Mechanisms of nitric oxide-mediated, neurogenic vasodilation in mesenteric resistance arteries of toad *Bufo marinus*

Brett L. Jennings and John A. Donald

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

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Jennings BL, Donald JA. Mechanisms of nitric oxide-mediated, neurogenic vasodilation in mesenteric resistance arteries of toad *Bufo marinus*. Am J Physiol Regul Integr Comp Physiol 298: R767–R775, 2010. First published January 13, 2010; doi:10.1152/ajpregu.00148.2009.—This study determined the role of nitric oxide (NO) in neurogenic vasodilation in mesenteric resistance arteries of the toad *Bufo marinus*. NO synthase (NOS) was anatomically demonstrated in perivascular nerves, but not in the endothelium. ACh and nicotine caused TTX-sensitive neurogenic vasodilation of mesenteric arteries. The ACh-induced vasodilation was endothelium-independent and was mediated by the NO/soluble guanylyl cyclase signaling pathway, inasmuch as the vasodilation was blocked by the soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one and the NOS inhibitors Nω-nitro-l-arginine methyl ester and Nω-nitro-l-arginine. Furthermore, the ACh-induced vasodilation was significantly decreased by the more selective neural NOS inhibitor Nω-(1-imino-3-butenyl)-l-ornithine. The nicotine-induced vasodilation was endothelium-independent and mediated by NO and calcitonin gene-related peptide (CGRP), inasmuch as pretreatment of mesenteric arteries with a combination of Nω-nitro-l-arginine and the CGRP receptor antagonist CGRP-(8 –37) blocked the vasodilation. Clostrimazole significantly decreased the ACh-induced response, providing evidence that a component of the NO vasodilation involved Ca2⁺-activated K⁺ or voltage-gated K⁺ channels. These data show that NO control of mesenteric resistance arteries of toad is provided by nitrigenic nerves, rather than the endothelium, and implicate NO as a potentially important regulator of gut blood flow and peripheral blood pressure.

amphibian; autonomic nervous system; nitric oxide synthase; endothelium

IN THE MAMMALIAN MESENTERIC vasculature, it is well established that the endothelium releases numerous vasoactive molecules, such as nitric oxide (NO), prostaglandins, endothelium-derived hyperpolarizing factor (EDHF), and endothelium-derived contracting factors, which contribute to the regulation of vascular tone (26). In addition, mesenteric arteries are innervated by sympathetic vasoconstrictor and primary sensory vasodilator nerves (71). NO is clearly important in maintaining the vasodilator tone of mesenteric arteries, inasmuch as inhibition of NO synthase (NOS) causes vasocostriction and increases blood pressure (20). Endothelial NOS (eNOS) is the predominant isoform responsible for NO generation in mesenteric arteries, but there is also evidence that neural NOS (nNOS) in perivascular nitrergic nerves generates NO to provide neurally derived vasodilation (2, 31, 63, 66).

In contrast to mammals, much less is known about the role of NO in the regulation of mesenteric vascular tone in amphibians. In the small intestine, NADPH-diaphorase histochemical staining and nNOS immunoreactivity (nNOS-IR) have been demonstrated in perivascular nerves (34, 35, 41, 49), but isoform-specific localization of NOS within the amphibian mesenteric vasculature has not been performed. There is evidence in amphibians that NO is involved in capillary fluid regulation, inasmuch as inhibition of NOS has been reported to decrease hydraulic conductivity (55) and capillary permeability (23, 24, 54) in the mesenteric circulation of the leopard frog *Rana pipiens*. Furthermore, substance P (SP) increased microvascular permeability in *R. pipiens* by increasing NO production (44). Although there is no definitive evidence establishing the site of NO production within the mesenteric circulation of amphibians, it was assumed, on the basis of the mammalian paradigm, that the capillary endothelium was the source of NO (55).

