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Endothelial and Neuronal Nitric Oxide Synthase Inhibitors Influences Angiotensin II Pressor Effect in Central Nervous System

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Abstract: The present study investigated the central role of angiotensin II and nitric oxide on arterial blood pressure (MAP) in rats. Losartan and PD123349 AT₁ and AT₂ (selective no peptides antagonists angiotensin receptors), as well as FK 409 (a nitric oxide donor), N^w-nitro-L-arginine methyl ester (L-NAME) a constitutive nitric oxide synthase inhibitor endothelial (eNOS) and 7-nitroindazol (7NI) a specific neuronal nitric oxide synthase inhibitor (nNOS) were used. Holtzman strain, (*Rattus norvegicus*) weighting 200-250 g were anesthetized with zoletil 50 mg kg⁻¹ (tiletamine chloridrate 125 mg and zolazepan chloridrate 125 mg) into quadriceps muscle and a stainless steel cannula was stereotaxically implanted into their Lateral Ventricle (LV). Controls were injected with a 0.5 µl volume of 0.15 M NaCl. Angiotensin II injected into LV increased MAP (19±3 vs. control 3±1 mm Hg), which is potentiated by prior injection of L-NAME in the same site 26±2 mm Hg. 7NI injected prior to ANG II into LV also potentiated the pressor effect of ANG II but with a higher intensity than L-NAME 32±3 mm Hg. FK 409 inhibited the pressor effect of ANG II (6±1 mm Hg). Losartan injected into LV before ANG II influences the pressor effect of ANG II (8±1 mm Hg). The PD 123319 decreased the pressor effects of ANG II (16±1 mm Hg). Losartan injected simultaneously with FK 409 blocked the pressor effect of ANG II (3±1 mm Hg). L-NAME produced an increase in the pressor effect of ANG II, may be due to local vasoconstriction and all at once by neuronal NOS inhibition but the main effect is of the 7-NIT an specific nNOS inhibitor. The AT₁ antagonist receptors improve basal nitric oxide (NO) production and release. These data suggest the involvement of constitutive and neuronal NOS in the control of arterial blood pressure induced by ANG II centrally, evolving AT₁ receptor-mediated vasoconstriction and AT₂ receptor-mediated vasodilatation. These results were confirmed by the experiment using FK 409.

Key words: CNS, L-NAME, 7-nitroindazol, angiotensin II receptor, arterial pressure

INTRODUCTION

Homeostasis of the body arterial pressure, extracellular volume and tonicity in mammals is a prerequisite for the functional control of life at all levels of body organization. Angiotensin II plays an important role in the maintenance of the homeostasis of the body by acting peripherally or centrally. ANG II increases the

firing rate of the magnocellular neurosecretory neurons of the supraoptic nucleus (SON). Neurons of the septal nuclei are also sensitive to angiotensin (Camargo and Saad, 1999). Central injection of angiotensin II (ANG II) produced pronounced responses increasing the arterial blood pressure (Reid, 1988). The neural circuit involving both median preoptic nucleus (MnPO) and organ vasculosum of the lamina terminalis (OVLT) has been

