Neurally-derived nitric oxide regulates vascular tone in pulmonary and cutaneous arteries of the toad, *Bufo marinus*

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Running title: Nitric oxide control of toad pulmo-cutaneous arteries

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Abstract

In this study, the role of NO in regulation of the pulmo-cutaneous vasculature of the toad, *Bufo marinus*, was investigated. *In vitro* myography demonstrated the presence of a neural NO signalling mechanism in both arteries. Vasodilation induced by nicotine was inhibited by the soluble guanylyl cyclase (GC) inhibitor, 1H-[1,2,4]Oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and the nitric oxide synthase (NOS) inhibitor, *N*^o^-*Nitro-L-arginine (L-NNA).* Removal of the endothelium had no significant effect on the vasodilation. Furthermore, pre-treatment with *N*^5^-(*1*-Imino-3-butenyl)-L-ornithine (vinyl-L-NIO), a more specific inhibitor of neural NOS (nNOS), caused a significant decrease in the nicotine-induced dilation. In the pulmonary artery only, a combination of L-NNA and the calcitonin gene-related peptide (CGRP) receptor antagonist, CGRP(8-37), completely blocked the nicotine-induced dilation. In both arteries, the vasodilation was also significantly decreased by glibenclamide, an ATP-sensitive K⁺ (K⁺\text{ATP}) channel inhibitor. Levcromakalim, a K⁺\text{ATP} channel opener, caused a dilation that was blocked by glibenclamide in both arteries. In the pulmonary artery, NO donor-mediated dilation was significantly decreased by pre-treatment with glibenclamide. The physiological data were supported by NADPH-diaphorase histochemistry and immunohistochemistry, which demonstrated NOS in perivascular nerve fibres but not the endothelium of the arteries. These results indicate that the pulmonary and cutaneous arteries of *B. marinus* are regulated by NO from nitrergic nerves rather than NO released from the endothelium. The nitrergic vasodilation in the arteries appears to be caused, in part, via activation of K⁺\text{ATP} channels. Thus, NO could play an important role in determining pulmo-cutaneous blood flow and the magnitude of cardiac shunting.

**Keywords:** endothelium; nitric oxide synthase; autonomic nervous system; amphibian; vasodilation
Introduction

In mammals, it is known that NO released from the vascular endothelium contributes to the maintenance of a low pulmonary vascular resistance and opposes hypoxic vasoconstriction (see [39]). In addition to the endothelium, nitrergic nerves are also thought to provide non-adrenergic, non-cholinergic vasodilation of the pulmonary vasculature [3, 27, 28, 42].

The mechanism of vascular NO signalling in amphibians is controversial because there are conflicting data on whether NO is derived from the endothelium or perivascular nerves. Initial studies on the presence of an endothelially-derived relaxing factor showed that ACh mediated an endothelium-dependent vasodilation in the systemic vasculature [25, 31], which was abolished by the NOS inhibitor, $N^\omega$-Nitro-L-arginine methyl ester (L-NAME), in leopard frog [25]. This suggested that NO released from the endothelium was responsible for mediating ACh-induced vasodilation in the aortae of frogs. In addition, studies of the microcirculation of various amphibian species (including toad, Bufo marinus) showed that capillary blood flow decreased following NOS inhibition, which indicated tonic NO control of the circulation that was attributed to an endothelial NO system [37, 38, 40]. However, we recently demonstrated that NO regulation of the lateral aortae, dorsal aorta and large veins of B. marinus occurred independently of the endothelium. Instead, NO signalling in these blood vessels occurred via nitrergic nerves [7, 8, 15].

Blood flow to the skin and lungs of amphibians is regulated by the resistance of the pulmo-cutaneous circulation and by systemic vascular resistance due to the incomplete separation of the right and left sides of the heart [17]. The external pulmonary artery distal to the branching of the cutaneous artery is very muscular and has been reported to form a distinct sphincter innervated by vagal cholinergic vasoconstrictor nerves [10, 13]. In addition, the pulmonary vasculature receives an adrenergic innervation via a vago-sympathetic nerve trunk, which mediates vasodilation [10]. In contrast, the cutaneous artery of B. marinus is not innervated.
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by cholinergic nerves but does contain adrenergic vasoconstrictor nerves [30, 45]. Thus, blood flow in the pulmo-cutaneous circulation can be finely regulated by the autonomic nervous system.

