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

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Running Head

Nitrite vasodilation in *R. marina* iliac arteries

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Abstract

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} 2236 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 vinyl-L-NIO (P = 0.0028) significantly reduced the NO_2^- vasodilation. The xanthine oxidoreductase inhibitor, allopurinol (P = 0.927), the NO-scavenger 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 cGMP concentration (P = 0.407). Plasma NO_2^- was found to be 0.86 ± 0.20 µmol.L^{-1}, while nitrate (NO_3^-) was 19.55 ± 2.55 µmol.L^{-1}. 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^-.

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 cyclic guanosine monophosphate (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 hyperpolarising-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 non-mammalian vertebrates, it has been suggested that similar mechanisms for NO signalling 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 oxidised to nitrite (NO_2^-) and nitrate (NO_3^-) (29). In humans, the NO_2^- concentration in the blood is approximately 0.05-0.3 μM, with the concentration of NO_3^- approximately 100-1000-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 up-

69 regulated in hypoxic (14) and acidotic (48, 49) states, when the oxygen-dependent
70 generation of NO from NOS is compromised. However, it is also clear that NO_2^- reduction to
71 NO to facilitate vasodilation can occur in normoxia (29).

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

82 In non-mammalian vertebrates, our understanding of NO_2^- reductase activity and its
83 role in vascular regulation is rudimentary. There is some evidence for an involvement of NO
84 generated from reduced plasma NO_2^- in regulating blood-flow redistribution during anoxia
85 in the turtle, *Trachemys scripta*, as indicated by increased concentrations of Fe-nitrosyl and
86 *N*-nitroso compounds in the blood (23). In rainbow trout, *Oncorhynchus mykiss*, infusion of
87 1 mM NO_2^- caused only minimal decreases in blood pressure (1), and there are no reports of
88 the vasoactive effects of NO_2^- in amphibians. Therefore, the aim of the current study was to
89 investigate whether arteries of the amphibian, *Rhinella marina*, possess the intrinsic ability
90 to reduce NO_2^- to NO to facilitate vasodilation. *Rhinella marina* aortae express NOS3 in the
91 endothelium (4) but do not display the classical endothelial NO signalling system, as applied

92 acetylcholine mediated a NO vasodilation that is not significantly affected by removal of the
93 endothelium and was attributed to activation of NOS1 expressed in perivascular, nitrergic
94 nerves (2, 3). In summary, this study provides the first systematic report that NO_2^- is
95 reduced to NO to facilitate vasodilation in arteries of a non-mammalian vertebrate.

96

97 **Material and Methods**

98 *Animals*

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

110

111 *Dual-wire myography*

112 Both left and right branches of the iliac artery were carefully dissected and freed of
113 connective tissue and placed into chilled physiological saline (mM: 115 NaCl, 3.2 KCl, 2.5

114 $CaCl_2$, 1.4 $MgSO_4$, 7 NaOH, 10 HEPES, 17 glucose, pH 7.4). The iliac artery was used for
115 experiments as it is an intermediate-sized vessel that has been shown to exhibit endothelial-
116 independent NO signalling in the same manner as other toad blood vessels (4). Isolated iliac
117 arteries were carefully cut into 3 mm lengths and mounted in a dual-wire myograph (420A,
118 Danish Myo Technology A/S, Aarhus, Denmark), which was connected to a Myo-Interface
119 (DMT) linked to a PowerLab data recorder (ADInstruments, NSW, Australia). Data were
120 recorded using LabChart 7 (ADInstruments). The myography chambers were filled with 5 mL
121 of physiological saline maintained at room temperature (~20 °C) and aerated. The vessels
122 were allowed to equilibrate for 15 min before a nominal tension of 2 mN was applied. The
123 optimal length-tension relationship was empirically determined by incrementally
124 lengthening and then constricting the vessels using osmotically balanced 60 mM KCl
125 physiological saline. The optimal length was assumed when active-force plateaued. Prior to
126 pharmacological treatment, vessels were pre-constricted using the prostaglandin H_2-
127 analogue and thromboxane A_2 mimic, U-46619 (Cayman Chemicals, MI, USA). Experiments
128 were conducted in a pairwise fashion with a matched control from the opposite iliac artery
129 from the same animal. If required, the endothelium of the iliac artery was disrupted by
130 rubbing the luminal surface several times with a 24-gauge needle.

131 Preliminary experiments revealed that pre-constricted iliac arteries vasodilated in
132 response to exogenously applied 1 mM sodium nitrite ($NaNO_2$). As the sodium ion
133 dissociates from the NO_2^- moiety in solution, the nomenclature of NO_2^- will be used
134 throughout this manuscript. Following preliminary experimentation, concentration-
135 response experiments were performed by the sequential addition of exogenous NO_2^- to
136 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. In order 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 (PO_2) 20.85 ± 0.06 % O_2 ~155 mmHg) and hypoxia (n=9; PO_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 PO_2 with an SI130 O_2 electrode and SI929 meter (Strathkelvin Instruments, North Lanarkshire, Scotland). Under these experimental conditions, bovine hemoglobin is approximately 10 % saturated with O_2 (46). However, for simplicity we have referred to 90% desaturated hemoglobin as deoxyHb throughout this manuscript; it would be expected that 90% desaturated hemoglobin would have significant reductase activity compared to oxyHb.

The second series of experiments involved the application of specific pharmacological inhibitors to the myography chamber prior to 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); N5-(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); 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 (Ann Arbor, MI, USA).

Serum NO_2^-/ NO_3^- assays

During surgery, blood was withdrawn via cardiac puncture and allowed to clot on ice for approximately 30 min (n=8). Blood was centrifuged at 10,000 x *g* for 5 min, the serum removed and snap frozen in liquid N_2, and stored at -80°C. Thawed samples were centrifuged at 10,000 x *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 µL of the filtrate was 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, Ann Arbor, MI, USA), according to the manufacturer's instructions. Data were read on a FLUOstar Omega fluorimeter (BMG LABTECH, VIC, Australia).

cGMP assay

In order 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 in order 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; non-specific 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 and 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 volumes of 0.1 N HCl with the aid of silica-zirconium beads and a TissueLyser II (Qiagen, Venlo, Netherlands) and centrifuged at 10,000 x *g* for 10 min. The supernatant was assayed for cGMP using colorimetric competitive immunoassay (Direct cGMP ELISA Kit, ENZO Life Sciences, Farmingdale, NY, USA) according to the manufacturer's instructions, and read on a FlexStation II spectrophotometer (Molecular Devices, CA, USA).

