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# Role of Angiotensin II and Nitric Oxide in the Rat Paraventricular Nucleus

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To investigate the mutual relationship between angiotensin II (Ang II) and nitric oxide (NO) in paraventricular nucleus (PVN), Ang II receptor type Ia ($AT_{1A}$), type Ib ($AT_{1B}$), endothelial constitutive nitric oxide synthase (ecNOS), and neuronal constitutive nitric oxide synthase (ncNOS) mRNA levels of rat PVN were measured after unilateral carotid artery ligation. $AT_{1A}$ and $AT_{1B}$ mRNA levels were markedly elevated 6 hrs after unilateral carotid artery ligation. Losartan injection (10 $\mu$g/0.3 $\mu$l) into the PVN augmented of the increment of $AT_{1A}$ and $AT_{1B}$ mRNAs It also increased ecNOS gene expression. In addition, $AT_{1B}$ mRNA levels increased after N-nitro-L-arginine methyl ester (L-NAME) injection (50 $\mu$g/0.3 $\mu$l) into the PVN. These results suggest that Ang II and NO in the rat PVN may interplay, at least in part, through regulation of gene expression of ecNOS and $AT_{1B}$, respectively.

Key Words: Angiotensin II, Nitric oxide, Hypothalamic paraventricular nucleus, Rat, Angiotensin II $AT_1$ receptors ($AT_{1A}$, $AT_{1B}$), Nitric oxide synthase (ecNOS, ncNOS)

## INTRODUCTION

The paraventricular nucleus (PVN) is an important integrative center for autonomic functions including regulation of cardiovascular activity (Dawson et al, 1998). The activity of PVN is affected either by autonomic projection from spinal cord, brainstem, and forebrain or by blood-born substances that gain access to the brain through circumventricular organs such as subfornical organ (SFO).

Angiotensin II (Ang II) is the main active component of the renin-angiotensin system. Two subtypes of Ang II receptors, $AT_1$ and $AT_2$, have been identified in the brain (Bottari et al, 1993). The $AT_1$ receptor is the predominant subtype found in the brain regions known to be affected by systemic Ang II, including PVN (Lebrun et al, 1995; Rowland et al, 1995) and SFO (McKinley et al, 1992; Lebrun et al, 1995; Rowland et al, 1995).

Recently, the brain is found to express higher levels of nitric oxide synthase (NOS) activity than any other tissues (Millatt et al, 1999). Indeed, NOS expression has been identified in all areas of the brain, with neuronal constitutive NOS (ncNOS) and endothelial constitutive NOS (ecNOS) being the predominant isoforms under normal conditions. Within the cerebral circulation, NO generated by ecNOS acts as a vasodilator, and is believed to play a protective role in maintaining blood flow during cerebral ischemia (Huang et al, 1996). Several studies (McLay et al, 1995; Olson et al, 1997; Ackermann et al, 1998; Luvara et al, 1998) have shown a mutual relation between the renin-angiotensin and the NO-generating systems in various organs. Despite of several in vivo and in vitro evidences regarding the countervailing influences between Ang II and NO in the blood pressure control, renal function, and cellular proliferation, there is no direct evidence to date addressing the mutual relationship between Ang II and NO within PVN, one of the important brain region for cardiovascular regulation.

To address this question, the gene expression of $AT_{1A}$, $AT_{1B}$, ecNOS, and ncNOS in rat PVN were measured after unilateral carotid artery ligation. The results of present study show that losartan and N-nitro-L-arginine methyl ester (L-NAME) treatment upregulate ecNOS and $AT_{1B}$ mRNA level, respectively.