While much is understood about NO control of the mammalian pulmonary circulation, the source and role of NO in amphibian pulmo-cutaneous circulation is not known and is the focus of the current study. We found no evidence for NOS in the endothelium of the pulmonary and cutaneous arteries of the toad, *B. marinus*, but nNOS was observed in perivascular nerves. Blood vessel myography showed that nitrergic nerves rather than the endothelium could provide NO regulation of vascular resistance in the toad pulmo-cutaneous circulation.

Methods

Animals. All experiments complied with Australian law on the use of animals for experimentation and were approved by the Animal Welfare Committee of Deakin University (Approval No. A5/2006). Toads, *B. marinus*, of either sex and a mass of 90-150 g, were purchased from a commercial supplier in Queensland, Australia. Toads were maintained at the Deakin University Animal House at 20-25 ºC and were not fed during captivity (up to 1 month), but had *ad libitum* access to water. Prior to experimentation, animals were sacrificed by decapitation, followed by pithing of the spinal cord.

Blood vessel physiology. After sacrifice, the pulmonary and cutaneous arteries were excised and placed in Mackenzie’s balanced salt solution (115.0 mM NaCl, 3.2 mM KCl, 20 mM NaHCO₃, 3.1 mM NaH₂PO₄·H₂O, 1.4 mM MgSO₄·7H₂O, 16.7 mM D-glucose and 1.3 mM CaCl₂·2H₂O; pH 7.2). For pulmonary arteries, individual rings of approximately 2-3 mm were mounted horizontally between two hooks for the measurement of isometric force and placed in a 50 ml organ bath. Blood vessel rings were bathed in 15 ml of Mackenzie’s
balanced salt solution, maintained at 22 °C and aerated with 95% O₂ and 5% CO₂. Force transducers (Grass-FT03) were linked to a PowerLab™ (ADInstruments) data collection system and a personal computer. An initial tension of 0.5 g was applied for at least 30 min to allow the vessels to equilibrate. Cutaneous arteries (internal diameter approximately 250 µm) were cut into individual rings of 2-3 mm, mounted horizontally between two pieces of 40 µm wire and attached to separate jaws of a dual wire myograph (Model 410A, Danish Myo Technology). The rings were bathed in 5 ml of Mackenzie’s balanced salt solution, maintained at 22 °C and aerated with 95% O₂ and 5% CO₂. Tension was placed on the cutaneous arteries by increasing the distance between the internal wires until they were flush against the vessel wall; vessels were left to equilibrate for at least 30 min. The myograph was linked to a Myo-Interface system, which was in turn attached to a PowerLab™ data collection system and a personal computer.

To determine the mechanisms of NO signalling, the pulmonary artery was pre-constricted with endothelin-1 (ET-1; 10⁻⁸ mol l⁻¹) and the cutaneous artery with the prostaglandin H₂-analogue, U-46619 (10⁻⁶ mol l⁻¹), and vasoconstriction was allowed to reach its maximum. The extent of vasodilation was determined as a percentage of the initial vasoconstriction. For experiments, matched controls were used from the same animal for comparison of drug effects. In some experiments, the endothelium was removed from the pulmonary artery by rubbing with a toothpick and from the cutaneous artery with a wire, which was verified with standard haematoxylin and eosin staining.

**Statistical analysis.** Data are expressed as mean ± one standard error (SE) of a minimum of five experiments, and statistical analysis was performed with paired samples t-tests using the SPSS (14.0) statistical package; a P value ≤ 0.05 was considered significant.
**NADPH-diaphorase histochemistry.** Pulmonary and cutaneous arteries were dissected free and immersed in PBS (0.01 mol l⁻¹ phosphate buffer and 0.15 mol l⁻¹ NaCl; pH 7.4). They were opened laterally and pinned endothelium-side up on dental wax, then fixed for 1 h in 4 % formaldehyde (pH 7.4) at 4 ºC. The arteries were washed in PBS (3 x 10 min) and removed from the dental wax. They were then stained in a NADPH-diaphorase mixture containing 1 mg/ml β-NADPH, 0.25 mg/ml nitroblue tetrazolium and 1 % Triton X-100 in 0.1 mol l⁻¹ Tris buffer (pH 8), for times ranging from 15-60 min at room temperature. The arteries were washed in PBS (3 x 5 min), mounted on slides in buffered glycerol (0.5 mol l⁻¹ Na₂CO₃ added drop-wise to 0.5 mol l⁻¹ NaHCO₃ to pH 8.6 combined 1:1 with glycerol), and observed using a point scanning laser confocal microscope (LSM 510 META; Zeiss). The descending aorta of crocodile, *Crocodylus porosus*, was used as a control to demonstrate the presence of NOS in the vascular endothelium [9].