Hemoglobin preparation and spectral properties

Ferrous bovine oxyhemoglobin was prepared from methemoglobin (Sigma, Castle Hill, Australia) by equilibrating a 25 x 2.5 cm Sephadex G-25 column (GE Life Sciences) with 20 mM phosphate buffer, 1 mM EDTA (pH 7.0) and 10% w/v 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 (Thermofisher, USA) 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, CA, USA). At each concentration of NO_2^- from 1 µM onwards, significant methemoglobin formation was observed reaching approximately 100% of total hemoglobin at 10 mM (data not shown).

204

205 *Molecular biology*

206 Primers based on *Xenopus laevis* and *X. tropicalis* globin mRNAs (NCBI accession numbers:
207 cytoglobin *cygb:* **NP_001087751.1**, neuroglobin *ngb:* **NM_001030351.1**, globin Y *gby:*
208 **BC128970.1**) were used to amplify *cygb*, *ngb*, and *gby* mRNAs, respectively, in *R. marina*.
209 Total RNA was extracted from *R. marina* brain by homogenising the tissue in TRIzol reagent
210 (Invitrogen, USA) with silica-zirconium beads using a Fastprep 20 benchtop homogeniser
211 (MPBio, USA). Total RNA was treated with DNAseI (Invitrogen, USA) prior to reverse
212 transcription into cDNA with Superscript II (Invitrogen, USA), using both random primers
213 (Promega Corporation, MA, USA) and Oligo $dT_{15\text{-}18}$. Specific globin cDNAs were amplified
214 using GoTaq G_2 Green Mastermix (Promega) and a series of overlapping gene specific
215 primers. To amplify the 3' UTR of *cygb* and *gby*, 3' rapid amplification of cDNA ends (3'
216 RACE) PCR was performed (Invitrogen, USA). Amplified PCR products were ligated into
217 pGEM-T easy (Promega) plasmids and transformed into highly competent *Escherichia coli*
218 (J109; Promega) cells. Colonies were selected for positive inserts using β-galactosidase
219 chromogenic detection with IPTG and X-gal (Adipogen, CA, USA). Positive insert colonies
220 were grown overnight in LB broth and the plasmid DNA harvested (PureYield® Miniprep Kit,
221 Promega). Plasmids were sequenced using M13 forward (5'-
222 CGCCAGGGTTTTCCCAGTCACGAC-3') and reverse (5'- TCACACAGGAAACAGCTATGAC-3')
223 primers and BigDye v3.1 chemistry (Applied Biosystems, CA, USA). Sequences were read at
224 the Ramaciotti Centre for Genomics (UNSW, Australia) using a 3500 series Genetic Analyser
225 (Applied Biosystems), and were identified by comparison to *X. laevis* and *X. tropicalis*
226 nucleotide sequences (blast.ncbi.nlm.nih.gov/Blast.cgi). Contigs were constructed in CAP3

(doua.prabi.fr/software/cap3) using 8 individually obtained sequences for each cDNA. From the cloned sequences, homologous primers for *R. marina cygb, ngb* and *gby* were designed using NCBI primer blast (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.

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

Gene	Primer	Sequence (5'-3')	Tm	Size (bp)	Accession
cygb	Forward	CAACTGTGAGGATGTTGGAGT	57	369	KU598827
cygb	Reverse	TATATGAGACTCCGAAGTTTGTTCC			
ngb	Forward	GTCTGGACTGCCTTTCTTCC	58	203	KU598828
ngb	Reverse	GCTCATGGACTGTACCACAC			
gby	Forward	AATATACGCGAATCCAGAGGAA	57	396	KU598829
gby	Reverse	TTGTGTAACAGCTGCCCAAG			
β-*act*	Forward	CATGGACTCAGGTGATGGTG	55	468	BC082343.1
β-*act*	Reverse	CCAGGGTACATTGTGGTTCC			

To obtain sufficient total RNA from *R. marina* iliac arteries, a single sample represented vessels pooled from two animals; we used 6 animals to make 3 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.

244

245 *Statistics*

246 Sigmoidal (logistic) concentration-response curves were fitted to $\log_{10}$ NO_2^- values for each
247 paired control and associated pharmacological or PO_2 treatment. The fitted curves were
248 used to estimate (1) whether fitted models differed from each other between groups
249 ("model"), (2) effective concentration to 50% of responses (EC_{50}) and (3) maximal dilation (%
250 of U46619). Differences in the above parameters were assessed by extra sum of squares *F*-
251 tests.

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

258

259 **Results**

260 *Dual-wire myography*

261 Exogenously applied NO_2^- caused a concentration-dependent vasodilation of the iliac artery
262 to 49% of the U-46619 preconstriction (Figs 1-4), which was completely abolished by the
263 application of ODQ (model, EC_{50} and maximum vasodilation all $P < 0.0001$; Figs 1B, 1C). The
264 NO_2^--induced vasodilation was significantly more sensitive under hypoxia (model $P = 0.0165$;
265 Fig 2A); 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 2B). In contrast, deoxyHb significantly (model $P = 0.0087$) altered maximum vasodilation (vasodilation $P = 0.0012$) but not the EC_{50} (EC_{50} $P = 0.1198$; Fig 2C). 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 2D). Neither L-NAME (Fig 3A; all $P = 0.3267$), nor L-NNA (Fig 3B; all $P = 0.6409$) significantly affected the NO_2^--induced vasodilation. However, vinyl-L-NIO (Fig 3C) significantly (model $P = 0.0028$) decreased the maximum vasodilation from 21 to 42% of U-46619 preconstriction (vasodilation $P = 0.0473$). C-PTIO (Fig 4A, model $P = 0.4787$), allopurinol (Fig 4B; model $P = 0.9279$), and disruption of the endothelium (Fig 4C; model $P = 0.094$) did not alter the NO_2^--induced vasodilation. A summary of the non-linear regression statistics is shown in Table 2.