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## METHODS

The experiments were performed using 10- to 12-week-old male Sprague-Dawley rats. Under pentobarbital sodium (50 mg/Kg, i.p.) anesthesia, rat was placed on a stereotaxic device and a stainless steel guide cannula (27 guage) was placed into the PVN (1.0 mm lateral, 1.8 mm posterior to the bregma, and 5.0 mm below the skull surface). The injector extended 1 mm beyond the end of the guide cannula. Injection into the PVN was given in a 0.3 $\mu$l volume over 20 sec with the use of a 32-guage internal cannula. Thirty min after either losartan (10 $\mu$g/0.3 $\mu$l) or L-NAME (50 $\mu$g/0.3 $\mu$l) injection through PVN cannula, a tight ligature was applied around common carotid artery of male Sprague-Dawley rat. Booster of antagonist was injected through PVN cannula every 30 min during experiment. The rat was decapitated either 6 hrs or 18 hrs after operation. Based on previous studies (Finke et al, 1993; Mies, 1994), which have reported an isolation of RNA from formalin fixed tissue sufficient as a template for the reverse transcription-polymerase chain reaction (RT-PCR), rat was perfused with 10% formalin for verifying cannula placement. Hypothalamus was, then, rapidly dissected out, frozen in liquid nitrogen, and stored at $-70^{\circ}$C until use.

*Total RNA isolation*

Total RNA was isolated according to the method described by Chomczynski & Sacchi (1987) with slight modifications. Frozen tissue was grounded to fine powder using the pestal and mortar in the presence of liquid nitrogen, and the denaturation solution (4 M guanidium thiocyanate, 25 mM sodium citrate pH 7, 0.5% sarcosyl, 0.2 M $\beta$-mercaptoethanol) was added (10 ml/g tissue). Homogenate was transferred to a fresh tube and 1/10 volume of 2 M sodium citrate (pH 4.0), 1 volume of water-saturated phenol and 1/5 volume of chloroform-isoamylalcohol (49 : 1) were sequentially added with gently mixing after each addition. The tube was kept on ice for 15 min and was centrifuged at 12,000 rpm for 15 min at 4°C. The upper aqueous phase was transferred to a fresh tube and reextracted with an equal volume of chloroform-isoamylalcohol (49 : 1). The supernatant was collected and mixed with 2.5 volume of absolute ethanol, and then placed at $-20^{\circ}$C for at least 1 hr. RNA was precipitated by centrifugation at 12,000 rpm for 15 min at 4°C. The pellet was washed in 75% ethanol. After drying in the air, RNA pellet was dissolved in DEPC-Q water and kept at $-20^{\circ}$C until needed. The concentration and purity of RNA were determined by measuring the absorbance at 260 nm and 280 nm.

*Reverse transcription PCR (RT-PCR)*

The nucleotide sequence of the primers and references are presented in Table 1. Total RNA (20 $\mu$g) was primed with oligo (dT) primers, and first strand cDNA was synthesized using Moloney murine leukemia virus (MMLV) reverse transcriptase in a 30 $\mu$l of reaction volume for 90 min at 37°C. The reaction mixture was heated for 5 min at 95°C to degrade template RNA and enzyme. In brief, 1 or 2 $\mu$l of each cDNA was amplified in a total volume of 25 $\mu$l containing PCR buffer (10 X polymerase standard buffer plus 5 mM $MgCl_2$ and 0.1% formamide), 200 $\mu$M of each dNTP, 20 pmole of each primers and 1.25 U of Taq DNA polymerase (Promega, USA). PCR was carried out using a Perkin-Elmer Cetus DNA Thermal Cycler as follows: 94°C for 45 sec, 58°C for 45 sec, 72°C for 1 min. PCR conditions are the same for all genes examined in this study except PCR cycles and the amount of template that were carefully selected according to the relative abundance of mRNAs in the PVN. After the end of PCR, one fifth of reaction mixture was separated on a 1.2% of agarose gel containing 0.5 $\mu$g/ml of ethidium bromide.

*Densitometric analysis*

The polaroid film was scanned using an Epson (ES-600) scanner with a resolution of 70 DPI (dot per inch) and 256 gray scale unit. In order to achieve a linear response curve, both brightness and contrast were finely tuned to the point where the gray scale unit of the background was within 20~40 and that of the darkest band was between 200~240. The resulting image was analysed from NIH-Image analysis program (written by Wayne Rasband, NIH, Betheda, MD). Using a selection box, the gray scale unit of various background areas was measured, subtracted from each band, and then, the mean density and total area of the band were calculated. The scale of each band was expressed by multiplying the mean density and the total area of the band. The resulting scale was used to quantify each band.

