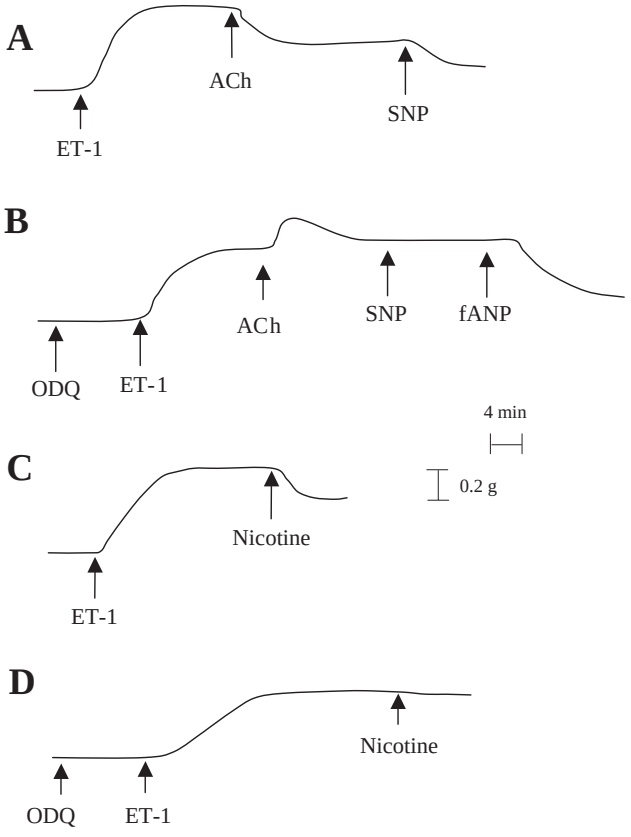

Fig. 5. Tension recordings showing the effect of ACh (A, B), SNP (A, B), frog ANP (B), and nicotine (C, D) on the toad lateral aorta with (B, D) or without (A, C) the soluble GC inhibitor, ODQ. The vessels were pre-treated with ODQ (10^{-5} M) for approximately 10 min before being constricted with ET-1 (10^{-8} M). At the peak of vasoconstriction, ACh (10^{-5} M) or nicotine (3×10^{-4} M) was administered. Acetylcholine caused a constriction in the preparation incubated with ODQ (B) and a vasodilation in the preparation without ODQ. Nicotine only caused a vasodilation in the preparation without ODQ. Sodium nitroprusside had no effect in the preparation with ODQ, but fANP induced a potent vasodilation (B; $N=5$).

neurotransmitters following NO blockade. In addition, the nicotine-mediated vasodilation was not affected by indomethacin (10^{-5} M), which indicated that prostaglandins are not involved in the response. Thus it was concluded that NO control of the dorsal aorta and the intestinal vein of short-finned eel is mediated by NO derived from perivascular, nitrergic nerves (Fig. 1B) rather than NO from the endothelium in which no NOS was demonstrated (Jennings et al., 2004). It was also shown in the short-finned eel that the endothelially mediated vasodilation induced by the calcium ionophore, A23187 (3×10^{-6} M), was significantly inhibited by indomethacin (10^{-5} M), which is indicative that the effect is mediated by cyclooxygenase-derived products (Jennings et al., 2004). This observation is consistent with previous studies examining endothelium-dependent vasodilation in teleost fish (see e.g. Olson and Villa, 1991).

In amphibians, the previous studies on vascular NO control focused on the presence of an endothelial NO system. In the American bullfrog, *R. catesbeiana*, Rumbaut et al. (1995) showed that capillary hydraulic conductivity was decreased when NOS was inhibited; hence, it was proposed that capillary endothelial cells synthesised and released NO. Further evidence for an endothelial NO system in amphibians was demonstrated in isolated aortic rings of the leopard frog, *R. pipiens* (Knight and Burnstock, 1996). These authors showed that an ACh-mediated vasodilation was abolished or greatly reduced by L-NAME (N^{ω}-Nitro-L-arginine methyl ester), or when the endothelium was removed. Since these responses were typical of mammalian blood vessels, it was concluded that amphibian blood vessels are regulated by an endothelial NO system.

In contrast to Knight and Burnstock (1996), we found that applied ACh (10^{-5} M) mediated a vasodilation in the large arteries of toad that was not dependent on the presence of an intact endothelium (Fig. 4), and was abolished in the presence of ODQ (10^{-5} M, Fig. 5) and L-NNA (10^{-4} M); the response was significantly reduced by L-N^5-(1-Imino-3-butenyl)-ornithine (vinyl-L-NIO, 10^{-4} M; a more specific

