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Nitrergic Pathways and L-Type Calcium Channel of MnPO Influencing Cardiovascular Homeostasis

[1-5]Wilson Abrão Saad, [4]Ismael Francisco Motta Siqueira Guarda, [3]Luiz Antonio de Arruda, Camargo, [1]Talmir Augusto Faria Brizola dos Santos, [1]Sylvio Simões and [6]William Abrão Saad

The median preoptic nucleus (MnPO) is one of most important site of the lamina terminalis implicated in the regulation of hydro electrolytic and cardiovascular balance. The purpose of this study was to determine the effect of L-Type calcium channel antagonist, nifedipine, on the increase of median arterial blood pressure (MAP) induce by angiotensin II (ANG II) injected into the MnPO. The influence of nitric oxide (NO) on nifedipine antipressor action has also been studied by utilizing N^W-nitro-L-arginine methyl ester (L-NAME) (40 μg 0.2 μL^{-1}) a NO synthase inhibitor (NOSI), 7-nitroindazole (7-NIT) (40 μg 0.2 μL^{-1}), a specific neuronal NO synthase inhibitor (nNOSI) and sodium nitroprusside (SNP) (20 μg 0.2 μL^{-1}) a NO donor agent. We have also investigated the central role of losartan and PD123349 (20 nmol 0.2 μL^{-1}), AT_1 and AT_2, respectively (selective non peptide ANG II receptor antagonists), in the pressor effect of ANG II (25 pmol 0.2 μL^{-1}) injected into the MnPO. Male Wistar rats weighting 200-250 g, with cannulae implanted into the MnPO were utilized. Losartan injected into the MnPO, prior to ANG II, blocked the pressor effect of ANGII. PD 123319 only decreased the pressor effect of ANG II. Rats pre-treated with either 50 μg 0.2 μL^{-1} or 100 μg 0.2 μL^{-1} of nifedipine, followed by 25 pmol 0.2 μL^{-1} of ANG II, decreased ANG II-pressor effect. L-NAME potentiated the pressor effect of ANG II. 7-NIT injected prior to ANG II into the MnPO also potentiated the pressor effect of ANGII but with less intensity than that of L-NAME. SNP injected prior to ANG II blocked the pressor effect of ANG II. The potentiation action of L-NAME and 7-NIT on ANG II-pressor effect was blocked by prior injection of nifedipine. The results described in this study provide evidence that calcium channels play important roles in central ANG II-induced pressor effect. The structures containing NO in the brain, such as MnPO, include both endothelial and neuronal cells, which might be responsible for the influence of nifedipine on the pressor effect of ANG II. These data have shown the functional relationship between L-Type calcium channel and a free radical gas NO in the MnPO, on the control of ANG II-induced pressor effect acting in AT_1 and AT_2 receptors.

Key words: Angiotensin II, rats, calcium channel, nitric oxide, blood pressure, MnPO

[1]Basic Institute of Biosciences-UNITAU, Taubaté, SP, Brazil
[2]Department of Exact and Natural Science UNIARA Araraquara SP Brazil
[3]Department of Physiology and Pathology, School of Dentistry, Paulista State University, UNESP Araraquara, S Brazil
[4]Department of Anesthesiology Clinic Hospital State of São Paulo, São Paulo, Brazil
[5]Department of Physiology, Federal University of São Carlos SP-Brazil
[6]Department of Gastroenterology, Clinic Hospital of University of São Paulo USP São Paulo Brazil

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For further information about this article or if you need reprints, please contact:

Wilson Abrão Saad
Department of Physiology and Pathology, School of Dentistry
Paulista State University, UNESP
Rua Humaitá, 1680
14801-903-Araraquara, SP, Brazil

Tel: +55(16) 3301-6488
Fax: +55(16) 3322-4118

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INTRODUCTION

Recents experiments of our laboratory demonstrated that the NO has interrelationship with AT_1 and AT_2 receptors of the CNS in the pressor effect of ANG II. The Influence of vasopressin receptor and nitric oxide on the water, sodium intake and arterial blood pressure induced by angiotensin injected into the third ventricle of the rat brain (Saad *et al*., 2006).

