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RESEARCH ARTICLE | *Cardiovascular and Renal Integration*

Vasoactivity of nitrite in the iliac artery of the toad *Rhinella marina*

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Forgan LG, Sofele M, McNeill BA, Cameron MS, Donald JA. Vasoactivity of nitrite in the iliac artery of the toad *Rhinella marina*. *Am J Physiol Regul Integr Comp Physiol* 314: R242–R251, 2018. First published October 18, 2017; doi:10.1152/ajpregu.00315.2016.— Nitrite (NO_2^-) causes vasodilation in mammals due to the formation of (nitric oxide) NO by endogenous NO_2^- reduction in the vascular wall. In this study, we determined if a similar mechanism operates in amphibians. Dual-wire myography of the iliac artery from *Rhinella marina* showed that applied NO_2^- caused a concentration-dependent vasodilation in normoxia (21% O_2; EC_{50}: 438 μM). Hypoxia (0.63% O_2) significantly increased the maximal dilation to NO_2^- by 5% ($P = 0.0398$). The addition of oxyhemoglobin significantly increased the EC_{50} ($P = 0.0144$; EC_{50}: 2,236 μM) but did not affect the maximal vasodilation. In contrast, partially deoxygenated hemoglobin (90% desaturation) did not affect the EC_{50} ($P = 0.1189$) but significantly ($P = 0.0012$) increased the maximal dilation to NO_2^- by 11%. The soluble guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) completely abolished the response to NO_2^- ($P < 0.0001$), and of the nitric oxide synthase inhibitors, only N^5-(1-imino-3-butenyl)-L-ornithine (vinyl-L-NIO; $P = 0.0028$) significantly reduced the NO_2^- vasodilation. The xanthine oxidoreductase inhibitor allopurinol ($P = 0.927$), the nitric oxide-scavenger 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (C-PTIO; $P = 0.478$), and disruption of the endothelium ($P = 0.094$) did not affect the NO_2^- vasodilation. Incubation of iliac arteries with 1 mM NO_2^- did not a cause a change in the cGMP concentration ($P = 0.407$). Plasma NO_2^- was found to be 0.86 ± 0.20 μmol/l, while nitrate (NO_3^-) was 19.55 ± 2.55 μmol/l. Both *cygb* and *ngb* mRNAs were expressed in the iliac artery, and it is possible that these globins facilitate NO_2^- reduction in hypoxia. In addition, NO_2^- intracellular disproportionation processes could be important in the generation of NO from NO_2^-.

amphibian; nitric oxide; nitrite; nitrite reductase; vascular

INTRODUCTION

Nitric oxide (NO) is produced from L-arginine and oxygen by nitric oxide synthase (NOS) enzymes, of which there are three isoforms; NOS1, NOS2, and NOS3 (15). NOS1 is structurally and functionally very similar to NOS3 in a range of vertebrates, while NOS2 has some distinctive characteristics (15). In mammals, the production of NO from NOS3 in endothelial cells, in addition to a range of other vasodilators and vasoconstrictors (11), is an important mechanism for regulating vascular tone, particularly in larger conduit vessels (30). Nitric oxide diffuses into the adjacent vascular smooth muscle cells and binds to soluble guanylyl cyclase (sGC), which gives rise to an increase in cGMP and activation of protein kinase G leading to smooth muscle relaxation (30). In addition to the actions mediated by sGC, NO can also act directly on potassium channels to cause hyperpolarizing-induced vasodilation, interact with heme-iron containing proteins, and modulate protein function through posttranslational modification by *S*-nitrosylation, *S*-glutathionylation, tyrosine nitration, or *S*-guanylation (10). In nonmammalian vertebrates, it has been suggested that similar mechanisms for NO signaling can occur, although there have been few studies to demonstrate this (13).

NO is highly labile as it is a one electron radical and is rapidly oxidized to nitrite (NO_2^-) and nitrate (NO_3^-) (29). In humans, the NO_2^- concentration in the blood is ~0.05–0.3 μM, with the concentration of NO_3^- ~100- to 1,000-fold greater than NO_2^- (29). Previously thought to be inactive by-products of NO metabolism, it is now clear that these nitrogenous species can be reduced back to NO and, therefore, act as an endocrine reservoir of NO in the plasma (38). In particular, there is evidence in mammalian blood vessels that the bioavailability of NO from NO_2^- and NO_3^- reduction is upregulated in hypoxic (14) and acidotic (48, 49) states, when the oxygen-dependent generation of NO from NOS is compromised. However, it is also clear that NO_2^- reduction to NO to facilitate vasodilation can occur in normoxia (29).

The mechanism of translocation of NO_2^- across cell membranes is not yet completely resolved. There is evidence that anion exchanger 1 (AE1) is involved in mediating the process in cells such as erythrocytes (24). Nitrite can also enter cells as HNO_2 by diffusing through the cell membrane (37). Regardless of the mechanism of transport, NO_2^- can be reduced to NO in cells by a range of intracellular processes including *1*) reduction by enzymes such as xanthine oxidoreductase (XOR) (19), hemoglobin (particularly deoxyhemoglobin; Ref. 29), myoglobin (33), cytoglobin (25), neuroglobin (33), and NOS3 (29); and *2*) nonenzymatic acidic disproportionation processes (29). Nitrite can also be formed indirectly through dietary NO_3^- reduction to NO_2^- by facultative microbes in the oral cavity, followed by protonation in the gastrointestinal tract (12).

In nonmammalian vertebrates, our understanding of NO_2^- reductase activity and its role in vascular regulation is rudimentary. There is some evidence for an involvement of NO generated from reduced plasma NO_2^- in regulating blood-flow redistribution during anoxia in the turtle *Trachemys scripta*, as indicated by increased concentrations of Fe-nitrosyl and *N*-

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nitroso compounds in the blood (23). In the rainbow trout *Oncorhynchus mykiss*, infusion of 1 mM NO_2^- caused only minimal decreases in blood pressure (1), and there are no reports of the vasoactive effects of NO_2^- in amphibians. Therefore, the aim of the current study was to investigate whether arteries of the amphibian *Rhinella marina* possess the intrinsic ability to reduce NO_2^- to NO to facilitate vasodilation. *Rhinella marina* aortas express NOS3 in the endothelium (4) but do not display the classical endothelial NO signaling system, as applied acetylcholine mediated a NO vasodilation that is not significantly affected by removal of the endothelium and was attributed to activation of NOS1 expressed in perivascular, nitrergic nerves (2, 3). In summary, this study provides the first systematic report that NO_2^- is reduced to NO to facilitate vasodilation in arteries of a nonmammalian vertebrate.