**Endothelial and nNOS immunohistochemistry.** The pulmonary and cutaneous arteries were fixed as described above except two different times were used: 2 h for arteries to be incubated with the nNOS antibody (polyclonal sheep, 1:4000, [1]) and 24 h for arteries to be incubated with the endothelial NOS (eNOS) antibody (polyclonal rabbit, 1:500, [21]). Arteries were unpinned and washed in PBS (3 x 10 min); arteries to be used for eNOS immunohistochemistry were further washed in DMSO (3 x 10 min) and then in PBS (5 x 2 min). Separate pieces of blood vessel were then incubated in primary antibody for 24 h at room temperature in a humid box. The vessels were washed in PBS (3 x 10 min) to remove any excess primary antibody, then incubated with secondary antibody for 2-3 h in a humid box as follows: FITC-conjugated goat anti-sheep IgG (1:200) for vessel segments incubated with anti-nNOS, or FITC-conjugated goat anti-rabbit IgG (1:200) for vessel segments incubated with anti-eNOS. The blood vessels were washed again in PBS (3 x 10 min),
mounted in buffered glycerol, observed and photographed as above. Immunohistochemical controls were performed by omission of the respective primary antibody.

**Materials.** ACh, atropine, sodium nitroprusside (SNP), l-NNA, levcromakalim, guanethidine monosulfate (1:1), bretylium tosylate, indomethacin, esculetin, clotrimazole, β-NADPH, reduced form, nitroblue tetrazolium and Triton X-100 were obtained from Sigma (St. Louis, USA); ET-1, rat atrial natriuretic peptide (rANP), and CGRP (8-37) were purchased from Auspep (Melbourne, Australia); nicotine was purchased from BDH chemicals (Poole, UK); ODQ, vinyl-l-NIO and glibenclamide were obtained from Alexis Biochemicals (San Diego, USA); U-46619 and l-Ν6-(1-iminoethyl)lysine (l-NIL) were purchased from Cayman Chemical (Ann Arbor, USA); tetrodotoxin (TTX) was purchased from Alomone labs (Jerusalem, Israel); nNOS and FITC-conjugated goat anti-sheep IgG antibodies were obtained from Chemicon (Melbourne, Australia); the eNOS antibody was purchased from BD Transduction Laboratories (San Jose, USA); and the FITC-conjugated goat anti-mouse IgG antibody was obtained from Zymed Laboratories (San Francisco, USA).