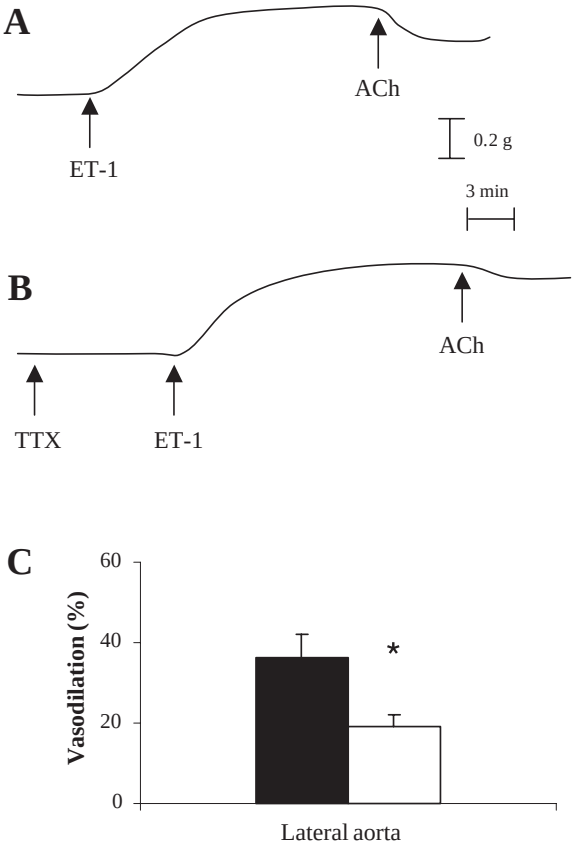

Fig. 6. Tension recordings showing the effect of ACh on the toad lateral aorta with (B) or without (A) the neurotoxin, TTX. The preparations were pre-treated with TTX (10^{-5} M) for approximately 10 min before being constricted with ET-1 (10^{-8} M). At the peak of vasoconstriction, ACh (10^{-5} M) was administered. The vasodilatory response was reduced in the presence of TTX. (C) Mean response of pre-constricted lateral aortae to ACh (10^{-5} M) with (□) or without (■) TTX (10^{-5} M). Note that TTX significantly reduced the ACh-mediated vasodilation ($P<0.05$, *denotes significant difference, $N=5$).

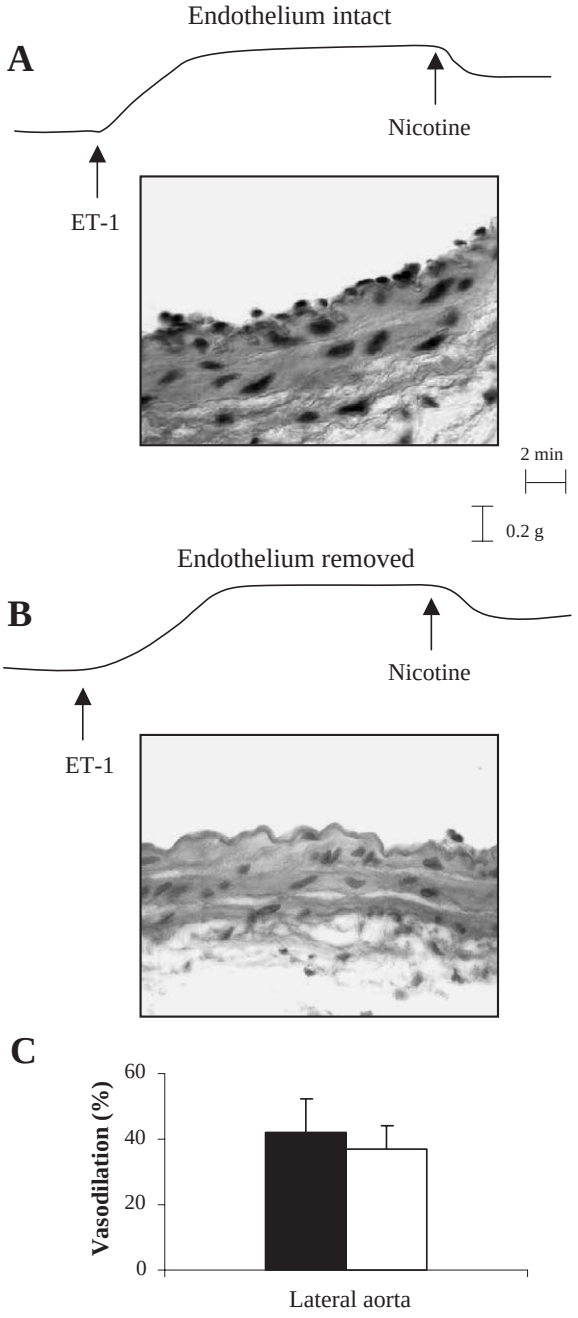

Fig. 7. Tension recordings showing the vasodilatory effect of nicotine on the toad lateral aorta with (A) or without (B) the endothelium. The preparations were exposed to ET-1 (10^{-8} M) until a maximum constriction was achieved and then nicotine (3×10^{-4} M) was added. (C) Mean response of nicotine on pre-constricted lateral aortae and dorsal aorta with (■) or without (□) the endothelium. Note that there is no significant difference in the nicotine-mediated dilation (lateral aortae $P=0.37$, $N=5$).