289 **Table 2.** Summary of non-linear 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**	2236	*P* = 0.0144*	43	*P* = 0.8906
-DeoxyHb	0.81	9	*P* = 0.0087*	1134	*P* = 0.1198	32	*P* = 0.0012**
OxyHb	0.82	9	*P* = 0.0006**	2236	*P* = 0.2146	43	*P* = 0.0061**
DeoxyHb	0.81	9		1134		32	
Control	0.39	8	*P* < 0.0001***	1540	*P* < 0.0001***	62	*P* < 0.0001***
ODQ	0.15	8		1716		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	1600	*P* = 0.9997	30	*P* = 0.3240
L-NAME	0.79	3		1599		47	
Control	0.88	6	*P* = 0.0028**	752	*P* = 0.5588	21	*P* = 0.0473*
Vinyl-L-NIO	0.69	6		1100		42	
Control	0.67	4	*P* = 0.0942	975	*P* = 0.6770	16	*P* = 0.4037
Endo -ve	0.70	4		1550		34	
Control	0.71	6	*P* = 0.4787	5374	*P* = 0.7853	68	*P* = 0.6007
C-PTIO	0.71	6		8724		49	

Control	0.71	4	*P* = 0.9279	1550	*P* = 0.9887	53	*P* = 0.9361
Allopurinol	0.54	4		1720		56	

290

291 *Nitrite and cGMP assays*

292 In the serum, the mean concentrations of NO_2^- and NO_3^- were 0.86 ± 0.20 µmol.L^{-1} and
293 19.55 ± 2.55 µmol.L^{-1}, respectively. Total NO_x (NO_2^- + NO_3^-) was 20.4 ± 2.62 µmol.L^{-1} (Fig 5),
294 and therefore, NO_3^- constituted the majority of NOx at 96%. The mean cGMP
295 concentrations of the control and 1 mM NO_2^- treated iliac arteries were 101.9 ± 6.16 pmol/g
296 and 91.82 ± 11.23 pmol/g, respectively, which were not significantly different from each
297 other *(P* = 0.400).

298

299 *Globin gene cloning and expression*

300 Two full-length globin cDNAs were cloned from the brain of *R. marina* as follows: *cygb* 540
301 bp with an open reading frame of 179 amino acids (GenBank Accession: KU598827); *gby* 471
302 bp with an ORF of 156 amino acids (KU598829). In addition, a partial *ngb* cDNA sequence
303 was obtained (211 bp, 70 amino acids, KU598828). The identity of globins was confirmed by
304 comparison with the respective *Xenopus* sequences; each had over 90% identity with both
305 *X. tropicalis* and *X. laevis*. *Cygb* (Fig. 6A) and *ngb* (Fig. 6B) mRNAs were expressed in the iliac
306 arteries of *R. marina*, but *gby* mRNA was not expressed in the iliac artery (Fig 6C).

307

308

309

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-characterised in mammalian blood vessels, and it is clear that although reduction can occur in normoxia, it is substantially up-regulated 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 aortae (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 aortae: 60 μM (21), 200 μM (31) and 686 μM (8). Following 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 µM to 345 µM NO_2^- without hemoglobin (0.27-fold decrease), and 2.2 mM to 1.1 mM with hemoglobin (0.47-fold decrease). However, the left-shift in the NO_2^- 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 (2007) showed a large increase in NO_2^- sensitivity from an EC_{50} of 686 µM (95% O_2) to 12 µM (1% O_2), representing a 57-fold decrease in the EC_{50} (8). Similarly, Isbell et al (2007) reported a 6-fold reduction in EC_{50} values from 60 µM to <10 µM for aortae at 2% O_2, 1% O_2 and anoxia, respectively (21). 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 utilising 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, in order to determine if these enzymes might function differently in amphibians compared to 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.*

354 *marina*, since application of allopurinol did not affect the NO_2^- mediated vasodilation. In
355 mammals, there is evidence for and against the involvement of XOR in vascular NO_2^-
356 responses (8, 39), and where implicated, the NO_2^- - reducing activity of XOR is only observed
357 in hypoxia. The non-specific NOS inhibitors, L-NAME and L-NNA, did not affect the NO_2^- -
358 mediated vasodilation, but curiously, the NOS1 specific inhibitor, vinyl-L-NIO, did
359 significantly reduce the vasodilatory response to NO_2^-. It is not clear why there would be a
360 difference between the NOS inhibitors. The large and intermediate-sized arteries of *R.*
361 *marina* express NOS1 in perivascular, nitrergic nerves (2, 3) and NOS3 in the endothelium
362 (5). Thus, it is possible that NOS1 may contribute to NO_2^- reduction in iliac arteries, at least
363 in part. Interestingly, Webb et al. (45) found that NOS3 inhibition with L-NAME had no
364 significant effect on vascular NO_2^- reductase activity in rat aortic supernatants, but it did
365 significantly inhibit NOS3 NO_2^- reductase activity in red blood cell preparations. Therefore,
366 there appears to be an anomaly about the function of NOS as a NO_2^- reductase in
367 mammalian blood vessels.

368 Myoglobin has been identified as a key NO_2^- reductase in mammalian blood vessels
369 (7, 18, 28, 41, 42, 44). Specifically, studies using homozygous myoglobin knockout ($myg^{-/-}$)
370 mice have shown decreased endogenous NO production and cGMP content in aortae, and
371 blunted NO_2^--induced vasodilation relative to wild-type mice ($myg^{+/+}$) (18, 32, 42).
372 Interestingly, amphibians do not possess the *mygb* gene but do contain the cytoglobin gene
373 (*cygb*), and it has been proposed that cytoglobin functionally replaces myoglobin in this
374 vertebrate class (16). In this study, we cloned mRNAs for *cygb*, *ngb*, *and gby*, and
375 demonstrated that *cygb* and *ngb* mRNAs were expressed in the iliac arteries of *R. marina*,
376 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.