MATERIALS AND METHODS

Animals. *Rhinella marina* of either sex were purchased from a commercial supplier (P. Douch, Mareeba, Australia) and maintained in custom housing in the Deakin University Animal House. The housing contained a water bath and a dry area that was fitted with a rainmaker and was maintained at 26°C; the animals had ad libitum access to the water. The water in the holding tanks was changed every 2 days. Lighting was set on 12:12-h light-dark cycle, and animals were fed once daily with crickets. Before experimentation, *R. marina* were anesthetized by immersion in 1% tricaine methanesulfonate (MS222) buffered to pH 7.5 until the animals were unresponsive to toe pinch stimuli and corneal stimulation. Animals were then humanely killed by removal of the heart; in some animals, blood was withdrawn by cardiac puncture before removal of the heart. All animal procedures were approved by the Deakin University Animal Ethics Committee (B15-2012).

Dual-wire myography. Both left and right branches of the iliac artery were carefully dissected and freed of connective tissue and placed into chilled physiological saline (mM: 115 NaCl, 3.2 KCl, 2.5 $CaCl_2$, 1.4 $MgSO_4$, 7 NaOH, 10 HEPES, and 17 glucose, pH 7.4). The iliac artery was used for experiments as it is an intermediate-sized vessel that has been shown to exhibit endothelial-independent NO signaling in the same manner as other toad blood vessels (4). Isolated iliac arteries were carefully cut into 3-mm lengths and mounted in a dual-wire myograph (420A; Danish Myo Technology, Aarhus, Denmark), which was connected to a Myo-Interface (DMT) linked to a PowerLab data recorder (ADInstruments, Bella Vista, NSW, Australia). Data were recorded using LabChart 7 (ADInstruments). The myography chambers were filled with 5 ml of physiological saline maintained at room temperature (~20°C) and aerated. The vessels were allowed to equilibrate for 15 min before a nominal tension of 2 mN was applied. The optimal length-tension relationship was empirically determined by incrementally lengthening and then constricting the vessels using osmotically balanced 60 mM KCl physiological saline. The optimal length was assumed when active-force plateaued. Before pharmacological treatment, vessels were preconstricted using the prostaglandin H_2-analogue and the thromboxane A_2 mimic U-46619 (Cayman Chemicals, Ann Arbor, MI). Experiments were conducted in a pairwise fashion with a matched control from the opposite iliac artery from the same animal. If required, the endothelium of the iliac artery was disrupted by rubbing the luminal surface several times with a 24-gauge needle.

Preliminary experiments revealed that preconstricted iliac arteries vasodilated in response to exogenously applied 1 mM sodium nitrite ($NaNO_2$). As the sodium ion dissociates from the NO_2^- moiety in solution, the nomenclature of NO_2^- will be used throughout this article. Following preliminary experimentation, concentration-response experiments were performed by the sequential addition of exogenous NO_2^- to produce concentrations in the myography chamber between 1 μM and 10 mM. Subsequent additions were made only after a vasodilatory plateau was reached after the previous application. To investigate the nature of the in vitro vasodilatory response, two experimental series were conducted. The first series was designed to examine the effects of normoxia [$n = 9$; atmospheric oxygen partial pressure (P_{O_2}) 20.85 ± 0.06% O_2 ~155 mmHg] and hypoxia ($n = 9$; P_{O_2} 0.63 ± 0.08% O_2; ~4.7 mmHg). The above experiments were then repeated in the presence of exogenous bovine hemoglobin (200 μM). Hypoxia was induced by aerating the myography chamber with N_2 gas and monitoring the P_{O_2} with an SI130 O_2 electrode and SI929 meter (Strathkelvin Instruments, North Lanarkshire, Scotland). Under these experimental conditions, bovine hemoglobin is ~10% saturated with O_2 (46). However, for simplicity we have referred to 90% desaturated hemoglobin as deoxyHb throughout this article; it would be expected that 90% desaturated hemoglobin would have significant reductase activity compared with oxyHb.

The second series of experiments involved the application of specific pharmacological inhibitors to the myography chamber before the addition of exogenous NO_2^-. These experiments were completed in normoxia and in the absence of hemoglobin. The pharmacological agents, the concentrations used, and targets were as follows: 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10 μM; sGC); N^{ω}-nitro -L-arginine methyl ester (L-NAME; 100 μM; all NOS); N^{ω}-nitro-L-arginine (L-NNA; 100 μM; all NOS); N^5-(1-imino-3-butenyl)-L-ornithine (vinyl-L-NIO; 100 μM; NOS1); 1H-pyrazolo[3,4-d] pyrimidin-4(2H)-one (allopurinol; 100 μM; XOR); and 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (C-PTIO; 200 μM; NO scavenger). All pharmacological agents were sourced from Cayman Chemicals.

Serum NO_2^-/NO_3^- assays. During surgery, blood was withdrawn via cardiac puncture and allowed to clot on ice for ~30 min ($n = 8$). Blood was centrifuged at 10,000 *g* for 5 min, and the serum was removed, snap frozen in liquid N_2, and stored at −80°C. Thawed samples were centrifuged at 10,000 *g* for 30 min at 4°C in a disposable centrifuge tube, and passed through a disposable 10-kDa MWCO polyethersulfone filter (Vivaspin20, Sigma Aldrich). Twenty microliters of the filtrate were assayed for NO_2^- and NO_3^- (NO_x) using NO_2^- reductase and 2,3-diaminonaphthalene chemistry (Nitrite/Nitrate Fluorometric Assay Kit; Cayman Chemicals), according to the manufacturer's instructions. Data were read on a FLUOstar Omega fluorimeter (BMG LABTECH, Mornington, VIC, Australia).

cGMP assay. To generate sufficient material to extract for the assay, cGMP production was determined in pooled dorsal aorta and iliac artery samples that were dissected as a single unit ($n = 7$). We have shown that the iliac artery, which bifurcates from the dorsal aorta, has a very similar profile for the regulation of vasodilation by NO (4). Therefore, it is reasonable to combine both vessels in the cGMP assay to markedly reduce the number of animals that would have needed to be used if the iliac artery was assayed in isolation. The vessels were freed of connective tissue and gently flushed with physiological saline to remove all visible traces of blood, and stored in saline for 1–2 h at 4°C. Vessels were incubated in physiological saline under normoxic conditions with either 0.5 mM 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione (IBMX; nonspecific phosphodiesterase inhibitor) alone or 0.5 mM IBMX and 1 mM NO_2^- for 20 min at 20.5 ± 0.1°C with constant gentle agitation. Vessels were blotted, snap frozen in liquid N_2, and stored at −80°C. Vessels were then ground into a fine powder under liquid N_2 using a mortar and pestle, extracted in 10 vol of 0.1 N HCl with the aid of silica-zirconium beads and a TissueLyser II (Qiagen, Venlo, The Netherlands) and centrifuged at 10,000 *g* for 10 min. The supernatant was assayed for cGMP using colorimetric competitive immunoassay (Direct cGMP ELISA Kit; ENZO Life Sciences, Farmingdale, NY) according to the manufacturer's instructions and read on a FlexStation II spectrophotometer (Molecular Devices).