**Results**

**Blood vessel physiology.** The physiological data are summarised in Table 1. Previously, we found that ACh (10^{-5} \text{ mol l}^{-1}) and nicotine (3 \times 10^{-4} \text{ mol l}^{-1}) caused a vasodilation in toad systemic arteries and veins, which was concluded to be due to the release of NO from perivascular, nitrergic nerves [7, 8, 15]. In toad pulmonary and cutaneous arteries, addition of ACh (10^{-5} \text{ mol l}^{-1}) always caused a vasoconstriction that was abolished by atropine (10^{-6} \text{ mol l}^{-1}; data not shown). The NO donor, SNP (10^{-4} \text{ mol l}^{-1}), induced a potent vasodilation, indicating the presence of a NO receptor in both the pulmonary and cutaneous arteries (Table 1).
In the pulmonary (Fig. 1a) and cutaneous (Fig. 1c) arteries, nicotine ($3 \times 10^{-4} \text{ mol l}^{-1}$) mediated a vasodilation. In both blood vessels, the soluble GC inhibitor, ODQ ($10^{-5} \text{ mol l}^{-1}$), caused a significant decrease in the nicotine-mediated vasodilation, but it was not completely abolished (Fig. 1b). ODQ abolished the SNP-mediated vasodilation, but had no effect on the dilation induced by rANP that is mediated via particulate GC (Fig. 1b). Pre-treatment of the arteries with the generic NOS inhibitor, L-NNA ($10^{-4} \text{ mol l}^{-1}$), significantly reduced the nicotine-mediated vasodilation in the pulmonary artery, and abolished the response to nicotine in the cutaneous artery (Fig. 1d). In the pulmonary artery only, pre-treatment with a combination of L-NNA ($10^{-4} \text{ mol l}^{-1}$) and the CGRP receptor antagonist, CGRP$_{(8-37)}$, completely abolished the nicotine-induced dilation. Interestingly, both ODQ and L-NNA alone caused a vasoconstriction prior to the addition of ET-1 or U-46619 (Figs. 1b, d). In addition, pre-incubation of the arteries with vinyl-L-NIO, a more specific inhibitor of nNOS, significantly decreased the nicotine-induced dilation (Fig. 2). Furthermore, there was no significant difference in the nicotine-induced vasodilation in pulmonary and cutaneous arteries that had the endothelium disrupted, compared to control arteries with the endothelium intact (Fig. 3); removal of the endothelium was verified with subsequent hematoxylin and eosin staining (data not shown). Levcromakalim ($10^{-5} \text{ mol l}^{-1}$), a $K^+$ATP channel opener, induced a dilation in both vessels that was blocked by glibenclamide ($10^{-5} \text{ mol l}^{-1}$; Figs. 4a, b); subsequent addition of trout CNP caused a vasodilation (Fig. 4b). In the pulmonary artery, the SNP-mediated dilation was also significantly decreased by pre-treatment with glibenclamide. Glibenclamide also caused a significant decrease in the nicotine-induced vasodilation in both the pulmonary and cutaneous arteries (Figs. 4c, d). Finally, the nicotine-induced vasodilation in the pulmonary and cutaneous arteries was not dependent on activation of the cyclooxygenase enzymatic pathway, as indomethacin had no effect on the response in both arteries (Table 1).
In the pulmonary artery, pre-incubation of the vessel with TTX, guanethidine, bretylium, esculetin, clotrimazole, and l-NIL had no effect on the nicotine-mediated vasodilation (Table 1); these drugs were not tested on the cutaneous artery.

**Presence and distribution of NOS.** In toad pulmonary and cutaneous arteries, no specific, perinuclear staining that could be attributed to NOS in vascular endothelial cells was observed, following processing for NADPH-diaphorase histochemistry (Fig. 5a; N = 3). Furthermore, no eNOS-immunoreactivity (IR) was observed in the arteries (Fig. 5c; N = 3). In contrast, vascular endothelial cells of the descending aorta of crocodile showed distinct perinuclear NADPH-diaphorase staining and eNOS-IR, as previously reported (Figs. 5b, d; N = 3, [9]). Positive NADPH-diaphorase staining and nNOS-IR was observed in perivascular nerve fibres in toad pulmonary and cutaneous arteries (Figs. 5e-g; N = 3). Both single nerve fibres and nerve bundles were observed in the arteries. A lack of staining was observed in tissues that were incubated in secondary antibody only (data not shown).

**Discussion**

The present study determined the nature of NO regulation in toad pulmonary and cutaneous arteries using nicotine as a stimulator of NO-mediated vasodilation, and extended our previous work on toad blood vessels by examining the intracellular mechanisms involved in vasodilation. We found using anatomical and physiological approaches, that NO-mediated vasodilation of the pulmonary and cutaneous arteries is probably provided by nitrergic nerves that can be activated by nicotine, as it is in toad systemic blood vessels [7, 8]. In both the pulmonary and cutaneous arteries, K^{+}_{ATP} channels appear to be involved in the NO-mediated vasodilation, but we found no evidence for the involvement of adrenergic nerves, the cyclooxygenase and lipoxygenase pathways, Ca^{2+}-activated K^{+} channels or inducible NOS.
Neurally-derived NO control of the systemic and pulmo-cutaneous circulation of *B. marinus* may play an important role in the regulation of vascular resistance and consequently blood flow into each vascular bed.

