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Role of brain nitric oxide in the cardiovascular control of bullfrogs

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ABSTRACT

The goal of the present study was to determine if nitric oxide (NO) acting on the brain of bullfrog (*Lithobates catesbeianus*) is involved in arterial pressure and heart rate (HR) control by influencing sympathetic activity. We investigated the effect of intracerebroventricular injections of L-NMMA (a nonselective NO synthase inhibitor) on mean arterial blood pressure (MAP), HR and cutaneous vascular conductance (CVC) of pelvic skin after intravenous injection of α or β adrenergic blockers, prazosin or sotalol, respectively. Arterial pressure was directly measured by a telemetry sensor inserted in the aortic arch of animals. L-NMMA increased MAP, but did not change HR. This hypertensive response was inhibited by the pre-treatment with prazosin, but accentuated by sotalol. The effect of L-NMMA on MAP was also inhibited by i.v. injections of the ganglionic blocker, hexamethonium. Thus, NO acting on the brain of bullfrog seems to present a hypotensive effect influencing the sympathetic activity dependent on α and β adrenergic receptors in the periphery.

1. Introduction

There is evidence that NO acts on the periphery as a vasodilator, not only in mammals (Rees et al., 1990), but also in amphibians and other vertebrates (Hoffmann and Romero, 2000; Donald and Broughton, 2005; Skovgaard et al., 2005). In amphibians, NO has been attributed to participate in physiological processes besides vascular resistance (Knight and Burnstock, 1996; Rea and Parsons, 2001; Broughton and Donald, 2002), such as microvascular permeability (Nguyen et al., 1995; Rumbaut and Huxley, 2002) and water uptake (Rea and Parsons, 2001; Rea et al., 2002). Much less is known, however, about the physiological role of encephalic NO in amphibians. We and others demonstrated previously that NO acting on brain of toads and frogs is involved in the reduction of preferred body temperature during hypoxia (Guerra et al., 2008) and the control of breathing (Hedrick et al., 1998; Gargaglioni and Branco, 2001), respectively. Moreover, nitrergic cells are reported to be ubiquitously distributed throughout the anuran brain (Bruning and Mayer, 1996; Huynh and Boyd, 2007), and there are NO-producing neurons in the hypothalamic regions and brainstem, including the *nucleus tractus solitarius* (NTS), of some anuran species (Bruning and Mayer, 1996; Munoz et al., 1996; Lazar and Losonczy, 1999) besides bullfrogs (Huynh and Boyd, 2007). To date, no study has investigated the possible role of brain NO in the cardiovascular control of amphibians. At least for mammals, a number of studies support the idea of a role for NO acting on specific encephalic nuclei, such as the paraventricular nuclei (Zhang et al., 1997; Zhang and Patel, 1998) and the NTS (Harada et al., 1993; Tseng et al., 1996), in decreasing sympathetic nerve activity, especially that which involves cardiovascular control (cf. Krukoff, 1999; Chen et al., 2001; Patel et al., 2001; Guo et al., 2009).

The sympathetic nervous system of anurans is organized such that its paravertebral chains extend from the second to the tenth spinal nerve, and a mixed sympatovagal trunk innervates the three-chambered heart (Nilsson, 2011). Adrenaline, released from the sympathetic nerves, is the major mediator of chronotropic and inotropic effects in the heart via activation of β-adrenergic receptors, namely β2 receptors (Herman and Sandoval, 1983; Herman and Mata, 1985). At least in bullfrogs, β2 receptors account for the majority of the receptors found in the atria and the ventricle (Herman et al., 1996). In terms of the peripheral vasculature, there is evidence for the vasoconstrictor effect of α-adrenergic receptors in anurans (Kimmel, 1992; Bianchi-da-Silva et al., 2000), which are activated by adrenaline, released from sympathetic nerves, and circulating noradrenaline (Azuma et al., 1965). In fact, adrenaline, noradrenaline and phenylephrine were all demonstrated to cause arterial pressure (AP) increases by activation of α receptors in amphibians (Erlij et al., 1965; Kirby and Burnstock, 1969; Lillo, 1979; Herman and Sandoval, 1983). Moreover, both α and β adrenergic receptors are present in the cutaneous vessels of frogs and contribute to vasoconstriction and vasodilation, respectively (Herman and Sandoval, 1983; Malvin and Riedel, 1990). It is also known that β receptors are important for water

