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Nitric oxide control of the dorsal aorta and the intestinal vein of the Australian short-finned eel *Anguilla australis*

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Summary

This study investigated the mechanisms by which nitric oxide (NO) regulates the dorsal aorta and the intestinal vein of the Australian short-finned eel *Anguilla australis*. NADPH diaphorase histochemistry and immunohistochemistry using a mammalian endothelial nitric oxide synthase (NOS) antibody could not demonstrate NOS in the endothelium of either blood vessel; however, NOS could be readily demonstrated in the endothelium of the rat aorta that was used as a control. Both blood vessels contained NADPH diaphorase positive nerve fibres and nerve bundles, and immunohistochemistry using a neural NOS antibody showed a similar distribution of neural NOS immunoreactivity in the perivascular nerves. *In vitro* organ bath physiology showed that a NO/soluble guanylyl cyclase (GC) system is present in the dorsal aorta and the intestinal vein, since the soluble GC inhibitor oxadiazole quinoxalin-1 (ODQ; 10^{-5} mol l^{-1}) completely abolished the vasodilatory effect of the NO donor, sodium nitroprusside (SNP; 10^{-4} mol l^{-1}). In addition, nicotine (3×10^{-4} mol l^{-1}) mediated a vasodilation that was not affected by removal of the endothelium. The nicotine-mediated dilation was blocked by the NOS inhibitor, N^{ω}-nitro-L-arginine (L-NNA; 10^{-4} mol l^{-1}), and ODQ (10^{-5} mol l^{-1}). More specifically, the neural NOS inhibitor, N^{ω}-propyl-L-arginine (10^{-5} mol l^{-1}), significantly decreased the dilation induced by nicotine (3×10^{-4} mol l^{-1}). Furthermore, indomethacin (10^{-5} mol l^{-1}) did not affect the nicotine-mediated dilation, suggesting that prostaglandins are not involved in the response. Finally, the calcium ionophore A23187 (3×10^{-6} mol l^{-1}) caused an endothelium-dependent dilation that was abolished in the presence of indomethacin. We propose the absence of an endothelial NO system in eel vasculature and suggest that neurally derived NO contributes to the maintenance of vascular tone in this species. In addition, we suggest that prostaglandins may act as endothelially derived relaxing factors in *A. australis*.

Key words: nitric oxide, neural nitric oxide synthase, soluble guanylyl cyclase, vasodilation, nicotine, *Anguilla australis*, prostaglandin.

Introduction

In mammals, nitric oxide (NO) is one of the most important regulators of vascular tone and consequently global blood pressure. In all mammalian blood vessels, NO is generated by a nitric oxide synthase (NOS) located in the vascular endothelium, which is called endothelial NOS (Moncada et al., 1991). Nitric oxide rapidly diffuses into the smooth muscle cells where it activates a soluble guanylyl cyclase (GC), which generates cGMP that mediates vasodilation (Denninger and Marletta, 1999). A second isoform of NOS, called neural or brain NOS, is located in many perivascular nerves (called nitrergic nerves) in the cranial and peripheral regions of the body (Young et al., 2000). In combination with the endothelium, nitrergic nerves provide a second mechanism for NO control of vascular tone in mammalian blood vessels (Toda, 2000).

The role of NO in the control of vascular tone in non-mammalian vertebrates has received less attention. Early comparative studies in avian (Hasegawa and Nishimura, 1991), reptilian (Knight and Burnstock, 1993), and amphibian (Rumbaut et al., 1995; Knight and Burnstock, 1996) species provided evidence that an endothelial NO system was present. Furthermore, there is evidence for endothelial NO signalling in teleost fish, which was obtained in perfused vascular beds in which vascular resistance was affected by the NO precursor L-arginine, and inhibition of NO synthase, the enzyme that generates NO (Nilsson and Söderström, 1997; Mustafa et al., 1997; Mustafa and Agnisola, 1998). The only study in teleost fish that has shown anatomically that an endothelial NO synthase is present is that of Fritsche et al. (2000), who showed immunoreactivity to endothelial NOS in developing zebrafish blood vessels. However, in some species of teleost fish, there is now convincing evidence that vasodilatory signalling molecules released by the endothelium are prostaglandins, rather than NO (Olson and Villa, 1991; Kågström and Holmgren, 1997; Park et al., 2000).

Recently, we demonstrated in the cane toad *Bufo marinus* that NO control of the large central arteries was mediated by NO generated from neural NOS that was located in the perivascular nerves (Broughton and Donald, 2002). Furthermore, no evidence for an endothelial NO system was found in *B. marinus* (Broughton and Donald, 2002). In the present study we use anatomical and physiological approaches to demonstrate that NO control of two blood vessels of the eel *Anguilla australis* is mediated by neural NOS in perivascular nerves.

Materials and methods

Animals

Australian short-finned eels *Anguilla australis* Richardson 1841, body mass 600–800 g and of either sex, were purchased from a commercial supplier (Eels Australis, Skipton, Victoria, Australia). *A. australis* were maintained within the laboratory at Deakin University in filtered recirculating containers of fresh water at 19–21°C and were not fed during captivity. Prior to experimentation, *A. australis* were anaesthetised with benzocaine (1:1000) and then killed by decapitation.

