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Dual mechanisms for nitric oxide control of large arteries in the estuarine crocodile *Crocodylus porosus*

Brad R. S. Broughton* and John A. Donald

School of Life and Environmental Sciences, Deakin University, Geelong, Victoria, 3217, Australia

*Author for correspondence at present address: Department of Cell Biology and Physiology, University of New Mexico Health Sciences Center, MSC 08-4750, 1 University of New Mexico, Albuquerque, NM 87131-0001, USA (e-mail: bbroughton@salud.unm.edu)

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Summary

In reptiles, accumulating evidence suggests that nitric oxide (NO) induces a potent relaxation in the systemic vasculature. However, very few studies have examined the source from which NO is derived. Therefore, the present study used both anatomical and physiological approaches to establish whether NO-mediated vasodilation is *via* an endothelial or neural NO pathway in the large arteries of the estuarine crocodile *Crocodylus porosus*. Specific endothelial nitric oxide synthase (NOS) staining was observed in aortic endothelial cells following nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) histochemistry and endothelial NOS immunohistochemistry (IHC), suggesting that an endothelial NO pathway is involved in vascular control. This finding was supported by *in vitro* organ bath physiology, which demonstrated that the relaxation induced by acetylcholine (10^{-5} mol l^{-1}) was abolished in the presence of the NOS inhibitor, *N*-omega-nitro-L-arginine (L-NNA; 10^{-4} mol l^{-1}), the soluble guanylyl cyclase inhibitor, 1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ; 10^{-5} mol l^{-1}), or when the endothelium was removed. Interestingly, evidence for a neural NO pathway was also identified in large arteries of the crocodile. Neural NOS was located in perivascular nerves of the major blood vessels following NADPH-d histochemistry and neural NOS IHC and in isolated aortic rings, L-NNA and ODQ, but not the removal of the endothelium, abolished the relaxation effect of the neural NOS agonist, nicotine (3×10^{-4} mol l^{-1}). Thus, we conclude that the large arteries of *C. porosus* are potentially regulated by NO-derived from both endothelial and neural NOS.

Key words: nitric oxide, nitric oxide synthase, endothelium, nitrergic nerves, blood vessel, vasodilation.

Introduction

The vascular endothelium of mammals is responsible for the production of paracrines that regulate vascular tone, including nitric oxide (NO). NO is synthesised from precursors by nitric oxide synthase (NOS), an enzyme with three isoforms: neural NOS (nNOS or NOSI), inducible NOS (iNOS or NOSII), and endothelial NOS (eNOS or NOSIII) (Förstermann et al., 1994). NO derived from the vascular endothelium provides vasodilator tone that is important in the control of blood pressure (Moncada et al., 1991). In addition, blood vessels in the cranial, pelvic and gut regions are innervated by nitrergic nerves that mediate vasodilation *via* NO neurotransmission (Young et al., 2000), and in combination with the endothelium, nitrergic nerves provide a second means of NO control of vascular tone (Toda and Okamura, 2003).

Many studies in non-mammalian vertebrates (birds, reptiles, amphibians and teleost fishes) have shown that NO mediates vasorelaxation (Olson and Villa, 1991; Knight and Burnstock, 1993; Knight and Burnstock, 1996; Nilsson and Soderstrom, 1997; Martinez-Lemus et al., 1999; Crossley et al., 2000; Axelsson et al., 2001; Broughton and Donald, 2002; Jennings et al., 2004; Broughton and Donald, 2005; Galli et al., 2005; Skovgaard et al., 2005), but there is some controversy as to whether the vascular endothelium synthesises and releases NO in fishes and amphibians. A number of studies have provided evidence that endothelial NO signalling is present in the vasculature of fishes (Mustafa and Agnisola, 1998; Fritsche et al., 2000; Pellegrino et al., 2002) and amphibians (Rumbaut et al., 1995; Knight and Burnstock, 1996). In contrast, in many species of teleost and elasmobranch fishes, there is physiological and anatomical evidence that an endothelial NO system is absent from the vasculature, and that the endothelium-derived relaxing factor is in fact a prostaglandin (Olson and Villa, 1991; Evans and Gunderson, 1998; Miller and Vanhoutte, 2000; Park et al., 2000; Donald et al., 2004; Jennings et al., 2004). Furthermore, it was found in the Australian short-finned eel *Anguilla australis* that NO control of the dorsal aorta and the intestinal veins was provided by nitrergic nerves and that there was no evidence for endothelial NOS or endothelial NO signalling (Jennings et al., 2004).

Similarly, we have shown that the large blood vessels of the cane toad *Bufo marinus* are regulated by NO that is derived from perivascular nerves and not the vascular endothelium (Broughton and Donald, 2002; Broughton and Donald, 2005), and that NOS is present in the perivascular nerves, but not the vascular endothelium of the American bullfrog *Rana catesbeiana* (Donald and Broughton, 2005). Thus, we proposed that perivascular nerves may be the primary means for providing NO regulation of blood vessels in teleost fishes and amphibians.