The influence of calcium channel in the pressor effect of ANG II has not yet study. Centrally ANG II elicits responses such as increased blood pressure, thirst, sodium appetite and the release of vasopressin (Reid, 1988; Saad *et al*., 2002a). Nitric oxide synthase such as your RNAm, are expressed into MnPO (Summy-Long *et al*., 2002). N^w-nitro-L-arginine methyl ester (L-NAME) reduces renal blood flow, urine flow rate and urinary sodium excretion (Naees *et al*., 1992). The influence of NO of the Central Nervous System (CNS) on several physiological parameters has been demonstrated (Saad *et al*., 2002b; Saad *et al*., 2003; Saad *et al*., 2004a). NO plays an important role in the hydromineral and cardiovascular regulation induced by ANG II (Saad *et al*., 1999). NO may facilitate the release of excitatory transmitters, possibly through a presynaptic cyclic GMP-dependent mechanism (Wu *et al*., 1997). L-NAME increases blood pressure which is at least in part salt sensitive (Hodge *et al*., 2002). Paraventricular neurons of the hypothalamus (PVN) have an important role in central regulatory mechanisms such as the temperature regulation, by releasing NO. Pharmacodynamic and pharmacokinetic studies of the 7-NIT also proved the pharmacological participation of NO in the CNS (Bush and Pollak, 2001).

It has been widely accepted that calcium ions are critically important for fast synaptic transmission, including the processes of neurotransmitter release from pre-synaptic terminals and post-synaptic receptor-mediated events (Ghosh and Greenberg, 1995). A major factor determining a neuronal Ca^{2+}-dependent signal is the opening of permeability pathways for Ca^{2+} in the cell membrane (Tsien *et al*., 1988). NMDA-type glutamate receptors seem to be concerned with the rapid behavioral actions of ANG II (Zhu and Herbert, 1997a).

Several data indicate that the MnPO is indeed the target of afferent from chemosensitive, osmosensitive and barosensitive systems concerned with fluid homeostasis and cardiovascular regulation (Gardiner and Stricker, 1985; O'neill and Brody, 1987; Cunningham *et al*., 2002; Yashuda *et al*., 2002). The neuronal circuit involving both the MnPO and the organ vasculosum of the lamina terminalis (OVLT) has been reported to play an important role in vasopressin secretion and thirst (Mangiapane *et al*., 1983). The injection of L-NAME a nitric oxide synthase inhibitor, into the MnPO, increased the Mean Arterial Pressure (MAP) (Saad *et al*., 2004a).

The influence of nifedipine L-Type calcium channel antagonist and NO in the CNS on the cardiovascular regulation has been previously demonstrated in our laboratory utilizing the Lateral Ventricle (LV) as the site of ANG II, nifedipine and L-NAME injection. The antidipsogenic actions of two L-type calcium channel blockers, verapamil and diltiazem injected into LV (Zhu and Herbert, 1997b) which bind to the phenylalkylamine and the benzothiazepine sites respectively did not significantly suppress ANG II-induce drinking behavior, however nifedipine injected prior to ANG II into LV suppress the dipsogenic effect of ANG II, suggesting that ANG II acts through a dihydropyridine-type calcium channel. These experiments verify whether the calcium channels has interrelationship with NO and ANG II receptors, of the MnPO, in the blood pressure regulation. The objective of this study determined the role of voltage-sensitive L-Type calcium channels in ANG II-induced pressor response when injected into the MnPO and the influence of NO, AT_1 and AT_2 ANG II receptors was also explored.

MATERIALS AND METHODS

This study was conducted in the Basic Institute of Biosciences-UNITAU, Taubaté, SP, BRAZIL and in the Department of Biology and Health Science UNIARA Araraquara SP BRAZIL in the year of 2004/2005.

All experiments were performed in adult male Wistar rats weighing 200-250 g. The animals were housed in individual metabolic cages. Food (Purina Rat Chow) and tap water were available *ad libitum*. The temperature was maintained at 22±2°C. The light cycle was held at 12:12 with lights on 06:00 h. All experiments were conducted during the light period, between 09:00 AM and 03:00 PM.

The Medical Ethics Committee of the Universidade Estadual Paulista UNESP approved all protocols in this study.

Experimental protocol

Experiment 1: There were six groups of rats: Rats were injected with the following: saline+saline 0.15 M NaCl (Group 1, n = 10). Saline+ANG II 25 pmol (Group 2 n = 9). Saline+Losartan (Group 3 n = 9). Saline+PD 123319 (Group 4 n = 8). Losartan+ANG II (Group 5 n = 8). PD 123319+ANG II (Group 6 n = 7). After the second injection, MAP was recorded during 30 min.

