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Angiotensin II acting on PVN induces sympathoexcitation and pressor responses via the PI3K-dependent pathway

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ABSTRACT

In vitro studies have shown that angiotensin II (ANG II), via activation of ANG II type 1 (AT_1) receptor plays an important role on the neural control of the blood pressure (BP), through an intracellular signalling pathway involving PI3K in the paraventricular nucleus of the hypothalamus (PVN). However, to the best of our knowledge, no *in vivo* study has been performed yet to unravel the functional role of ANG II and its interaction with PI3K pathways in the neural control of circulation of non-anesthetized animals. Here, we demonstrate that exogenous ANG II microinjected into the PVN in anaesthetic-free animals evokes an increase in sympathetic nerve activity and BP in a PI3K-dependent manner.

1. Introduction

The central nervous system (CNS) plays an essential role in the pathogenesis of neurogenic hypertension, mainly because of the increase in sympathetic nerve activity (SNA) that contributes to the augmented peripheral vascular resistance and blood pressure (BP) (Esler, 2000; Dibona, 2004). The CNS network that controls the circulation is influenced by various blood-born substances. One such substance is angiotensin II (ANG II), which accesses the brain directly or through circumventricular organs (CVOs). The CVOs in turn project to the paraventricular nucleus of the hypothalamus (PVN), a key integrative site of neuroendocrine and autonomic signals involved on the BP regulation and blood volume as well as plasma osmolality (Antunes et al., 2006).

The PVN is composed of two distinct neuronal populations: (i) the magnocellular division containing neuroendocrine neurons that synthesize and release vasopressin and oxytocin in to the circulation as well as in specific brain regions; and (ii) the parvocellular neurons that project to premotor sympathetic neurons in the rostral ventral lateral medulla (RVLM) and the intermediolateral cell column (IML) of the spinal cord (Swanson and Sawchenko, 1980, Pyner and Coote, 2000).

There is a large body of evidence showing that centrally, the action of ANG II plays an important role in the neural control of BP because of its interaction with the AT_1 receptors in specific brain areas associated with autonomic and cardiovascular control, like the PVN (Allen et al., 1998, Yang and Raizada, 1999; Zhu et al., 2002, McKinley et al., 2003). *In vitro* studies performed in primary neuronal cultures prepared from the hypothalamus and brainstem of the spontaneously hypertensive rat (SHR) demonstrated that the activation of AT_1 receptors with ANG II stimulates noradrenergic neuromodulation through intracellular signalling involving the phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB or Akt) (Yang et al., 1996). However, to the best of our knowledge, no *in vivo* study has been performed to unravel the functional role of ANG II and its interaction with the PI3K pathways in the autonomic control of circulation within the PVN of non-anesthetized animals. Thus, our study hypothesis is that ANG II acting in the PVN elicits pressor and sympathetic-mediated responses via intracellular PI3K pathway activation. In fact, here we demonstrated that ANG II microinjected into the PVN evokes an increase in SNA and BP of Wistar rats in a PI3K-dependent manner.

2. Material and methods

2.1. Ethical approval

All experimental procedures were performed in accordance with the Ethical Principles in Animal Research mandated by the Brazilian College of Animal Experimentation and were approved by the Ethical Committee for Animal Research of ICB/USP (Protocol 105-50/2007). *In vivo* implanted rats were killed by an overdose of sodium pentobarbital (>60 mg/kg of body weight) given intravenously.

2.2. Animals

Wistar male rats (17–20 weeks old) were used for *in vivo* studies and juvenile (3–4 weeks old) for *in situ* studies. The animals were housed

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individually, and kept at a constant temperature of 22 °C–24 °C and a relative humidity of 50%–60% under a controlled light/dark cycle (12/12 h) with normal rat chow and drinking water *ad libitum*.

2.3. PVN microinjection and blood pressure recording in conscious animals

Rats were anesthetized with a mixture of ketamine chloride (100 mg/kg, i.p.) and xylasin chloride (20 mg/kg, i.p.), and placed in a stereotaxic head frame (David Kopf Instruments, USA) with the incisor bar set at −3.3 mm below the interaural point. Bilateral stainless steel guide cannulas (0.6 mm o.d., 15 mm length) were placed through a 2 mm burr hole drilled over the sagittal midline suture overlying the PVN (AP = −1.2 mm from bregma, ML = ±0.3 mm from sagittal venous sinus, DV = −4.8 mm from skull surface) according to coordinates derived from a rat brain atlas (Paxinos and Watson, 2007). Cannulae were fixed to the skull with dental acrylic resin (Clássico Artigos Odontológicos, Campo Limpo Paulista, SP, Brazil), anchored by two small stainless steel watch screws. After the surgical procedures, all animals received a prophylactic, broad-spectrum antibiotic (penicillin and streptomycin, 1,200,000 UL Fort Dodge, Campinas, SP, Brazil) and a non-steroidal anti-inflammatory drug, ketoprofen (Biofen 1%; Biofarma Química e Farmacêutica LTDA, Jaboticabal, SP, Brazil) subcutaneously. Three days after guide cannulae implantation, the rats were re-anesthetized with a mixture of ketamine and xylasin chloride, and the femoral artery catheterized (PE-10 connected to PE-50; Clay Adams, Parsippany, NJ, USA) for measurement of pulsatile arterial pressure (PAP), mean arterial pressure (MAP) and heart rate (HR). The catheters were tunnelled and exteriorized in the back of the neck so that MAP and HR could be monitored under conscious freely moving conditions. The cardiovascular parameters were acquired using a digital data system (PowerLab, ADInstruments, New South Wales, Australia). The microinjections into the PVN were performed in conscious, freely moving rats using a 1 μL syringe (Hamilton, Reno, NV, USA) connected by a polyethylene tube PE-10 to an injection needle (33 gauge, Small Parts, Miami Lakes, FL, USA) 3.5 mm longer than the guide cannula, and the volume injected was always 100 nL.