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. (1999) (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 in order to induce vasodilation in iliac arteries of *R. marina*. Previous studies have found that *R. marina* is tolerant of hypercapnia in the 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 3-fold 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, anaesthesia 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 approximately 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 0.05-0.3 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*

423 data, and it is therefore possible that the theoretical NO_2^- concentration used *in vitro* may
424 not reflect true intracellular concentrations, which may be due to diffusion-limitation across
425 the cell membrane as a result of the relatively poor circulation of fluid in the vessel
426 chamber. This is in contrast to the intact circulation, which maintains maximal diffusion
427 gradients to drive movement across the cell membrane. Another point to consider is the
428 fact that red blood cells are present *in vivo* and the NO_2^- reducing capacity of partially
429 oxygenated Hb may generate more NO for a given concentration of plasma NO_2^-. Certainly,
430 the relatively lower titre of NO_2^- required to induce significant vasodilation in perfused
431 human and primate forearm antecubital veins and brachial arteries suggests that, *in vivo*,
432 the pharmacokinetics differ from those observed *in vitro* (6, 9).

433

434 *NO_2^- vasoctivity and the sGC/cGMP Pathway*

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

445 Alternatively, following NO_2^- reduction, the NO signalling cascade acted via sGC but
446 independently of cGMP utilising mechanisms such as nitrosylation of sGC by the addition of
447 a nitrosyl ion (NO^-) (36) or NO reacting with a thiol group on sGC to form *S*-nitroso (SNO)
448 residues that may go on to participate in nitrosation reactions (20). We also cannot exclude
449 the possibility that 8-nitro-cGMP was formed from 8-nitro-GTP through the actions of sGC
450 (35), which would not have been detected in our assay. Certainly 8-nitro-GTP can be readily
451 derived *in vivo* from GTP nitration by NO-derived reactive nitrogen species (17).

452

453 *Perspectives and significance*

454 For many years, NO_2^- was thought be an inactive by product of NO metabolism. However, it
455 is now known that the *in vitro* application of NO_2^- to tissues, including blood vessels, elicits
456 biological responses that are attributed to NO, due to cells possessing endogenous NO_2^-
457 reductase mechanisms that generate NO. Accordingly, plasma NO_2^- is considered an
458 endocrine source of NO, which has been found to be important in hypoxia where the
459 generation of NO by NOS is compromised by limited O_2 supply. In the cardiovascular
460 system, this is exemplified by data demonstrating that dietary NO_x or infusion with NO_x have
461 improved tissue blood flow in various animal models of hypoxia and ischemia. In this study,
462 we provide the first evidence that NO_2^- is a vasodilator in a non-mammalian vertebrate (*R.*
463 *marina*), most likely by the endogenous reduction of NO_2^- to NO in the vascular smooth
464 muscle. Thus, it appears that the capacity for vascular reduction of NO_2^- to NO appeared
465 early in the tetrapods and preceded the emergence of endothelial NO signalling, which is
466 not a characteristic of amphibian blood vessels (4, 5). This study also found that hypoxia
467 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 is physiologically well-tolerated (40); this hypoxia tolerance may explain the absence of hypoxic vasodilation in amphibians (34).

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Figure legends

Fig 1 Representative tension recordings from isolated *R. marina* iliac arteries showing concentration-dependent NO_2^--mediated vasodilation. Vessels were pre-constricted with U-46619 (10^{-6} M) until stable. The values for NO_2^- are indicated by arrows and shown as $\log_{10}$ M. **A** demonstrates NO_2^--mediated vasodilation under normoxic (~21% O_2) conditions. **B** demonstrates total abolition of the NO_2^--mediated vasodilation in the presence of the sGC inhibitor, ODQ (10^{-5} M). **C** Concentration-dependent vasodilation of isolated *R. marina* iliac arteries in response to NO_2^- versus the complete abolition of the response following application of ODQ. Sigmoidal concentration-response curves are fitted to the data. The *P*-value indicate that the fitted curves differ significantly as determined by an *F-test*. Data in **C** are mean ± SEM. n=8.

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

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** shows the control versus L-NAME, n=3. **B** shows the control versus L-NNA, n=4. **C** shows the control versus vinyl-L-NIO, n=6. Data are shown as a percentage of net U-46619 pre-constriction force. Sigmoidal concentration-response curves are fitted to the data. The *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 mean ± SEM.

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** shows control versus C-PTIO, n=6; **B** shows the control versus allopurinol, n=4; **C** shows endothelium intact versus endothelial removal, n=4. Data are shown as a percentage of the U-46619 pre-constriction force. Sigmoidal concentration-response curves are fitted to the data. The *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 mean ± SEM.