The majority of physiological studies in amphibians on NO control of the circulation have focused on large-conductance blood vessels. Recently, we provided evidence that the systemic (8, 9) and pulmonary (29) arteries of the toad *Bufo marinus* lack an endothelial NO system and, instead, NO control is provided by nitrergic nerves. In addition, we showed that toad cutaneous arteries are similarly regulated by NO released from nitrergic nerves (29). However, it is unknown whether an endothelial or a neural NO signaling system exists in small-resistance arteries of the circulation of amphibians. A comparison between NO regulation of large and small systemic vessels of *B. marinus* is essential to establish whether NO plays a role in regulating blood pressure and tissue blood flow. Therefore, the aim of this study was to determine whether mesenteric resistance arteries of *B. marinus* are regulated by NO generated by eNOS and/or nNOS and to investigate potential intracellular mechanism(s) of NO signaling.

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. AS/2006/1). Toads (*B. marinus*) of both sexes and 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°C and were not fed during captivity (up to 1 mo) but had ad libitum access to water. Before experimentation, the animals were killed by decapitation and then pithed. Sprague-Dawley rats (*Rattus norvegicus*) of both sexes and a mass of 200–300 g were obtained from a breeding colony at the Deakin University Animal House. The animals were housed in rat boxes and maintained at 23°C on a 12:12-h light-dark cycle; they had ad libitum access to water and rat chow. Before experimentation, rats were euthanized with a carbon dioxide overdose, according to standard operating procedures at Deakin University.

NADPH-diaphorase histochemistry. The small intestine and attached mesentery or isolated mesenteric arteries were dissected free and placed in ice-cold PBS. Then the mesentery attached to the small intestine was stretched and pinned out on dental wax. All tissues were

Address for reprint requests and other correspondence: B. L. Jennings, Dept. of Pharmacology, College of Medicine, Univ. of Tennessee Health Science Centre, Memphis, TN 38163 (e-mail: bjennin6@uthsc.edu).

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fixed for 1 h in 4% formaldehyde (pH 7.4) at 4°C and then washed three times (10 min each) in PBS. Isolated mesenteric arteries were cryoprotected overnight in a solution containing PBS, 30% sucrose, and 0.1% sodium azide, placed into molds containing optimum cutting temperature (OCT) TissueTek, and frozen at −80°C. Sections (12 μm) were cut on a cryostat (Microm HM 550 OMP), transferred to silane-subbed slides, and allowed to air dry for 30 min. Whole-mount and sectioned preparations were stained in an NADPH-diaphorase mixture containing 1 mg/ml β-NADPH, 0.25 mg/ml nitro blue tetrazolium, and 1% Triton X-100 in 0.1 mol/l Tris buffer (pH 8) for 15 min at 37°C. This mixture, which is light-sensitive, was kept in darkness. Tissues were again washed three times (5 min each) in PBS and covered with buffered glycerol (0.5 mol/l Na2CO3 added drop-by-drop to 0.5 mol/l NaHCO3 to pH 8.6 combined 1:1 with glycerol). Tissues were evaluated for NADPH-diaphorase staining (formazan precipitates) using a light microscope (Zeiss) and photographed with a digital imaging system (Spot 35 camera system) (6). Mesenteric arteries from rats were used as a control to demonstrate the presence of NOS in the vascular endothelium, as has been previously demonstrated (1, 4).

nNOS immunohistochemistry. Whole mounts of mesentery and mesenteric arteries were fixed for 2 h in 4% paraformaldehyde and then washed in PBS. Pieces of blood vessel and mesentery were incubated with an nNOS antibody (polyclonal sheep, 1:4,000 dilution) (3) overnight at 4°C in a humid box. The nNOS antibody is raised against an epitope sequence of rat nNOS (aa 1409-1429) that is 90% similar to the corresponding region of Xenopus laevis nNOS (access no. NP_001079155). Tissues were washed three times (10 min each) in PBS for removal of any excess primary antibody and incubated with FITC-conjugated donkey anti-sheep IgG (1:200 dilution) and 1% Triton X-100 in 0.1 mol/l Tris buffer for 1 h. Sections were then washed in PBS, mounted in buffered glycerol (0.5 mol/l Na2CO3 added drop-by-drop to 0.5 mol/l NaHCO3 to pH 8.6 combined 1:1 with glycerol), and observed and photographed using a point-scanning confocal laser microscope (LSM 510 META, Zeiss). Immunohistochemical controls were performed by omission of primary antibody.