NADPH diaphorase histochemistry

The dorsal aorta, the intestinal vein and the gut were dissected free and immersed in phosphate-buffered saline (PBS; 0.01 mol l^{-1} phosphate buffer, 0.15 mol l^{-1} NaCl, pH 7.4) at 4°C. Each vessel was opened and pinned out endothelium side up on dental wax, prior to fixing for 2 h in 4% formaldehyde (pH 7.4) at 4°C. The blood vessels were washed in 0.01 mol l^{-1} PBS (3×10 min) and removed from the dental wax. They were then stained in a NADPH diaphorase mixture containing 1 mg ml^{-1} β-NADPH, 0.25 mg ml^{-1} Nitroblue Tetrazolium (NBT), 1% Triton X-100 in 0.1 mol l^{-1} Tris buffer, pH 8, for periods ranging from 15 to 60 min at room temperature. This mixture was kept in the dark, as it is light sensitive. The vessels were then washed in 0.01 mol l^{-1} PBS and mounted on slides in buffered glycerol (0.5 mol l^{-1} Na_2CO_3 added dropwise to 0.5 mol l^{-1} $NaHCO_3$ to pH 8.6, combined 1:1 with glycerol). Blood vessels were observed under a light microscope (Axioskop 20, Zeiss, Germany) and photographed with a digital colour system (Spot 35 Camera System, Diagnostic Instruments, USA). Control experiments were performed on the myenteric plexus of the gut of *A. australis*, because positive NADPH diaphorase staining has been previously demonstrated in the myenteric plexus of fish (Li et al., 1993).

Immunohistochemistry

Blood vessels were fixed as described above. They were unpinned, washed in 0.01 mol l^{-1} PBS (3×10 min), incubated in dimethyl sulphoxide (3×10 min) and washed in 0.01 mol l^{-1} PBS (5×2 min). The vessels were then incubated in either sheep anti-neural NOS (1:4000; Anderson et al., 1995) or mouse anti-endothelial NOS (1:1000; O'Brien et al., 1995) for 24 h at room temperature in a humid box. The following day, tissues were washed in 0.01 mol l^{-1} PBS (3×10 min) to remove any excess antibody and incubated in FITC-conjugated goat anti-sheep IgG (1:200) or FITC-conjugated goat anti-mouse IgG (1:200) (Zymed Laboratories, San Francisco, USA) for 3–4 h at room temperature in a humid box. The blood vessels were then washed in 0.01 mol l^{-1} PBS (3×10 min), and mounted in buffered glycerol. Blood vessels were observed under a fluorescence microscope (Zeiss) using a FITC filter and photographed as above.

Organ bath physiology

After decapitation, the dorsal aorta and the intestinal vein were excised and placed in Cortland's ringer solution (124.1 mmol l^{-1} NaCl, 5.1 mmol l^{-1} KCl, 12 mmol l^{-1} $NaHCO_3$, 0.41 mmol l^{-1} NaH_2PO_4, 0.29 mmol l^{-1} $MgSO_4$, 7.8 mmol l^{-1} D[+]glucose and 2.5 mmol l^{-1} $CaCl_2$, pH 7.2). Individual rings of approximately 4–5 mm in length were mounted horizontally between two hooks for the measurement of isometric force, and placed in an organ bath. The rings were bathed in 15 ml of Cortland's ringer solution, which was maintained at 19°C and aerated with air. The force transducer (FT03, Grass Instruments, USA) was linked to a PowerLab™ data collection system and a personal 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 a pin, and the extent of removal was determined using NADPH histochemistry. The extent of removal was also substantiated by the use of the calcium ionophore, A23187, which mediates vasodilation in the presence of an intact endothelium, but has no effect when the endothelium is disrupted. Prior to administering various vasodilatory substances, each vessel was pre-constricted with endothelin-1 (10^{-8} mol l^{-1}), and vasoconstriction was allowed to reach its maximum. Previous studies have demonstrated that endothelin-1 at 10^{-8} mol l^{-1} elicits an appropriate vasoconstriction for studies of vasodilatory mechanisms (Minerds and Donald, 2001; Broughton and Donald, 2002). The extent of vasodilation was determined for each vasodilator, by scoring the degree of relaxation as a ratio, having assigned relaxation to pre-constriction levels at 100%. In experiments, matched controls were used from the same animal for comparison of drug effects. Data are expressed as mean ± one standard error (S.E.M.) of five or more experiments, and statistical analysis was performed by independent *t*-tests using the SPSS (11.5) statistical package; a P value ≤0.05 was considered significant.

Materials

Sodium nitroprusside (SNP), acetylcholine (ACh), N^{ω}-nitro-L-arginine (L-NNA), indomethacin, calcium ionophore A23187, β-nicotinamide adenine dinucleotide phosphate, reduced form (β-NADPH), NBT and Triton X-100 were obtained from Sigma (St Louis, USA). Endothelin-1 (ET-1) and rat atrial natriuretic peptide (rANP) were purchased from Auspep (Melbourne, Australia), and oxadiazole quinoxalin-1 (ODQ) was obtained from Alexis (San Diego, USA). Nicotine

was purchased from BDH chemicals and N^{ω}-propyl-L-arginine was obtained from Cayman chemicals. The NOS antibodies were obtained from Chemicon (Melbourne, Australia).

Results

NADPH diaphorase histochemistry

In the dorsal aorta, no specific, perinuclear staining could be observed in the endothelium following processing for NADPH diaphorase histochemistry (Fig. 1A, *N*=5). Similar results were observed in the intestinal vein (not shown). The absence of specific NADPH diaphorase staining was observed in all preparations incubated for varying times up to 60 min. In contrast, endothelial cells of the rat aorta showed distinct perinuclear staining, indicating the presence of endothelial NOS (eNOS; Fig. 1B, *N*=5), as has been previously demonstrated (O'Brien et al., 1995). However, in *A. australis* NADPH diaphorase staining could be observed in perivascular nerve fibres of both the dorsal aorta (Fig. 2A, *N*=5), the intestinal vein (Fig. 2B, *N*=5), and the myenteric plexus of the gut (used as a control). Positive NADPH diaphorase staining was observed in both single, varicose fibres and nerve bundles. In the intestinal vein, occasional cell bodies were observed in the nerve bundles (Fig. 2B).