The question then arises as to when vascular endothelial NO signalling first appeared in vertebrate evolution. In 1986 (prior to the discovery of biological NO), it was demonstrated that the acetylcholine (ACh)-mediated relaxation in the descending aorta of the spectacled caiman *Caiman crocodylus* was endothelium-dependent, which suggested that an endothelial-derived relaxing factor was involved in regulating vascular tone in reptiles (Miller and Vanhoutte, 1986). More recently, it was found that the ACh-mediated relaxation in isolated, pre-constricted aortic rings of the garter snake *Thamnophis sirtalis parietalis* was abolished or greatly reduced by the NOS inhibitor, N^{ω}-nitro-L-arginine methyl ester (L-NAME), or when the endothelium was removed (Knight and Burnstock, 1993). It was thus concluded that ACh was mediating vasorelaxation following the synthesis of NO by an endothelial NOS. Furthermore, endothelial NO signalling has been clearly demonstrated in the arteries of chickens (see Hasegawa and Nishimura, 1991; Martinez-Lemus et al., 1999; Le Noble et al., 2000). Similarly to amphibians (Broughton and Donald, 2002; Broughton and Donald, 2005) and teleost fish (Jennings et al., 2004), neural NOS immunoreactivity (IR) has also been found in the perivascular nerves of reptilian blood vessels, including the estuarine crocodile *Crocodylus porosus* (Karila et al., 1995; Olsson and Gibbins, 1999; Axelsson et al., 2001; Donald and Broughton, 2005). Therefore, there is the potential for vascular control by NO derived from the endothelium and perivascular nerves of reptiles. A number of studies have shown that a NO tonus is present in the circulation of various reptilian species (see Skovgaard et al., 2005).

This study investigated the NO control of the large arteries of the crocodile, *C. porosus*. We used both NADPH-d histochemistry and immunohistochemistry (IHC) to determine whether endothelial and/or neural NOS are present in the large arteries, and *in vitro* organ bath physiology to examine the mechanism of NO control. Our findings show, for the first time, both endothelial and neural NO control of systemic blood vessels in a non-mammalian vertebrate.

Materials and methods

Animals

Estuarine crocodiles *Caiman porosus* Schneider 1801, of either sex, body mass 600–900 g, were purchased from Cairns Crocodile Farm (Queensland, Australia). The crocodiles were housed in 2 m diameter circular tanks containing freshwater, and a dry area for basking. The room was kept at a constant temperature of 30°C and contained a UV light that was on a 12:12 h light:dark cycle (lights on at 07:00 h). The tanks were cleaned weekly, and the animals were fed rat pups twice a week. Prior to experimentation, the animals were sacrificed by an intramuscular injection of ketamine hydrochloride (100 μl 100^{-1} g), followed by decapitation and pithing. All experiments were approved by the Animal Welfare Committee of Deakin University (approval A21/2000). The animals were acquired and transported under permit from the Australian Department of Environment and Sustainability (permit numbers 10001872 and 10002555).

NADPH-d histochemistry

The right aorta, dorsal aorta, aortic anastomosis and vena cava were dissected free and immersed in phosphate buffered saline (PBS, 0.01 mol l^{-1} phosphate buffer and 0.15 mol l^{-1} NaCl; pH 7.4) at 4°C. Prior to fixation, the blood vessels that were prepared as whole-mounts were opened and pinned out on dental wax, endothelium side up. All blood vessels were fixed in 4% formaldehyde (pH 7.4) at 4°C for 1 h. They were then washed in 0.01 mol l^{-1} PBS (3×10 min) and either removed from the dental wax (whole-mounts) or cryoprotected overnight in a solution of 0.01 mol l^{-1} PBS containing 30% sucrose for cryostat sectioning. The cryoprotected tissues were mounted in an OCT Tissue-Tek (Bayer Diagnostics, Puteaux, France) mould and frozen in liquid nitrogen. Sections were then cut at 12 μm on a Reichardt-Jung cryostat (Heidelberg, Germany, and thaw-mounted onto 0.1% gelatinised slides. The sectioned blood vessels and whole-mounts were stained in a NADPH-d 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), at room temperature, for 15 min at 37°C; this mixture was kept in the dark as it is light sensitive (Beesley, 1995). The tissues were then washed in 0.01 mol l^{-1} PBS (3× 2 min) and mounted 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). Both tissue sections and whole-mounts were observed under a light microscope (Zeiss, Oberkochen, Germany) and were photographed with a digital colour system (Spot 35 Camera System, USA).

Endothelial and neural NOS immunohistochemistry

The right aorta, dorsal aorta and aortic anastomosis were fixed as whole-mount preparations, as described above. The blood vessels were unpinned, washed in 0.01 mol l^{-1} PBS (3×10 min), incubated in DMSO (3×10 min) and washed in 0.01 mol l^{-1} PBS (5×2 min). The blood vessels were then incubated in a polyclonal antibody raised against mouse endothelial NOS (1:1000) (O'Brien et al., 1995), or a polyclonal antibody raised against sheep neural NOS (1:4000) (Anderson et al., 1995), for 24 h at room temperature in a humid box. The following day, the tissues were washed in 0.01 mol l^{-1} PBS (3×10 min) to remove any excess antibody, and were incubated in a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or FITC-conjugated goat anti-sheep IgG (1:200) (Zymed Labratories, San Francisco, CA,

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), mounted in buffered glycerol, and observed under a fluorescence microscope (Zeiss) using a FITC filter, and photographed as above.