inhibitor of nNOS). These observations indicated that in toad the ACh-induced vasodilation occurred via NOS that was not located in the vascular endothelium, which is consistent with the anatomical studies that revealed an absence of NOS staining in the endothelium of arteries and veins (see above). In the next experiment, it was found that TTX (10^{-5} M) abolished or significantly reduced the ACh-mediated vasodilation (Fig. 6), which indicates that the effect of ACh is most likely due to activation of the nitrergic nerves in the aortae (Fig. 1C). In endothelium-denuded aortae of toad, nicotine (3×10^{-4} M) caused a vasodilation that was similar in magnitude to that observed in aortae with the endothelium intact (Fig. 7), but was abolished by ODQ (10^{-5} M) and significantly reduced in the presence of L-NNA (10^{-4} M, Fig. 8). However, the nicotine-mediated vasodilation was not affected by TTX (10^{-5} M). Previous studies in mammals have shown that nicotine-mediated vasodilation in some blood vessels may occur independently of the generation of action potentials (see Toda and Okamura, 2003). An essentially identical suite of data was obtained on the NO control of toad veins (Broughton and Donald, 2005). For example, in the ventral abdominal vein and vena cava of toad, nicotine (3×10^{-4} M) induced a vasodilation that was abolished by ODQ (10^{-5} M) and L-NNA (10^{-4} M), but was unaffected by the removal of the endothelium.

These physiological data, in combination with the anatomical demonstration of neuronal NOS in the peri-

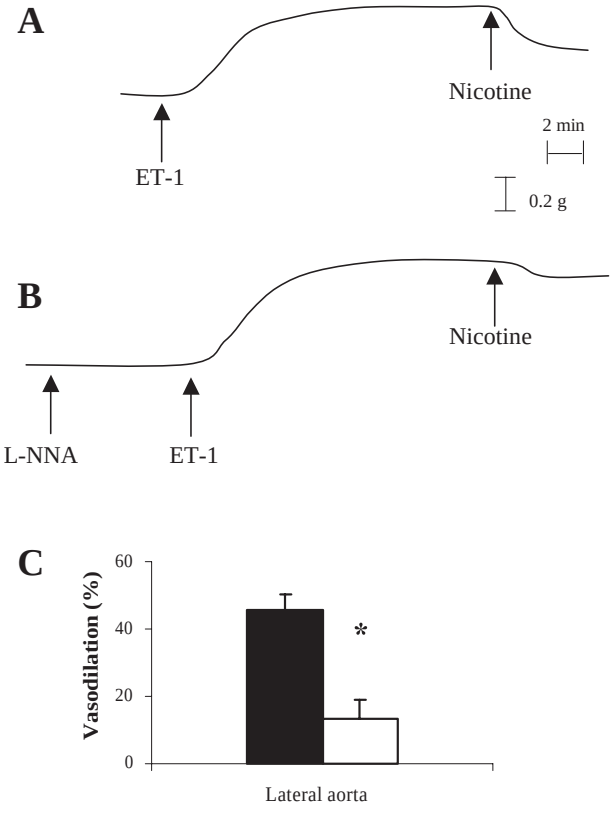

Fig. 8. Tension recordings showing the effect of nicotine on the toad lateral aorta with (B) or without (A) the NOS inhibitor, L-NNA. The preparations were pre-treated with L-NNA (10^{-4} M) for approximately 10 min before being constricted with ET-1 (10^{-8} M). At the peak of vasoconstriction, nicotine (3×10^{-4} M) was administered, which only caused a marked vasodilation in the preparation without L-NNA (B; $N=5$). (C) Mean response of pre-constricted lateral aortae to nicotine (3×10^{-4} M) with (□) or without (■) L-NNA (10^{-4} M; $N=5$).

vascular nerves (Fig. 1A,B), strongly suggest that the large arteries and veins of short-finned eel and toad receive a functional innervation by nitrergic nerves, which mediates NO control of the vascular smooth muscle. It remains to be determined as to whether smaller arterioles are regulated by a similar neural NO signalling system. The small arterioles are the main site of variable resistance in the systemic circulation and, therefore, it is important to determine how they are regulated by NO. A number of in vivo studies have shown that NO can regulate vascular resistance in lower vertebrates, which is probably occurring at the level of the microcirculation (see e.g. Galli et al., 2005; Rea and Parsons, 2001). We could not find any physiological evidence for endothelial NO signalling in fish and amphibians. However, the anatomical studies were able to demonstrate endothelial NOS in the blood vessels of blue-tongue lizard, pigeon and rat, and therefore, it is tempting to speculate that endothelial NOS first appeared in the blood vessels of reptiles. Further evidence for this claim comes from experiments on the effects of NOS inhibition on basal tone in isolated vascular rings of reptiles, birds and mammals. In each of these vertebrate classes, the addition of a NOS inhibitor to the vascular ring causes a marked increase in basal tone (reptiles, Knight and Burnstock, 1993; birds, Martinez-Lemus et al., 1999; Le Noble et al., 2000; mammals: see Moncada et al., 1991). In contrast, similar effects are generally not observed in vascular rings of fish (e.g., Jennings et al., 2004) and amphibians (see, e.g., Broughton and Donald, 2002). Therefore, it is possible that NO only regulates basal tone in those animals that contain an endothelial NO system. However, it is essential that other approaches such as molecular analysis are used to ultimately determine in which class of vertebrates a vascular endothelial NO signalling pathway first appeared.

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