Dual-wire myography. After the animals were killed, mesenteric arteries (~200 μm ID) were dissected from branches of the superior mesenteric artery. Arteries were cleaned of any surrounding connective tissue 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). Arteries were cut into individual 2- to 3-mm rings, which were mounted horizontally between two pieces of 40-μm wire, which, in turn, were 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 and maintained at 22°C and aerated with 95% O2-5% CO2. Tension was placed on the arteries by increasing the distance between the internal wires until they were flush against the vessel wall, and the arteries were left to equilibrate for 30 min. The myograph was linked to a Myo-Interface system, which was attached to a PowerLab data collection system and a personal computer.

To determine vasodilator mechanism(s), arteries were preconstricted 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 U-46619-induced vasoconstriction. For experiments, matched controls were used from the same animal for comparison of drug effects. In some experiments, the artery was rubbed with a wire for removal of the endothelium from the artery, which was verified with standard hematoxylin-eosin staining.

Statistical analysis. Values are means ± SE of a minimum of five experiments. Statistical analysis was performed with paired-samples t-tests using the SPSS (version 14.0) statistical package. P ≤ 0.05 was considered significant.

Materials. Sodium nitroprusside (SNP), ACh, atropine, Nω-nitro-L-arginine methyl ester (i-NAME), Nε-nitro-L-arginine (i-NNA), indomethacin, esculentin, guanethidine monosulfate, clotrimazole, levorenmakalin, β-NADPH (reduced form), nitro blue tetrazolium, and Triton X-100 were obtained from Sigma (St. Louis, MO); rat atrial natriuretic peptide (rANP) and calcitonin gene-related peptide (CGRP)- (8–37) from Auspep (Melbourne, Australia); nicotine from BDH Chemicals (Poole, UK); 3-morpholinosydnonimine (SIN-1), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). N5-(1-imino-3-butenyl)-l-ornithine (viny-l-NIO), and glibenclamide from Alexis Biochemicals (San Diego, CA); U-46619 and L-Nω-(1-iminoethyl)lysine (i-NIL) from Cayman Chemical (Ann Arbor, MI); TTX from Alomone Labs (Jerusalem, Israel); and the nNOS and FITC-conjugated donkey anti-sheep IgG antibodies from Chemicon (Melbourne, Australia).

RESULTS

Presence and distribution of NOS in the mesenteric circulation. No specific NADPH-diaphorase staining was evident in the endothelium of toad mesenteric arteries (Fig. 1A; n = 3). In contrast, positive NADPH-diaphorase staining, typical of eNOS, was observed in the endothelium of rat mesenteric arteries, as has been previously reported (Fig. 1B) (1, 4). In contrast to the endothelium of toad, positive NADPH-diaphorase histochemical staining was observed in nerve fibers located throughout the mesentery (Fig. 1C; n = 3). Specifically, NADPH-diaphorase-positive varicose perivascular nerve fibers were observed running parallel to mesenteric blood vessels (Fig. 1D). nNOS-IR was observed in single varicose fibers in the outer media and adventitia of the blood vessels (Fig. 1, E and F; n = 3). No specific immunoreactivity was observed in tissues that were incubated in secondary antibody only (results not shown).

Dual-wire myography. The physiological data are summarized in Table 1.

NO/cGMP signaling in toad mesenteric arteries. The NO donors SNP (10−4 mol/l; Fig. 2A) and SIN-1 (10−4 mol/l; Fig. 2B) mediated vasodilation of mesenteric arteries, indicating the presence of an NO receptor. The SNP and SIN-1 vasodilations were blocked after preincubation with the soluble GC inhibitor ODQ (10−5 mol/l; Fig. 2C); generally, addition of ODQ to the bath caused a constriction. Subsequent addition of rANP (10−8 mol/l), which mediates its effects through a particulate GC, caused vasodilation in the presence of ODQ (Fig. 2C).