**Table 1.** Primer sequences for $AT_{1A}$, $AT_{1B}$, ecNOS, ncNOS and GAPDH used in this study

| Gene | Primer sequence | Product size | Reference |
|---|---|---|---|
| $AT_{1A}$ | 5' GCCTGCAAGTGAAGTGATTT 3'<br>5' TTTAACAGTGGCTTTGCTCC 3' | 388 bp | Kitami et al, 1992 |
| $AT_{1B}$ | 5' GCACACTGGCAATGTAATGC 3'<br>5' GTTGAACAGAAGAAGTGACC 3' | 204 bp | Kitami et al, 1992 |
| ecNOS | 5' TACGGAGCAGCAAATCCAC 3'<br>5' GATCAAAGGACTGCAGCCTG 3' | 591 bp | Ujiie et al, 1994 |
| ncNOS | 5' ATACCAGCCTGATCCATGGA 3'<br>5' CTCCAGGAGGGTGTCCAC 3' | 540 bp | Bredt et al, 1991 |
| GAPDH | 5' ATCAAATGGGGTGATGCTGGTGCT3'<br>5' CAGGTTTCTCCAGGCGGCATGTCAG 3' | 504 bp | Tso et al, 1985 |

*Ang II receptor densities: receptor binding assay*

Immediately after decapitation, hypothalamus was removed and homogenized in ice-cold Tric-HCl buffer (50 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 7.2). After centrifugation at 600 g for 10 min, the supernatant was re-centrifuged at 48,000 g for 20 min. The supernatant was discarded and the pellet resuspended in Tris-HCl buffer. Total protein level of each homogenate preparation was determined in duplicate using the Bradford method and then diluted to a final concentration, 100 $\mu$g/100 $\mu$l. For binding studies, hypothalamus tissue membranes, 100 $\mu$l, were incubated with 400 $\mu$l incubation buffer (Tris-HCl buffer containing 1 mM bacitracin, 10 mM O-phenanthroline, 0.1% bovine serum albumin) containing 200 pM $^{125}$I-Sar$^1$, Ile$^8$-Ang II for 90 min at room temperature. Determinations were performed in triplicate. Non-specific, $AT_1$-specific, and $AT_2$ specific bindings were investigated in replicate tubes by co-incubation with 1 $\mu$M cold Ang II, 1 $\mu$M losartan, and 1 $\mu$M PD123319, respectively. All incubations were terminated by rapid filtration and washing with ice-cold Tris-HCl using a Brandel cell harvester and Whatman GF/B filters pre-soaked overnight in 1.5% BSA. Bound radioligand was quantified using a gamma counter (Beckmann, efficiency > 67%).

*Statistical analysis*

All data are presented as means ± SE. Unpaired *t*-test was used for the comparison of means of different groups. Differences were considered significant if $p < 0.05$.

**Table 2.** Changes in hypothalamic $AT_1$-specific density after carotid artery ligation

| Group | % change in specific binding |
|---|---|
| Sham | 100.0 ± 15.2 |
| Unilateral + 6 hrs | 75.8 ± 23.8 |
| Bilateral + 6 hrs | 68.7 ± 18.2 |
| Unilateral + 18 hrs | 115.8 ± 10.3 |

Sham, unilateral, and bilateral denote sham-operated, unilateral and bilateral carotid artery ligated groups, respectively. Data represent a percentage compared to sham values. Values are mean ± SE (n=6).

## RESULTS

The hypothalamic Ang II receptor density 6 hrs after either unilateral or bilateral carotid artery ligation decreased about 25 to 30% even though it failed to get statistical significance (Table 2). At this time, the ratio of $AT_1/AT_2$ was not different from that of sham-operated control, and $AT_1$ was the predominant subtype. Eighteen hrs after unilateral carotid artery ligation Ang II receptor density slightly increased compared to that of sham-operated control. In addition, $AT_2$ density was increased even though it was still lower