Experiment 2: There were six groups of rats. Rats were injected with the following: saline 0.15 M NaCl, (Group 1, n = 9). Saline+ANGII 25 pmol (Group 2, n = 9). Saline+Nifedipine 50 μg (Group 3; n = 8). Nifedipine 50 μg followed 10 min later by 25 pmol ANGII (Group 4, n = 7). Nifedipine 100 μg followed 10 min later by 25 pmol ANGII (Group 5, n = 7) and Sodium nitroprussite 20 μg followed 10 min later by 25 pmol ANGII (Group 6, n = 7). After the second injection, MAP was recorded during 30 min.

Experiment 3: There were seven groups of rats. Rats were injected with the following: (saline 0.15 M NaCl, (Group 1, n = 10). Saline+ANGII 25 pmol (Group 2, n = 10). Nifedipine 100 μg followed 10 min later by 25 pmol ANGII (Group 3, n = 9). L-NAME 40 μg followed 10 min later by 25 pmol ANGII (Group 4, n = 9). 7-NIT 40 μg followed 10 min later by 25 pmol ANGII (Group 5, n = 8). L-NAME 40 μg+Nifedipine 100 μg followed 10 min later by 25 pmol ANGII (Group 6, n = 7). 7-NIT 40 μg+Nifedipine 100 μg followed 10 min later by 25 pmol ANGII (Group 7, n = 6). After the second injection, MAP was recorded during 30 min.

Drugs

- Saline 0.15M NaCl (control)
- PD123319 and losartan (20 nmol 0.2 μL^{-1}) purchased from DuPont, Merck, (Wilmington, DE USA)
- Saline 0.15M NaCl (control)
- Angiotensin II (25 pmol 0.2 μL^{-1}) purchased from Sigma (Chemical Co., St. Louis, MO)
- Nifedipine (50 μg 0.2 μL^{-1} or 100 μg 0.2 μL^{-1}) purchased from Sigma (Chemical Co., St. Louis, MO)
- L-NAME (40 μg 0.2 μL^{-1}) purchased from Sigma (Chemical Co., St. Louis, MO)
- 7-Nitroindazole (7-NIT) (40 μg 0.2 μL^{-1}) purchased from Sigma (Chemical Co., St. Louis, MO)
- Sodium Nitroprusside (20 μg 0.2 μL^{-1}) purchased from Sigma (Chemical Co., St. Louis, MO)

Cerebral surgery: Male Holtzman rats weighing 250-300 g were anesthetized with zoletil 50 mg kg^{-1} (tiletamine chloridrate 125 mg and zolazepan chloridrate 125 mg) into quadriceps muscle. A stainless steel cannula with 10 and 12 mm long and 0.7 mm OD was implanted into the MnPO according to the coordinates of Paxinos and Watson (1986) atlas rat brain. The cannulae were fixed to the skull with the aid of jeweler screws and dental acrylic resin and protected with a stiletto. The experiments started after 5 days of the brain surgery.

Vascular catheter: After the animals recovery from brain surgery (5 days) PE-10 polyethylene tubing connected to PE-50 tubing was inserted into the abdominal aorta through the femoral artery under zoletil 50 mg kg^{-1} (tiletamine chloridrate 125 mg and zolazepan chloridrate 125 mg) anaesthesia. The polyethylene tube was tunneled subcutaneously to the back of the rat and externalized at the dorsal cervical region. Catheters were fill with heparinized saline and plugged with 23-G obturators. Rats recovered from surgery (vascular catheter) for a minimum of 24 h before beginning of testing.

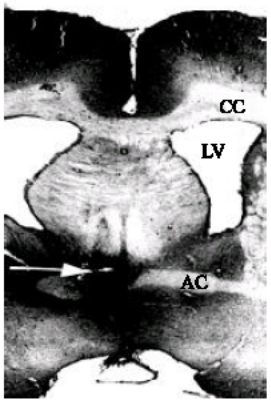

Fig. 1: Photomicrograph of a hematoxylin stained transverse section of the rat brain showing site of injection into the MnPO (arrow). CC Corpus Callosum; LV Lateral Ventricle; AC Anterior commissure

Drug injection: The drugs were injected into the MnPO by using a Hamilton micro syringe (5 μL) connected by a PE-10 polyethylene tubing (25 cm) to a needle (0.3 mm o.d.), which was introduced into the brain through the cannula previously fixed to the animals' head. The volume of injection was always 0.2 μL injected over a period of 30-60 sec.

Arterial blood pressure recordings: Direct mean arterial blood pressure (MAP) was record in anaesthetized and unrestrained rats. The animal was remove from the home cage and placed in a test cage, without access to food or water. The previously implanted catheter was connected to a Statham (P23 Db) pressure transducer (Statham-Gould, Valley View, OH) coupled to a multi channel recorded (PowerLab multirecord). This program permits the acquisition of cardiovascular data by computer.