Endothelial and neural NOS immunohistochemistry

To specifically identify the types of NOS present in the blood vessels, eNOS and neural NOS (nNOS) antibodies were used (*N*=3). In the dorsal aorta and the intestinal vein, no eNOS immunoreactivity was found (Fig. 1C), but it could be readily demonstrated in endothelial cells of the rat aorta (Fig. 1D). In both the dorsal aorta (Fig. 2C) and intestinal vein (Fig. 2D), nNOS immunoreactivity was observed in nerve bundles and single fibres in a similar pattern to that observed using NADPH diaphorase histochemistry. In addition, NADPH diaphorase staining and specific nNOS immunoreactivity were colocalised in the same neural structures, demonstrating that the NADPH diaphorase reaction was staining positively for nNOS.

In vitro organ bath physiology

Blood vessels were preconstricted with ET-1 (10^{-8} mol l^{-1}), which induced a potent and long-lasting effect that was allowed to reach its maximum. Following this, various substances associated with vasodilator mechanisms were added to the baths. In tetrapods, ACh has been used to indirectly activate NOS to generate a NO-mediated vasodilation; however, in fish, ACh generally causes vasoconstriction in peripheral blood vessels. This was verified in *A. australis* in which ACh always caused vasoconstriction regardless of whether or not the vessel had been preconstricted with ET-1 (10^{-8} mol l^{-1}) (*N*=5, results not shown). In the dorsal aorta and the intestinal vein, the NO donor, SNP (10^{-4} mol l^{-1}), induced a dilation of 52.27±9.43% and 94.12±8.79%, respectively (*N*=5, Fig. 3). In addition, the application of

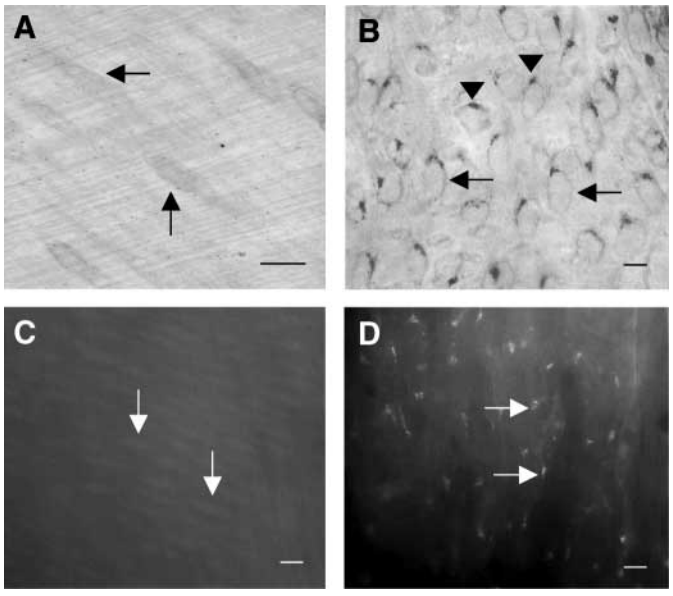

Fig. 1. Photomicrographs showing whole-mount preparations of eel dorsal aorta (A,C) and rat aorta (B,D) following processing for NADPH diaphorase histochemistry (A,B) and endothelial nitric oxide synthase (eNOS) immunohistochemistry (C,D). In the rat aorta, punctate eNOS-positive staining (arrowheads) occurred around the nuclei (arrows) of the endothelial cells, which was demonstrable with both techniques (B,D). In contrast, no eNOS-positive staining was observed around the nuclei of the endothelial cells in the eel dorsal aorta (A,C). Scale bars, 10 μm.

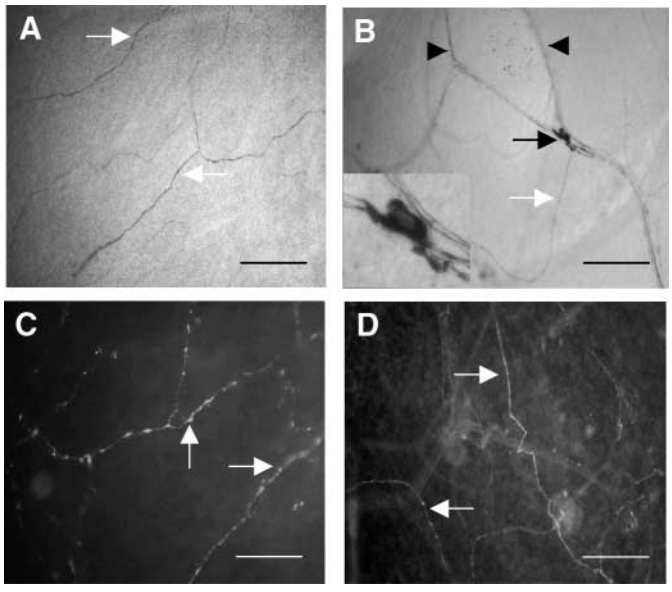

Fig. 2. Photomicrographs showing whole-mount preparations of eel dorsal aorta (A,C) and intestinal vein (B,D) following processing for NADPH diaphorase histochemistry (A,B) and neural nitric oxide synthase (nNOS) immunohistochemistry (C,D). Using both techniques a plexus of nNOS-positive perivascular nerve bundles (white arrows) was observed in the outer layers of the wall of each vessel. In addition, some larger nNOS-positive nerve bundles (B, arrowheads) were observed. Within the nerve bundles, nNOS-positive cell bodies were observed (B, black arrow). Inset, high power magnification of a nerve cell body. Scale bars, 100 μm.

nicotine (3×10^{-4} mol l^{-1}) mediated a vasodilation in the dorsal aorta (73.86±14.84%) and intestinal vein (70.09±6.24%; *N*=5; Fig. 4). Preincubation of the vessels with the soluble GC inhibitor, ODQ (10^{-5} mol l^{-1}), blocked the dilation induced by both SNP (10^{-4} mol l^{-1}) and nicotine (3×10^{-4} mol l^{-1}). However, the addition of rat ANP (10^{-8} mol l^{-1}), which mediates dilation through a particulate GC, caused a marked vasodilation (*N*=5, Fig. 5B). This indicates that both SNP and nicotine mediate vasodilation *via* a soluble GC.

