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Immunohistochemical and functional characterization of nitric oxide signaling pathway in isolated aorta from *Crotalus durissus terrificus*

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ABSTRACT

We characterized the nitric oxide (NO)-cyclic GMP-phosphodiesterase-5 (PDE5) pathway in *Crotalus durissus terrificus* aorta. Concentration responses curves to acetylcholine (ACh), sodium nitroprusside (SNP), BAY41-2272 (soluble guanylyl cyclase [sGC] stimulator), BAY60-2770 (sGC activator) and tadalafil (PDE5 inhibitor) were constructed in phenylephrine (10 μM)-precontracted tissues with intact (E^+) or denuded (E^-) endothelium. ACh (0.0001–10 μM) and SNP (0.0001–10 μM) relaxed aorta, which were reduced by the NO synthase (L-NAME,100 μM) or the sGC inhibitors (ODQ, 10 μM). Tadalafil (0.0001–10 μM) relaxed E^+ rings with potency (pEC_{50}) and maximal response (E_{max}) values of 7.34 ± 0.02 and 105 ± 8%, respectively. E^- or ODQ treatment significantly ($P<0.05$) reduced tadalafil relaxations (66 ± 18% and 71 ± 7%, respectively). BAY41-2272 (0.0001–300 nM) produced concentration-dependent relaxations in E^+ rings, which were reduced by addition of either ODQ or L-NAME (16.0- and 5.2-fold rightward shifts, respectively). The relaxation of BAY60-2770 was markedly potentiated by ODQ and L-NAME (41.0- and 9.7-fold leftward shifts, respectively), whereas in E^- the pEC_{50} values were shifted by 7-fold to the right. Immunohistochemistry, followed validation by transcriptomic analysis, revealed the presence of eNOS in endothelium, whereas nNOS was observed only in perivascular nerves. sGC and PDE5 were expressed in smooth muscle. Thus, NO–sGC–PDE5 pathway is evolutionarily present in *Crotalus sp.* vessels, and has a remarkable degree of functional similarity to mammalian vessels.

1. Introduction

In mammals, nitric oxide (NO) is an important signaling molecule that is generated by NO synthase (NOS). Soluble guanylyl cyclase (sGC) is the physiological receptor of NO (Arnold et al., 1977; Poulos, 2006). In the presence of NO, sGC is activated to convert guanosine triphosphate (GTP) to cyclic guanosine 3′, 5′ monophosphate (cGMP), which in turn has a direct influence on various biological processes, including those of the cardiovascular and neural systems. The biological effects of cGMP are mediated by three major types of intracellular effectors, namely: cGMP-dependent protein kinases I and II, cGMP-gated ion channels and cGMP-regulated phosphodiesterases (PDEs) (Beavo, 1995). Mammalian PDEs are categorized into 11 families based on sequence homology, enzymatic properties and sensitivity to inhibitors. PDE5A plays a prominent role in cGMP breakdown including visual response, smooth muscle relaxation, platelet aggregation, fluid homeostasis, immune response and cardiac contractility (Francis et al., 2001). Few studies have investigated the role of PDEs in the cardiovascular system from non-mammalian vertebrates (Gauthier and Soustre, 1992; Wasser et al., 1997).

Studies in birds (Hasegawa and Nishimura, 1991), reptiles (Broughton and Donald, 2007) and amphibians (Rumbaut et al., 1995; Knight and Burnstock, 1996) have provided evidence that an endothelial NO system is present in the vasculature, although this evidence is controversial. For instance, in eel aorta, immunoreactivity for nNOS is found in the outer layers of the vessel wall, and no eNOS staining is observed in endothelial cells (Jennings et al., 2004). In aorta from the toad *Bufo marinus*, relaxations to acetylcholine (ACh) occurs even when the endothelium was removed and neither NADPH-diaphorase positive nor NOS-immunoreactive cells in endothelium was observed (Broughton and Donald, 2002). However, in isolated aorta from leopard frog, ACh-induced relaxations are inhibited by the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester (L-NAME) (Knight and Burnstock, 1996).

