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

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Jennings BL, Donald JA. Neurally-derived nitric oxide regulates vascular tone in pulmonary and cutaneous arteries of the toad, Bufo marinus. Am J Physiol Regul Integr Comp Physiol 295: R1640–R1646, 2008. First published August 27, 2008; doi:10.1152/ajpregu.00057.2008.—In this study, the role of nitric oxide (NO) in regulation of the pulmocutaneous vasculature of the toad, Bufo marinus was investigated. In vitro myography demonstrated the presence of a neural NO signaling 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, and the NO synthase (NOS) inhibitor, N\(^{\text{μ}}\)-nitro-L-arginine methyl ester 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 signaling in these blood vessels occurred via nitricergic nerves (7, 8, 15).

Blood flow to the skin and lungs of amphibians is regulated by the resistance of the pulmocutaneous 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 vasconstrictor nerves (10, 13). In addition, the pulmonary vasculature receives an adrenergic innervation via a vagosympathetic nerve trunk that mediates vasodilation (10). In contrast, the cutaneous artery of B. marinus is not innervated by cholinergic nerves but does contain adrenergic vasconstrictor nerves (30, 45). Thus, blood flow in the pulmocutaneous 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 pulmocutaneous 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 neural NOS (nNOS) was observed in perivascular nerves. Blood vessel myography showed that nitricergic nerves, rather than the endothelium, could provide NO regulation of vascular resistance in the toad pulmocutaneous vasculature.

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 weighing 90–150 g, were purchased from a commercial supplier in Queensland, Australia. Toads were maintained at the Deakin University Animal House at 20–25°C and were not fed during captivity (up to 1 mo) but had ad libitum access to water. Prior to experimentation, animals were killed by decapitation, followed by pithing of the spinal cord.

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IN MAMMALS, IT IS KNOWN THAT nitric oxide (NO) released from the vascular endothelium contributes to the maintenance of a low pulmonary vascular resistance and opposes hypoxic vasoconstriction (see Ref. 39). In addition to the endothelium, nitricergic nerves are also thought to provide nonadrenergic, noncholinergic vasodilation of the pulmonary vasculature (3, 27, 28, 42).

The mechanism of vascular NO signaling 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 NO synthase (NOS) inhibitor N\(^{\text{μ}}\)-nitro-L-arginine methyl ester 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 signaling in these blood vessels occurred via nitricergic nerves (7, 8, 15).
Blood vessel physiology. After death, the pulmonary and cutaneous arteries were excised and placed in Macken’s balanced salt solution in mM: NaCl, 13.6; KCl, 2.0; NaHCO3, 3.1; NaH2PO4, 1.4; MgSO4.7H2O, 1.7; glucose, 1.3; CaCl2, 2H2O; pH 7.2. For pulmonary arteries, individual rings of 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 Macken’s balanced salt solution, maintained at 22°C, and aerated with 95% O2-5% 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 ~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 Macken’s balanced salt solution maintained at 22°C and aerated with 95% O2-5% 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 that was, in turn, attached to a PowerLab data collection system and a personal computer.

To determine the mechanisms of NO signaling, the pulmonary artery was preconstricted with endothelin-1 (ET-1; 10−8 mol/l) and the cutaneous artery with the prostaglandin H2-analog U-46619 (10−6 mol/l), 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 by rubbing with a wire, which was verified with standard hematoxylin and cosin staining.

Statistical analysis. Data are expressed as means ± 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 (0.1 mol/l phosphate buffer and 0.15 mol/l NaCl; pH 7.4). They were opened laterally and pinned endothelium side up on dental wax, and then fixed for 1 h in 4% formaldehyde (pH 7.4) at 4°C. The arteries were washed in PBS (3 × 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 Tris buffer (pH 8) for times ranging from 15–60 min at room temperature. The arteries were washed in PBS (3 × 5 min), mounted on slides in buffered glycerol (0.5 mol/l Na2CO3 added drop-wise to 0.5 mol/l NaHCO3 to pH 8.6, combined 1:1 with glycerol), and observed using a point scanning laser confocal microscope (model LSM 510 META; Zeiss). The descending aorta of crocodile, Crocodylus porosus, was used as a control to demonstrate the presence of NO 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, 1:4,000, (1)] and 24 h for arteries to be incubated with the endothelial NO synthase (eNOS) antibody [polyclonal rabbit, 1:500, (21)]. Arteries were unpinned and washed in PBS (3 × 10 min); arteries to be used for eNOS immunohistochemistry were further washed in DMSO (3 × 10 min) and then in PBS (5 × 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 × 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 × 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, levromakalim, 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, MO); ET-1, rat atrial natriuretic peptide, and calcitonin gene-related peptide (CGRP) receptor antagonist CGRP(R1641), were purchased from Auspep (Melbourne, Australia); nicotine was purchased from BDH Chemicals (Poole, UK); H1-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one (ODQ), N6-(1-imino-3-butenyl)-l-ornithine (vinyl-L-NIO), and glibenclamide were obtained from Alexis Biochemicals (San Diego, CA); U-46619 and l-N6-(1-iminoethyl)lysine (l-NIL) were purchased from Cayman Chemical (Ann Arbor, MI); 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, CA); and the FITC-conjugated goat anti-mouse IgG antibody was obtained from Zymed Laboratories (San Francisco, CA).