Fig 5 Box plots of serum NO_2^-, NO_3^- and total NOx measured by a fluorimetric assay. **A** shows NO_2^-. **B** shows NO_3^- and **C** shows total NO_2^- ± 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** indicates 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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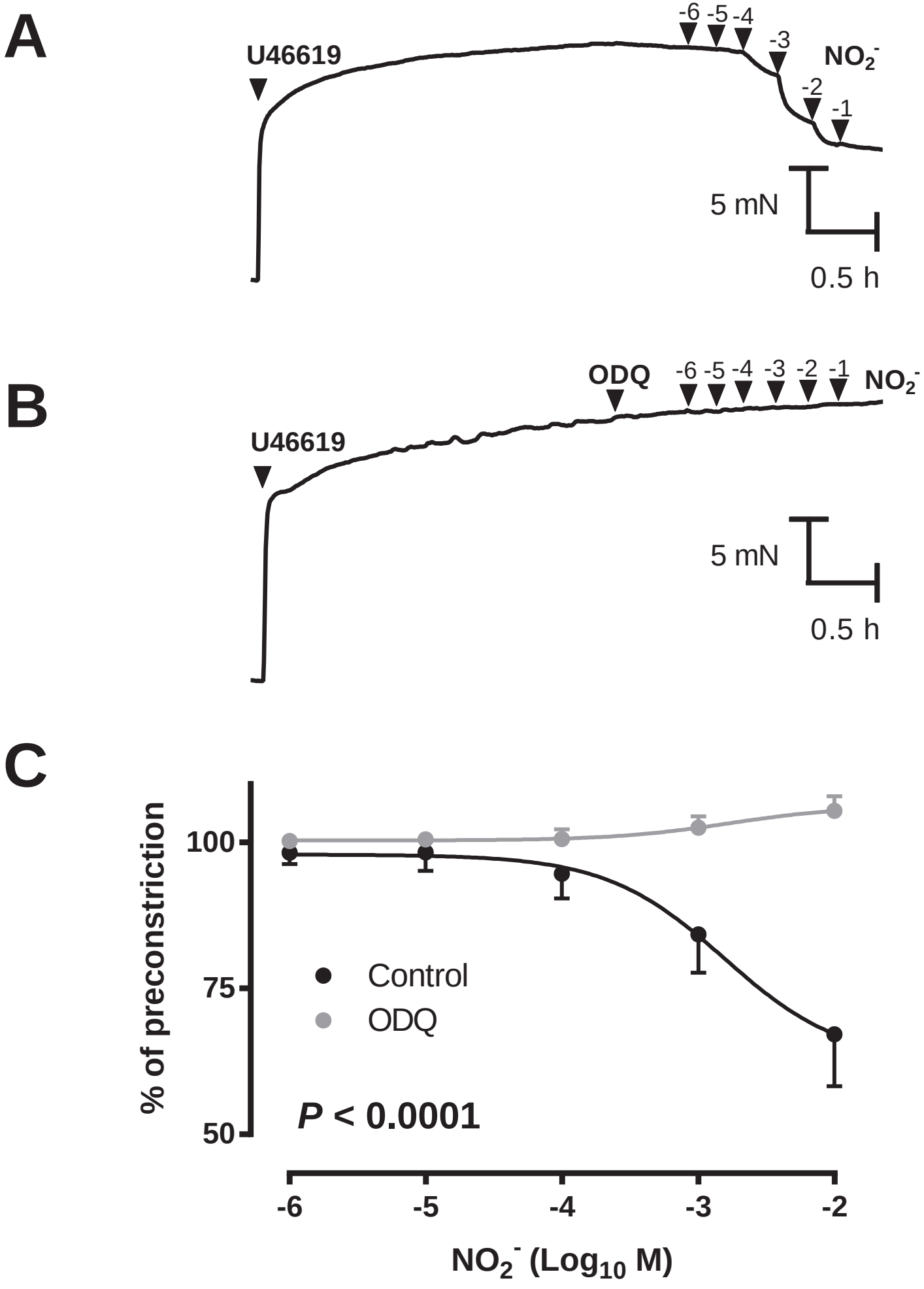
667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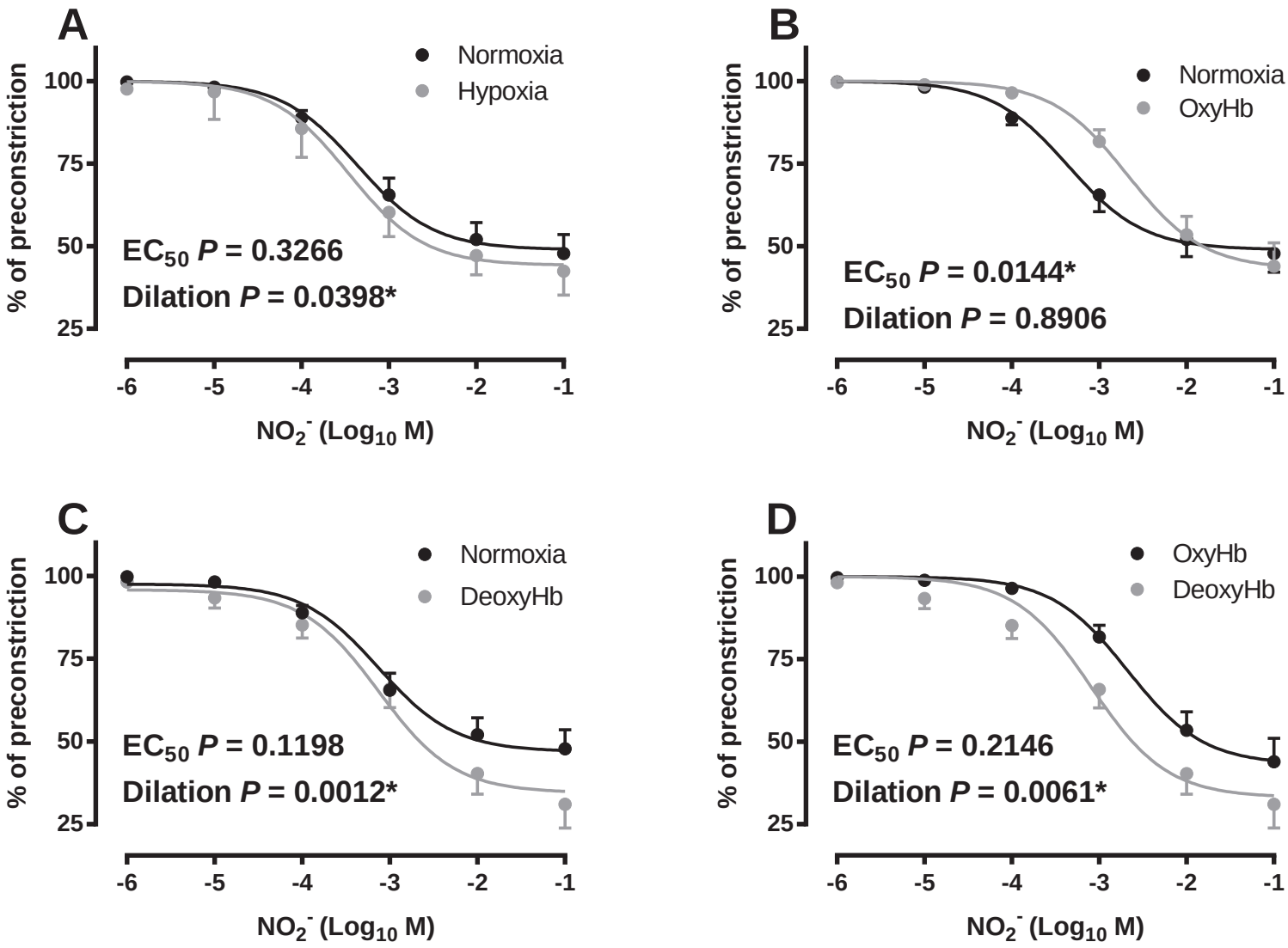
-3
-2
-1
NO2-
5 mN
0.5 h
B
U46619
ODQ
-6 -5 -4 -3 -2 -1
NO2-