ACh-induced vasodilation. In toad mesenteric arteries, ACh (10−5 mol/l), a known activator of nNOS in toad large systemic arteries (8), caused a vasodilation (Fig. 3A); this response was blocked by preincubation with the muscarinic receptor antagonist atropine (10−6 mol/l; Table 1). The ACh-induced vasodilation was completely blocked after preincubation of mesenteric arteries with ODQ (10−5 mol/l; Fig. 3B); subsequent addition of rANP (10−8 mol/l) caused vasodilation (Fig. 3B). Preincubation of mesenteric arteries with the nonspecific NO inhibitors L-NNAME (10−4 mol/l; Fig. 3C) and L-NNA (10−4 mol/l; Table 1) also inhibited the ACh-induced vasodilation, but SNP (10−4 mol/l), which acts independently of NOS, mediated vasodilation. Removal of the endothelium had no effect on the ACh-induced vasodilation (Fig. 3D). Preincubation with vinyl-l-NIO (10−5 mol/l), a selective NOS inhibitor in mammalian NO signaling systems, caused a significant decrease in the ACh-induced vasodilation (Fig. 3E), but inhibition of inducible NOS with i-NIL (10−5 mol/l) had no effect (Table 1).

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Preincubation of the mesenteric arteries with a combination of indomethacin (10^{-5} mol/l) and esculetin (10^{-5} mol/l), inhibitors of cyclooxygenase and lipoxygenase, respectively, had no effect on the ACh-induced vasodilation (Table 1). Blockade of voltage-gated Na^{+} channels with TTX (10^{-6} mol/l) significantly decreased the ACh-induced vasodilation (Table 1), and antagonism of sympathetic neurotransmission with guanethidine (10^{-6} mol/l) had no effect on the vasodilation (Table 1). Preincubation with clotrimazole (10^{-5} mol/l), a nonspecific K^{+} channel inhibitor, caused a significant decrease in ACh-induced vasodilation of the mesenteric arteries (Fig. 4), but the ATP-sensitive K^{+} (K_{ATP}) channel inhibitor glibenclamide (10^{-5} mol/l) did not affect the vasodilation after addition of ACh (10^{-5} mol/l; Table 1). Interestingly, levromakalim (10^{-5} mol/l), a K_{ATP} channel opener, did not affect vascular tone.

Nicotine-induced vasodilation. Nicotine has previously been used to activate nNOS in toad large arteries (17, 29). Nicotine (10^{-4} mol/l) caused vasodilation of the mesenteric arteries (Fig. 5A), which was significantly decreased after preincubation with ODQ (10^{-5} mol/l; Fig. 5B). Subsequent addition of SNP (10^{-4} mol/l) to the ODQ-treated vessels was without effect, but rANP (10^{-8} mol/l) mediated vasodilation. Inhibition of NOS with L-NNA (10^{-4} mol/l) also significantly decreased the vasodilation (Fig. 5C). A combination of L-NNA (10^{-4} mol/l) and the CGRP receptor antagonist CGRP-(8–37) (10^{-6} mol/l) completely blocked the response to nicotine (10^{-4} mol/l); in fact, a constriction was observed (Fig. 5D). SNP (10^{-4} mol/l) caused vasodilation of mesenteric arteries treated with L-NNA + CGRP-(8–37). Finally, the nicotine-induced vasodilation was not affected by disruption of the endothelium (Fig. 5E).
Table 1. Summary of the vasodilatory responses of toad mesenteric arteries to various treatments associated with the NO signaling system

<table>
<thead>
<tr>
<th>Treatment</th>
<th>SNP</th>
<th>SIN-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ACh</td>
<td>Nicotine</td>
</tr>
<tr>
<td>Control</td>
<td>45 ± 3</td>
<td>58 ± 6</td>
</tr>
<tr>
<td>Control</td>
<td>41 ± 6</td>
<td>Not tested</td>
</tr>
<tr>
<td>ODQ*</td>
<td>44 ± 4</td>
<td>55 ± 7</td>
</tr>
<tr>
<td>Control</td>
<td>34 ± 2</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

Control Not tested

**DISCUSSION**

NO signals via activation of soluble GC and generation of the second messenger cGMP, which in blood vessels generally mediates vasodilation (56). Intriguingly, NO-mediated vasodilation is not ubiquitous in all vertebrates, because it is not observed in chondrichthyans and lungfishes; the first group in which it is generally observed is the bony fishes (47). NO-mediated vasodilation is always found in amphibian blood vessels, inasmuch as the NO donor SNP is a generic vasodilator (8, 9, 29, 57). The presence of an NO/cGMP signaling system was confirmed in toad mesenteric arteries, inasmuch as SNP and SIN-1 caused vasodilation, which was blocked by the soluble GC inhibitor ODQ.