Hemoglobin preparation and spectral properties. Ferrous bovine oxyhemoglobin was prepared from methemoglobin (Sigma, Castle Hill, Australia) by equilibrating a 25 × 2.5 cm Sephadex G-25 column (GE Life Sciences) with 20 mM phosphate buffer, 1 mM EDTA (pH 7.0), and 10% wt/vol sodium dithionite (Sigma). The hemoglobin eluate was dialysed overnight to remove the dithionite, then O_2 saturated by aeration, and stored at −80°C. Oxyhemoglobin concentration was measured at 280 nm using a Nanodrop 1000 (Thermo Fisher) and referenced to BSA standards. As NO_2^- is a known oxidant of hemoglobin, the oxidation state was assessed in a 200 μM solution of hemoglobin after sequential additions of NO_2^- (data not shown). All samples were scanned immediately in sealed N_2-flushed quartz cuvettes. Wavelength scans were performed on a Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). At each concentration of NO_2^- from 1 μM onwards, significant methemoglobin formation was observed reaching ~100% of total hemoglobin at 10 mM (data not shown).

Molecular biology. Primers based on *Xenopus laevis* and *Xenopus tropicalis* globin mRNAs (National Center for Biotechnology Information Accession No.: cytoglobin *cygb:* NP_001087751.1, neuroglobin *ngb:* NM_001030351.1, and globin Y *gby:* BC128970.1) were used to amplify *cygb*, *ngb*, and *gby* mRNAs, respectively, in *R. marina*. Total RNA was extracted from the *R. marina* brain by homogenizing the tissue in TRIzol reagent (Invitrogen) with silica-zirconium beads using a Fastprep 20 benchtop homogenizer (MPBio). Total RNA was treated with DNAseI (Invitrogen) before reverse transcription into cDNA with Superscript II (Invitrogen), using both random primers (Promega) and oligo $dT_{15–18}$. Specific globin cDNAs were amplified using GoTaq G_2 Green Mastermix (Promega) and a series of overlapping gene-specific primers. To amplify the 3′-untranslated region of *cygb* and *gby*, 3′-rapid amplification of cDNA ends (3′-RACE) PCR was performed (Invitrogen). Amplified PCR products were ligated into pGEM-T easy (Promega) plasmids and transformed into highly competent *Escherichia coli* (J109; Promega) cells. Colonies were selected for positive inserts using β-galactosidase chromogenic detection with IPTG and X-gal (Adipogen). Positive insert colonies were grown overnight in Luria-Bertani broth, and the plasmid DNA was harvested (PureYield Miniprep Kit; Promega). Plasmids were sequenced using M13 forward (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and reverse (5′-TCACACAGGAAACAGCTATGAC-3′) primers and BigDye v3.1 chemistry (Applied Biosystems). Sequences were read at the Ramaciotti Centre for Genomics (University of New South Wales) using a 3500 series Genetic Analyzer (Applied Biosystems), and were identified by comparison to *X. laevis* and *X. tropicalis* nucleotide sequences (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Contigs were constructed in CAP3 (http://doua.prabi.fr/software/cap3) using eight individually obtained sequences for each cDNA. From the cloned sequences, homologous primers for *R. marina cygb, ngb*, and *gby* were designed using National Center for Biotechnology Information primer blast (https://www.ncbi.nlm.nih.gov/). As a control, a partial β-actin mRNA was amplified using primers based on *X. tropicalis* (43). The primer sequences, melting temperatures, and product sizes are listed in Table 1.

To obtain sufficient total RNA from *R. marina* iliac arteries, a single sample represented vessels pooled from two animals; we used six animals to make three independent cDNAs. Total RNA was extracted as above, with the exception that the pooled iliac arteries were first ground into a fine powder with a mortar and pestle under liquid N_2. Complementary DNA synthesis and PCR were performed as described above using the melting temperatures listed in Table 1. As a control, the homologous *R. marina* primers were used in parallel with the sequence verified *R. marina* globin plasmids constructed above.

Statistics. Sigmoidal (logistic) concentration-response curves were fitted to $\log_{10}$ NO_2^- values for each paired control and associated pharmacological or P_{O2} treatment. The fitted curves were used to estimate *1*) whether fitted models differed from each other between groups ("model"), *2*) effective concentration to 50% of responses (EC_{50}), and *3*) maximal dilation (% of U46619). Differences in the above parameters were assessed by extra sum of squares *F*-tests.

Box and whisker plots were created from the NO_x values, and a Student's *t*-test was used to determine whether there were differences in cGMP concentration between the control and NO_2^--treated groups. $P < 0.05$ was considered statistically significant throughout. A Shapiro-Wilk normality test was used to confirm that data followed a Gaussian distribution. Data are shown as means ± SE unless otherwise stated. Statistical analysis was completed in Prism 6 (GraphPad Software).

RESULTS

Dual-wire myography. Exogenously applied NO_2^- caused a concentration-dependent vasodilation of the iliac artery to 49% of the U-46619 preconstriction (Figs. 1–4), which was completely abolished by the application of ODQ (model, EC_{50}, and maximum vasodilation all $P < 0.0001$; Fig. 1. *B* and *C*). The NO_2^--induced vasodilation was significantly more sensitive under hypoxia (model $P = 0.0165$; Fig. 2*A*); the EC_{50} was marginally left shifted from 438 to 345 μM (EC_{50} $P = 0.3266$), and the maximal dilation was 5% greater than normoxia (vasodilation $P = 0.0398$). The addition of oxyhemoglobin significantly affected the response (model $P = 0.0144$), causing a right shift in the data from an EC_{50} of 438 μM to 2.24 mM (EC_{50} $P = 0.0144$). However, the maximal vasodilation was not affected (dilation $P = 0.8906$; Fig. 2*B*). In contrast, deoxyHb significantly (model $P = 0.0087$) altered

Table 1. *Primers, reaction melting temperatures, amplicon sizes, and accession numbers for the globin expression experiment*