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reported to play an important role in vasopressin secretion and thirst (Mckinley *et al.*, 1992). Electrical stimulation of the basal forebrain causes the release of arginine-vasopressin (Szcypanska-Sadowska *et al.*, 1982). This effect seems to be produced by the activation of functional ANG II receptors within the SON since it is reversibly blocked by the angiotensin peptide antagonist salarasin (Jhamandas *et al.*, 1989). Application of the non-peptide type 1 angiotensin antagonist DuP753 blocks the ANG II-induced depolarization in the SON. In contrast, application of the type 2 antagonist PD123177 was ineffective in blocking this response (Yang *et al.*, 1982). Utilization of nitric oxide synthase (NOS) inhibitors to probe the role of NO in various central nervous system processes requires use of an inhibitor selective for neuronal NOS and is facilitated by knowledge of the pharmacokinetics of the inhibitor. This study was undertaken to elucidate the disposition of the selective neuronal NOS, inhibitor 7-nitroindazole (Mark and Gray, 2001). NO synthase inhibitors have been used widely to determine the role of endogenous NO. Several studies have shown that NO may function as a neurotransmitter or a neuromodulator. Recognition of the role of nitric oxide in cell-to-cell communication has changed the concept of traditional neurotransmission. N-methyl-D-aspartate receptors mediate the dipsogenic response and c-Fos expression induced by intracerebroventricular (icv) infusion of angiotensin II (ANG II) (Zhu and Herbert, 1997). It has been demonstrated that NO may facilitate the release of excitatory transmitters, possibly through a presynaptic cyclic GMP-dependent mechanism (Wu *et al.*, 1997). The influence of NO on angiotensin pressor effects has been demonstrated (Saad *et al.*, 1999; Camargo and Saad, 1999; Saad *et al.*, 2002a). Also these effects implicated the participation of septal area (Saad *et al.*, 2002b, c). Treatment with L-NAME increases blood pressure that is at least in part salt sensitive (Hodge *et al.*, 2002).

7-Nitriindazol (7NI) inhibits the cerebellum Nitric Oxide synthase (NOS) in rats (Moorer *et al.*, 1990). Intracerebroventricular injection of 7NI demonstrated that, NO of the paraventricular neurons of the hypothalamus, has important role in the central regulatory mechanisms such as the temperature regulation (Wang *et al.*, 2003). Pharmacodynamic and pharmacokinetics studies of the 7NI also proved the pharmacological NO participation in the CNS (Bush and Pollack, 2001). The systemic application of ibersartan and losartan abolished the ANG II central responses in increasing arterial pressure (Camargo and Saad, 1999). The role of renin-angiotensin system in the control of arterial blood pressure had been demonstrated (Thunhorst and Johnson, 1994). The treatment with

losartan reversed the blood pressure increase. The role of NO, found in circumventricular structures surround the third ventricle in the hydro mineral and cardiovascular regulation has been demonstrated (Saad *et al.*, 2003). Several data indicate that the MnPO is indeed the target of afferent from chemo sensitive and barosensitive systems concerned with fluid homeostasis and cardiovascular regulation (Yashuda *et al.*, 2000). Since the circumventricular structures of the central nervous system are involved in the control of arterial blood pressure we investigated whether the pressor response induced by LV injection of ANG II could be mediated by angiotensin AT₁ and AT₂ and endogenous endothelial and neuronal NO.

MATERIALS AND METHODS

Subjects: Holtzman rats weighing 200-250 g with canulae implanted unilaterally into LV unilaterally were used. The animals were housed in individual metabolic cages. Food (Purina Rat Chow) and tap water is available, ad libitum, for the duration of the experiments. The room 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.

Cerebral cannula: The animal were anesthetized with zoletil 50 mg kg⁻¹ (tiletamine chloridrate 125 mg and zolazepan chloridrate 125 mg) into quadriceps muscle and implanted unilaterally on the left side with 10 and 12 mm long and 0.7 mm OD stainless steel cannulae into the LV, according to the coordinates of the Paxinos and Watson (1986) rat brain atlas. The stereotaxic coordinates for the LV were obtained from Paxinos and Watson (1986) rat brain atlas. The coordinates were: 0.92 mm caudal to bregma, 1.3 mm lateral to midline and 3.4 mm bellow the duramater. The cannulae were fixed to the skull with the aid of jeweler screws and dental acrylic resin and protected with a stylet. Rats recovered from surgery for a minimum of 5 days beginning of testing.

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). The polyethylene tube was tunneled subcutaneously to the back of the rat and externalized at the dorsal cervical region. Catheters were filled 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.

Central drugs injections: Injections into LV were made using 10 μ L Hamilton syringes connected by polyethylene tubing (PE-10) to 30-gauge injection cannulas. At the time of testing, the obturator was removed and the injection cannula introduced into the chronically implanted guide cannula. The injection cannula was 0.2 mm longer than the guide cannula. The injection volume was 0.5 μ L delivered over 20 to 30 sec. After injection, the styles were replaced and the rats were placed back into the cage. The dose of the drugs was base in several works of the laboratory and the literature.