In vitro organ bath physiology

After sacrifice, segments of the right aorta and dorsal aorta were excised and placed in Mackenzie's balanced salt solution (115 mmol l^{-1} NaCl, 3.2 mmol l^{-1} KCl, 20 mmol l^{-1} $NaHCO_3$, 3.1 mmol l^{-1} NaH_2PO_4, 1.4 mmol l^{-1} $MgSO_4$, 16.7 mmol l^{-1} D[+] glucose and 1.3 mmol l^{-1} $CaCl_2$; pH 7.2–7.3), which was maintained at 30°C. 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 Mackenzie's balanced salt solution, which was aerated with 95% O_2 and 5% CO_2. Tension was recorded by force transducers (Grass-FT03, West Warwick, USA) connected through a PowerLab™ (ADI Instruments, Castle Hill, Australia) data acquisition system to a PC computer. 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 deliberately removed by gently rotating the blood vessel on a fine toothpick, and the extent of removal was determined using Haematoxylin and Eosin staining (see below). Prior to administering various vasorelaxant substances, each vessel was pre-contracted with endothelin 1 (ET-1, 10^{-8} mol l^{-1}), and vasocontraction was allowed to reach its maximum. The extent of vasorelaxation was determined for each relaxant by scoring the degree of relaxation as a ratio by assigning a relaxation to pre-contraction levels as 100%. In all experiments from the one animal, an additional ring from the same blood vessel was used as a matched control for the comparison of drug effects. Data are expressed as mean ± 1 standard error (s.e.m.) of five experiments from five animals, and statistical analysis was performed with paired *t*-tests using the SPSS (10.0) statistical package; $P \leq 0.05$ was considered significant.

Haematoxylin and Eosin staining

After *in vitro* organ bath physiology, the aortic rings from *C. porosus* with the endothelium removed and the matched controls with the endothelium intact were fixed and sectioned as described above. The vessel sections were stained with Haematoxylin and Eosin to verify the presence or absence of the endothelium.

Materials

Sodium nitroprusside, ACh, L-NNA, atropine, β-NADPH, NBT and Triton X-100 were obtained from Sigma (St Louis, MO, USA). Nicotine was purchased from BDH chemicals (Melbourne, Australia) and ET-1 and rat atrial natriuretic peptide (rANP) were obtained from Auspep (Melbourne, Australia). Oxadiazole quinoxalin-1 was purchased from Alexis (San Diego, CA, USA), and the NOS antibodies were obtained from Chemicon (Melbourne, Australia).

Results

NADPH-d histochemistry and endothelial NOS and neural NOS IHC

Similar patterns of staining were observed in the endothelium of the right and dorsal aortae, aortic anastomosis and vena cava following NADPH-d histochemistry (*N*=5; Fig. 1A–C). Each vascular endothelial cell appeared to contain a small patch of intense, perinuclear staining in both whole-mount preparations and blood vessel sections, indicating the presence of NOS; the NADPH-d staining pattern was similar to that observed in the blood vessels of mammals (see O'Brien et al., 1995). The frequency and staining intensity of the patches was less in the vena cava in comparison to the large arteries and the aortic anastomosis (Fig. 1B). Furthermore, in the dorsal aorta and the aortic anastomosis, a similar pattern of staining was observed in the endothelial cells following endothelial NOS IHC (*N*=3; Fig. 1D).

Upon scanning through the various layers of the blood vessels following NADPH-d histochemistry, positive staining was also observed in the perivascular nerve fibres (*N*=5; Fig. 2A,B). This observation was confirmed with IHC, which showed specific neural NOS-IR in the perivascular nerves of each blood vessel (*N*=5; Fig. 2C,D); the neural NOS-IR showed the same distribution pattern as that of the NADPH-d staining. Therefore, a single description is provided for the observations made using both techniques. A moderate to dense plexus of neural NOS-positive nerves was observed in all blood

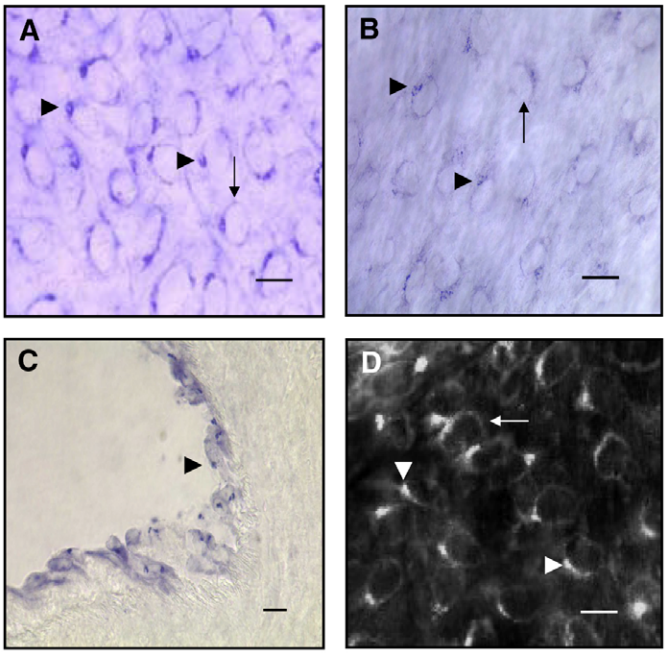

Fig. 1. Photomicrographs showing whole-mount (A,B,D) and sectioned (C) preparations of the crocodile right aorta (A), vena cava (B), aortic anastomosis (C) and dorsal aorta (D) following processing for NADPH-d histochemistry (A–C) and endothelial NOS IHC (D). In all vessels, punctate endothelial NOS-positive staining (arrowheads) occurred around the nuclei (arrows) of the endothelial cells. Scale bars, 10 μm.