The type and location of NOS isoforms and, therefore, the potential source of endogenous NO in toad mesenteric arteries were investigated using NADPH-diaphorase histochemistry and immunohistochemistry. No positive NADPH staining was significantly decreased the nicotine-induced vasodilation (Table 1), and blockade of sympathetic neurotransmission with guanethidine (10⁻⁶ mol/l) caused a significant increase in the vasodilation (Fig. 6). The nicotine-induced vasodilation was not affected after preincubation with clotrimazole (10⁻⁵ mol/l; Table 1) or glibenclamide (10⁻⁵ mol/l; Table 1).

**Fig. 2.** Representative tension recordings showing the vasodilatory effect of the NO donor compound sodium nitroprusside (SNP, 10⁻⁴ mol/l; A) and 3-morpholinosydnonimine (SIN-1, 10⁻⁴ mol/l; B) and the effect of the soluble GC inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinolin-1-one (ODQ, 10⁻⁵ mol/l; C) on toad mesenteric artery. Artery was preincubated with ODQ for ~10 min before preconstriction with U-46619 (10⁻⁶ mol/l). ODQ completely blocked vasodilations induced by SNP and SIN-1; subsequent addition of rat atrial natriuretic peptide (rANP, 10⁻⁸ mol/l), which mediates vasodilation via a particulate guanylyl cyclase, caused a marked vasodilation (C). Addition of ODQ caused an initial vasoconstriction before preconstriction.
observed in the endothelium of the mesenteric arteries, which provides further evidence that amphibian vascular endothelial cells do not express eNOS/NOS. Recent genomic and expressed sequence tag (EST) analyses have demonstrated that eNOS is apparently not present in fish but first appears in amphibians \textit{X. laevis}, accession no. AW765292 (Washington University Xenopus EST project, 1999); \textit{Ambystoma tigrinum}, accession no. CN053573 (53). Recently, our laboratory sequenced a full-length eNOS cDNA from the Western clawed frog \textit{Silurana tropicalis} (accession no. FJ665981) and a partial eNOS cDNA from \textit{B. marinus} (accession no. GU138862). Furthermore, in \textit{S. tropicalis}, eNOS mRNA is not expressed in systemic blood vessels (Trajanovska and Donald, unpublished observations), further supporting our contention that eNOS or NO derived from the endothelium does not play a role in vascular regulation in amphibians. In contrast to the endothelium, positive NADPH staining was always observed in perivascular nerves of mesenteric blood vessels, and nNOS-IR nerves showed a pattern very similar to that of NADPH-positive nerves. The presence of nitrogentic nerves in toad mesenteric arteries is consistent with our previous studies in large systemic blood vessels (8, 9) and the pulmocutaneous vasculature (29) of this species; the functionality of the nerves in the mesenteric resistance arteries was determined with myography.

\textit{ACh-} and nicotine-induced vasodilation of toad mesenteric arteries. Previously, \textit{ACh} was found to cause the release of NO from perivascular nitrogentic nerves in toad systemic vasculature (8, 9), presumably by neuronal signaling that activates nNOS. In toad mesenteric arteries, application of \textit{ACh} also mediated vasodilation that was solely attributed to NO generation of cGMP, because it was completely blocked by the soluble GC inhibitor ODQ and the nonspecific NOS inhibitors L-NAME and L-NNA. Given the presence of nitrogentic nerves in the mesenteric arteries and the fact that removal of the endothelium had no effect on the \textit{ACh}-induced vasodilation, it is then probable that \textit{ACh} is inducing NO neurotransmission and subsequent vasodilatation. This is further supported by the observation that preincubation of the arteries with the more selective nNOS inhibitor \textit{N}5-(1-imino-3-butenyl)-l-ornithine (vinyl-L-NIO, 5) significantly decreased the response to \textit{ACh}. Any role for inducible NOS and prostaglandin/leukotriene synthesis was discounted by the use of selective inhibitors that had no effect on the \textit{ACh} vasodilatation. In the mammalian mesenteric vasculature, \textit{ACh} mediated an endothelium-dependent vasodilation that was due to NO and an endothelium-independent vasodilation that was due to \textit{ACh} binding to muscarinic receptors on CGRP-containing nerves causing CGRP release (61). In toad, the mesenteric blood vessels are innervated by CGRP-containing nerves (39), but