The addition of the non-specific NOS inhibitor, L-NNA (10^{-4} mol l^{-1}), abolished the vasodilatory effect of nicotine (3×10^{-4} mol l^{-1}) in the aorta and the vein (*N*=5). However, when SNP (10^{-4} mol l^{-1}) was applied to the vessels in the presence of L-NNA a marked vasodilation was observed (Fig. 5C). This result was expected as SNP is a NO donor, and does not require NOS for the production of NO.

In the next series of experiments, the endothelium was removed from the blood vessels, and was verified by subsequent NADPH staining. In endothelium-denuded dorsal

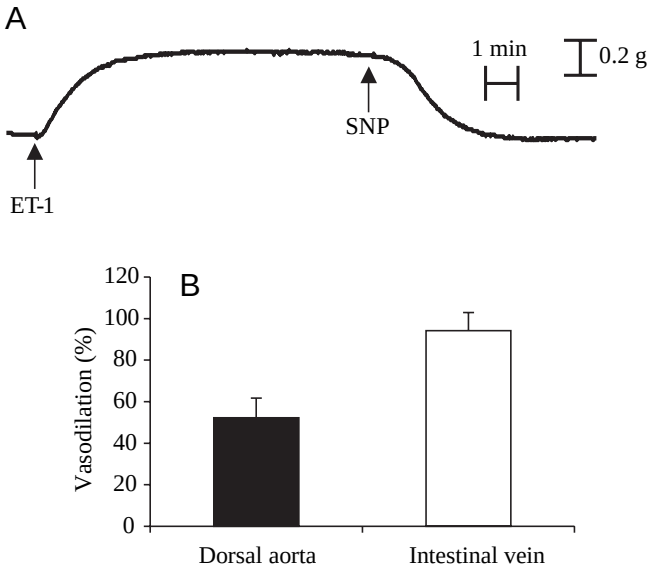

Fig. 3. (A) Tension recording showing the vasodilatory effect of sodium nitroprusside (SNP) on the intestinal vein. The vessels were preconstricted with endothelin-1 (ET-1; 10^{-8} mol l^{-1}) and at the point of maximum constriction, SNP (10^{-4} mol l^{-1}) was added, which caused a marked dilation. Similar results were observed in the dorsal aorta. (B) Mean responses (% vasodilation) of preconstricted dorsal aorta and intestinal vein to SNP (10^{-4} mol l^{-1}; *N*=5).

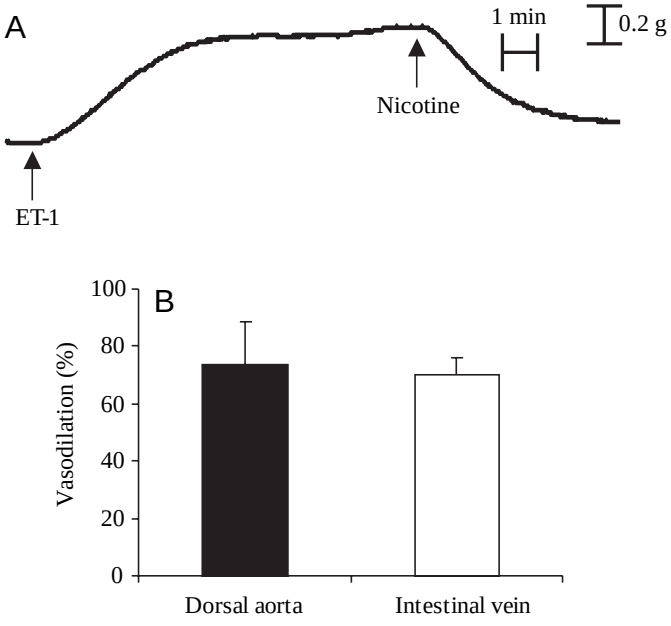

Fig. 4. (A) Tension recording showing the vasodilatory effect of nicotine on the dorsal aorta. The vessels were preconstricted with endothelin-1 (ET-1; 10^{-8} mol l^{-1}) and at the point of maximum constriction, nicotine (3×10^{-4} mol l^{-1}) was added, which caused a marked dilation. Similar results were observed in the intestinal vein. (B) Mean responses (% vasodilation) of preconstricted dorsal aorta and intestinal vein to nicotine (3×10^{-4} mol l^{-1}; *N*=5).

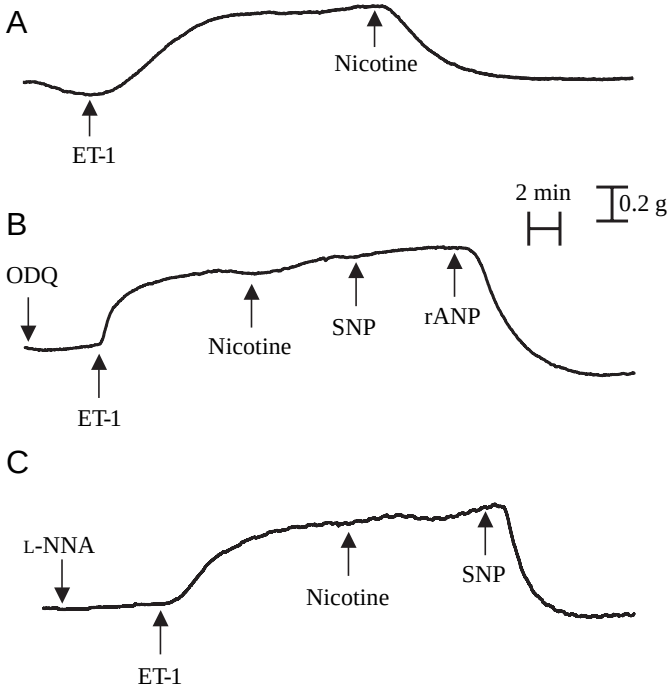

Fig. 5. Tension recordings from the intestinal vein showing the vasodilatory effect of nicotine (A) and its effect in the presence of the soluble guanylyl cyclase (GC) inhibitor ODQ (B) and the NOS inhibitor, L-NNA (C). Vessels were pre-incubated with ODQ (10^{-5} mol l^{-1}) or L-NNA (10^{-4} mol l^{-1})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