Drugs: ANG II purchased from Sigma (Chemical Co., St. Louis, MO) and dissolved in saline (0.15 M NaCl) at 10 nmol/0.5 μ L.

PD123319 and losartan purchased from DuPont, Merck, Wilmington, DE USA and dissolved in saline (0.15 M NaCl), at 20 nmol/0.5 μ L.

FK 409 (From AN Epstein Lab. Philadelphia Joseph Leidy Laboratories Philadelphia PA. USA) dissolved in saline (0.15 M NaCl) at 5 μ g/0.5 μ L.

N^G-nitro-L-arginine methyl ester (L-NAME) purchased from Sigma (Chemical Co., St. Louis, MO), dissolved in saline (0.15 M NaCl) at 10 μ g/0.5 μ L.

7-nitroindazol (7-NIT) (Tocris Cookson Inc, Ballwin, MO, USA) dissolved in saline (0.15 M NaCl) at 10 μ g/0.5 μ L.

Arterial blood pressure recordings: Direct mean arterial blood pressure (MAP) was recorded in anaesthetized and unrestrained rats. The animal was removed 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 recorder (Dataq multirecord USA). This program permits the acquisition of cardiovascular data by computer.

Experimental procedures

Lateral ventricle injections of losartan, PD 123319, FK 409, L-NAME on the mean arterial pressure (MAP) effect-ANG II injection into LV: Hotzman rats were implanted with cannulae into LV. Following a recover period animals were randomly assigned to one of eight treatment conditions: Vehicle SAL + SAL (n = 12); vehicle + ANG II (n = 12); losartan+ANG II (n = 9); PD 123319 + ANG II (n = 9); FK409+ANG II (n = 8); L-NAME + ANG II (n = 7); L-NAME+losartan + ANG II (n = 8); L-NAME + PD123319 + ANG II (n = 7) and losartan + FK409 + ANG II (n = 7). These substances were injected into the LV in order to investigated the participation of the AT₁, AT₂ ANG II receptors, eNOSI in

mean arterial pressure induced by ANG II injection into LV. Antagonist of eNOSI or vehicle pretreatment were administered into LV. Fifteen minutes following the drug injections, ANG II (10 nmol/0.5 μ L) was administered into the LV. MAP was recorded at each 15 min during 120 min following ANG II administration into the LV. Vehicle was 0.15 M NaCl.

Lateral ventricle injections of losartan, PD 123319, FK 409, 7NI on the mean arterial pressure (MAP) effect of ANG II injection into LV: Hotzman rats were implanted with cannulae into LV. Following a recover period animals were randomly assigned to one of eight treatment conditions: Vehicle SAL + SAL (n = 12); vehicle + ANG II (n = 12); losartan + ANG II (n = 9); PD 123319 + ANG II (n = 9); FK409 + ANG II (n = 8); 7NI + ANG II (n = 9); 7NI + losartan + ANG II (n = 8); 7NI + PD123319 + ANG II (n = 7) and losartan + FK409 + ANG II (n = 7). These substances were injected into the LV in order to investigated the participation of the AT₁, AT₂ ANG II receptors, eNOSI in mean arterial pressure induced by ANG II injection into LV. Antagonist of nNOSI or vehicle pretreatment were administered into LV. Fifteen min following the drug injections, ANG II (10 nmol/0.5 μ L) was administered into the LV. MAP was recorded at each 15 min during 120 min following ANG II administration into the LV. Vehicle was 0.15 M NaCl.

Histology: At the end of the experiments, the rats were anesthetized with ether and given at 0.5 μ L injection of fast green dye via the intracranial cannula, followed by perfusion with saline and buffered formalin. The brains were removed, fixed in 10% formalin, frozen to 25°C and cut into 20-30 μ m coronal sections and cut into 20-30 μ m coronal sections and stained with hematoxylin-eosin. Only animals in which the injection was placed in the LV were use in this study.