![Fig. 3. Representative tension recordings showing the vasodilatory effect of \textit{ACh} (10^{-5} mol/l; A) on the mesenteric artery, which is completely blocked in the presence of ODQ (10^{-5} mol/l; B) and the NOS inhibitor \textit{N}6-nitro-l-arginine methyl ester (l-NAME, 10^{-4} mol/l; C) but unaffected after endothelial disruption (D) and significantly decreased in the presence of the more selective nNOS inhibitor \textit{N}6-(1-imino-3-butenyl)-l-ornithine (vinyl-L-NIO, E). In B, C, and E, blood vessels were preincubated with the inhibitors for \textdegree{}10 min; in D, endothelium was disrupted before preconstriction with U-46619 (10^{-6} mol/l). In B, initial application of ODQ to the bath caused a vasoconstriction before addition of U-46619, and subsequent addition of rANP (10^{-8} mol/l) caused vasodilation. In C, subsequent addition of SNP (10^{-4} mol/l), which acts independently of NOS, also mediated vasodilation.](http://ajpregu.physiology.org/)

![Fig. 4. Representative tension recordings showing the vasodilatory effect of \textit{ACh} (10^{-5} mol/l; A) on the mesenteric artery, which is significantly decreased in the presence of the nonspecific K+ channel inhibitor clotrimazole (10^{-5} mol/l; B). Artery was preincubated with clotrimazole for \textdegree{}10 min before preconstriction with U-46619 (10^{-6} mol/l).](http://ajpregu.physiology.org/)
ACh does not appear to cause a similar release of CGRP (see below).

In addition to ACh, nicotine also facilitates the release of NO from perivascular nitrergic nerves in toad conductance vessels (17, 29). In the mesenteric arteries, nicotine caused an endothelium-independent vasodilation, because it was unaffected by pretreatment with indomethacin + esculetin or removal of the endothelium. The nicotine-induced vasodilation was found to have two components. The first component was mediated by NO, because the majority of the vasodilation was inhibited by both ODQ and l-NNA. Interestingly, nicotine-induced vasodilation of large-conductance vessels and the cutaneous artery of toad was entirely caused by NO (17, 29). Subsequently, pre-incubation of toad mesenteric arteries with a combination of l-NNA and the CGRP receptor antagonist CGRP-(8–37) completely blocked the nicotine-induced vasodilation; in fact, addition of nicotine now caused a vasoconstriction. The evidence for neurally mediated CGRP vasodilation is supported by previous data from toad showing that CGRP-containing nerves occur in mesenteric arteries and that CGRP is a vasodilator of mesenteric blood vessels (50). In addition, the nicotine-induced vasodilation of toad pulmonary artery was due to NO and CGRP (29). It is curious that nicotine appears to specifically stimulate the release of CGRP, but not SP, from toad mesenteric artery perivascular nerves, inasmuch as CGRP and SP are colocalized in the nerves (39). A similar situation was observed in the mammalian carotid arterial bed, in which CGRP and SP are colocalized in sensory nerves (67), but nicotine-induced vasodilation was mediated by the release of CGRP, rather than SP (51).

To determine whether the vasodilatory responses induced by ACh and nicotine in toad mesenteric arteries involved neurogenic mechanisms, TTX was used to inhibit voltage-gated Na+ channels. Preincubation of the mesenteric artery with TTX significantly decreased the vasodilation caused by ACh and nicotine, providing further evidence that the NO vasodilation is due to activation of nitrergic nerves. ACh-induced NO-mediated vasodilation of toad conductance arteries is also TTX-sensitive (17), but TTX does not affect nicotine-induced NO vasodilation in toad systemic and pulmonary arteries (17, 29).