aortae, the application of nicotine (3×10^{-4} mol l^{-1}) caused a similar dilation (76.22±7.42%) to that observed in control vessels with an intact endothelium (76.95±12.01%; P=0.94, N=5; Fig. 6). Similar results were observed in the intestinal vein (endothelium denuded 77.35±12.50%; endothelium intact 74.50±12.10%; P=0.90, N=5; Fig. 6C). These results indicate that removal of the endothelium does not significantly affect the nicotine-mediated vasodilation in the dorsal aorta and the intestinal vein. In the dorsal aorta incubated with N^{ω}-propyl-L-arginine (PLA, 10^{-5} mol l^{-1}), a specific nNOS inhibitor (Zhang et al., 1997), the vasodilatory effect of nicotine (3×10^{-4} mol l^{-1}) was significantly reduced compared to control blood vessels (PLA 27.80±11.25%; control 72.36±11.23%; P<0.05, N=5; Fig. 7). A similar result was obtained in endothelium-denuded dorsal aortae (P<0.05, N=5; not shown). Furthermore, incubation of the intestinal vein with PLA (10^{-5} mol l^{-1}) significantly reduced the nicotine-mediated vasodilation (PLA 45.89±13.76%; control 80.11±16.81%; P<0.05, N=5; Fig. 7). A similar result was obtained in endothelium-denuded intestinal vein (P<0.05, N=5; not shown). These results suggest that PLA significantly inhibits the nicotine-mediated vasodilation in the dorsal aorta and the intestinal vein with or without an intact endothelium.

In the dorsal aorta, preincubation with the cyclo-oxygenase inhibitor, indomethacin (10^{-5} mol l^{-1}), did not significantly reduce the vasodilation to nicotine (indomethacin 71.49±9.41%; control 66.55±7.40; P=0.63, N=5; Fig. 8). Similar results were also observed in the intestinal vein (indomethacin 70.68±10.97; control 82.88±5.70; P=0.20, N=5; Fig. 8). These results suggest that nicotine is not stimulating the production of cyclo-oxygenase metabolites to mediate vasodilation in the dorsal aorta and the intestinal vein.

In the dorsal aorta and the intestinal vein, the calcium ionophore, A23187 (3×10^{-6} mol l^{-1}), mediated a significant dilation (N=3; Fig. 9A). In both vessels, pre-incubation with indomethacin (10^{-5} mol l^{-1}) significantly reduced the dilation induced by the calcium ionophore (P<0.05, N=3; Fig. 9B); in

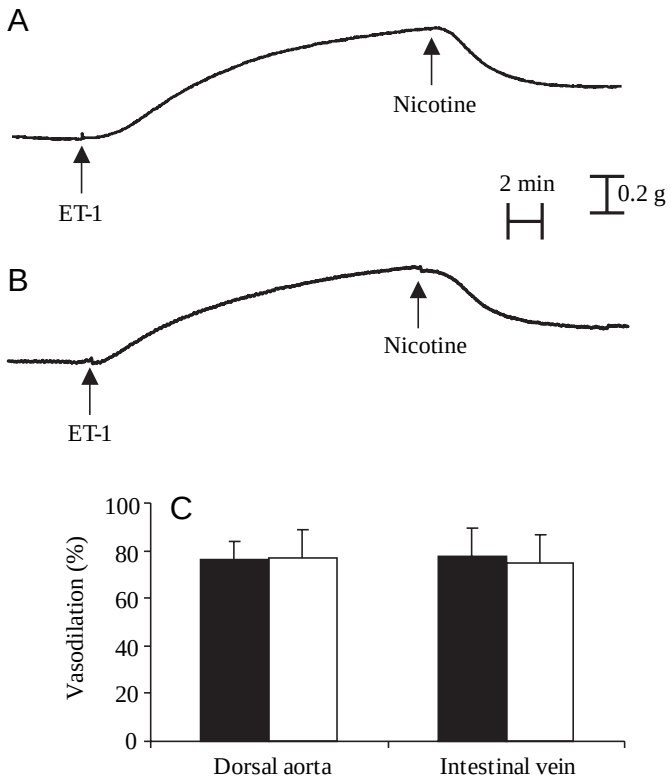

Fig. 6. Tension recordings showing the vasodilatory effect of nicotine on the dorsal aorta with an intact endothelium (A) and without an endothelium (B). Vessels were pre-constricted with endothelin-1 (ET-1; 10^{-8} mol l^{-1}), and at the point of maximal vasoconstriction nicotine (3×10^{-4} mol l^{-1}) was administered. Similar results were observed in the intestinal vein. (C) Mean response (% vasodilation) of nicotine on pre-constricted dorsal aorta and intestinal vein with the endothelium removed (filled bars) and with an intact endothelium (open bars). Note that there is no significant difference in the nicotine-mediated dilation (dorsal aorta, P=0.944; intestinal vein, P=0.902; N=5).