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absorption in the ventral pelvic skin (Viborg and Rosenkilde, 2004). No study has addressed the possible influence of brain NO on peripheral adrenergic activity in amphibians.

Based on the considerations above, our aim was to investigate if brain NO is involved in the control of AP and HR via peripheral adrenergic and/or noradrenergic activity. By using a telemetry sensor to measure AP directly, we investigated the effect of brain NO inhibition, induced by intracerebroventricular injection of L-NMMA, on the peripheral α- and β-adrenergic influences on AP, HR and skin blood flow in bullfrogs.

2. Material and methods

2.1. Animals

We used bullfrogs, *Lithobates catesbeianus* (Shaw, 1802), of both sexes, weighing 180–350 g. Frogs were obtained from the bullfrog farm (CAUNESP) at the College of Agricultural and Veterinarian Sciences — UNESP, São Paulo state, Brazil. In our laboratory at the Department of Animal Morphology and Physiology (UNESP), all the animals were maintained at 25 °C in containers, with free access to tap water from an artesian well and a basking area, for at least two weeks before experimentation. The animals were fed *Tenebrio molitor* larvae and/or commercial carnivorous fish food two to three times per week. The study was conducted with the approval of the local Animal Care and Use Committee (Protocol 008519-10).

2.2. Surgical procedures

The animals were anesthetized by submergence in an aqueous 0.3% solution of 3-aminobenzoic acid ethyl ester (MS-222, Sigma, St. Louis, USA) buffered to pH 7.7 with sodium bicarbonate. After loss of the eye reflex, the animals were fixed to a David Kopf stereotaxic apparatus (Model 900 Small Animal Stereotaxic, Tujunga, CA, USA). The skin covering the skull was removed with the aid of a bone scraper and an opening was made in the skull above the telencephalon using a small drill (LB100, Beltec, Araraquara, Brazil). A guide cannula, prepared from a hypodermic needle segment, 14 mm in length and 0.55 mm in outer diameter, was attached to the tower of the stereotaxic apparatus and lowered into the lateral cerebral ventricle. These coordinates were adapted from the encephalic atlas of anurans (Donkelaar, 1998). The displacement of the meniscus in a water manometer confirmed correct positioning of the cannula within the lateral ventricle. The orifice around the cannula was filled with a paste consisting of a mixture of equal parts of paraffin and glycerin. The cannula was attached to the bone with stainless steel screws and acrylic cement. A tight-fitting stylet was kept inside the guide cannula to prevent occlusion and infection. The experiments were performed seven days after brain surgery.

To measure pulsatile arterial pressure (PAP) and body temperature, the frogs were instrumented with a telemetry transmitter (TR43P, Telemetry Research, New Zealand). This telemetry system, originally developed for rat instrumentation, was adapted for use in anurans as follows: the animal was positioned in lateral decubitus, and an incision was made in skin and muscles at the left side of the abdomen to expose the left aortic arch (Fig. 1). The aortic arch was isolated using surgical threads to diminish blood flow, and a catheter, connected to the telemetry transmitter, was inserted into a tiny hole made in the artery using a needle (25 × 0.6 mm). The catheter was fixed to the artery wall with 2-octyl cyanoacrylate (Dermabond® Topical Skin Adhesive, Johnson & Johnson, Brazil) surgical glue to allow uninterrupted blood flow in the cannulated vessel. A surgical mesh joined the vessel with the catheter and was fixed to the surrounding tissue with surgical glue to reinforce the whole structure. The body of the telemetry transmitter, which houses the temperature sensor, was carefully placed inside the abdominal cavity and sutured to the obliquus internus muscle.