In mammals, the precise cellular pathways activated by nicotine to cause NO vasodilation are unclear (65); thus it is not possible to explain why the nicotine effects in the toad vasculature show variable TTX sensitivity.
Previous studies in various mammalian vascular beds have reported that the initial response to nicotine is a transient contraction that is reversed to a relaxation in the presence of various antagonists. For example, in canine lingual arteries, nicotine-induced vasoconstriction is converted to vasodilation in the presence of adrenergic and purinergic blockade (46), and in monkey mesenteric arteries, nicotine-induced vasoconstriction was reversed to an NO-mediated vasodilation by phentolamine and guanethidine (64). Thus, in mammalian vascular beds, nicotine-induced vasoconstriction is dominant, and vasodilation is observed after blockade of vasoconstriction (18). However, in toad mesenteric arteries, the dominant effect of nicotine is NO/CGRP-mediated vasodilation, and vasoconstriction is only revealed after inhibition of the vasodilator signaling pathways.

Another interesting point of difference between mammals and toad is the interaction between adrenergic and nitricergic mechanisms in vascular control. In various mammalian blood vessels, there is evidence that endothelium-independent, NO-mediated vasodilation induced by nicotine is dependent on perivascular adrenergic nerves, because the vasodilation is abolished by guanethidine and chemical sympathectomy (18, 32, 59, 70). It is proposed that nicotine binds to receptors on adrenergic terminals, causing the release of norepinephrine (NE), which then binds to adrenergic receptors on adjacent nitricergic nerves to initiate the release of NO. In rat mesenteric arteries, a different mechanism is found. In these vessels, it is postulated that NO is acting presynaptically to regulate NE release, because in endothelium-denuded mesenteric arteries, inhibition of nNOS augmented the adrenergic vasoconstriction upon nerve stimulation, which was concluded to be due to attenuation of NO inhibiting NE release from adrenergic nerves (22). It appears in toad mesenteric arteries that the nicotine-induced NO vasodilation is a direct effect that is not dependent on interaction with adrenergic nerves, because pre-treatment of the arteries with guanethidine did not attenuate the NO vasodilation, as it does in mammals; in fact, a larger vasodilation was observed.

The present data, in combination with our previous studies, clearly show that NO control of toad (and teleost fish) blood vessels is provided by nNOS in nitricergic perivascular nerves and that an endothelial NO signaling system is absent. In fact, in fish and amphibians, there is strong evidence that prostanoids are key endothelial vasodilators, instead of NO (19, 28, 48). Interestingly, NO control of toad blood vessels shows similarities to the NO signaling phenotype of eNOS knockout mice. For example, the ACh-induced NO vasodilation of toad mesenteric arteries resembles the ACh-induced dilation of pial arterioles from eNOS knockout mice, in which the source of NO is attributed to activation of nNOS in perivascular nerves. It was proposed that nNOS compensates for the loss of eNOS in pial arterioles, inasmuch as evidence for a role for nNOS is not apparent in wild-type mice (36, 37); a similar finding was reported in the coronary circulation of eNOS knockout mice (13). Moreover, in eNOS knockout mice, neurally derived NO (25) and endothelially derived prostaglandins (12, 60) have been reported to compensate for the lack of endothelially derived NO in different vascular beds, including the mesenteric circulation (12). Thus it appears that the phenotype for vasodilation in eNOS knockout mice shows remarkable similarity to that of lower vertebrates, where neurally derived NO and endothelially derived prostaglandins are the primary vasodilators before the evolution of endothelial NO signaling via eNOS in the higher vertebrates.