Gene/Primer	Sequence (5′–3′)	Melting Temperatures	Size, bp	Accession No.
cygb				
Forward	CAACTGTGAGGATGTTGGAGT	57	369	KU598827
Reverse	TATATGAGACTCCGAAGTTTGTTCC			
ngb				
Forward	GTCTGGACTGCCTTTCTTCC	58	203	KU598828
Reverse	GCTCATGGACTGTACCACAC			
gby				
Forward	AATATACGCGAATCCAGAGGAA	57	396	KU598829
Reverse	TTGTGTAACAGCTGCCCAAG			
β-*act*				
Forward	CATGGACTCAGGTGATGGTG	55	468	BC082343.1
Reverse	CCAGGGTACATTGTGGTTCC			

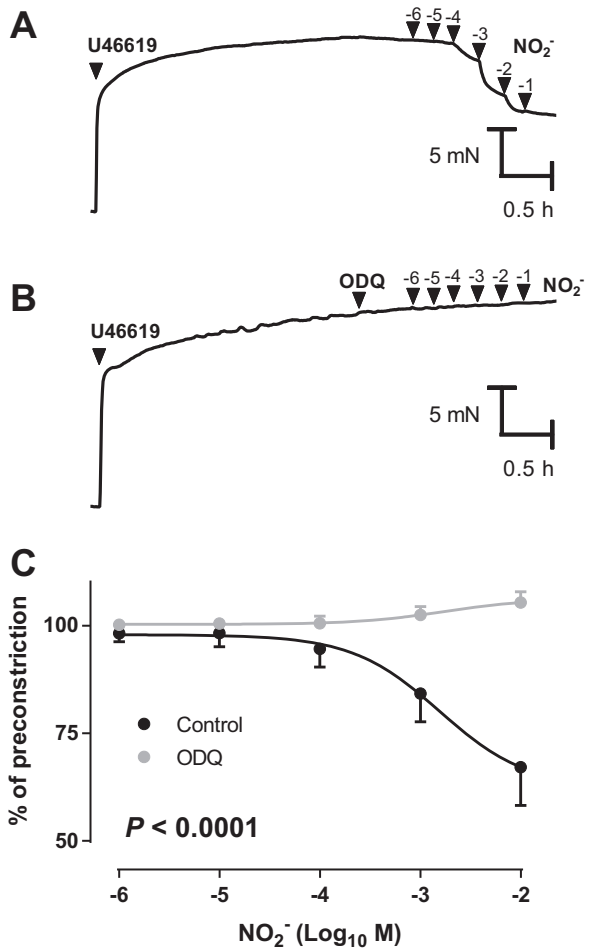

Fig. 1. Representative tension recordings from isolated *Renilla marina* iliac arteries showing concentration-dependent NO_2^--mediated vasodilation. Vessels were preconstricted with U-46619 (10^{-6} M) until stable. The values for NO_2^- are indicated by arrows and shown as $\log_{10}$ M. *A*: NO_2^--mediated vasodilation under normoxic (~21% O_2) conditions. *B*: total abolition of the NO_2^--mediated vasodilation in the presence of the soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; 10^{-5} M). *C*: concentration-dependent vasodilation of isolated *R. marina* iliac arteries in response to NO_2^- vs. the complete abolition of the response following application of ODQ. Sigmoidal concentration-response curves are fitted to the data. *P* values indicate that the fitted curves differ significantly as determined by an *F*-test. Data in *C* are means ± SE $n = 8$.

maximum vasodilation (vasodilation $P = 0.0012$) but not the EC_{50} (EC_{50} $P = 0.1198$; Fig. 2*C*). Comparison of the oxyhemoglobin and deoxyHb curves revealed a significantly (model $P = 0.0006$) greater sensitivity to NO_2^- in the deoxyHb treatment, which was a result of a greater degree of vasodilation (vasodilation $P = 0.0061$), rather than EC_{50} (EC_{50} $P = 0.2146$; Fig. 2*D*). Neither L-NAME (Fig. 3*A*; all $P = 0.3267$) nor L-NNA (Fig. 3*B*; all $P = 0.6409$) significantly affected the NO_2^--induced vasodilation. However, vinyl-L-NIO (Fig. 3*C*) significantly (model $P = 0.0028$) decreased the maximum vasodilation from 21 to 42% of U-46619 preconstriction (vasodilation $P = 0.0473$). C-PTIO (Fig. 4*A*, model $P = 0.4787$), allopurinol (Fig. 4*B*; model $P = 0.9279$), and disruption of the endothelium (Fig. 4*C*; model $P = 0.094$) did not alter the NO_2^--induced vasodilation. A summary of the nonlinear regression statistics is shown in Table 2.

Nitrite and cGMP assays. In the serum, the mean concentrations of NO_2^- and NO_3^- were 0.86 ± 0.20 and 19.55 ± 2.55 μmol/l, respectively. Total NO_x (NO_2^- + NO_3^-) was 20.4 ± 2.62 μmol/l (Fig. 5), and, therefore, NO_3^- constituted the majority of NOx at 96%. The mean cGMP concentrations of the control and 1 mM NO_2^--treated iliac arteries were 101.9 ± 6.16 and 91.82 ± 11.23 pmol/g, respectively, which were not significantly different from each other ($P = 0.400$).

Globin gene cloning and expression. Two full-length globin cDNAs were cloned from the brain of *R. marina* as follows: *cygb* 540 bp with an open reading frame of 179 amino acids (GenBank Accession No.: KU598827); *gby* 471 bp with an open reading frame of 156 amino acids (KU598829). In addition, a partial *ngb* cDNA sequence was obtained (211 bp, 70 amino acids, KU598828). The identity of globins was confirmed by comparison with the respective *Xenopus* sequences; each had over 90% identity with both *X. tropicalis* and *X. laevis*, *Cygb* (Fig. 6*A*), and *ngb* (Fig. 6*B*) mRNAs were expressed in the iliac arteries of *R. marina*, but *gby* mRNA was not expressed in the iliac artery (Fig. 6*C*).

DISCUSSION

NO_2^- is vasoactive in R. marina iliac arteries. In mammals, reduction of NO_2^- in the blood provides a mechanism for NO production and vasodilation, particularly during hypoxia when the oxygen-dependent generation of NO from NOS is compromised (29). The mechanisms of NO_2^- reduction have been well characterized in mammalian blood vessels, and it is clear that although reduction can occur in normoxia, it is substantially upregulated in hypoxia to promote vasodilation and tissue perfusion to restore tissue oxygenation and ameliorate acidosis (44). This process is facilitated, in part, by proteins that only act as NO_2^- reductases in hypoxia and acidosis (29).

In this study, we show for the first time in amphibians that applied NO_2^- is a vasodilator of the iliac arteries of *R. marina* and that the response is abolished by the sGC inhibitor ODQ as it is in mammals (33). Previously, we have shown that the NO donor SNP is a potent vasodilator of blood vessels from *R. marina* and that the response was abolished by ODQ (2, 3). Taken together, it is reasonable to conclude that NO_2^- is being translocated into the vascular smooth muscle cells of the iliac artery of *R. marina*, where it is reduced by an endogenous mechanism to NO, which then mediates vasodilation via sGC.