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The role of NO pathway in the control of vascular tone in reptile has received less attention than mammals. The majority of the studies have focused on *in vivo* and *in vitro* assays. For instance, the NO-donor sodium nitroprusside (SNP) led to a large systemic vasodilatation in reptiles, including rattlesnake, turtles and lizards, indicating a potential role for NO in regulating reptile vascular tone (Galli et al., 2005). Acetylcholine induces concentration-dependent relaxations in isolated aorta from estuarine crocodile *Crocodylus porosus* that is sensitive to NOS and sGC inhibitors (Broughton and Donald, 2007). To date, no studies have explored the presence and functionality of sGC and PDE5 in reptile vessels. Therefore, we have characterized by functional assays in vitro, immunohistochemistry and transcriptomic analysis the complete NO-cGMP-PDE5 pathway in the aorta from the rattlesnake *Crotalus durissus terrificus*.

2. Material and methods

2.1. Experimental animals

All experimental procedures using rattlesnakes were approved by an institutional Animal Care and Use Committee (CEUA/UNICAMP: 1655-1) and were done in accordance with the Ethical Principles for Animal Research adopted by the Brazilian College for Animal Experimentation (COBEA). The use of these animals was authorized by the Brazilian Institute of Environment (IBAMA) (Sisbio: 18020-1). Male *Crotalus durissus terrificus* (body mass weight: 400–750 g) were provided by the serpentarium of the Center for the Study of Nature at the University of Vale do Paraiba (UNIVAP, São José dos Campos, SP, Brazil).

2.2. Functional studies

The animals were first anesthetized with isoflurane followed by ketamine (100 mg/kg, i.p) and xilazine (10 mg/kg, i.p.). The aorta was dissected out and cut into eight rings of approximately 3 mm in length after removal of surrounding fat and connective tissues. In some ring preparations, the endothelium was removed by gently rubbing of the intimal surface with a fine-tipped forceps. Each ring was suspended between two wire hooks and mounted under 10 mN passive tension in 10-ml organ chambers filled with Krebs' solution of the following composition (mM): 118 NaCl, 25 $NaHCO_3$, 5.6 glucose, 4.7 KCl, 1.2 KH_2PO_4, 1.17 $MgSO_4$, 2.5 $CaCl_2$ at 27 °C, pH 7.4 and aerated continuously with 95% O_2 and 5% CO_2 (Knight and Burnstock, 1995, 1996, Yoshinaga et al., 2007; Mesquita et al., 2008). To record the development of isometric tension, hooks were fixed to the bottom of the chamber and to a force transducer (AdInstruments, MA, USA) connected to a PowerLab 4/30 data-acquisition system (Software Chart, version 7.0; ADInstruments, Colorado Springs, MA, USA). All rings were allowed to equilibrate for 45 min. Bathing solution was replaced every 15 min, and baseline tension adjusted when necessary. The absence of endothelium was verified by the lack of responsiveness to acetylcholine (ACh, 3 μM) in vessels precontracted with phenylephrine (PE, 10 μM). Tissues that did not relax to ACh were considered as endothelium denuded. Rings were then washed several times to restore tension to baseline.

2.3. Concentration-response curves

In the first set of experiments, concentration response curves to ACh (0.0001–10 μM) or SNP (0.0001–10 μM) were constructed in endothelium-intact (E+) or -denuded (E-) preparations in the absence and in the presence of N^{ω}-nitro-L-arginine methyl ester (L-NAME, 100 μM) or the sGC inhibitor 1*H*-[1,2,4] oxadiazolo [4,3,-*a*]quinoxalin-1-one (ODQ, 10 μM). The second set of experiments consisted of assessing whether the presence of endothelium interferes with the aorta relaxations induced by tadalafil (PDE5 inhibitor; 0.0001–10 μM), BAY 41–2272 (sGC stimulator, 0.0001–30 μM) or BAY 60–2770 (sGC activator, 0.001–3000 nM). Hence, concentration responses curves to tadalafil, BAY 41–2272 or BAY 60–2770 were constructed in E^+ and E^-, as well as in the presence of either ODQ (10 μM) or L-NAME (100 μM) (Teixeira et al., 2006).