Prior to this study, there were no physiological data regarding the role of NO in the control of the pulmonary vasculature of amphibians. Initially, we demonstrated the presence of a NO receptor in the pulmonary and cutaneous arteries of toad, as SNP mediated a marked dilation that was blocked by the soluble GC inhibitor, ODQ. Subsequently, both NADPH-diaphorase histochemistry and immunohistochemistry using specific eNOS and nNOS antibodies showed that both arteries contained perivascular, nitrergic nerves, but NOS could not be localised to the endothelium. However, both techniques demonstrated punctate, perinuclear staining in the descending aorta of *C. porosus*, in which eNOS has been previously demonstrated [9]. These observations are also consistent with our earlier studies on toad systemic blood vessels [7, 8]. Furthermore, the techniques used in this study have demonstrated eNOS and nNOS in the pulmonary vasculature of mammals [18, 19, 26, 43, 48]. In other amphibian species, nNOS-immunoreactive perivascular nerves have been shown in the respiratory tract [6, 49].

We have previously shown that the nicotine-induced vasodilation of systemic arteries of crocodile [9], toad [15] and eel [24] was due to NO because it was blocked by ODQ and L-NNA, but was not affected by disruption of the endothelium. These blood vessels contain perivascular, nitrergic nerves; therefore, we concluded that nicotine was activating the nerves to facilitate NO neurotransmission, as has been reported in various mammalian vascular beds (see [46]). In toad pulmonary and cutaneous arteries, nicotine also caused a vasodilation that was blocked or mostly reduced by both ODQ and L-NNA, respectively. Interestingly, pre-incubation of the arteries with ODQ or L-NNA prior to the application of ET-1 or U-46619 caused a vasoconstriction, which indicated that the vessels have an endogenous NO tonus; such an effect was not observed in toad systemic blood vessels [7, 8]. The response of the
arteries to nicotine was unaffected by removal of the endothelium or inhibition of the cyclooxygenase pathway with indomethacin. The lack of effect of endothelial disruption on the nicotine response is consistent with observations in toad systemic blood vessels [15], and the absence of histologically demonstrable NOS in the endothelium of the pulmonary and cutaneous arteries. Furthermore, the selective nNOS inhibitor, vinyl-L-NIO [5], significantly decreased the nicotine-induced vasodilation in the pulmonary and cutaneous arteries. Vinyl-L-NIO does not inhibit iNOS and has a much lower affinity for eNOS than nNOS [5]. Given that we are proposing that toad blood vessels do not express eNOS, the vinyl-L-NIO inhibition of the nicotine-induced vasodilation provides compelling evidence that nNOS generation of NO in perivascular nerves is responsible for the vasodilation. In the pulmonary artery, the response to nicotine was not affected by inhibition of inducible NOS with L-NIL [32], providing further evidence for our conclusion.

In the pulmonary artery, there was a small residual non-nitrergic vasodilation to nicotine. Previously, nicotine has been shown to stimulate the release of CGRP in various vascular beds of mammals [4, 35, 36, 44, 47] and the dorsal aorta of a chondrichthyan fish [23], and CGRP is a known dilator of toad pulmonary vasculature [12]. Pre-incubation of the toad pulmonary artery with a combination of L-NNA and the CGRP receptor antagonist, CGRP(8-37), completely blocked the nicotine-induced dilation, indicating that both NO and CGRP are responsible for the dilation.