Data analysis: Results are reported as mean \pm standard error of the mean (SEM) for the indicated experiments. Statistical analysis was subjected ANOVA followed by the Neuman-Keuls post-hoc test. Differences were considered significant at p<0.05.

RESULTS

Effect of ANG II injected into LV on the Mean Arterial Pressure (MAP) and its antagonism by eNOSI (L-NAME) and AT₁ and AT₂ ANG II receptors inhibitors: Microinjections of ANG II into the LV caused an increased in MAP compared to control (19 \pm 2 mm Hg vs. 3 \pm 1 mmHg) (n = 12), p<0.05. LV microinjection of L-NAME

Table 1: Effect of pretreatment with losartan, PD123319, FK 409, L-NAME and Losartan+FK 409 or vehicle (saline) into the LV on mean arterial pressure evoked by injection of ANG II into the LV

SAL + SAL	SAL + ANG II	LNA + ANG II	LOS + ANG II	PD + ANG II	LNA + LOS + ANG II	PD + LOS + ANG II	FK409 + LOS + ANG II
3±1 ⁺	19±2*	26±2**	8±1 ⁺	16±1**	11±1**	15±2	3±1 (mm Hg)

*p<0.05 vs SAL+ANG II; **p<0.05 vs SAL+SAL

Table 2: Effect of pretreatment with losartan, PD123319, FK 409, 7-nitroindazol and Losartan+FK 409 or vehicle (saline) into the LV on mean arterial pressure evoked by injection of ANG II into the LV

SAL + SAL	SAL + ANG II	7NI + ANG II	LOS + ANG II	PD + ANG II	7-NI + LOS + ANG II	7NI + PD + ANG II	FK409 + LOS + ANG II
3±1 ⁺	21±2*	32±2**	9±1 ⁺	16±1**	7±1**	12±2**	2±1 (mm Hg)

*p<0.05 vs SAL+ANG II; **p<0.05 vs SAL+SAL

prior to ANG II, potentiated the increase MAP response seen when ANG II was injected alone (26±2 mm Hg) (n = 7), p<0.05. LV microinjection of losartan prior to ANG II decrease the effect of ANG II in MAP (8±1 mm Hg) (n = 9) p<0.05. LV microinjection of PD 123319 prior to ANG II decreased the pressor effect of ANG II (16±1.0 mm Hg (n = 9) p<0.05 vs. ANG II. LV microinjection of a combination of L-NAME and losartan prior to ANG II decreased the pressor action of ANG II (11±1 mm Hg) (n = 8) p<0.05. LV microinjection of a combination of PD 123319 and losartan prior to ANG II decreased the pressor effect of ANG II (15±2 mm Hg) (n = 7) p<0.05. FK with combination with losartan abolished the increased in MAP induce by ANG II injected into LV (3±1 mm Hg) (n = 7) p<0.05 (Table 1).

Effect of ANG II injected into LV on the mean arterial pressure and its antagonism by nNOS1 7-nitroindazol and AT₁ and AT₂ ANG II receptors inhibitors:

Microinjections o ANG II into the LV caused an increased in MAP compared to control (21±2 mm Hg vs. 3±1 mm Hg) (n = 12), p<0.05. LV microinjection of 7NI prior to ANG II, potentiated the increase MAP response seen when ANG II was injected alone (32±2 mm Hg) (n = 12), p<0.05. LV microinjection of losartan prior to ANG II abolished the increase in MAP seen when ANG II was microinjected alone (9±1 mm Hg) (n = 9) p<0.05. LV microinjection of PD 123319 prior to ANG II decreased the pressor effect of ANG II (16±1.0 mm Hg (n = 9) p<0.05 vs. ANG II. LV microinjection of a combination of 7NI and losartan prior to ANG II blocked the pressor action of ANG II (7±1 mm Hg) (n = 8) p<0.05. LV microinjection of a combination of 7NI and PD 123319 decreased the pressor effect of ANG II (12±2 mm Hg) (n = 7) p<0.05. FK with combination with losartan abolished the increased in MAP induce by ANG II injected into LV (2±1 mm Hg) (n = 7) p<0.05 (Table 2).