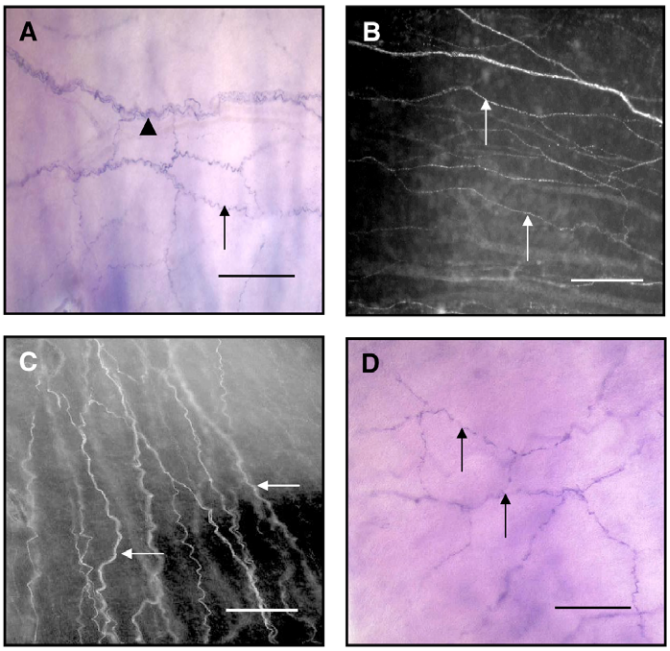

Fig. 2. Photomicrographs showing wholemount preparations of the crocodile right aorta (A), vena cava (B), aortic anatomosis (C) and dorsal aorta (D) following processing for NADPH-d histochemistry (A,D) and neural NOS IHC (B,C,). A moderate plexus of neural NOS-positive perivascular nerve fibres (arrows) was observed in the outer layers of the wall of each vessel. In addition, some neural NOS-positive perivascular nerve bundles (arrowhead) were observed. Scale bars, 100 μm.

Table 1. *Summary of the vasodilatory responses of the crocodile right and dorsal aortae to various treatments associated with the NO signalling system*

	Vasodilation (%)	
Treatment	Right aorta	Dorsal aorta
SNP	109.8±7	94±7.1
ACh	71.7±7.6	79.6±4.4
Nicotine	32.8±3.6	38.2±1.8
SNP	109.2±10.5	91.9±4.3
ODQ/SNP	No dilation	No dilation
ACh	71.7±7.6	79.6±4.4
ODQ/ACh	Constriction	Constriction
Nicotine	32.8±3.6	38.2±1.8
ODQ/Nicotine	No dilation	No dilation
ACh	78.6±5.8	86.3±3.9
L-NNA/ACh	Constriction	No dilation
Nicotine	32.8±3.6	38.2±1.8
L-NNA/Nicotine	No dilation	No dilation
ACh	77.2±5	90.3±3.4
Endo denuded/ACh	Constriction	Constriction
Nicotine	39.3±6.9	42.8±6.2
Endo denuded/Nicotine	42±4.6	39.1±6.3
ACh	78.6±5.8	86.3±3.9
Atropine/ACh	No dilation	No dilation

Endo denuded, endothelium denuded.
% vasodilation values are means ± s.e.m. (*N*=5).

vessels. All vessels contained neural NOS-positive nerve bundles (arrowhead, Fig. 2A) and single, varicose nerve fibres (arrows, Fig. 2A–D), although there was no distinct pattern in the distribution of perivascular nerves.

In vitro organ bath physiology

The right aorta and dorsal aorta were pre-contracted with ET-1 (10^{-8} mol l^{-1}). At the peak of contraction various chemicals associated with the relaxation mechanisms of NO were added. A summary of the data for all *in vitro* organ bath experiments is given in Table 1.

In the right and dorsal aortae, the NO donor, SNP (10^{-4} mol l^{-1}), induced a potent relaxation (*N*=5, Fig. 3A,D). To activate a putative endothelial NO system, ACh was used as an agonist because previous studies in mammals (see Moncada et al., 1991) and reptiles (see Knight and Burnstock, 1993) have shown that ACh indirectly stimulates the release of NO from endothelial NOS. In contrast, nicotine was used to stimulate the release of NO from nitrergic nerves, because previous studies in mammals (see Toda and Okamura, 2003), amphibians (Donald and Broughton, 2005) and teleost fish (Jennings et al., 2004) have shown that nicotine indirectly stimulates the release of NO from perivascular nerves. In the right and dorsal aortae, both applied ACh (10^{-5} mol l^{-1}; Fig. 3B,D) and nicotine (3×10^{-4} mol l^{-1}; Fig. 3C,D) produced a relaxation (*N*=5). In both aortae, the addition of the soluble guanylyl cyclase inhibitor, ODQ (10^{-5} mol l^{-1}), completely abolished the relaxation effect of SNP (10^{-4} mol l^{-1}; Fig. 4B), applied ACh (10^{-5} mol l^{-1}; Fig. 4B) and nicotine (3×10^{-4} mol l^{-1}; Fig. 5B), in comparison to the control vessels (*N*=5; Fig. 4A and Fig. 5A); ACh now caused a contraction, whereas SNP and nicotine had no effect. Rat ANP (10^{-8} mol l^{-1}), which mediates vasodilation *via* a particulate guanylyl cyclase (Winquist et al., 1984), caused a potent vasodilation in the presence of ODQ (rANP was only added to the vessels with ODQ; Fig. 4B, Fig. 5B).