Following the demonstration that nicotine activated NO signalling in the pulmonary and cutaneous arteries, we then investigated potential intracellular mechanisms by which NO may mediate vasodilation. One mechanism of NO-mediated vasodilation that has been demonstrated in various mammalian blood vessels is activation of $K^+_{\text{ATP}}$ channels by cGMP. For example, NO-mediated vasodilation in pial arteries [2] and retinal arterioles [22] of pig have been shown to be dependent on activation and opening of $K^+_{\text{ATP}}$ channels, as they are
blocked by glibenclamide. Furthermore, it has also been suggested that arachidonic acid-mediated vasodilation in human pulmonary arteries that is attributed to NO also involves $K^+_{\text{ATP}}$ channels [20]. In contrast, other studies have found that NO-mediated vasodilation in mammalian pulmonary arteries occurred independently of $K^+_{\text{ATP}}$ channels, as glibenclamide had no effect on the vasodilation [16, 41, 50]. In toad pulmonary and cutaneous arteries, we showed that the $K^+_{\text{ATP}}$ channel activator, levcromakalim, caused a vasodilation that could be blocked with glibenclamide. In the pulmonary artery, glibenclamide significantly decreased the SNP vasodilation, which provides evidence that $K^+_{\text{ATP}}$ channels are involved in NO signalling, in at least the pulmonary artery. Subsequently, we demonstrated that glibenclamide caused a significant decrease in the nicotine-induced vasodilation in both the pulmonary and cutaneous arteries. Since l-NNA abolished the nicotine-induced vasodilation in the cutaneous artery, it can be concluded that $K^+_{\text{ATP}}$ channels are involved in the NO-mediated vasodilation in this artery. However, in the pulmonary artery, the nicotine-induced vasodilation is attributed to NO and CGRP and glibenclamide only caused a small decrease in the response to nicotine. Thus, it is not possible to conclude if $K^+_{\text{ATP}}$ channels play a role in the nicotine-induced NO-mediated vasodilation of the pulmonary artery, but given the effect of glibenclamide on the SNP vasodilation, it is likely that neurally-derived NO signalling in toad pulmo-cutaneous arteries does involve $K^+_{\text{ATP}}$ channels.

In addition to $K^+_{\text{ATP}}$ channels, we investigated the possible involvement of other pathways in the nicotine-induced vasodilation in the pulmonary artery (Table 1). However, we could find no evidence that the generation of action potentials, activation of the lipoxygenase enzyme pathway or opening of Ca$^{2+}$-activated $K^+$ channels were involved in the response. Furthermore, the response was unaffected by the inhibitors of adrenergic neurotransmission, guanethidine and bretylium, indicating that nicotine is not activating sympathetic adrenergic
nerves to mediate vasodilation. It has been previously demonstrated that sympathetic, adrenergic nerves mediate vasodilation in the pulmonary artery of *B. marinus* [10].

It is well-known that the pulmonary artery of amphibians is under a strong cholinergic constrictor tone, as activation of perivascular cholinergic neurons in the vagus by electrical stimulation caused vasoconstriction [10, 12, 13, 45]. In contrast, the cutaneous artery of amphibians is devoid of cholinergic nerves [30, 45]. We found that ACh caused an atropine-sensitive vasoconstriction in both the pulmonary and cutaneous arteries of toad. This provides further evidence for a lack of endothelial NO signalling in amphibians since ACh causes NO-mediated vasodilation in the mammalian pulmonary vasculature [11, 14, 29]. Interestingly, in toad systemic blood vessels, ACh activates perivascular nitrergic nerves to mediate vasodilation in a similar fashion to nicotine [7]; clearly, a similar signalling pathway is absent in the pulmonary and cutaneous arteries. The toad pulmo-cutaneous vasculature is also innervated by adrenergic nerves that mediate vasodilation in the pulmonary artery [10] and vasoconstriction in the cutaneous artery [45]. Furthermore, peptide immunohistochemistry has shown neuropeptide Y-, substance P-, CGRP-, somatostatin-, and galanin-IR in perivascular nerves of the pulmonary and/or cutaneous artery [33, 34]. This study clearly demonstrated that the pulmonary and cutaneous arteries of toad are well-supplied with nitrergic nerves and it would be interesting to determine if the nitrergic nerves are sympathetic, parasympathetic or sensory in nature.

**Perspective**

In mammals, NO produced by the endothelium is now considered to be one of the most important regulators of blood pressure [39]. However, the role of NO in vascular regulation of lower vertebrates has been debated, because the presence of eNOS has not been unequivocally demonstrated in fishes and amphibians. We have performed a series of
analyses in fishes and amphibians and have found that nNOS is expressed in perivascular
cells and that eNOS is absent from the endothelium; this is consistent with physiological
findings that vascular NO signalling is provided by nitrergic nerves [15]. In contrast,
endothelial NO signalling is demonstrable in reptiles and birds [9, 15]. Therefore, it is
probable that neurally-based vascular NO signalling evolved in fish and amphibians and that
endothelial NO signalling first appeared in the amniotic vertebrates.