DISCUSSION

LV microinjection of losartan prior to ANG II abolished the increase in MAP. LV microinjection of PD 123319 prior to ANG II decreased the pressor effect of

ANG II. While the combination of L-NAME and losartan prior to ANG II blocked the pressor action of ANG II, microinjection of a combination of L-NAME and PD 123319 only decreased the pressor effect of ANG II. These results showed that the AT₁ receptors are more important than the AT₂ receptors in the effect of NO in the central regulation of MAP. It has been demonstrated that NO pays an important role in the cardiovascular regulation (Saad *et al.*, 1999, 2002a). FK409 with combination with losartan abolished the increased in MAP induce by ANG II. AT₁ receptors are found in several brain regions such as the hypothalamic paraventricular and supraoptic nucleus, the lamina terminalis, lateral parabrachial nucleus, ventro lateral medulla and nucleus of the solitary tract, which are know to have roles in the regulation of the cardiovascular system. Therefore these results show that ANG II-induced pressure response was dependent on AT₁ and AT₂. In the MnPO ANG II via AT₁ receptors mediates cardiovascular responses to an acute increase in CSF sodium as well as the chronic pressor responses to high sodium intake in Spontaneous Hypertensive Rats (SHR). These results are strongly supported by the experiments of Camara and Osborn (2001). There is functional evidence that ANG II exerts excitatory effects on neurons of the PVN and that these effects are antagonized by CGP 42112A (Felix *et al.*, 1991).

The results of the present study clearly demonstrated that ANG II need NO to induce increase in arterial blood pressure. NO play an important role in this response. In these experiments microinjections of ANG II into the LV caused an increased in Mean Arterial Pressure (MAP) that is potentiated by 7NI and L-NAME. Treatment with L-NAME induces an increase in blood pressure. The action of L-NAME may be due a local vasoconstriction. Further, the salt-sensitive component appears to be ANG II-dependent, as it was associated with increasing plasma ANG II levels and could be reversed by treatment with an ANG II receptor antagonist (Hodge *et al.*, 2002). 7NI injected Intracerebroventricular demonstrated that neuronal Nitric Oxide Synthase (nNOS) induce synthesis of NO in the paraventricular neurons of the hypothalamus, that is important in the central

regulatory mechanisms such as the temperature regulation (Wang *et al.*, 2003). Pharmacodynamic and pharmacokinetics studies of the 7NI proved the pharmacological NO participation in the CNS in many physiological functions (Bush and Pollack, 2001). Several studies demonstrated that endogenous NO might function as a neurotransmitter and/or neuromodulator in various organs, since the presence of an intact endothelium inhibits electrical stimulation induced-nor epinephrine release from adrenergic nerves (Cohen and Weisbrod, 1998).

The present study also investigated the role of 7 NI, nNOSI, injected into the LV on the pressor effect of ANG II. The major findings of the study is that L-NAME produced an increase in the pressor effect of ANG II and this effect may be due to local vasoconstriction and all at once by neuronal NOS inhibition, but the main effect is that of the 7-NI, a specific nNOSI, having more effective effect in raise to a power increase in MAP induce by ANG II. NO synthase inhibitors enhanced norepinephrine release via a prejunctional mechanism in the rat-tail artery (1989; Vo *et al.*, 1991) and from rat heart sympathetic nerves (Schwartz *et al.*, 1996). The present study noted that changes in these parameters induced by L-NAME were abolished by LH-lesion (Saad *et al.*, 2004a). Thus NO probably has a role as inhibitory modulator of norepinephrine secretion into MnPO that is controlled by LH. The presence of nitric oxide in many structures of the central nervous system has been described (Arnaiz *et al.*, 1999; Bredt *et al.*, 1991; Ignarro *et al.*, 1987; Rees *et al.*, 1989). Nitric oxide centrally or systemically plays an important role in the regulation of MAP and heart rate (Rees *et al.*, 1989). The present results support that 7NI influence the mechanism of neuronal NO of the structures surrounding the ventricle in the regulation of arterial pressure. The experiments utilizing FK409 confirmed these results. We also propose that endogenous NO functions tonically as an inhibitory modulator of brain noradrenergic neurotransmission (Saad *et al.*, 2004a) Altogether, these findings suggest that neurons surrounding LV could increase blood flow in these structures partly via a local NO relay neuron whereby the freely diffusing gas would be the direct smooth muscle vasodilator agent have been demonstrated (Ignarro *et al.*, 1987) These results suggest that NO generated may act to regulate the arterial pressure and heart rate but it may not play an essential role in eliciting the responses of these variables to osmotic or prostaglandin E (2) stimuli as previously described by (Yamaguchi *et al.*, 2000). The results of the present study are consistent with other hypothesis that NO is a tonic inhibitor of sympathetic nervous system tone, possibly in part through an influence on dopamine synthesis or release as has been demonstrated in the studies of