The concentration range of NO_2^- that facilitates vasodilation in the iliac arteries of *R. marina* is similar to values obtained in in vitro myography studies using normoxic rodent aortas (8, 21, 32). For example, the EC_{50} values obtained in this study for NO_2^- in normoxic iliac arteries (438 μM) are in the range of some EC_{50} values reported in rodent aortas: 60 μM (21), 200 μM (31), and 686 μM (8). After the experiments in normoxia, we then demonstrated a small but significant effect of hypoxia on NO_2^- -induced vasodilation, which decreased the EC_{50} from 438 to 345 μM NO_2^- without hemoglobin (0.27-fold decrease) and 2.2 to 1.1 mM with hemoglobin (0.47-fold decrease). However, the left shift in the concentration-response curve during hypoxia in *R. marina* is substantially smaller in magnitude than that reported in rodents (8, 21, 26). For example, Dalsgaard et al. (8) showed a large increase in NO_2^- sensitivity from an EC_{50} of 686 μM (95% O_2) to 12 μM (1% O_2),

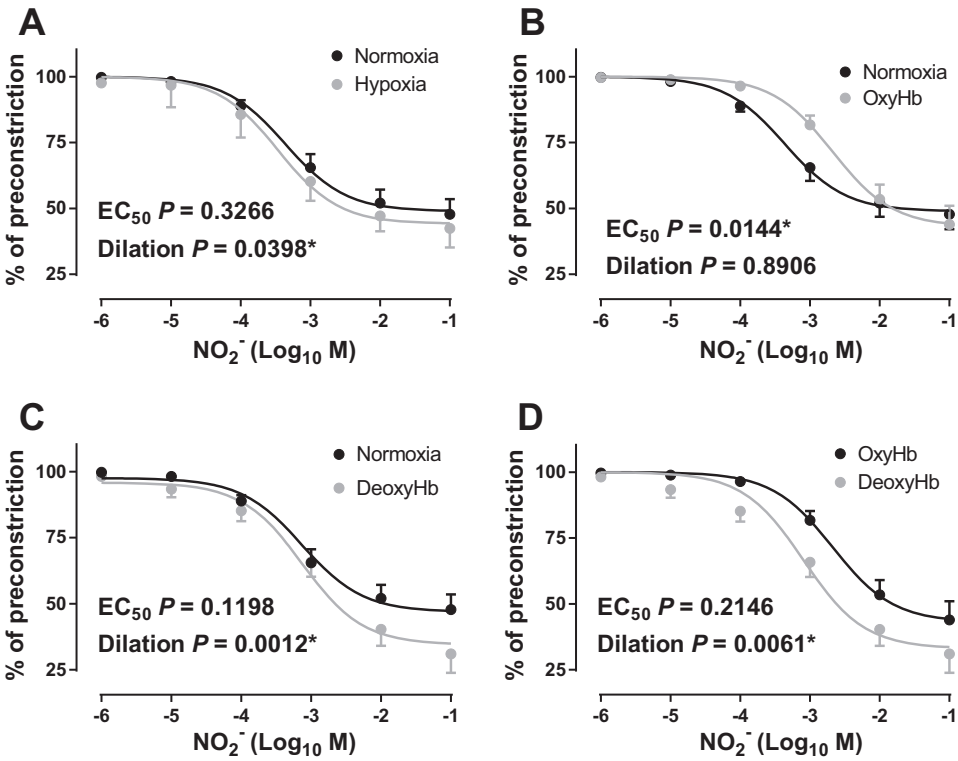

Fig. 2. Concentration-dependent vasodilation of isolated *R. marina* iliac arteries in response to the application of NO_2^-. *A*: normoxia vs. hypoxia (0.63% O_2), $n = 9$. *B*: effect of 200 μM oxyhemoglobin (OxyHb) on NO_2^--mediated vasodilation, $n = 9$. *C*: effects of 200 μM partially deoxyhemoglobin (DeoxyHb) $n = 8$. *D*: OxyHb vs. DeoxyHb, $n = 9$. Data are shown as a percentage of net U-46619 preconstriction force. Sigmoidal concentration-response curves are fitted to the data. *P* values indicate if the fitted sigmoidal curves differ significantly as determined by an *F-test*. Data are means ± SE.

representing a 57-fold decrease in the EC_{50}. Similarly, Isbell et al. (21) reported a sixfold reduction in EC_{50} values from 60 μM to <10 μM for aortas at 2% O_2, 1% O_2, and anoxia, respectively. The small effect of hypoxia on vascular NO_2^- reduction in *R. marina* might be due to the fact that amphibian blood vessels do not show classic hypoxic vasodilation (34), potentially involving NO.

NO_2^- reduction in R. marina iliac arteries. The iliac arteries of *R. marina* clearly possess endogenous NO_2^- reductase capacity, but, unlike mammals, hypoxia only had a small effect on the NO_2^- -mediated vasodilation. This suggests that the mammalian paradigm for the vascular role of NO_2^- in hypoxia utilizing hypoxia-sensitive NO_2^- reductases may not be applicable in amphibians. In this study, the mechanisms of NO_2^- reduction were not definitively demonstrated but a number of pathways were investigated.

Given the novelty of this study in amphibians, we tested the effect of the inhibition of XOR and NOS in normoxia to determine if these enzymes might function differently in amphibians compared with mammals and play a role in normoxia. There was no evidence for the involvement of XOR in the reduction of NO_2^- to NO in the iliac arteries of *R. marina*, since application of allopurinol did not affect the NO_2^--mediated vasodilation. In mammals, there is evidence for and against the involvement of XOR in vascular NO_2^- responses (8, 39), and where implicated, the NO_2^--reducing activity of XOR is only observed in hypoxia. The nonspecific NOS inhibitors L-NAME and L-NNA did not affect the NO_2^- -mediated vasodilation, but curiously, the NOS1-specific inhibitor vinyl-L-NIO did significantly reduce the vasodilatory response to NO_2^-. It is not clear why there would be a difference between the NOS inhibitors. The large and intermediate-sized arteries of *R. marina* express NOS1 in the perivascular, nitrergic nerves (2, 3) and NOS3 in the endothelium (5). Thus it is possible that NOS1 may contribute to NO_2^- reduction in iliac arteries, at least in part. Interestingly, Webb et al. (45) found that NOS3 inhibition with L-NAME had no significant effect on vascular NO_2^- reductase activity in rat aortic supernatants, but it did significantly inhibit NOS3 NO_2^-reductase activity in red blood cell preparations. Therefore, there appears to be an anomaly about the function of NOS as a NO_2^- reductase in mammalian blood vessels.