5 mN
0.5 h
C
% of preconstriction
100
75
50
Control
ODQ
P < 0.0001
-6
-5
-4
-3
-2
NO2- (Log10 M)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Hypoxia
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-5
-4
-3
-2
-1
NO2- (Log10 M)
B
Normoxia
OxyHb
% of preconstriction
100
75
50
25
EC50 P = 0.0144*
Dilation P = 0.8906
-6
-5
-4
-3
-2
-1
NO2- (Log10 M)
C
Normoxia
DeoxyHb
% of preconstriction
100
75
50
25
EC50 P = 0.1198
Dilation P = 0.0012*
-6
-5
-4
-3
-2
-1
NO2- (Log10 M)
D
OxyHb
DeoxyHb
% of preconstriction
100
75
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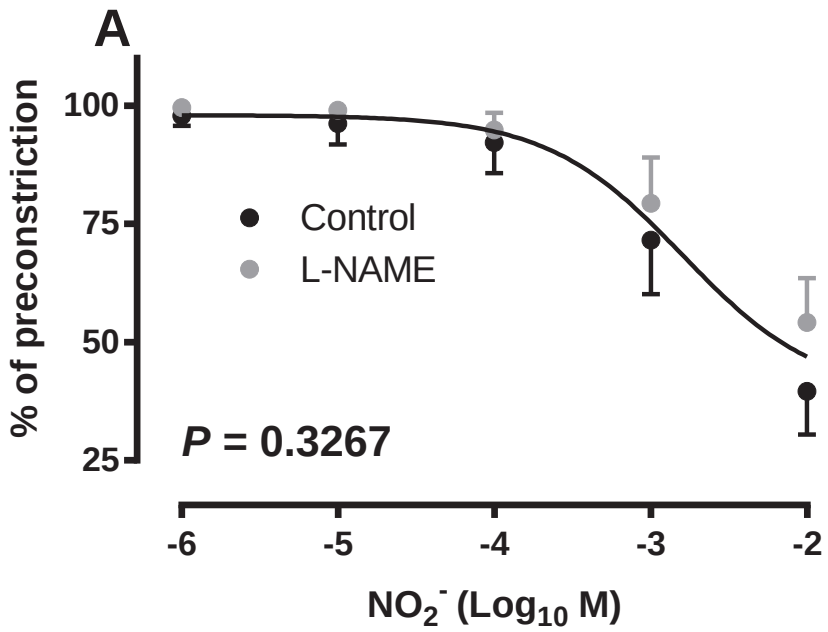
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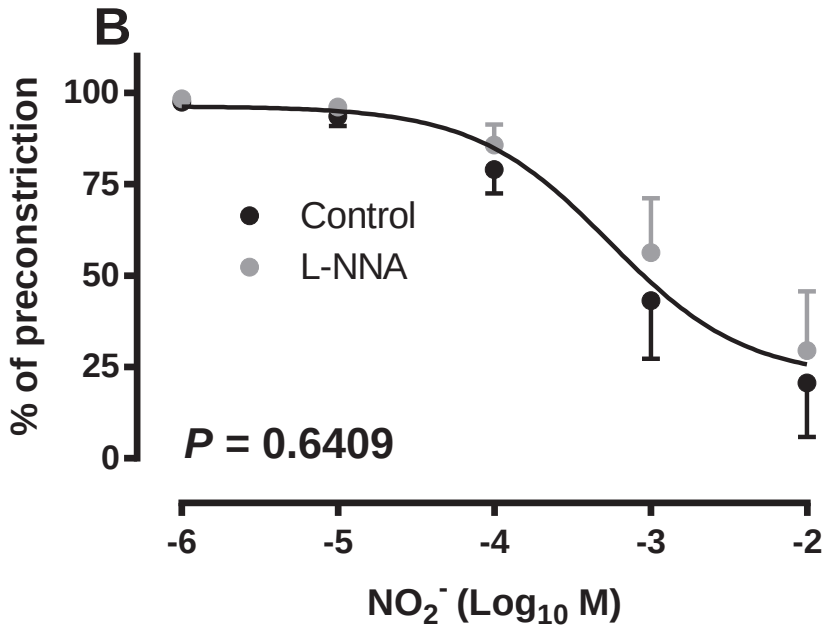
NO2- (Log10 M)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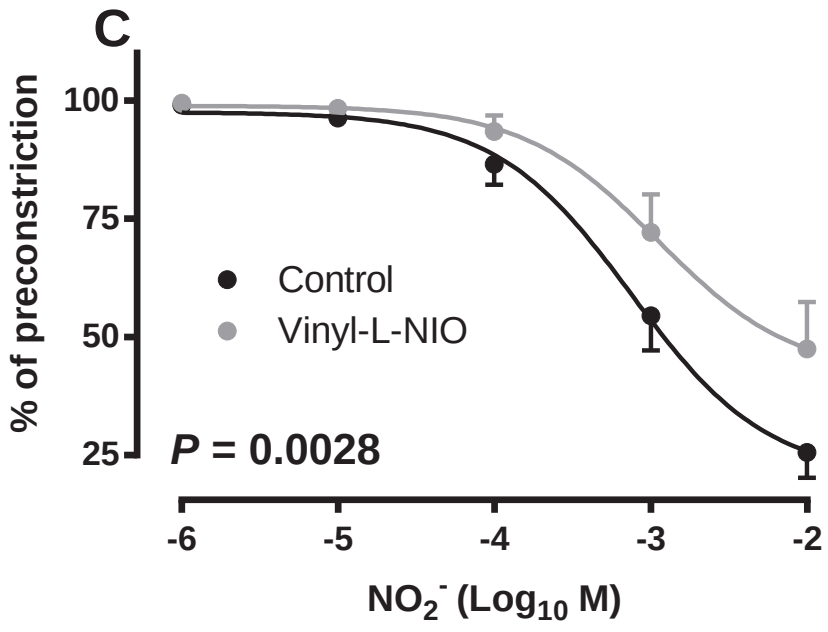
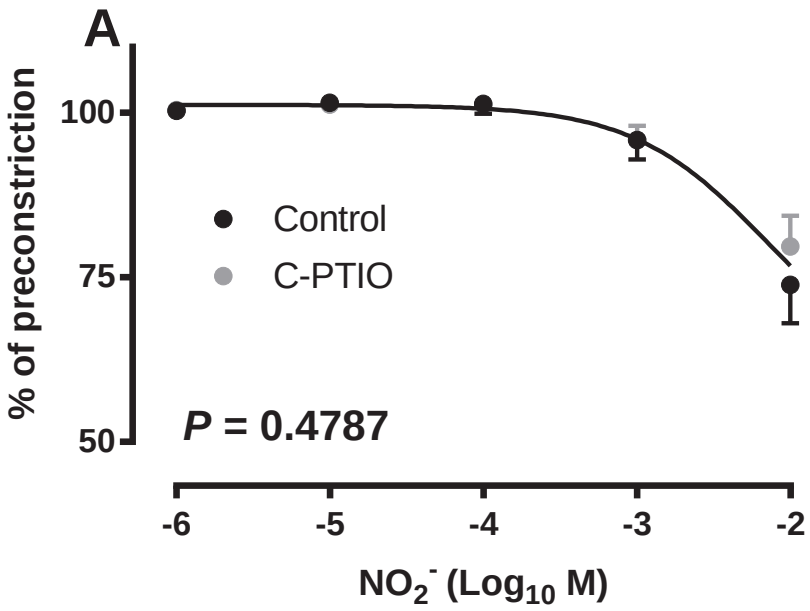
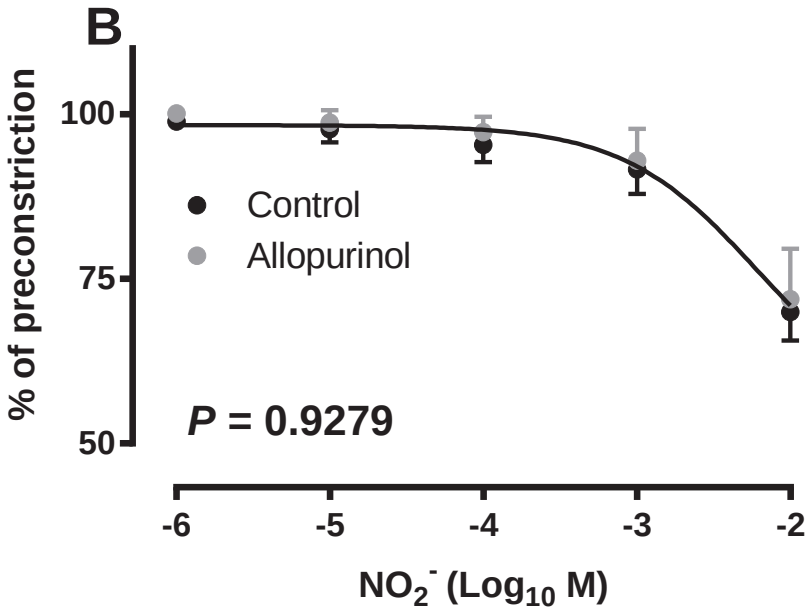
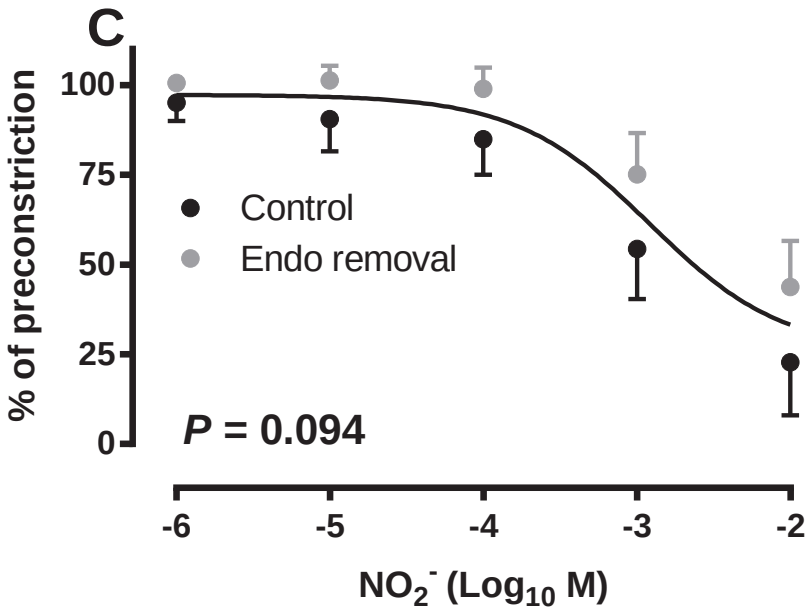
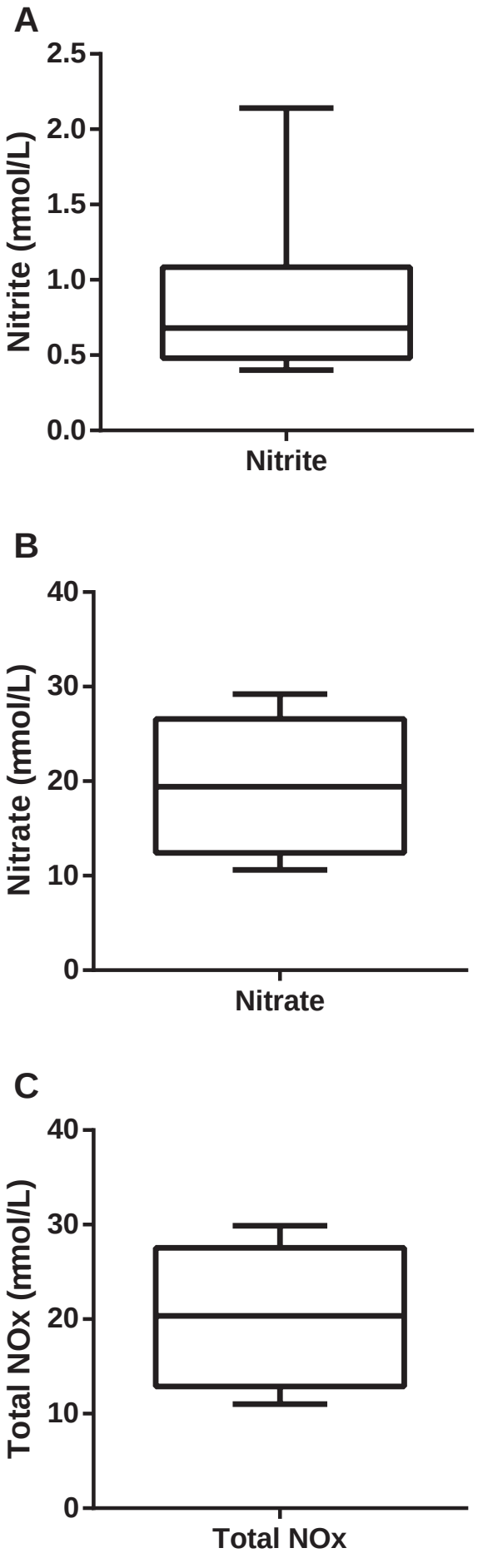
75
50
25
0
Control
L-NNA
P = 0.6409
-6
-5
-4
-3
-2
NO2- (Log10 M)