After the surgery, the frogs were treated with a prophylactic antibiotic (enrofloxacin, Flotril®; Schering-Plough, 5.0 mg kg^{-1}, s.c.) and an analgesic (Flunixina Meglumina, Banamine®; Schering-Plough, 1.0 mg kg^{-1}, s.c.), according to recommended dosages for amphibians (Gentz, 2007; Smith, 2007). Five days after guide cannula and telemetry transmitter implantations, the animals were again anesthetized (see protocol above) and a polyethylene cannula (PE50) was implanted in the femoral vein for the administration of adrenergic agonists and antagonists. For measurements of regional skin blood flow (SkBF), a laser Doppler probe (MSP100XP Standard Surface Probe, ADInstruments®, Sydney, Australia) was sutured to the pelvic surface skin. After the surgery, the animals were again treated with antibiotic and analgesic agents (see above).

2.3. Intracerebroventricular (i.c.v.) microinjections

Microinjections were performed via a thin dental needle (Mizzy, 30 Gauge), which was inserted until its tip was 0.4 mm below the guide cannula. A volume of 1 μL was injected over a period of 45 s with a 5-μL Hamilton syringe using a microinjection pump (model 310, Stoelting Co., IL, USA). The movement of an air bubble inside the PE-10 polyethylene tubing connecting the microsyringe to the dental needle confirmed drug flow. Thirty minutes were allowed to elapse before the injection needle was removed from the guide cannula to avoid reflux and interference in PAP recordings.

At the end of each experiment, 1 μL of 2% Evans blue solution was microinjected into the lateral ventricle. The animals were euthanized by submergence in an aqueous 0.025% solution of benzocaine hydrochloride buffered to pH 7.7 with sodium bicarbonate (AVMA, 2007). Upon dissection, we observed that the dye had diffused into the periventricular tissue and spread along the ventricular system.

2.4. Determination of mean arterial blood pressure (MAP), heart rate (HR) and body temperature (T_b)

Pulsatile AP and T_b from each animal were continuously monitored during all experimental protocols by telemetry transmission. The sensor implanted within the animal's abdominal cavity detects, amplifies and transmits PAP and T_b data to a receiver (Telemetry Research Receiver, Auckland, New Zealand). This signal is transmitted to a system of data acquisition and analysis (PowerLab System, ADInstruments®/Chart Software, version 7.3, Sydney, Australia). The telemetry transmitters have been calibrated by the manufacturer using a Mensor 6100 series reference pressure sensor (pressure range from 760 to 1040 mm Hg; temperature range from 22 to 40 °C). Heart rate was calculated by counting the peaks of the PAP recording. Mean arterial pressure was automatically calculated as MAP = 2/3 DP + 1/3 SP (DP = diastolic pressure; SP = systolic pressure) from the pulsatile arterial pressure recording in real-time using the cyclic measurements tool from the Chart Software.

2.5. Measurements of skin blood flow (SkBF) and calculation of cutaneous vascular conductance (CVC)

Skin BF was continuously monitored in real-time using a laser Doppler probe (MSP100XP Standard Surface Probe, ADInstruments®, Sydney, Australia) sutured to the pelvic surface skin. Signals from the laser Doppler probe were monitored by a blood flow meter (ML191, ADInstruments®, Sydney, Australia) connected to a computer (PowerLab System, ADInstruments®/Chart™ Software, Sydney, Australia). This methodology was based on previous studies in toads (Viborg and Rosenkilde, 2004; Viborg and Hillyard, 2005; Viborg et al., 2006) and crocodiles (Seebacher and Franklin, 2007). The flow meter was calibrated in a colloidal solution of suspended latex spheres of standard size and concentration, according to recommendations in the manufacturer's manual (MLA191 calibration kit, ADInstruments).