Myoglobin has been identified as a key NO_2^- reductase in mammalian blood vessels (7, 18, 28, 41, 42, 44). Specifically, studies using homozygous myoglobin knockout ($myg^{-/-}$) mice have shown decreased endogenous NO production and cGMP content in aortas and blunted NO_2^--induced vasodilation relative to wild-type mice ($myg^{+/+}$) (18, 32, 42). Interestingly, amphibians do not possess the *mygb* gene but do contain the cytoglobin gene (*cygb*), and it has been proposed that cytoglobin functionally replaces myoglobin in this vertebrate class (16). In this study, we cloned mRNAs for *cygb*, *ngb*, and *gby* and demonstrated that *cygb* and *ngb* mRNAs were expressed in the iliac arteries of *R. marina*, which provides evidence that there are globins expressed in the iliac arteries that could act as endogenous NO_2^- reductases. However, the functionality of cytoglobin is similar to myoglobin in that the NO_2^--reducing properties of the protein are only observed in hypoxia or anoxia (25, 44). Therefore, it is unlikely that cytoglobin and/or neuroglobin are contributing to the NO_2^--mediated vasodilation of the iliac arteries of *R. marina* in normoxia. The globins, in addition to XOR and NOS, could play a role in the small left shift of the NO_2^- concentration-response curve observed in hypoxia.

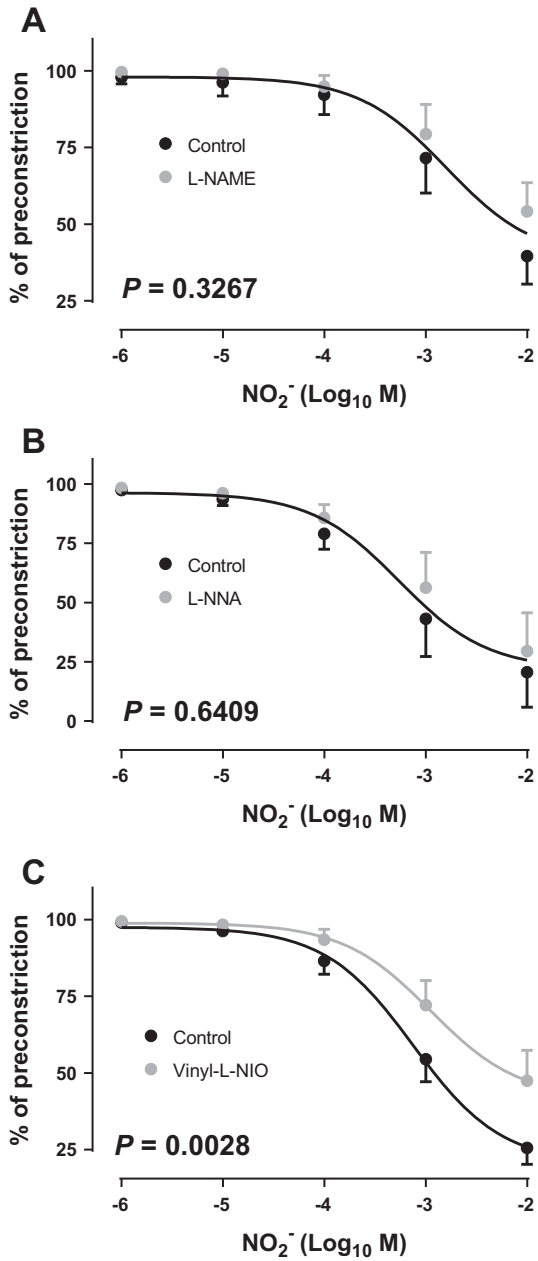

Fig. 3. Concentration-dependent vasodilation of isolated *R. marina* iliac arteries in response to the application of NO_2^-, in the presence of selected inhibitors. *A*: control vs. N^{ω}-nitro -L-arginine methyl ester (L-NAME; $n = 3$.) *B*: control vs. N^{ω}-nitro-L-arginine (L-NNA; $n = 4$). *C*: control vs. N^5-(1-imino-3-butenyl)-L-ornithine (vinyl-L-NIO), $n = 6$. Data are shown as a percentage of net U-46619 preconstriction force. Sigmoidal concentration-response curves are fitted to the data. *P* values indicate if the fitted sigmoidal curves differ significantly as determined by an *F*-test. A single curve is fitted in cases where no significance was determined. Data are means ± SE.

An alternative pathway for NO formation from NO_2^- that does not involve an enzyme is through the process of NO_2^- disproportionation, as described by Zweier et al. (48). In this scenario, the acidic intracellular environment of cells permits NO_2^- to be in equilibrium with HNO_2, which can enter a reaction pathway leading to NO formation (48). This mechanism has been demonstrated in rat cardiomyocytes during acidosis and is suggested as an alternative route for NO formation from NO_2^- (49). It is reasonable to consider that NO_2^- disproportionation may occur in the iliac artery vascular smooth muscle cells of *R. marina* leading to NO formation and vasodilation. This mechanism could also explain the relatively high NO_2^- concentrations needed to generate NO to induce vasodilation in iliac arteries of *R. marina*. Previous studies have found that *R. marina* is tolerant of hypercapnia in the