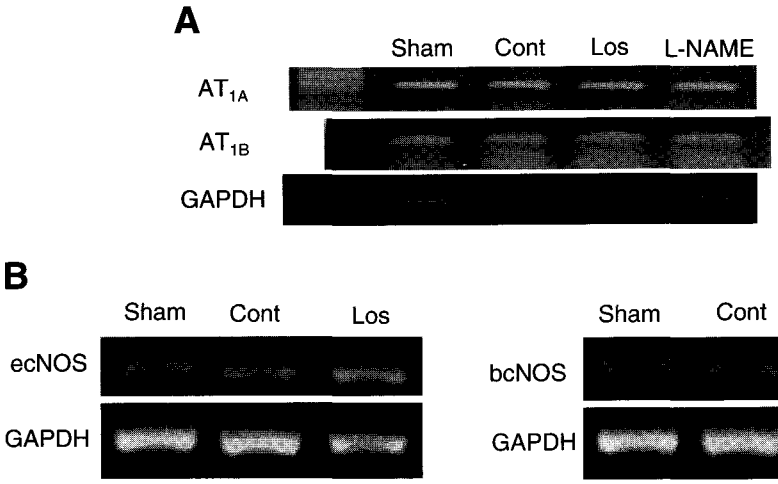


**Fig. 1.** The representitive ethidium bromide-staining gel of PCR products for $AT_{1A}$ (A, top pannel), $AT_{1B}$ (A, middle pannel), ecNOS (B, top left pannel), and ncNOS (B, top right pannel) in the hypothalamus from the rats 6 hrs after unilateral carotid artery ligation.

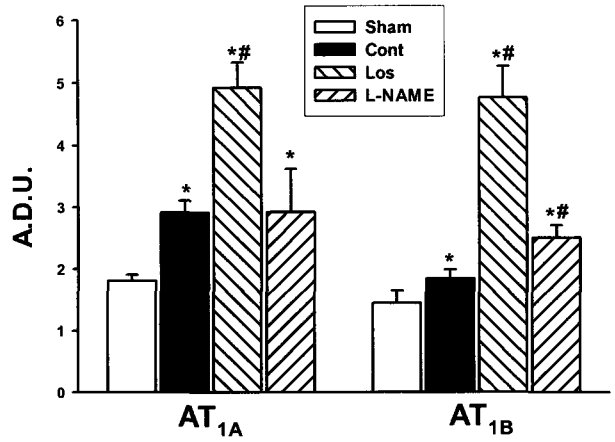


**Fig. 2.** Densitometric analysis of the PCR products for $AT_{1A}$ and $AT_{1B}$ corrected for GAPDH in each rat. The values were expressed in arbitary densitometric unit (A.D.U.). Sham, Cont, Los, L-NAME denote sham group and carotid artery ligated group without or with either losartan or L-NAME treatment, respectively. $^{*}<0.05$, compared to sham values. $^{\#}<0.05$, compared to carotid artery ligated values without treatment (n=4 in each group).

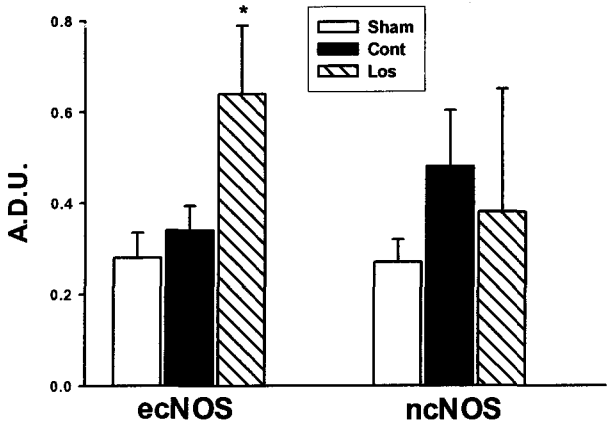


**Fig. 3.** Densitometric analysis of the PCR products for ecNOS and ncNOS corrected for GAPDH in each rat. The values were expressed in arbitary densitometric unit (A.D.U.). Sham, Cont, and Los denote sham group and carotid artery ligated group without or with losartan treatment, respectively. $^{*}<0.05$, compared to sham values (n=4 in each group).

than $AT_1$ density.