Table 1. Summary of the vasodilatory responses of toad pulmonary and cutaneous arteries to various treatments associated with the NO signaling system

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Pulmonary Artery</th>
<th>Cutaneous Artery</th>
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<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>
</tr>
<tr>
<td>Nicotine + ODQ*</td>
<td>13.12 ± 4.14; P &lt; 0.05</td>
<td>18.96 ± 6.35; P &lt; 0.05</td>
</tr>
<tr>
<td>Nicotine + L-NNA*</td>
<td>53.36 ± 4.45</td>
<td>43.53 ± 4.68</td>
</tr>
<tr>
<td>Nicotine + L-NIO*</td>
<td>9.96 ± 2.66; P &lt; 0.05</td>
<td>No response</td>
</tr>
<tr>
<td>Nicotine + glibenclamide</td>
<td>59.97 ± 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>
</tr>
<tr>
<td>Nicotine + Endo removal</td>
<td>44.91 ± 8.75</td>
<td>51.64 ± 6.53</td>
</tr>
<tr>
<td>Nicotine + L-NIL/</td>
<td>61.13 ± 8.66; NS</td>
<td>56.08 ± 2.17; NS</td>
</tr>
<tr>
<td>CGRP(R1641)</td>
<td>54.58 ± 4.05</td>
<td>Not tested</td>
</tr>
<tr>
<td>Levromakalim</td>
<td>44.19 ± 6.12</td>
<td>46.69 ± 8.04</td>
</tr>
<tr>
<td>Nicotine + glibenclamide</td>
<td>No response</td>
<td>No response</td>
</tr>
<tr>
<td>Nicotine + glibenclamide*</td>
<td>55.47 ± 7.02</td>
<td>51.69 ± 3.67</td>
</tr>
<tr>
<td>Nicotine + glibenclamide**</td>
<td>41.36 ± 4.62; P &lt; 0.05</td>
<td>33.41 ± 1.79; P &lt; 0.05</td>
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<tr>
<td>Nicotine + glibenclamide***</td>
<td>72.64 ± 6.17</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine + glibenclamide****</td>
<td>47.74 ± 4.52; P &lt; 0.05</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine + glibenclamide*****</td>
<td>59.53 ± 2.91</td>
<td>53.22 ± 2.87</td>
</tr>
<tr>
<td>Nicotine + glibenclamide******</td>
<td>55.61 ± 6.53; NS</td>
<td>45.81 ± 7.22; NS</td>
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<tr>
<td>Nicotine + glibenclamide*******</td>
<td>38.80 ± 7.69</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine + glibenclamide********</td>
<td>52.71 ± 8.21; NS</td>
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<td>Nicotine + glibenclamide*********</td>
<td>45.70 ± 6.39</td>
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<td>Nicotine + guanethidine</td>
<td>56.70 ± 11.58; NS</td>
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<tr>
<td>Nicotine + guanethidine*</td>
<td>53.94 ± 8.82</td>
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<tr>
<td>Nicotine + bretylium</td>
<td>58.68 ± 6.23; NS</td>
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<tr>
<td>Nicotine + esculetin</td>
<td>48.96 ± 1.73; NS</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine + clotrimazole</td>
<td>53.28 ± 4.76</td>
<td>Not tested</td>
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<tr>
<td>Nicotine + clotrimazole*</td>
<td>53.24 ± 6.92; NS</td>
<td>Not tested</td>
</tr>
<tr>
<td>Nicotine + L-NIL</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>
</table>

Values are calculated as % initial constriction and displayed as means ± SE percentages of at least 5 experiments. SNP, sodium nitroprusside; NO, nitric oxide; ODQ, H1-(1,2,4)oxadiazolo(4,3-a)quinoxalin-1-one; L-NNA, N6-nitro-L-arginine; vinyl-L-NIO, N6-(1-imino-3-butenyl)-L-ornithine; CGRP(R1641), calcitonin gene-related peptide receptor antagonist; L-N6-(1-iminoethyl)lysine, L-NIL. *Significant difference; NS, not significant.
Results