Histology: At the end of the experiments, the rats were anesthetized with ether and perfused with saline and buffered formalin. The brains were removed, fixed in 10% formalin, frozen to -25°C and cut into 20-30 μm coronal sections. Subjects were included in the analysis only if their cannula was in the lateral medial portion of MnPO (Fig. 1). We examined every section throughout the

cannula path and eliminated all animals with cannula that perforated the ependyma, detected by histology and Evans blue.

Statistical analysis: The results are reported as mean±SEM. The ANOVA and Newman-Keuls post-hoc test were used to determine the significance. The values were considered statistically significant with 5% level ($p<0.05$).

RESULTS

Effect of AT_1 and AT_2 ANG II receptor antagonists on the pressor effect of ANG II: Microinjections of ANG II into the MnPO caused an increase in MAP compared to control (29±3 mmHg vs. 5±1 mmHg, $p<0.05$). The injection of saline+losartan and saline+PD 123319 into MnPO produced no change in MAP (5±1 mmHg and 4±1 mmHg, $p>0.05$). The micro-injection of losartan into MnPO prior to ANGII abolished the increase in the MAP as it was previously seen when ANG II was micro-injected alone (5±1 mmHg, $p<0.05$). The micro-injection of PD 123319 in to MnPO prior to ANG II decreased the pressor effect of ANG II with less intensity than that of losartan (8±1 mmHg, $p<0.05$) Table 1.

Effects of nifedipine and sodium nitroprusside on the increase of MAP induced by the injection of ANG II into the MnPO: Microinjections of ANG II into the MnPO cause an increase in MAP compared to control (28±2 mmHg vs. control 5±1 mmHg, $p<0.05$). The micro-injection of saline+nifedipine into the MnPO caused no change in the MAP (7±1 mmHg). Nifedipine 50 μg injected into the MnPO followed by ANG II decreased the pressor effect (8±1 mmHg, $p<0.01$). Nifedipine 100 μg injected into the MnPO followed by ANG II blocked the pressor effect (3±0.5 mmHg, $p<0.01$). The injection of SNP also blocked the pressor effect of ANG II (8±1 mmHg, $p<0.01$). SNP injected alone into the MnPO produced no change in the MAP Table 2.

Effect of nifedipine, L-NAME and 7-NIT on ANG II pressor effects: The MAP of rats injected with ANG II into MnPO produced an increase in the MAP 29±1 vs control 6±2 mmHg. Nifedipine injected prior to ANG II produced a decrease in the pressor effect 4±1 mmHg ($p<0.001$). The MAP of rats injected with L-NAME prior to ANGII was 35±1 mmHg ($p<0.001$). The MAP of rats injected with 7-NIT prior to ANGII was 32±1 mmHg ($p<0.001$). Nifedipine 100 μg prior to L-NAME followed by ANG II blocked the pressor effect (7±1 mmHg, $p<0.05$). Rats injected with nifedipine 100 μg prior to 7-NIT followed by ANG II decreased the pressor effect (5±2 mmHg, $p<0.001$) (Table 3).

DISCUSSION

These results showed the important participation of L-Type calcium channel and NO in the regulation of arterial pressure induced by ANG II injected into MnPO. These results also showed that the MnPO is more sensitive to the action of these drugs than when injected into the LV as previously demonstrated in our laboratory.

In the MnPO ANG II via AT_1 receptors mediates cardiovascular responses to an acute increase in cerebrospinal fluid sodium as well as the chronic pressor responses to high sodium intake in spontaneously hypertensive rats (Budzikowski and Leenen, 2001). The micro-injection of losartan into MnPO prior to ANGII abolished the MAP increase. The micro-injection of PD 123319 into MnPO prior to ANG II partially reduced the pressor effect of ANG II. There is functional evidence that ANG II exerts excitatory effects on neurons of the PVN and that these effects are antagonized by CGP 42112A (AT_2 receptor antagonist) (Felix *et al.*, 1991). These results showed that AT_1 and AT_2 receptors of the MnPO are important for the role of ANG II on central regulation of MAP. These findings are supported by the results of McKinley *et al.* (2003) showing that angiotensin AT_1, AT_2 and AT_4 receptors are also plentiful in the brain. In the MnPO ANG II via AT_1 receptors mediated cardiovascular