**Cellular mechanisms of ACh- and nicotine-induced vasodilation of toad mesenteric arteries.** In arterial smooth muscle, one of the major intracellular facilitators of NO-mediated vasodilation is K^+^ channels (43). In mammalian mesenteric arteries, it has been shown that NO-mediated vasodilation is caused, in part, by opening of Ca^2+^-activated K^+^ (K_{Ca}) (11, 30, 38, 52, 62) and voltage-gated K^+^ (K_V) (27, 68) channels. In toad, clotrimazole, an inhibitor of large-conductance K_{Ca} (BK_{Ca}) channels, intermediate-conductance K_Ca (IK_{Ca}) channels, and K_V channels (58) significantly decreased the ACh-induced vasodilation that is attributed to NO. Although the exact K^+^ channel(s) involved in the NO-induced vasodilation of toad mesenteric arteries was not elucidated, opening of IK_{Ca} channels can tentatively be discounted as follows. In the mammalian vasculature, it is widely accepted that opening of IK_{Ca} channels is involved in EDHF-mediated vasodilation (10). However, in this study, the involvement of EDHF can be excluded, because the ACh-induced vasodilation is not dependent on an intact endothelium and is solely due to NO. Thus it would appear that ACh-induced, NO-mediated vasodilation of toad mesenteric arteries involves the opening of BK_{Ca} and/or K_V channels. Since ACh- and NO donor-mediated vasodilations are completely inhibited by ODQ in toad mesenteric arteries, it is most likely that opening of the ion channel(s) is cGMP-dependent, as has been previously reported in mammalian mesenteric arteries (11, 27, 69).

NO released from nitricergic nerves after addition of nicotine would presumably mediate vasodilation in the same manner as that released by ACh stimulation, but preincubation of the toad mesenteric artery with clotrimazole did not significantly decrease the nicotine-induced vasodilation. This could be due to the fact that nicotine causes the release of two vasodilators, NO and CGRP, whereby CGRP signals via an intracellular mechanism different from NO. For example, CGRP has been shown to dilate mammalian mesenteric arteries independently of K_{Ca} and K_V channels (33). In toad, it is possible CGRP caused a K_{Ca}/K_V channel-independent vasodilation that resulted in a net insignificant effect of clotrimazole, despite the fact that the NO-mediated vasodilation (as demonstrated with ACh) is inhibited by clotrimazole.

Opening of K_{ATP} channels has also been reported to be involved in NO-mediated (21, 40) and CGRP-mediated (7, 42) vasodilation of the mammalian mesenteric artery. However, in toad mesenteric arteries, inhibition of K_{ATP} channels with glibenclamide had no effect on the vasodilation induced by ACh or nicotine. In fact, levcromakalim, a K_{ATP} channel opener, had no effect on vascular tone, suggesting that toad mesenteric arteries may lack K_{ATP} channels. Interestingly, levcromakalim caused vasodilation of the toad pulmonary and cutaneous arteries (29), which demonstrates a heterogenous distribution of K_{ATP} channels, as well as distinct cellular mechanisms underlying vascular regulation, in toad.

**Perspective**

The evolution of autonomic nervous system control of the systemic circulation has focused on the role of sympathetic, adrenergic vasoconstrictor nerves in maintaining vascular tone
and, consequently, blood pressure. In fishes, the blood vessels of cyclostomes and chondrichthyes have few adrenergic nerves, and, intriguingly, NO-mediated vasodilation is not apparent in these vertebrate classes (47). In contrast, the first vertebrate class in which blood vessels have an extensive sympathetic innervation is the teleost fishes (16), and NO vasodilation first appears in this group (47). In fishes, the eNOS gene or cDNA cannot be found in any genome or EST database, but nNOS is ubiquitous in vertebrates and is the key NOS in the gene or cDNA cannot be found in any genome or EST database, but nNOS is ubiquitous in vertebrates and is the key NOS in the blood vessels of eel and toad (8, 9, 28, 29). It appears that nNOS expression in vertebrate perivascular nerves occurred in parallel with the development of sympathetic innervation, thus providing a mechanism for rapid vasodilation to oppose sympathetic vasoconstriction. The next step in understanding the evolution of NO vasodilation is to utilize the fish and amphibian genomes, in combination with physiology, to delineate the coevolution of the cellular mechanisms underlying vasodilation, such as the role of K⁺ channels; K⁺ channels have been implicated in cGMP-mediated vasodilation in fish (15), amphibians (present study; Ref. 29) and birds (45), but the types of channels have only been deduced from pharmacological experiments.

**REFERENCES**

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**DISCLOSURES**

No conflicts of interest are declared by the author(s).

**GRANTS**

**NITRIC OXIDE CONTROL OF TOAD MESENTERIC VASCULATURE**