Neural control of the pulmo-cutaneous circulation in amphibians appears to be designed to
provide reciprocal perfusion of the pulmonary and cutaneous circuits. Blood flow in the
pulmonary artery is predominately controlled by cholinergic nerves but as mentioned the
cutaneous artery is devoid of cholinergic nerves. Therefore, activation of the cholinergic
neurons will decrease pulmonary blood flow, which will divert blood to the cutaneous circuit
if systemic resistance is unchanged [45]. Thus, blood perfusion can be matched to pulmonary
or cutaneous gas exchange. This study is the first to demonstrate the presence of a NO system
in the pulmo-cutaneous vasculature of an amphibian species that could provide neurally-
mediated vasodilation. In the pulmonary artery, the nitrergic nerves would oppose the
cholinergic vasoconstrictor nerves that play a critical role in cardiac shunting [17].
Furthermore, nitrergic nerves in the cutaneous artery could provide a mechanism for rapid
vasodilation that would permit increased blood flow to the skin for respiration when
pulmonary vascular resistance is high.

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**Table 1**: Summary of the vasodilatory responses of toad pulmonary and cutaneous arteries to various treatments associated with the NO signalling system. Values are calculated as a percentage of initial constriction and displayed as mean percentages ± SE of at least 5 experiments. * denotes significant difference; NS, not significant

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pulmonary artery</th>
<th>Cutaneous artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP</td>
<td>71.41 ± 4.97</td>
<td>47.90 ± 4.36</td>
</tr>
<tr>
<td>Nicotine</td>
<td>65.65 ± 10.23</td>
<td>46.46 ± 6.18</td>
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<td>Nicotine + ODQ*</td>
<td>13.12 ± 4.14; *P &lt; 0.05</td>
<td>18.96 ± 6.35; *P &lt; 0.05</td>
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<tr>
<td>Nicotine</td>
<td>53.36 ± 4.45</td>
<td>43.53 ± 4.68</td>
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<tr>
<td>Nicotine + L-NNA*</td>
<td>9.96 ± 2.66; *P &lt; 0.05</td>
<td>No response</td>
</tr>
<tr>
<td>Nicotine</td>
<td>56.97 ± 3.06</td>
<td>52.42 ± 3.54</td>
</tr>
<tr>
<td>Nicotine + Vinyl-L-NIO*</td>
<td>17.41 ± 2.75; *P &lt; 0.05</td>
<td>25.54 ± 5.20; *P &lt; 0.05</td>
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<td>Nicotine</td>
<td>44.91 ± 8.75</td>
<td>51.64 ± 6.53</td>
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<tr>
<td>Nicotine + Endo removal</td>
<td>61.13 ± 8.66; NS</td>
<td>56.08 ± 2.17; NS</td>
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<tr>
<td>Nicotine</td>
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<td>Not tested</td>
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<tr>
<td>Nicotine + L-NNA/CGRP(8-37)</td>
<td>No response</td>
<td>No response</td>
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<tr>
<td>Levcromakalim</td>
<td>44.19 ± 6.12</td>
<td>46.69 ± 8.04</td>
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<tr>
<td>Levcro. + Glibenclamide</td>
<td>No response</td>
<td>No response</td>
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<tr>
<td>Nicotine</td>
<td>55.47 ± 7.02</td>
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<td>Nicotine + Glibenclamide*</td>
<td>41.36 ± 4.62; *P &lt; 0.05</td>
<td>33.43 ± 1.79; *P &lt; 0.05</td>
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<td>55.61 ± 6.53; NS</td>
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<td>54.98 ± 8.06</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine + Esculetin</td>
<td>48.96 ± 1.73; NS</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine</td>
<td>53.28 ± 4.76</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine + Clotrimazole</td>
<td>53.24 ± 6.92; NS</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine</td>
<td>53.83 ± 10.99</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine + L-NIL</td>
<td>56.57 ± 4.69; NS</td>
<td>Not tested</td>
</tr>
</tbody>
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Figure Legends

Fig. 1. Representative tension recordings showing the effect of nicotine (3 x 10^{-4} mol l^{-1}) on the pulmonary (A, B) and cutaneous (C, D) arteries preconstricted with ET-1 (10^{-8} mol l^{-1}) and U-46619 (10^{-6} mol l^{-1}), respectively, and the effect of inhibition of NO signalling. In the pulmonary artery (B), the soluble GC inhibitor, ODQ (10^{-5} mol l^{-1}) caused constriction, markedly reduced the nicotine-induced vasodilation and blocked the effect of SNP (10^{-4} mol l^{-1}); subsequent addition of rANP (10^{-8} mol l^{-1}) that acts via particulate GC caused a vasodilation. A similar effect was observed in the cutaneous artery. In the cutaneous artery (D), the NOS inhibitor, L-NNA (10^{-4} mol l^{-1}) caused constriction and completely blocked the nicotine-induced vasodilation; subsequent addition of SNP (10^{-4} mol l^{-1}) caused a marked vasodilation.