Nonlinear regression analysis to determine the pEC_{50} was done using GraphPad Prism (GraphPad Software, San Diego, CA) with the constraint that = 0. All concentration-response data were evaluated for a fit to a logistics function in the form: $E = E_{max}/([1 + (10^c/10^x)^n] +$; where *E* is the effect above basal; E_{max} is the maximum response produced by ACh, SNP, BAY 41–2272, BAY 60–2770 or tadalafil; *c* is the logarithm of the EC_{50}, the concentration of drug that produces a halfmaximal response; *x* is the logarithm of the concentration of the drug; the exponential term, *n*, is a curve-fitting parameter that defines the slope of the concentration-response line; and is the response observed in the absence of added drug.

2.4. Immunohistochemistry

Aortas from three specimens (n = 3) were fixed by immersion in phosphate-buffered formalin (10%) for 24 h. After macroscopic examination, aortas were cut in rings, all of which were dehydrated, cleared in xylene, and embedded in paraffin. Transversal sections (4 μm) were cut and stained for eNOS, nNOS, sGC and PDE5 by an immunoperoxidase method. Briefly, three levels of 4-μm thick sections were obtained from each paraffin block, placed on silanized slides, deparaffinized in xylene and rehydrated. Endogenous peroxidase activity was quenched by incubating the slides with 3% H_2O_2 for 10 min. Antigen retrieval was performed by heating slides in citrate buffer (10 mM, pH 6.0) at 95 °C for 30 min. As primary polyclonal antibodies, we used anti-eNOS (polyclonal, Abcam, diluted at 1:50), anti-nNOS (polyclonal, Abcam, diluted 1:200), anti-sGC (polyclonal, Abcam, diluted at 1:50) and anti-PDE5 (polyclonal rabbit, Abcam, diluted at 1:10). Antigen-antibody binding was detected using the Advance system (Dako). Staining was achieved using 3,3-diamonobenzidine tetrahydrochlride (Sigma-Aldrich, St. Louis, MO, USA) and counterstaining using Mayer's haematoxilin. All reactions were performed using appropriate positive (mouse aorta tissue) and negative (omission of the primary antibody) controls. Microscopic examination and photomicrographic documentation of immunostained sections were performed using a bright field microscope (Leica DM5000 B) connected to a digital camera (Leica DFC360 FX, Leica, Germany). (Capel et al., 2011)

2.5. Transcriptome profile of the Crotalus durissus aorta

Isolation and purification of aorta and brain RNA transcripts were performed with Trizol (Invitrogen). Briefly, the tissues were homogenized with Trizol, followed by addition of 20% chloroform and centrifugation. The isolated RNA was pelleted with isopropyl alcohol and washed with 75% ethanol. The cDNA was sequenced with Roche 454 GS FLX Titanium equipment at Helixxa Bases for Life (Campinas, São Paulo, Brazil). Aorta and brain library comprised 310,381 and 364,708 reads with an average size of reads size of 290 and 293 bp, respectively. Assemblies were executed using a combination of Newbler Assembler and iAssembler. Aorta and brain library reads were grouped, respectively, into 59,790 and 87,841 unigenes, including singlet sequences. We searched genes for NOS, sGC and PDE5 in snake aorta unigenes, whereas in the brain library just NOS was searched. All protein sequences used in comparisons against snake unigenes were taken from UniProt. The comparisons were performed using BLAST (tblastn), and unigenes with e-values better than e-10 against. We then used blastx to compare each candidate to the NR protein database of GenBank (database containing virtually all publicly known proteins from all species). If the best NR hit of an eNOS candidate was with a eNOS of any species, we consider the candidate