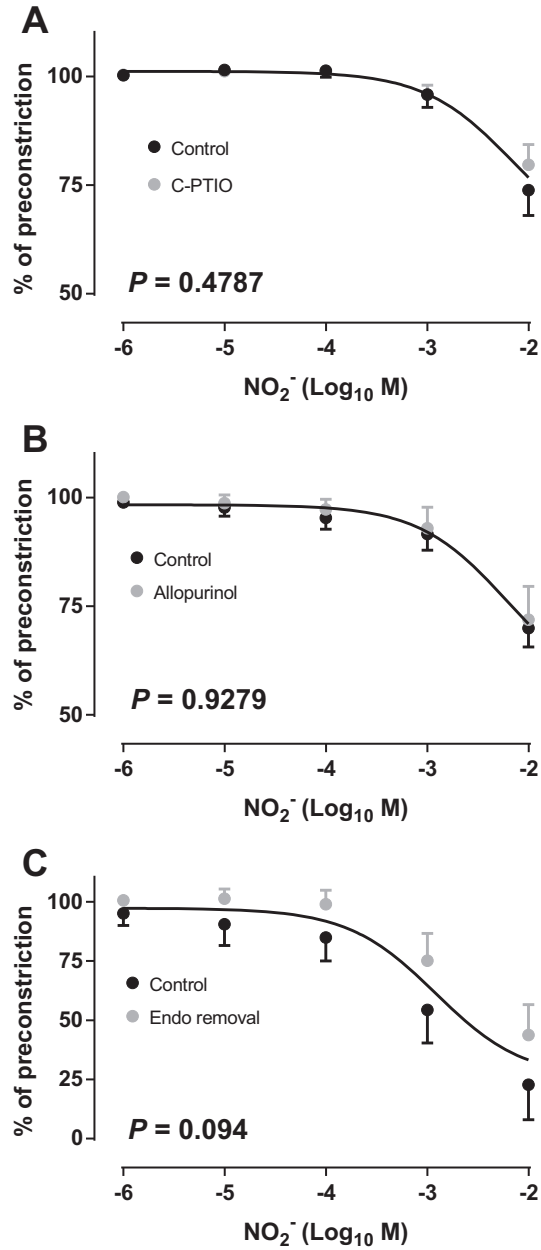

Fig. 4. Concentration-dependent vasodilation of isolated *R. marina* iliac arteries in response to the application of NO_2^- in the presence of selected inhibitors and after endothelial removal. *A*: control vs. 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide (C-PTIO), $n = 6$. *B*: the control vs. allopurinol, $n = 4$. *C*: endothelium intact vs. endothelial removal, $n = 4$. Data are shown as a percentage of the U-46619 preconstriction force. Sigmoidal concentration-response curves are fitted to the data. *P* values indicate if the fitted sigmoidal curve differ significantly as determined by an *F*-test. A single curve is fitted in cases where no significance was determined. Data are means ± SE.

Table 2. *Summary of nonlinear regression statistics from myography experiments*

Treatment	R_2	n	P Value (Model)	EC_{50}, μM	P Value (EC_{50})	Dilation,%max	P Value (Dilation)
Normoxia	0.99	9	—	438	—	49	—
Hypoxia	0.99	9	$P = 0.0165$*	345	$P = 0.3266$	44	$P = 0.0398$*
OxyHb	0.82	9	$P = 0.0081$**	2,236	$P = 0.0144$*	43	$P = 0.8906$
DeoxyHb	0.81	9	$P = 0.0087$*	1,134	$P = 0.1198$	32	$P = 0.0012$**
OxyHb	0.82	9	$P = 0.0006$**	2,236	$P = 0.2146$	43	$P = 0.0061$**
DeoxyHb	0.81	9		1,134		32	
Control	0.39	8	$P < 0.0001$***	1,540	$P < 0.0001$***	62	$P < 0.0001$***
ODQ	0.15	8		1,716		105	
Control	0.74	4	$P = 0.6409$	423	$P = 0.6419$	18	$P = 0.6921$
L-NNA	0.69	4		719		25	
Control	0.81	3	$P = 0.3267$	1,600	$P = 0.9997$	30	$P = 0.3240$
L-NAME	0.79	3		1,599		47	
Control	0.88	6	$P = 0.0028$**	752	$P = 0.5588$	21	$P = 0.0473$*
Vinyl-L-NIO	0.69	6		1,100		42	
Control	0.67	4	$P = 0.0942$	975	$P = 0.6770$	16	$P = 0.4037$
Endo -ve	0.70	4		1,550		34	
Control	0.71	6	$P = 0.4787$	5,374	$P = 0.7853$	68	$P = 0.6007$
C-PTIO	0.71	6		8,724		49	
Control	0.71	4	$P = 0.9279$	1,550	$P = 0.9887$	53	$P = 0.9361$
Allopurinol	0.54	4		1,720		56	

ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; L-NNA, N^{ω}-nitro-L-arginine; L-NAME, N^{ω}-nitro -L-arginine methyl ester; vinyl-L-NIO, N^{5}-(1-imino-3-butenyl)-L-ornithine; C-PTIO, 2-(4-carboxyphenyl)-4,5-dihydro-4,4,5,5-tetramethyl-1H-imidazolyl-1-oxy-3-oxide. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

natural environment (40), and toads exposed to hypercapnia showed a marked decrease in intracellular pH in brain and skeletal muscle (40). A markedly lower intracellular pH would enhance NO production via NO_2^- disproportionation processes and potentiate local vasodilation.

In in vitro experiments in mammals, Hb has been successfully used to ("mop-up") any NO formed in NO_2^- reduction experiments, thereby acting as an indicator of NO formation from NO_2^- (21). In normoxia, we observed an approximately threefold increase in the EC_{50} in response to NO_2^- application, with the addition of hemoglobin to the vessel chamber. This provides further evidence that the iliac arteries of *R. marina* are able to reduce NO_2^- to NO that freely diffuses through the vessel wall and binds to the heme moiety of Hb, thereby reducing the availability of NO for vasodilation. We also found that the response to NO_2^- was altered by incubating the iliac arteries in deoxyHb, potentially due to the protein now acting as a NO_2^- reductase in addition to binding NO. The experiments with Hb in *R. marina* show that, in vivo, the oxygenation state of Hb will be a factor in regulating the bioavailability of NO from reduced NO_2^-, as has been demonstrated in mammals (21).

Plasma nitrite. The mean serum NO_2^- value of 0.86 μM in *R. marina* was higher than that previously reported for the toad *Bufo woodhousei fowleri* and frog *Rana pipiens* in which the NO_2^- values were found to be in the range 0.1 to 0.2 μM (47). It is difficult to directly compare the two studies because, and as discussed by Williams et al. (47), factors such as diet, anesthesia, and blood sampling regime can affect the values, in addition to the type of assay used to measure NO_2^-. Furthermore, the tube type used to process the samples can contribute to variability in the NO_2^- values (22).

Paradoxically, the EC_{50} for NO_2^- in *R. marina* is ~500-fold higher than the resting serum NO_2^- value of 0.86 μM. Blood NO_2^- in rodents and humans is also much lower than reported EC_{50} values for in vitro studies with values in the literature of between 0.05 and 0.3 μM NO_2^- (29). The disparity between in vitro EC_{50} values and serum NO_2^- concentrations is reduced in mammals during hypoxia and acidosis in which the sensitivity to NO_2^- is increased (48, 49). However, the above EC_{50} values are calculated from in vitro data, and it is therefore possible that the theoretical NO_2^- concentration used in vitro may not reflect true intracellular concentrations, which may be due to diffusion-limitation across the cell membrane as a result of the relatively poor circulation of fluid in the vessel chamber. This is in contrast to the intact circulation, which maintains maximal diffusion gradients to drive movement across the cell membrane. Another point to consider is the fact that red blood cells are present in vivo and the NO_2^- reducing capacity of partially oxygenated Hb may generate more NO for a given concentration of plasma NO_2^-. Certainly, the relatively lower titer of NO_2^- required to induce significant vasodilation in perfused human and primate forearm antecubital veins and brachial arteries suggests that, in vivo, the pharmacokinetics differ from those observed in vitro (6, 9).