Blood vessel physiology. The physiological data are summarized in Table 1. Previously, we found that ACh (10^{-5} mol/l) and nicotine (3 \times 10^{-4} mol/l) 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, the addition of ACh (10^{-5} mol/l) always caused a vasoconstriction that was abolished by atropine (10^{-6} mol/l; data not shown). The NO donor, SNP (10^{-4} mol/l), 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} mol/l) mediated a vasodilation. In both blood vessels, the soluble guanylyl cyclase (GC) inhibitor, ODQ (10^{-5} mol/l), caused a significant decrease in the nicotine-induced vasodilation, but it was not completely abolished (Fig. 1B). ODQ abolished the SNP-mediated vasodilation, but had no effect on the dilation induced by rat atrial natriuretic peptide that is mediated via particulate GC (Fig. 1B). Pretreatment of the arteries with the generic NOS inhibitor, L-NNA (10^{-4} mol/l), significantly reduced the nicotine-induced vasodilation in the pulmonary artery, and abolished the response to nicotine in the cutaneous artery (Fig. 1D). In the pulmonary artery only, pretreatment with a combination of L-NNA (10^{-4} mol/l) and the CGRP receptor antagonist CGRP(8-37) 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. 1, B and D). In addition, preincubation 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 with control arteries with the endothelium intact (Fig. 3); removal of the endothelium was verified with subsequent hematoxylin and eosin staining (data not shown). Levromakalim (10^{-5} mol/l), a K^+ ATP channel opener, induced a dilation in both vessels that was blocked by glibenclamide (10^{-5} mol/l; Figs. 4, A and B); subsequent addition of trout C-type natriuretic peptide caused a vasodilation (Fig. 4B). In the pulmonary artery, the SNP-mediated dilation was also significantly decreased by pretreatment with glibenclamide. Glibenclamide also caused a significant decrease in the nicotine-induced vasodilation in both the pulmonary and cutaneous arteries (Figs. 4, C and 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, preincubation of the vessel with TTX, guanethidine, bretylium, esculetin, clotrimazole, and...
l-NIL had no effect on the nicotine-induced 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. 5, B and D; n = 3, (9)]. Positive NADPH-diaphorase staining and nNOS-IR was observed in perivascular nerve fibers in toad pulmonary and cutaneous arteries (Figs. 5, E–G; n = 3). Both single nerve fibers 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<sup>+</sup>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<sup>2+</sup>-activated K<sup>+</sup> channels, or inducible NOS (iNOS). Neurally-derived NO control of the systemic and pulmocutaneous 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, nitriergic nerves, but NO could not be localized 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-
vasodilation in the pulmonary and cutaneous arteries. 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 pulmonary arteries that is attributed to NO also involves K^+_ATP channels (20). In contrast, other studies have found that NO-mediated vasodilation in mammalian pulmonary arteries occurred independently of K^+_ATP channels, as glibenclamide had no effect on the vasodilation (16, 41, 50). In toad pulmonary and cutaneous arteries, we showed that the K^+_ATP channel activator, levromakalim, caused a vasodilation that could be blocked with glibenclamide. In the pulmonary artery, glibenclamide significantly decreased the SNP vasodilation, which provides evidence that K^+_ATP channels are involved in NO signaling, 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^+_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 whether K^+_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 signaling in toad pulmocutaneous arteries does involve K^+_ATP channels.

In the pulmonary artery, there was a small residual nonnitrergic 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 chondrychthyan fish (23), and CGRP is a known dilator of toad pulmonary vasculature (12). Preincubation of the toad pulmonary artery with a combination of L-NNA and CGRP(8-37) blocked the nicotine-induced vasodilation, indicating that both NO and CGRP are responsible for the dilation.

Following the demonstration that nicotine activated NO signaling in the pulmonary and cutaneous arteries, we then investigated potential intracellular mechanisms by which NO may mediate vasodilation. One mechanism of NO-mediated vasodilation has been demonstrated in various mammalian blood vessels is activation of K^+_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^+_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^+_ATP channels (20). In contrast, other studies have found that NO-mediated vasodilation in mammalian pulmonary arteries occurred independently of K^+_ATP channels, as glibenclamide had no effect on the vasodilation (16, 41, 50). In toad pulmonary and cutaneous arteries, we showed that the K^+_ATP channel activator, levromakalim, caused a vasodilation that could be blocked with glibenclamide. In the pulmonary artery, glibenclamide significantly decreased the SNP vasodilation, which provides evidence that K^+_ATP channels are involved in NO signaling, 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^+_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 whether K^+_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 signaling in toad pulmocutaneous arteries does involve K^+_ATP channels.
In addition to K$_{ATP}$ 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 signaling 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 nitricergic nerves to mediate vasodilation in a similar fashion to nicotine (7); clearly, a similar signaling pathway is absent in the pulmonary and cutaneous arteries. The toad pulmocutaneous 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 nitricergic nerves, and it would be interesting to determine whether the nitricergic nerves are sympathetic, parasympathetic, or sensory in nature.

**Perspective and Significance**

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 nerves and that NOS is absent from the endothelium; this is consistent with physiological findings that vascular NO signaling is provided by nitricergic nerves (15). In contrast, endothelial NO signaling is demonstrable in reptiles and birds (9, 15). Therefore, it is probable that neurally-based vascular NO signaling evolved in fish and amphibians and that endothelial NO signaling first appeared in the amniotic vertebrates.

Neural control of the pulmocutaneous 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 signaling system in the pulmocutaneous vasculature of an amphibian species that could provide neurally-mediated vasodilation. In the pulmonary artery, the nitricergic nerves would oppose the cholinergic vasoconstrictor nerves that play a critical role in cardiac shunting (17). Furthermore, nitricergic nerves in the cutaneous artery could provide a mechanism for rapid vasodilation, which would permit increased blood flow to the skin for respiration when pulmonary vascular resistance is high.

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