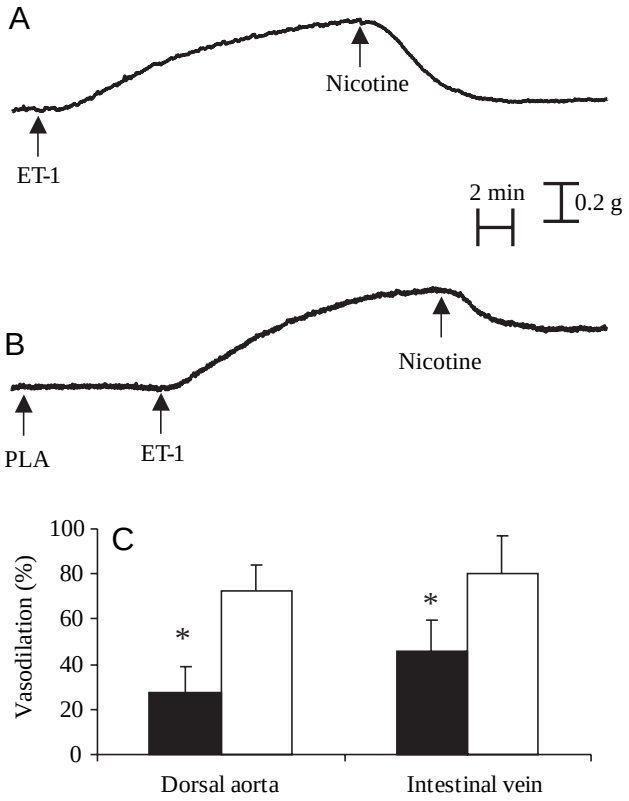

Fig. 7. Tension recordings from the dorsal aorta showing the vasodilatory effect of nicotine (A), and its effect in the presence of the specific nNOS inhibitor N^{ω}-propyl-L-arginine (PLA) (B). Vessels were pre-incubated with PLA (10^{-5} mol l^{-1}) for approximately 10 min prior to being pre-constricted with endothelin-1 (ET-1; 10^{-8} mol l^{-1}). At the point of maximal vasoconstriction, nicotine (3×10^{-4} mol l^{-1}) was added. Note that the vasodilation is reduced in the presence of PLA. (C) Mean response (% vasodilation) of nicotine on pre-constricted dorsal aorta and intestinal vein in the in the presence of (filled bars) and in the absence of PLA (open bars). Note that PLA significantly reduced the nicotine-mediated dilation (dorsal aorta and intestinal vein, P<0.05; *denotes significant difference; N=5).

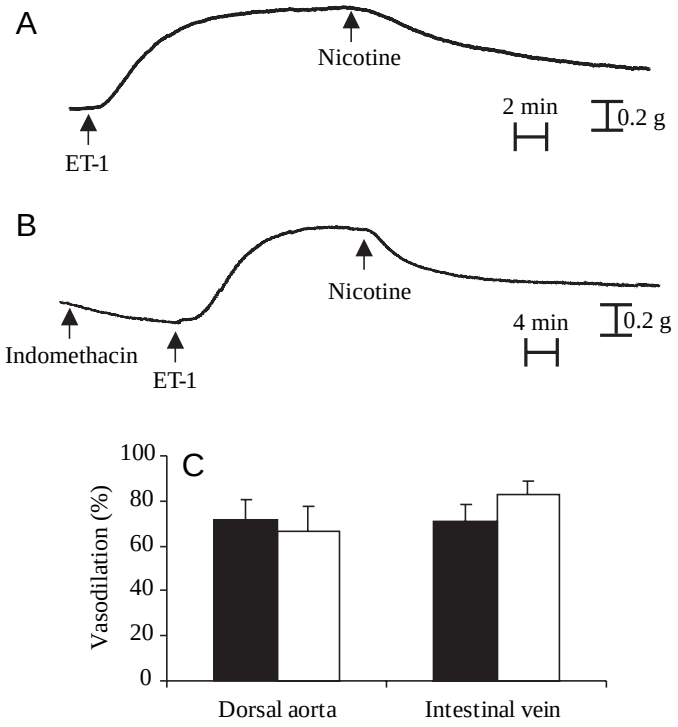

Fig. 8. Tension recordings from the dorsal aorta showing the vasodilatory effect of nicotine (A) and its effect in the presence of the cyclo-oxygenase inhibitor, indomethacin (B). Vessels were pre-incubated with indomethacin (10^{-5} mol l^{-1}) for approximately 10 min prior to being pre-constricted with endothelin-1 (ET-1; 10^{-8} mol l^{-1}). At the point of maximal vasoconstriction, nicotine (3×10^{-4} mol l^{-1}) was administered. (C) Mean response (% vasodilation) of nicotine on pre-constricted dorsal aorta and intestinal vein in the absence of (filled bars) and in the presence of indomethacin (open bars). Note that there is no significant difference in the nicotine-mediated dilation (dorsal aorta, P=0.63; intestinal vein, P=0.20; N=5).