2.4. Nerve recordings and microinjection into the PVN in an arterially perfused rat preparation

In the protocol for sympathetic nerve recording combined with microinjection into the PVN, we used a decorticated, arterially perfused *in situ* rat preparation (DAPR). The detailed methodological procedures for the DAPR preparation were performed as previously described by Antunes et al., 2006, and are outlined here in brief. The preparation was perfused at flow rates of 28 ± 2 mL/min using a roller pump (Watson Marlow 505S, UK) with artificial cerebrospinal fluid containing an oncotic agent (PEG 20,000, 1.5%; Sigma, St. Louis, USA), gassed with carbogen (95% O_2 and 5% CO_2), warmed to 32 °C and filtered using a nylon screen (pore size: 25 μm). The lumbar sympathetic chain (L2 − L3) was visualized and dissected through a binocular microscope and recordings made from the distal cut end using a bipolar glass suction electrode. Signals were AC-amplified (NL104, Neurolog, UK) and band-pass filtered (100 Hz–3 kHz) and displayed on a computer using the Spike 2 software (Cambridge Electronic Design, Cambridge, UK). For the microinjections into the PVN, the head of the DAPR was fixed with ear bars and a nasal clamp mounted on the perfusion chamber. A three-barrelled glass micropipette (external tip diameter 10–30 μm) was placed into the PVN using a 3-D micromanipulator. The volume microinjected unilaterally (100 nL) was determined by viewing the movement of the meniscus through a binocular microscope fitted with a pre-calibrated eyepiece reticule. All data were acquired using biopotential AC amplifiers and filters (Neurolog, Digitimer Ltd., UK) and collected using a CED 1401A–D interface (CED, Cambridge Electronic Design, Cambridge, UK) and a computer running Spike 2 software (CED) with custom-written scripts for data acquisition and on- and off-line analyses. Lumbar sympathetic nerve activity (LSNA) was displayed as a moving average (200 ms time constant) and measured as the area of LSNA, both 10 s before and 10 s after ANG II injection into the PVN. To standardize the data across preparations, LSNA changes were expressed as a percentage of the basal values (before injection into the PVN). The noise for LSNA was assessed by application of hexamethonium, a ganglionic blocker (100 mM–0.5 mL) into the perfusate (final concentration: 5 mM) at the end of each experiment.

2.5. Histological analysis

At the end of all experiments Evan's Blue dye (2% *w*/*v*) was microinjected (100 nL) to mark the drug injection sites, and brains were removed and fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline and 20% sucrose. Coronal sections (40 μm of thickness) were cut using a cryostat (CM1900, Leica, Switzerland) and thaw-mounted on gelatin-subbed glass slides. Brain sections were visualized under light microscopy (dark-field) and the injection sites mapped according to the rat brain atlas by Paxinos and Watson (2007). Only data in which the microinjections were confirmed to be within the PVN were considered in the statistical analyses.

2.6. Statistical analyses

One-way ANOVA for repeated measures followed by Bonferroni's (blood pressure data), and Friedman's (nerve recording data) post hoc test were used. The results presented are expressed as mean ± standard error mean (SEM), and "*n*" is the number of animals of each group. Statistical significance levels were set at $^{*}p \leq 0.05$. All statistical analyses were conducted using Prism V 5.0 (GraphPad Software, Inc.; La Jolla, CA, USA).

3. Results

3.1. Pressor and sympathoexcitatory responses induced by injection of ANG II into the PVN involve PI3K activation

Fig. 1A shows representative traces of changes in the PAP, MAP and HR elicited by unilateral microinjection of ANG II (10 μM; 100 nL) before and after LY294002, a selective PI3K inhibitor (50 μM; 100 nL), into the PVN of conscious rat. Microinjection of ANG II into the PVN evoked a significant increase in the MAP (Δ: 13 ± 1 mmHg, $n = 5$), such a response was attenuated at 5 (Δ: 4 ± 1 mmHg; $p < 0.05$) and recovered 30 min (Δ: 11 ± 1 mmHg, $n = 5$), following LY294002 microinjection (Fig. 1B). Vehicle control injection failed to affect the ANG II-induced hypertension when microinjected in the PVN at the same time course evaluated (ΔMAP: 12 ± 1 mmHg; 5 min: 12 ± 2 mmHg, and 30 min: 11 ± 1 mmHg, $n = 4$). No significant changes were observed in heart rate (data not shown). Fig. 1C depicts a representative photomicrograph of the site of unilateral microinjection into the PVN of conscious rats.

In a separate group of rats, we performed a set of experiments in the DARP model to determine the effects of ANG II microinjected into the PVN on LSNA. Fig. 2A shows representative traces of changes in LSNA elicited by microinjection of ANG II (10 μM; 100 nL) in comparison to its basal value (before injection) previously (control), and 5 and 30 min after pre-treatment with LY294002 into the PVN. In the Fig. 2B, we can observe that the increase in the LSNA (14 ± 8%, $n = 4$) evoked by unilateral microinjection of ANG II into the PVN of the DAPR was abolished by pre-treatment with LY294002 at 5 min (−2 ± 1%, $p < 0.05$) and partially recovered 30 min later (7 ± 5%). Misplaced microinjections of ANG II, LY294002 or vehicle control located outside of the PVN (reticular thalamic nucleus and peduncular part of the lateral hypothalamus) had no significant effect on MAP and LSNA. Fig. 2C is a representative photomicrograph of the injections' site into the PVN.