Table 1: Effect of nifedipine, L-NAME and 7-NIT on ANGII pressor effects

	SAL + SAL	SAL + ANGII	SAL + LOS	SAL + PD	LOS + ANGII	PD + ANGII
(mmHg)	5±1$^+$	29±3*	5±1$^+$	4±1$^+$	5±1$^+$	8±1^{+*}

$^+p<0.05$ vs SAL+ANGII; *$p<0.05$ vs SAL+SAL

Table 2: Effects of nifedipine and sodium nitroprusside on the increase of MAP induced by the injection of ANGII into the MnPO

	SAL + SAL	SAL + ANGII	SAL + NIF	NIF (50 μg) + ANGII	NIF (100 μg) + ANGII	SAL + SNP	SNP + ANGII
(mmHg)	5±1$^+$	28±2*	7±1$^+$	8±1$^+$	3±1$^+$	4±1$^+$	8±1^{*+}

$^+p<0.05$ vs SAL+ANGII; *$p<0.05$ vs SAL+SAL

Table 3: Effect of nifedipine, L-NAME and 7-NIT on ANGII pressor effects

	SAL + SAL	SAL + ANGII	NIF + ANGII	LNA + ANGII	7NI + ANGII	NIF (100 μg) + LNA + ANGII	NIF (100 μg) + 7NI + ANGII
(mmHg)	6±2$^+$	29±1*	4±1$^+$	35±1^{*+}	32±1^{*+}	7±1$^+$	5±2$^+$

$^+p<0.05$ vs SAL+ANGII; *$p<0.05$ vs SAL+SAL

responses to an acute increase in CSF sodium as well as the chronic pressor responses to high sodium intake in SHR. Furthermore, the salt-sensitive component appears to be ANGII-dependent, as it was associated with increase of plasma ANG II levels and could be reversed by the treatment with ANG II receptor antagonist (Thunhorst and Johnson, 1994).

It has been demonstrated that NO plays an important role in the cardiovascular regulation (Saad *et al*., 2002a). FK409, a NO donor agent, combined with losartan, abolished the increase in the MAP induced by ANG II (Saad *et al*., 2002b). L-NAME induced an increase in blood pressure, which is at least in part salt sensitive. L-NAME produced an increase in the pressor effect of ANGII and this effect may be due to local vasoconstriction and all at once by neuronal NOS inhibition. We have also investigated the role of 7-NIT (nNOSI) injected into the MnPO in the pressor effect of ANG II. 7-NIT has less effective potentiation effect on ANG II pressor effect than L-NAME when injected into the MnPO. Pharmacodynamic and pharmacokinetic studies of the 7-NIT proved the neural pharmacological participation of NO in the CNS in many physiological functions (Bush and Pollack, 2001). This let us to postulate that the eNOSl and nNOS in the MnPO are involved in the pressor effect of ANG II.

Angiotensinergic neural pathways and calcium channels are important in neural function and may have important homeostatic roles, particularly related to cardiovascular function by involving NO. The activation of glutamate-NMDA receptors can increase intracellular Ca^{2+} concentration, which in turn acts on calmodulin to evoke nitric oxide syntheses (Garthwaite, 1991; Dawson and Snyder, 1994). In most neurons of the central nervous system, there are at least two major classes of calcium channel: voltage sensitive calcium channels (VSCCs) and receptor-operated calcium channels (ROC). Nifedipine may have interfered with Ca^{2+} influx in the presynaptic terminals, where L-type calcium channels play important roles in the modulating presynaptic neurotransmitter release (Miller, 1998; Ghosh and Greenberg, 1995). It may also have altered Ca^{2+}-dependent signal events in postsynaptic neurons since previous studies demonstrated the permissive effects of VSCCs on NMDA receptor-mediated Ca^{2+} influx (Bumashev, 1996). ANG II into MnPO may acts primarily on AT_1 receptors that increase intracellular Ca^{2+} concentration, which in turn acts on calmodulin to evoke nitric oxide synthesis. Central NO-cGMP pathway is involved in ANGII-induced drinking behavior (Zhu and Herbeert, 1997b). The NOS and your RNAm is plentiful found into MnPO (Summy-Long *et al*., 2002). In the MnPO, the influence of L-Type calcium channel and NO utilizing cGMP pathways on ANG II pressor effect can explain part of these results as has been demonstrated for the water intake (Zhu and Herbert, 1997a). An interaction between L-Type calcium channel and a free radical gas (NO) in the MnPO on the control of ANG II-induced pressor effect acting primarily in AT_1 and secondary in AT_2 receptors, respectively may be postulated. Also we may infer that ANGII act in AT_1 receptor opening calcium channels and inhibit the NO release.

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