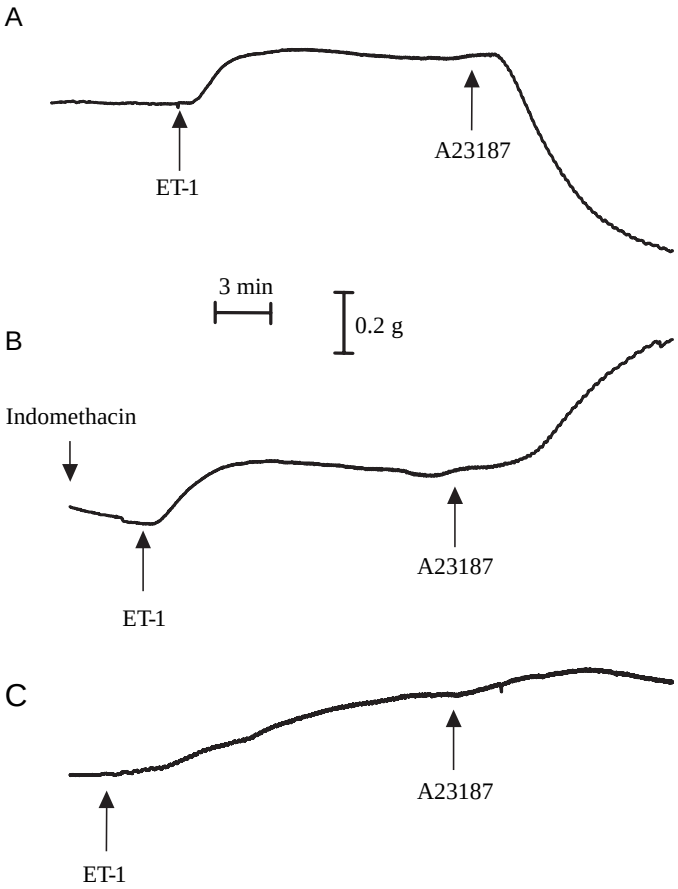

Fig. 9. Tension recordings from the intestinal vein showing the vasodilatory effect of the calcium ionophore, A23187 (A), and its effect in the presence of the cyclo-oxygenase inhibitor, indomethacin (B), and in the absence of an intact endothelium (C). Vessels were pre-incubated with indomethacin (10^{-5} mol l^{-1}) for approximately 10 min prior to being constricted with endothelin-1 (ET-1; 10^{-8} mol l^{-1}), and at the point of maximal vasoconstriction, the ionophore (3×10^{-6} mol l^{-1}) was added. Pre-incubation with indomethacin or disruption of the endothelium abolished the dilation induced by the addition of the ionophore (3×10^{-6} mol l^{-1}; N=3).

fact a vasoconstriction was now observed. In addition, removal of the endothelium in both blood vessels abolished the dilation induced by the calcium ionophore (P<0.05, N=3; Fig. 9C). These results suggest that the calcium ionophore stimulates the production of endothelium dependent, cyclo-oxygenase derived metabolites to induce vasodilation in the dorsal aorta and intestinal vein of *A. australis*.

Discussion

The present study demonstrates, using both anatomical and physiological techniques, that NO control of the dorsal aorta and the intestinal vein of *A. australis* is mediated by neurally derived NO rather than NO derived from the endothelium. Anatomically, it was shown using NADPH diaphorase histochemistry and immunohistochemistry that NOS was not found in the endothelium, but was demonstrated in the perivascular nerves of the blood vessels. These data suggested that NO could only be derived from nerves rather than the endothelium. The physiological experiments supported the anatomical findings because they showed, using a range of approaches, that the vasodilation to nicotine was due to NO derived from neural sources rather than the endothelium. This is the first study to demonstrate that a nNOS system is involved in the NO regulation of the vasculature of fish.

The current study demonstrated the absence of an endothelial NO system in *A. australis*, which is in accordance with a number of previous studies in teleost fishes. These studies have proposed that prostaglandins released from the endothelium are responsible for mediating vasodilation (Olson and Villa, 1991; Sverdrup et al., 1994; Farrell and Johansen, 1995; Kågström and Holmgren, 1997; Miller and Vanhoutte, 2000; Park et al., 2000). However, the presence of an endothelial NO system in fish vasculature remains controversial because it has been reported that some vascular beds are dilated by NO purportedly released by the

endothelium (Nilsson and Söderström, 1997; Mustafa et al., 1997; Mustafa and Agnisola, 1998). Furthermore, it has been demonstrated that elasmobranchs lack a NO system and instead it is proposed that prostaglandins are the endothelium-dependent vasodilators in this group (Evans and Gunderson, 1998; Evans, 2000).

NADPH diaphorase histochemistry and immunohistochemistry are two anatomical techniques that have been used previously to identify eNOS in the vasculature of mammals (Beesley, 1995). In the present study, these techniques demonstrated an absence of NOS in the endothelium in both the dorsal aorta and intestinal vein of *A. australis*. However, in the endothelium of the rat aorta (used as a control), perinuclear staining was observed as previously demonstrated in mammals (O'Brien et al., 1995). This suggests that the dorsal aorta and intestinal vein of *A. australis* do in fact lack an eNOS. The only study to report the presence of eNOS in teleost blood vessels is that of Fritsche et al. (2000), who demonstrated using immunohistochemistry and a different eNOS antibody to that used in this study, that eNOS immunoreactivity was present in the dorsal vein of the developing zebrafish. Thus, there seems to be conflicting data on the presence of eNOS that could reflect a dichotomy between developing and adult fish. In contrast to the endothelium, NADPH diaphorase histochemistry readily demonstrated NOS staining in the perivascular nerves of the dorsal aorta and intestinal vein of *A. australis*, which was supported by the use of immunohistochemistry and a mammalian nNOS antibody. The staining patterns of the two techniques were identical, revealing both nerve bundles and single, varicose nerve fibres in the blood vessels. It has been previously reported that nNOS is located within autonomic nerves that innervate peripheral blood vessels of a number of species of teleost fishes (Brunning et al., 1996; Esteban et al., 1998; Jiminez et al., 2001). Taken together, our data suggest that the vascular endothelium of *A. australis* is incapable of synthesising NO, but that NO may be released from perivascular nerves to regulate vascular tone in eels.