(Moses and Hull, 1999) Ascending pathways from medial hypothalamus or LH to AV3V region were suggested by studies from our laboratory using cholinergic activation of these nuclei (Valadão *et al.*, 1992). The results of these studies are strongly supported by others studies (Chicada *et al.*, 2000), demonstrating that NO acts directly in the brain to reduce the systemic blood pressure and that the endogenous NO pathway may play a role in cardiovascular and autonomic regulation by modulating neuronal activities in discrete regions of the brain and LH having an important role in the regulation of these variables. Saad *et al.* (2004a) recently demonstrated that NO of the supraoptic nucleus influences the arterial blood pressure induced by ANG II and showed an interaction between supraoptic nucleus and septal area in the control of water, sodium intake and arterial blood pressure induced by injection of angiotensin II (Saad *et al.*, 2004b). The participation of arginine vasopressin receptors and angiotensin receptor subtypes on the water and arterial pressure induced by vasopressin injected into the lateral septal area of the rat has been demonstrated.

These data, suggest that structures surrounding the cerebral ventricles may release ANG II, which acts as a neurotransmitter resulting in postsynaptic effects, which in turn influence blood pressure this effect is controlled by inhibition nNO induced by 7NI. Chang *et al.* (2001) have supported the results of the present study. They found that 7NI injected into the organum vasculosum laminae terminalis of rat brain cause 98 mm Hg increase in blood pressure. The participation of NO in reflector control of the cardiovascular system by the medullary neurons within n. tractus solitarii, dorsal nucleus of the vagus nerve, n. ambiguus and the lateral reticular nucleus has been studied (Shapoval *et al.*, 2003). These authors conclude, by utilizing sodium nitroprusside, L-arginine as well as by intraperitoneal injection of 7-nitroindazol, that stimulation of nNOS activity in the populations of the medullary neurons resulted in both remarkable shifts in the arterial pressure level and in inhibiting the chemo receptor reflector responses. The mechanism of ANG II may utilize vasopressin that in turn is controlled by NO. The participation of arginine vasopressin receptors and angiotensin receptor subtypes on the water and arterial pressure induced by vasopressin injected into the lateral septal area of the rat has been demonstrated (Saad *et al.*, 2004c) In conclusion, angiotensinergic neural pathways are important in neural function and may have important homeostatic roles, particularly related to cardiovascular function by utilizing NO. The main finding of this study is that 7NI (nNOSI) has more effective effect on ANG II in increase blood pressure than L-NAME; these results allowed us to infer

that in the central area of the brain the neuronal NO plays a more important role than endothelial NO in the regulation of MAP induced by ANG II. This finding is strongly supported by Obst *et al.* (2004). They conclude that AT₂ receptors deletion and concomitant up-regulation of the AT₁ receptor is associated with up-regulation of nNOS and iNOS did not changed blood pressure. By other hand we cannot exclude that L-NAME may had the higher effect in endothelial inhibition of NO but the effect on neuronal NO cannot be discharged.

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