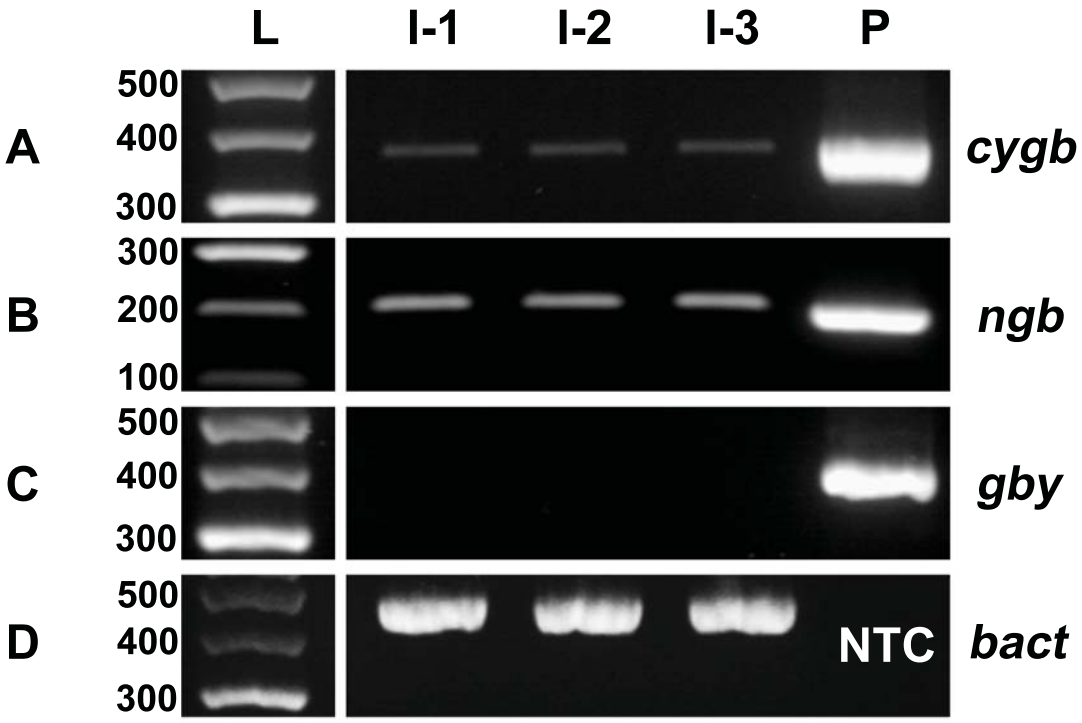

C
% of preconstriction
100
75
50
25
Control
Vinyl-L-NIO
P = 0.0028
-6
-5
-4
-3
-2
NO2- (Log10 M)


A
% of preconstriction
100
75
50
Control
C-PTIO
P = 0.4787
-6
-5
-4
-3
-2
NO2- (Log10 M)


B
% of preconstriction
100
75
50
Control
Allopurinol
P = 0.9279
-6
-5
-4
-3
-2
NO2- (Log10 M)


C
% of preconstriction
100
75
50
25
0
Control
Endo removal
P = 0.094
-6
-5
-4
-3
-2
NO2- (Log10 M)


A
2.5
2.0
1.5
1.0
0.5
0.0
Nitrite (mmol/L)
Nitrite
B
40
30
20
10
0
Nitrate (mmol/L)
Nitrate
C
40
30
20
10
0
Total NOx (mmol/L)
Total NOx


L
I-1
I-2
I-3
P
A
500
400
300
cygb
B
300
200
100
ngb
C
500
400
300
gby
D
500
400
300
NTC
bact