To establish if the ACh- and nicotine-mediated vasodilations were dependent on the presence of an intact endothelium, the endothelium was removed from both aortae, which was subsequently verified by Haematoxylin and Eosin staining (Fig. 6 and Fig. 7). In the endothelium-denuded right and dorsal aortae, the vasodilation induced by ACh (10^{-5} mol l^{-1}) was abolished in comparison to vessels with the endothelium intact; ACh now caused a vasoconstriction (*N*=5; Fig. 6). In contrast, in the endothelium-denuded right aorta, the vasodilation induced by nicotine (3×10^{-4} mol l^{-1}) was not significantly different to the control vessels that had the endothelium intact (*P*=0.52, *N*=5); a similar effect was observed in the dorsal aorta (*P*=0.63, *N*=5; Fig. 7). In both

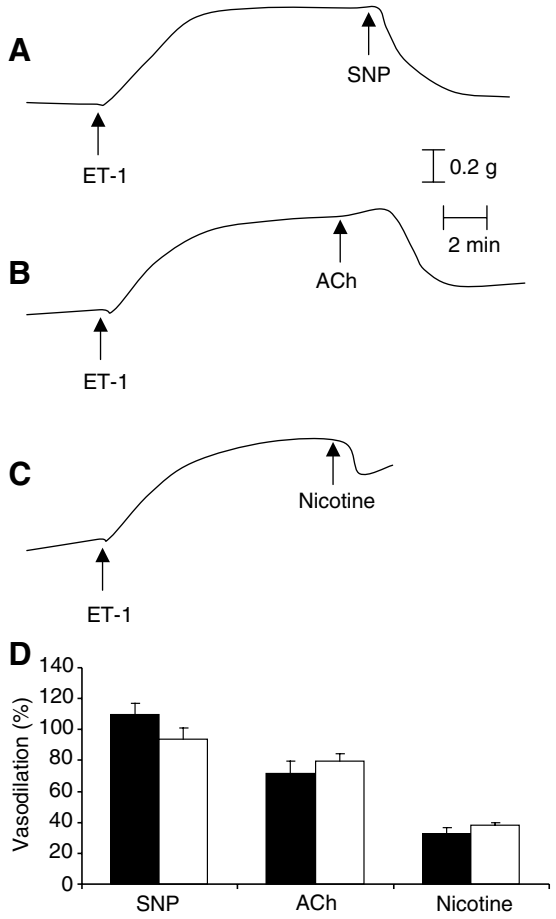

Fig. 3. Tension recordings showing the effect of SNP (A), ACh (B) and nicotine (C) on the crocodile right aorta (A,C) and dorsal aorta (B). The vessels were incubated with ET-1 (10^{-8} mol l^{-1}) until a maximal constriction was achieved, and then either SNP (10^{-4} mol l^{-1}), ACh (10^{-5} mol l^{-1}), or nicotine (3×10^{-4} mol l^{-1}) was added. All three chemicals caused a marked vasodilation. (D) Mean response of pre-constricted right aorta (filled bars) and dorsal aorta (open bars) to SNP (10^{-4} mol l^{-1}), ACh (10^{-5} mol l^{-1}) and nicotine (3×10^{-4} mol l^{-1}). Values are means ± s.e.m., N=5.

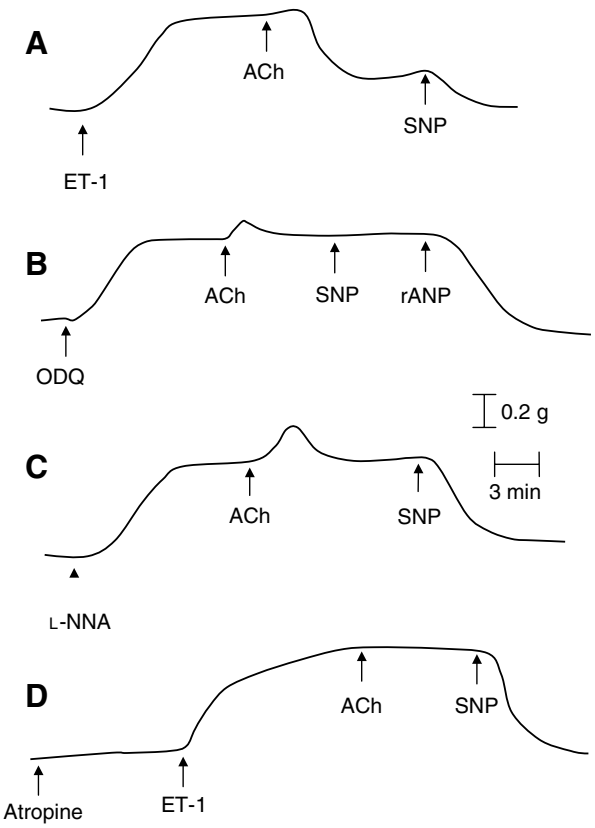

Fig. 4. Tension recordings showing the effect of ACh (A–D), SNP (A–D) and rANP (B) on the crocodile right aorta in the presence of ODQ (B), L-NNA (C) and atropine (D). The aortae were pre-treated with ODQ (10^{-5} mol l^{-1}), L-NNA (10^{-4} mol l^{-1}) or atropine (10^{-6} mol l^{-1}) for approximately 10 min before being constricted with ET-1 (10^{-8} mol l^{-1}). Once maximal constriction was achieved, ACh (10^{-5} mol l^{-1}) was added, followed by SNP (10^{-4} mol l^{-1}); rANP was added to vessels pre-treated with ODQ only. The ACh-mediated vasodilation was abolished in the presence of ODQ, L-NNA and atropine, whereas the SNP-mediated vasodilation was only abolished by ODQ. Rat ANP induced a potent vasodilation in the presence of ODQ (N=5).

aortae, the ACh-mediated vasodilation was abolished in the presence of the muscarinic receptor antagonist, atropine (10^{-6} mol l^{-1}; N=3; Fig. 4D); however, SNP-mediated vasodilation was not effected. The effect of atropine on nicotine-mediated vasodilation was not determined.