The presence of nitrergic nerves in the dorsal aorta and intestinal vein suggested that NO is a key regulator of vascular tone, but it was important to determine physiologically whether the nerves provide NO control of the blood vessels. Donald et al. (2003) demonstrated the presence of nitrergic nerves in the vasculature of the giant shovelnose ray *Rhinobatus typus*, but they could not stimulate the production of NO, nor could they demonstrate the presence of a NO receptor *via* use of the NO donor, SNP. These data indicated that despite the presence of nitrergic nerves, NO does not contribute to the maintenance of vascular tone in *R. typus*. The present study, however, demonstrates that SNP mediated a dilation in both the dorsal aorta and intestinal vein that could be blocked by ODQ, suggesting that NO stimulates the production of cGMP *via* a soluble GC. This observation is consistent with a number of previous studies that have shown that the vascular smooth muscle of teleost fish contains a NO receptor that mediates vasodilation (Small et al., 1990; Small and Farrell, 1990; Olson and Villa, 1991; Hylland and Nilsson, 1995; Kågström and Holmgren, 1996; McGeer and Eddy, 1996; Mustafa et al., 1997; Miller and Vanhoutte, 2000). In contrast to these studies, Pellegrino et al. (2002) reported a vasoconstrictive effect of NO in the branchial circulation of *A. anguilla* using the NO donors, SNP and SIN-1, at a range of concentrations. These authors also demonstrated that the vasoconstriction was due to the activation of soluble GC, as pre-incubation with ODQ prevented the constriction induced by the NO donors. This result was substantiated with the use of the cGMP analog 8-bromo cGMP, which caused a dose-dependent vasoconstriction. This was the first study in fish to suggest that NO stimulates the production of cGMP, and elicits a subsequent vasoconstriction. In addition, a vasoconstrictory effect of NO has been shown in the dogfish shark *Squalus acanthias* (Evans and Gunderson, 1998; Evans, 2000). Interestingly, a vasoconstriction mediated by cGMP signalling has recently been demonstrated in murine splenic vessels (Andrews and Kaufman, 2003).

Acetylcholine has been used as a pharmacological tool to indirectly stimulate the production of NO *via* nNOS in mammals (Meng et al., 1998) and amphibians (Broughton and Donald, 2002). However, it is clear that ACh generally causes vasoconstriction in peripheral blood vessels of fish, and a similar result was observed in this study. Previously, it has been suggested in mammals that nicotine can specifically stimulate nitrergic nerves to produce NO (Toda and Okamura, 1990; Toda et al., 1997, 1998), but it is apparent that the action of nicotine is more complex than initially thought. Recently, it has been shown that in mammalian cerebral blood vessels, the nicotine-induced NO-mediated vasodilation is dependent on an intact sympathetic innervation (Zhang et al., 1998; Lee et al., 2000; Si and Lee, 2001). These authors proposed that nicotine does not directly stimulate the production of NO from nitrergic nerves, but instead, it binds to nicotinic receptors on sympathetic nerves to release noradrenaline. Noradrenaline then binds to adrenoceptors situated on neighbouring nitrergic nerves, which stimulates the production and release of NO from these nerves. In *A. australis*, it was demonstrated that nicotine induced a vasodilation that was mediated *via* a soluble GC, as ODQ completely abolished the dilation. Subsequently, it was then shown that nicotine was stimulating the production of NO *via* NOS, as the NOS inhibitor, L-NNA, abolished or significantly inhibited the dilation. In addition, pre-incubation with the specific nNOS inhibitor, PLA, significantly inhibited the vasodilation caused by the application of nicotine, which provided evidence that nNOS was generating NO. Thus, it was demonstrated that in *A. australis* nicotine can be used to stimulate the production of NO from nitrergic nerves of blood vessels, but the exact mechanism of nicotine-mediated vasodilation is not known and warrants further study.

We also demonstrated that the endothelium did not contribute to the nicotine-mediated vasodilation, as disruption of the endothelium did not alter the effect of nicotine. Recently, Donald et al. (2003) demonstrated that in *R. typus* nicotine induced an endothelium-dependent vasodilation due to the

production of prostaglandins, because it was inhibited by the cyclo-oxygenase inhibitor, indomethacin. However, pre-incubation of the blood vessels from *A. australis* with indomethacin did not affect the nicotine-mediated vasodilation. This study also looked at the possible role of endothelium-dependent vasodilation due to the release of prostaglandins. Our results demonstrated that vessels pre-constricted with ET-1, dilated after the addition of the calcium ionophore, A23187. In addition, it was shown that the vasodilation was due to the production of cyclo-oxygenase derived products, as pre-incubation of the blood vessels with indomethacin significantly inhibited the vasodilatory response to A23187. Subsequently, it was demonstrated that the dilation was endothelium-dependent, as removal of the endothelium abolished the response. These results demonstrate that cyclo-oxygenase derived products are endothelium-dependent vasodilators in *A. australis*.

The demonstration of NO control of vascular smooth muscle of *A. australis* by nitrergic nerves is consistent with a recent study that demonstrated a similar mechanism in the toad, *B. marinus* (Broughton and Donald, 2002). Furthermore, in both species, an endothelial NO system could not be demonstrated. In light of the findings in other species it seems that there has been a clear evolutionary progression in NO control of vertebrate blood vessels. The elasmobranch vasculature contains nitrergic nerves but not endothelial NOS, and the vascular smooth muscle apparently lacks a soluble GC that mediates vasodilation (Evans and Gunderson, 1998; Evans, 2000; Donald et al., 2003). In most teleost fish and amphibians, a soluble GC that mediates vasodilation is present in the vascular smooth muscle. We have provided evidence that the source of NO for the soluble GC in specific blood vessels of *A. australis* and *B. marinus* is the perivascular nitrergic nerves. Future work will be directed towards determining how general the mechanism of vascular regulation by nitrergic nerves is in teleost fish and amphibians.

List of abbreviations

ACh	acetylcholine
ANP	atrial natriueretic peptide
eNOS	endothelial NOS
ET-1	endothelin-1
GC	guanylyl cyclase
L-NNA	N^{ω}-nitro-L-arginine
NBT	Nitroblue Tetrazolium
nNOS	neural NOS
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	oxadiazole quinoxalin-1
PBS	phosphate-buffered saline
PLA	N^{ω}-propyl-L-arginine
sGC	soluble guanylyl cyclase
SNP	sodium nitroprusside
β-NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form

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