NO_2^- vasoctivity and the sGC/cGMP pathway. In the current study, 1 mM NO_2^- did not significantly increase the cGMP concentration in a pooled tissue sample of iliac arteries and dorsal aortas, compared with control vessels incubated in physiological saline alone. This is a curious observation given that 1 mM NO_2^- induced a vasodilation that was blocked by ODQ, which is indicative of NO signaling via sGC. It is pertinent that our previous work has assumed that NO-induced vasodilation in *R. marina* blood vessels is cGMP dependent (2, 3), but this has not been demonstrated by measurement of cGMP. Interestingly, previous studies in rodent aortas have also shown that ODQ significantly attenuated or abolished the NO_2^--mediated vasodilation without concomitant increases in cGMP (7, 26, 27). Thus there is a precedent for the observations of the current study.

Alternatively, following NO_2^- reduction, the NO signaling cascade acted via sGC but independently of cGMP utilizing mechanisms such as nitrosylation of sGC by the addition of a

nitrosyl ion (NO^-) (36) or NO reacting with a thiol group on sGC to form *S*-nitroso (SNO) residues that may go on to participate in nitrosation reactions (20). We also cannot exclude the possibility that 8-nitro-cGMP was formed from 8-nitro-GTP through the actions of sGC (35), which would not have been detected in our assay. Certainly 8-nitro-GTP can be readily derived in vivo from GTP nitration by NO-derived reactive nitrogen species (17).

Perspectives and Significance

For many years, NO_2^- was thought be an inactive by product of NO metabolism. However, it is now known that the in vitro application of NO_2^- to tissues, including blood vessels, elicits biological responses that are attributed to NO, due to cells possessing endogenous NO_2^- reductase mechanisms that generate NO. Accordingly, plasma NO_2^- is considered an endocrine source of NO, which has been found to be important in hypoxia where the generation of NO by NOS is compromised by limited O_2 supply. In the cardiovascular system, this is exemplified by data demonstrating that dietary NO_x or infusion with NO_x improved tissue blood flow in various animal models of hypoxia and ischemia. In this study, we provide the first evidence that NO_2^- is a vasodilator in a nonmammalian vertebrate (*R. marina*), most likely by the endogenous reduction of NO_2^- to NO in the vascular smooth muscle. Thus it appears that the capacity for vascular reduction of NO_2^- to NO appeared early in the tetrapods and preceded the emergence of endothelial NO signaling, which is not a characteristic of amphibian blood vessels (4, 5). This study also found that hypoxia had little effect on the NO_2^--induced vasodilation in *R. marina*, which is in contrast to the situation in mammals. *R. marina* use burrowing and estivation in response to extremes in environmental conditions, which can cause marked acidosis and hypoxia that are physiologically well tolerated (40); this hypoxia tolerance may explain the absence of hypoxic vasodilation in amphibians (34).

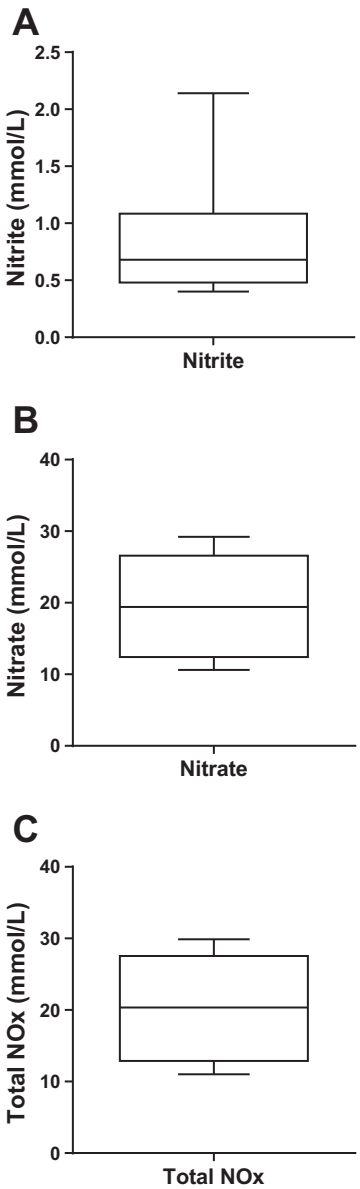

Fig. 5. Box plots of serum NO_2^-, NO_3^-, and total NOx measured by a fluorimetric assay. *A*: NO_2^-. *B*: NO_3^-. *C*: total $NO_2^- \pm NO_3^-$ (NOx) concentration. Boxes represent the quartile range, and whiskers show minimum and maximum values. All $n = 8$.

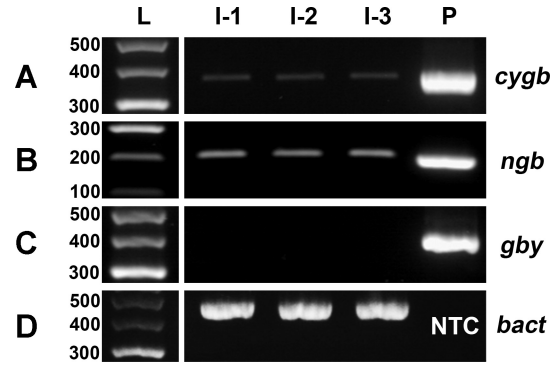

Fig. 6. Gel electrophoresis images of PCR products resolved on 1.5% agarose gels. The expression of mRNA for cytoglobin (*cygb*) (*A*) and neuroglobin (*ngb*) (*B*) was present in three separate iliac artery extracts (I-1, I-2, and I-3). However, globin Y (*gby*) (*C*) mRNA was not detected in iliac arteries. *β-act* mRNA expression is shown for each cDNA in *D*. P, amplification of PCR product from a control plasmid containing the gene of interest; L, molecular weight marker; NTC, no template control. Irrelevant lanes between the ladder and samples have been removed as indicated by the white spaces.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.G.F. and J.A.D. conceived and designed research; L.G.F., M.S., B.A.M., and M.S.C. performed experiments; L.G.F. and B.A.M. analyzed data; L.G.F., M.S.C., and J.A.D. interpreted results of experiments; L.G.F. and B.A.M. prepared figures; L.G.F. drafted manuscript; L.G.F., B.A.M., M.S.C., and

J.A.D. edited and revised manuscript; L.G.F., M.S., B.A.M., M.S.C., and J.A.D. approved final version of manuscript.

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