To determine if both the ACh- and nicotine-mediated vasodilations were *via* NOS, the vessels were pre-treated with L-NNA. The addition of L-NNA caused a constriction in the aortic rings with or without the endothelium, although the constriction was more potent in the vascular rings with the endothelium present (data not shown). The vasodilation induced by ACh (10^{-5} mol l^{-1}) was abolished by L-NNA (10^{-4} mol l^{-1}) in both the right and dorsal aortae with an intact endothelium; however, SNP produced a potent vasodilatory response (N=5; Fig. 4C). Furthermore, in the endothelium-denuded right and dorsal aortae, the vasodilation induced by nicotine (3×10^{-4} mol l^{-1}) was abolished in the presence of L-NNA (10^{-4} mol l^{-1}; N=5; Fig. 5C).

Discussion

Although several studies have shown that NO mediates relaxation in the vasculature of reptiles (Knight and Burnstock, 1993; Crossley et al., 2000; Skovgaard et al., 2005), little is understood as to whether NO is generated by an endothelial and/or neural NOS. Interestingly, the present study demonstrates, using both anatomical and physiological approaches, that both NO signalling mechanisms are capable of regulating the large arteries of the estuarine crocodile *C. porosus*.

NADPH-d histochemistry revealed punctate, perinuclear staining in the endothelial cells of the right and dorsal aortae,

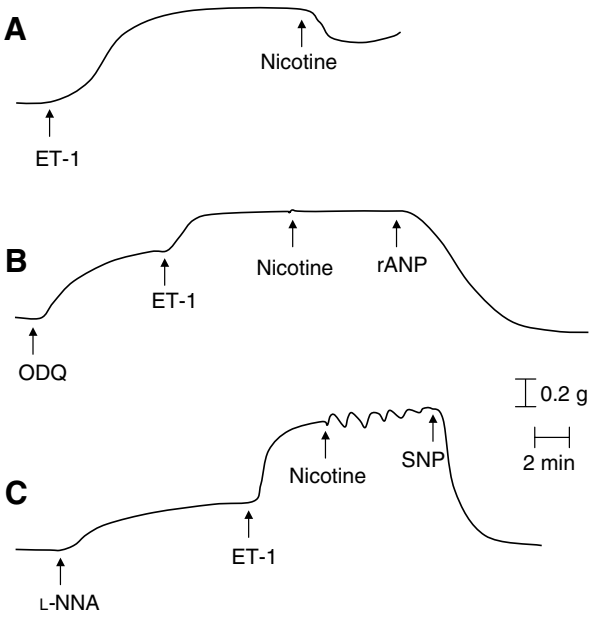

Fig. 5. Tension recordings showing the effect of nicotine (A–C), rANP (B) and SNP (C) on the crocodile dorsal aorta in the presence of ODQ (B) and L-NNA (C). The aortae were pre-treated with ODQ (10^{-5} mol l^{-1}) or L-NNA (10^{-4} mol l^{-1}) for approximately 10 min before being constricted with ET-1 (10^{-8} mol l^{-1}). Once maximal constriction was achieved, nicotine (3×10^{-4} mol l^{-1}) was added, followed by either rANP (10^{-8} mol l^{-1}), which was added to the vessels pre-treated with ODQ, or SNP (10^{-4} mol l^{-1}), which was added to the vessels pre-treated with L-NNA. The nicotine-mediated vasodilation was abolished in the presence of ODQ or L-NNA, but rANP and SNP both induced a potent vasodilation in the presence of ODQ and L-NNA, respectively (N=5).

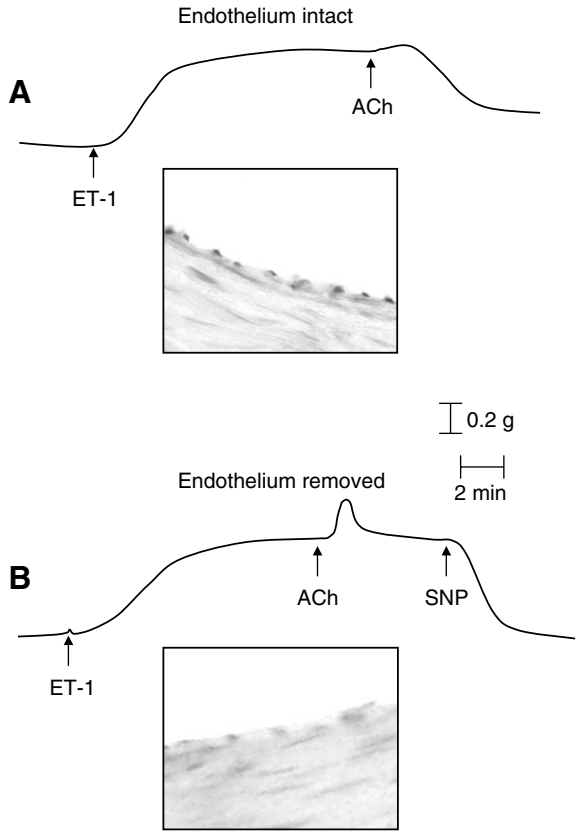

Fig. 6. Tension recordings showing the effect of ACh on the crocodile right aorta with the endothelium intact (A) and with the endothelium removed (B). Haematoxylin and Eosin staining was used to verify that the endothelium was present or removed (see inset). The preparations were exposed to ET-1 (10^{-8} mol l^{-1}) until a maximum constriction was achieved, and then ACh (10^{-5} mol l^{-1}) was added. Acetylcholine only induced a vasodilation in the vessels with the endothelium intact (N=5).