After common carotid artery ligation, mRNA levels of $AT_{1A}$ and $AT_{1B}$ were increased compared to those of sham-operated control (Fig. 1 and Fig. 2). The treatment of losartan induced further elevation of $AT_{1A}$ and $AT_{1B}$ mRNA levels (Fig. 2). $AT_{1B}$ gene expression was also increased by L-NAME treatment, but $AT_{1A}$ mRNA level was not affected by L-NAME (Fig. 2). Losartan treatment increased the gene expression of ecNOS profoundly, but it had no effects on gene expression of ncNOS following carotid artery ligation (Fig. 3).

## DISCUSSION

The present study demonstrated that the receptor

density of $AT_1$ decreased and the gene expression of $AT_{1A}$ and $AT_{1B}$ of rat PVN increased 6 hrs after unilateral carotid artery ligation. In addition, the elevation of $AT_{1B}$ mRNA levels was augmented by L-NAME treatment and the gene expression of ecNOS was increased by losartan treatment.

Both $AT_{1A}$ and $AT_{1B}$ mRNA levels were upregulated after carotid artery ligation. It might be a compensatory response to the decrease of $AT_1$ density by carotid artery ligation. Losartan treatment-induced augmentation of the increase of $AT_1$ mRNA level might also be due to the decrease of the number of active Ang II receptor by receptor blockade. L-NAME-induced upregulation of $AT_{1B}$ mRNA levels in hypothalamus was well matched with previous reports (Ichiki et al, 1998; Usui et al, 1998), which were done in other tissues. The NO-induced decrease of Ang II receptors was first reported in rat vascular smooth muscle cells by Cahill et al (1995). Ichiki et al (1998) also demonstrated a profound inhibition of $AT_1$ receptor mRNA expression in vascular smooth muscle cells exposed to a NO donor. They showed that the inhibitory effect of NO on $AT_1$ expression might occur at the transcriptional level. However, the transcription factor(s) that mediate this effect remain to be identified. In the rat adrenal gland, one week-treatment with the NOS inhibitor L-NAME enhanced both the mRNA expression and the number of $AT_1$ receptors, but did not influence the $AT_2$ receptor number (Usui et al, 1998). In contrast, one-week L-NAME treatment increased the number of both $AT_1$ and $AT_2$ receptors in the rat heart, with both receptor subtypes returning to baseline levels after 4 weeks of L-NAME treatment (Katoh et al, 1998). Under this experimental condition, injection of L-NAME into the PVN augmented the gene expression of $AT_{1B}$, but not increased that of $AT_{1A}$. It therefore appears that NO may affect the expression of the Ang II receptor subtypes in a tissue specific manner.

The ecNOS mRNA level was increased by losartan treatment. Timmermanns et al (1993) demonstrated an evolving course dissociation of Ang II and glomerular receptor density. They suggested that the down regulation of receptor density may represent the elevation of Ang II and its augmented activity. Under present experimental conditions, $AT_1$ density showed a tendency to decrease 6 hrs after carotid artery ligation compared to that of sham-operated control. It is therefore, supposed to increase Ang II activity. Olson et al (1997) first demonstrated a stimulatory effect of Ang II on ecNOS mRNA, protein, and NO production in cultured bovine pulmonary artery endothelial cells. They proposed that this effect might be a protective mechanism whereby lower pulmonary arterial pressure might be maintained in systemic hypertension. In Ang II infusion studies, Hennington et al (1998) reported that an acute infusion increased kidney ecNOS mRNA levels, but had no effect on ecNOS protein levels. Since this study was performed with whole-homogenized kidneys, the localization of these changes within the kidney remains to be determined. In addition, neither of these studies (Olson et al, 1997; Hennington et al, 1998) addressed the question of which Ang II receptor mediates the changes in ecNOS expression observed in response to Ang II. The results of the present study demonstrated that $AT_1$ may be involved in the regulation of ecNOS gene expression after carotid artery ligation in rat PVN.

In conclusion, the present study has shown here that the $AT_{1A}$ and $AT_{1B}$ mRNA levels of rat PVN increased after unilateral carotid artery ligation. In addition, the gene expression of ecNOS was increased by losartan and the elevation of $AT_{1B}$ mRNA level was augmented by L-NAME treatment. These results suggest that Ang II and NO in the rat PVN may interplay, at least in part, through regulation of gene expression of ecNOS and $AT_{1B}$, respectively.

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