Fig. 2. Representative tension recordings showing the vasodilatory effect of nicotine (3 x 10^{-4} mol l^{-1}; A, C) on the pulmonary (A, B) and cutaneous (C, D) arteries, and the effect of the nNOS inhibitor, vinyl-L-NIO (10^{-5} mol l^{-1}; B, D). The arteries were pre-incubated with vinyl-L-NIO for 10 min prior to pre-constriction with ET-1 (10^{-8} mol l^{-1}) or U-46619 (10^{-6} mol l^{-1}). Vinyl-L-NIO inhibited the effect of nicotine in both arteries.

Fig. 3. Representative tension recordings showing the vasodilatory effect of nicotine (3 x 10^{-4} mol l^{-1}; A, C) on the pulmonary (A, B) and cutaneous (C, D) arteries, and the effect of disruption of the endothelium (B, D). The arteries were denuded of endothelium prior to pre-constriction with ET-1 (10^{-8} mol l^{-1}) or U-46619 (10^{-6} mol l^{-1}). In both arteries, the vasodilation induced by nicotine was unaffected by disruption of the endothelium.
Fig. 4. Representative tension recordings showing the vasodilatory effect of levkromakalim (A) on the cutaneous artery, which is abolished after pre-incubation with the $K^+_\text{ATP}$ channel inhibitor, glibenclamide (B); subsequent addition of tCNP caused a vasodilation. C and D show the response to nicotine (C) and its effect in the presence of the $K^+_\text{ATP}$ channel inhibitor, glibenclamide (D) on the cutaneous artery. The artery was pre-incubated with glibenclamide ($10^{-5} \text{ mol l}^{-1}$) for 10 min prior to being pre-constricted with U-46619 ($10^{-6} \text{ mol l}^{-1}$). The nicotine-mediated dilation was reduced in the presence of glibenclamide.

Fig. 5. Photomicrographs showing whole-mount preparations of toad pulmonary (A, C, E, F) and cutaneous (G) arteries and crocodile aorta (B, D) following processing for NADPH-diaphorase histochemistry (A, B, E), eNOS (C, D), or nNOS (F, G) immunohistochemistry. In the crocodile aorta, punctate NOS-positive staining (arrowheads) occurred around the nuclei (arrows) of the endothelial cells, which was demonstrable with both techniques (B, D). In contrast, no NOS-positive staining was observed around the nuclei of the endothelial cells in the toad pulmonary artery (A, C). NOS-positive perivascular nerves (E) were observed in the outer layers of the wall of pulmonary artery, and nNOS-IR was observed in perivascular nerves in both the pulmonary (F) and cutaneous (G) arteries. Staining was observed in both single fibres (arrows) and nerve bundles (arrowheads). Scale bars, (A-D, 10 µm; E-G, 50 µm).
Figure 1

A

ET-1

Nicotine

0.2g

2 min

B

ODQ

ET-1

Nicotine

SNP

rANP

C

U-46619

Nicotine

2 min

1 mN

D

L-NNA

U-46619

Nicotine

SNP
Figure 2

A

Nicotine

ET-1

3 min 0.1g

B

Nicotine

ET-1

Vinyl-L-NIO

C

Nicotine

U-46619

1 min 1 mN

D

Nicotine

U-46619

Vinyl-L-NIO
Figure 3

A

Nicotine

ET-1

2 min

0.1 g

B

Nicotine

ET-1

C

Nicotine

U-46619

1 min

1 mN

D

Nicotine

U-46619
Figure 4

A

U-46619

Levcromakalim

4 min

Levcromakalim

1mN

B

U-46619

Glibenclamide

tCNP

2 min

C

Nicotine

U-46619

1mN

D

Nicotine

U-46619

Glibenclamide
Figure 5