aortic anastomosis, and vena cava of *C. porosus*, which was similar to the NADPH-d staining observed in the endothelial cells of mammalian blood vessels (see O'Brien et al., 1995) and the pigeon dorsal aorta (Donald and Broughton, 2005). More specifically, similar staining was observed in the dorsal aorta and aortic anastomosis following endothelial NOS IHC. These findings indicated that the large blood vessels of *C. porosus* are potentially regulated by NO synthesised and released from an endothelial NOS located in the endothelial cells. The lower frequency of stained patches in the vena cava compared to the arteries may be due to different characteristics of endothelial cells in veins compared to arteries, which would be consistent with observations in the mammalian circulation (Isogai et al., 1991).

Furthermore, NADPH-d staining was observed in nerve bundles and single varicose fibres, which indicated that the nerves were NOS positive. The staining pattern using a specific antibody to neural NOS was identical to that obtained using NADPH-d histochemistry, which indicated that the NADPH-d staining was specific for neural NOS. In *C. porosus*, the presence of neural NOS in perivascular nerves was previously reported in the gastrointestinal vasculature (Karila et al., 1995; Olsson and Gibbins, 1999). Moreover, Axelsson et al. (Axelsson et al., 2001) demonstrated that NOS was present in the perivascular nerves of the aortic anastomosis, but the distribution of NOS was diffuse and difficult to interpret in the photomicrographs of the tissue sections. In contrast, the use of tissue whole-mounts in the present study demonstrated that a moderate-to-dense plexus of perivascular nitrergic nerves is present in the vasculature of *C. porosus*. Therefore, the systemic vasculature of *C. porosus* is potentially modulated by NO derived from both endothelial and neural NOS. To the best of our knowledge, this is the first study to show that both endothelial and neural NOS are present in the same blood vessel in a non-mammalian vertebrate.

Although the anatomical findings indicated that both an endothelial and neural NO system are present in the large arteries of *C. porosus*, it was imperative to determine whether NO derived from endothelial and/or neural NOS was involved in regulating vascular tone. Two different pharmacological tools were used to stimulate the release of endothelially and/or

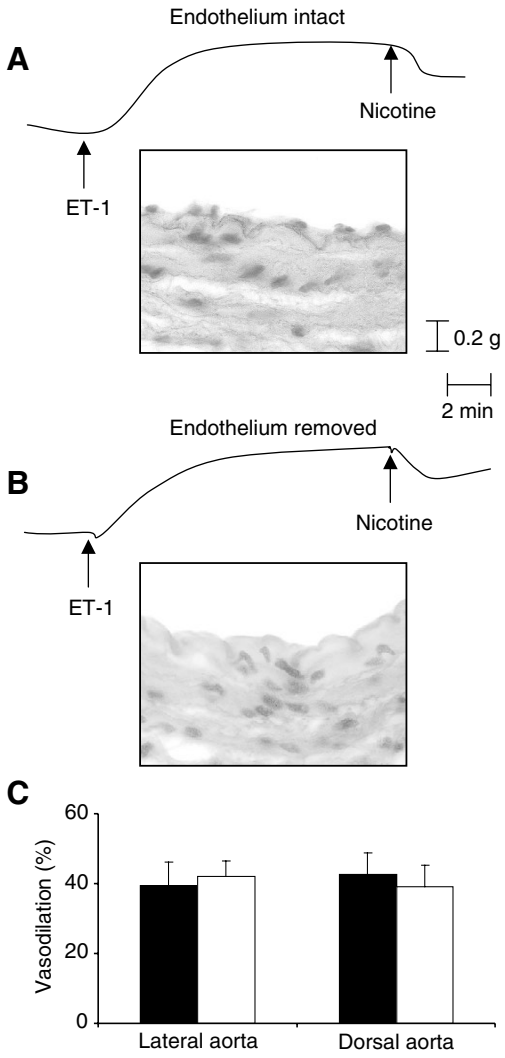

Fig. 7. Tension recordings showing the vasodilatory effect of nicotine on the crocodile right aorta with the endothelium intact (A) and with the endothelium removed (B). Haematoxylin and Eosin staining was used to verify that the endothelium was present or removed (see inset). The preparations were exposed to ET-1 (10^{-8} mol l^{-1}) until a maximum constriction was achieved and then nicotine (3×10^{-4} mol l^{-1}) was added. Nicotine induced a vasodilation in both preparations. The lower graph shows the mean response of nicotine on pre-constricted right and dorsal aortae with the endothelium intact (filled bars) or the endothelium removed (open bars). There is no significant difference in the nicotine-mediated vasodilation with or without the endothelium (right aorta, P=0.52, dorsal aorta, P=0.63, N=5).

neurally derived NO. Acetylcholine was used as an agonist for activating the endothelial NO system, as it is well-documented in mammals that ACh activates endothelial NOS to produce NO (see Moncada et al., 1991). The nicotinic receptor agonist, nicotine was used to stimulate the release of NO from perivascular, nitrergic nerves. Previously, nicotine has been shown to specifically cause the release of NO from nerves in the vasculature of various mammals (see Toda and Okamura, 2003) and non-mammalian vertebrates (Jennings et al., 2004; Donald and Broughton, 2005).

In the right and dorsal aortae of *C. porosus*, the ACh-mediated relaxation was endothelium-dependent and abolished in the presence of atropine, L-NNA and ODQ. These data indicated that ACh mediates relaxation by activating a similar endothelial NO signalling cascade to that found in mammalian blood vessels (Moncada et al., 1991). These findings are consistent with those reported by Knight and Burnstock (Knight and Burnstock, 1993), who demonstrated that the ACh-mediated relaxation in the dorsal aorta of *T. sirtalis parietalis* was abolished or significantly reduced in the presence of L-NNA, or when the endothelium was removed. Furthermore, ACh-mediated relaxation that is probably due to NO was reported in isolated, vascular rings of the chicken aorta (Hasegawa and Nishimura, 1991) and pulmonary artery (Martinez-Lemus et al., 1999). The presence of an endothelial NO system in the vasculature of both *C. porosus* and birds is to be expected because it is thought that birds evolved from a crocodilian-like ancestor (Hedges, 1994). Overall, it appears that NO derived from endothelial NOS plays an important role in maintaining tone in the large arteries of *C. porosus*. Interestingly, when the endothelium was removed from the vasculature of *C. porosus*, ACh induced a contraction even though nitrergic nerves had been shown to be present in these vessels. This is in contrast to our previous findings in the toad *B. marinus*, in which ACh induced a relaxation in the endothelium-denuded central aortae (Broughton and Donald, 2002). One possible explanation for this difference is that the muscarinic receptors are only located on the endothelial cells in *C. porosus*, whereas in *B. marinus*, it is likely that they are located on the perivascular, nitrergic nerves.

The presence of nitrergic nerves in the large arteries of *C. porosus* suggested that neurally derived NO was involved in regulating vascular tone, in addition to that derived from the endothelium. In the right and dorsal aortae, the nicotine-mediated relaxation was abolished by ODQ and L-NNA, but the removal of the endothelium had no significant effect. This suggested that nicotine, acting independently of the endothelium, activated neural NOS to synthesise and release NO. This subsequently induced a relaxation *via* a soluble guanylyl cyclase, which is the first demonstration of neurally based NO signalling in reptilian blood vessels. The findings are similar to those previously reported in the large systemic blood vessels of *B. marinus* (Broughton and Donald, 2002; Broughton and Donald, 2005) and *A. australis* (Jennings et al., 2004), which suggests that nitrergic nerves may play a significant role in regulating the large blood vessels of lower vertebrates. This is the first study to demonstrate both endothelial and neural NO control of large systemic blood vessels in any non-mammalian vertebrate species. However, it remains to be determined how the dual NO systems contribute to vascular control *in vivo* in *C. porosus*.

It is well documented that NO contributes to basal tone in the circulation of mammals (Moncada et al., 1991). In the present study, L-NNA increased the basal tonus in both the right

and dorsal aortae with or without the endothelium being present; however, the response was less in endothelium-denuded vessels than when the endothelium was intact. This suggested that in the absence of an endothelial NO system, NO-derived from another source, which is likely to be from neural NOS located in perivascular nerves, is also able to contribute to basal tone. The presence of a basal NO tonus has also been reported in the dorsal aorta of *T. sirtalis parietalis* (Knight and Burnstock, 1993) and in the aortic anastomosis of *C. porosus* (Axelsson et al., 2001), but not in isolated coeliac or mesenteric arteries of the latter species (Kågström et al., 1998). Furthermore, *in vivo* injection of the NOS inhibitor, L-NAME, caused an elevation in blood pressure in American alligator *Alligator mississippiensis* (Platzack et al., 2002), the varanid lizard *Varanus exanthematicus*, ball python *Python regius*, and turtle *Trachemys scripta*, but not the rattlesnake *Crotalus durissis* (see Skovgaard et al., 2005). Thus, it appears that there is a tonic release of NO from endothelial and/or neural NOS in the circulation of some reptiles. Skovgaard et al. have proposed that the NO regulation of the reptilian circulation is correlated with circulatory anatomy and lung complexity, where it is more developed in species with separated systemic and pulmonary circulation (Skovgaard et al., 2005). Accordingly, it would be intriguing to examine the *in vitro* mechanism of NO control of blood vessels in the different reptilian groups.

The discovery that NO is derived from the endothelium and nitrergic nerves in the large arteries of *C. porosus* is important for our understanding of vascular NO control in this species; however, further studies are required to determine if endothelial and/or neural NO signalling mechanisms are present in smaller resistance vessels that control blood flow distribution and overall blood pressure. Nitric oxide is clearly an important regulator of the crocodilian circulation since in *C. porosus* it was found to regulate vascular tone in the aortic anastomosis (Axelsson et al., 2001) and play an important role in buffering blood pressure against changes in heart rate during cooling (Seebacher and Franklin, 2004). However, in both studies the source from which NO was produced and released was not determined. Therefore, future studies of cardiovascular regulation in *C. porosus* will need to consider that NO regulation could be provided by both endothelial and/or neural NOS systems.

List of abbreviations

ACh	acetylcholine
eNOS (NOSIII)	endothelial NOS
ET-1	endothelin 1
FITC	fluorescein isothiocyanate
IHC	immunohistochemistry
iNOS (NOSII)	inducible NOS
IR	immunoreactivity
L-NAME	N^{ω}-nitro-L-arginine methyl ester
L-NNA	*N*-omega-nitro-L-arginine
NADPH-d	nicotinamide adenine dinucleotide phosphate-diaphorase
NBT	nitroblue tetrazolium
nNOS (NOSI)	neural NOS
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	1H-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one
PBS	phosphate buffered saline
rANP	rat atrial natriuretic peptide
SNP	sodium nitroprusside

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