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Role of Brain Angiotensin AT_1 Receptor in the Carbachol-Induced Natriuresis and Expression of nNOS in the Locus Coeruleus and Proximal Convoluted Tubule

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Summary

Central administration of losartan effectively blocked the increase of blood pressure and drinking response induced by angiotensin II (Ang II) or carbachol. However, the relationship between angiotensin AT_1 receptors and the natriuresis induced by brain cholinergic stimuli is still not clear. The purpose of the study is to reveal the role of brain angiotensin AT_1 receptor in the carbachol-induced natriuresis and expression of neuronal nitric oxide synthase (nNOS) in the locus coeruleus (LC) and proximal convoluted tubule (PCT). Our results indicated that 40 min after intracerebroventricular (ICV) injection of carbachol (0.5 μg), urinary sodium excretion was significantly increased to 0.548±0.049 $\mu mol \cdot min^{-1} \cdot 100\ g^{-1}$. Immunohistochemistry showed that carbachol induced an increase of neuronal nitric oxide synthase immunoreactivity (nNOS-IR) in the LC and renal proximal tubular cells. After pretreatment with losartan (20 μg), carbachol-induced urinary sodium excretion was reduced to 0.249±0.067 $\mu mol \cdot min^{-1} \cdot 100\ g^{-1}$. The same was true for carbachol-induced increase of nNOS-IR in the LC and PCT. The present data suggest that ICV cholinergic stimulation could induce a natriuresis and upregulate the activity of nNOS in the LC and PCT. The blockade of AT_1 receptors might downregulate the effects induced by carbachol in the LC and PCT. Consequently, we provide a new evidence that brain angiotensinergic pathway and NO-dependent neural pathway contribute to the natriuresis following brain cholinergic stimulation and thus play an important role in the regulation of fluid homeostasis. Furthermore, the final effect of nitric oxide on proximal tubular sodium reabsorption participated in the natriuresis induced by brain cholinergic stimulation.

Key words

AT_1 receptor • Neuronal nitric oxide synthase • Carbachol • Locus coeruleus • Proximal convoluted tubule

Introduction

Injection of cholinergic agonists into the lateral cerebral ventricle, medial septal area (MSA), subfornical organ (SFO) was found to induce natriuresis (Fitts and Simpson 1986, Colombari *et al.* 1992a,b). Similar effects were obtained after injection of angiotensin II (Ang II) into the anteroventral third ventricle region (AV3V), paraventricular nucleus (PVN), SFO and MSA (Fitts and Simpson 1986, De Arruda Camargo and Saad 1999, Camargo and Saad 2001, Saad *et al.* 2002). Previous studies have revealed that the central administration of losartan, a specific AT_1-receptor blocker, effectively blocked the increase of blood pressure and drinking

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response induced by Ang II or carbachol (Colombari *et al.* 1992a,b, De Arruda Camargo and Saad 1999, Thunhorst and Johnson 1993, Saad *et al.* 1997, 2004). These data implicated that the brain angiotensinergic pathway interacted with the brain cholinergic pathway in the control of drinking and blood pressure. However, it is not clear whether the angiotensin AT_1 receptor is related to the natriuresis induced by brain cholinergic stimuli.

Locus coeruleus (LC), an important integrated site in the pons, regulates sympathetic nerve activity, fluid balance and arginine vasopressin (AVP) release, affecting sodium excretion *via* renal nerves and water excretion *via* AVP release at rest and during volume challenges (De Luca *et al.* 1990, McCann *et al.* 1997). Cholinergic stimulation in the SFO, lateral preoptic area (LPO), ventromedial hypothalamus (VMH), LC and septal area resulted in natriuresis and diuresis (Fitts and Simpson 1986, Colombari *et al.* 1992a,b, De Luca *et al.* 1990). Neuronal nitric oxide synthase (NOS)-positive neurons were found in the LC (Xu *et al.* 1998) and NO-dependent neural pathway was involved in the natriuresis induced by blood volume expansion (Li *et al.* 2003). However, it is not clear whether NO-dependent neural pathway is involved in the renal sodium excretion induced by ICV injection of cholinergic agonist.

The primary role of the kidney is to maintain the volume and electrolyte composition of body fluids and, in this way, to regulate the blood pressure. Recent studies indicated that renal nitric oxide is an important factor in the control of urinary sodium excretion. NO might enhance natriuresis by inhibiting transport along the nephron as well as by altering renal hemodynamics (Manning and Hu 1994, Krier and Romero 1998, Noonan and Banks 1999). Renal medullary infusion of 7-nitro-indazole (7-NI), a specific inhibitor of nNOS, decreased medullary nNOS activity by 37 % and increased arterial blood pressure in Sprague-Dawley rats on high sodium intake but not on normal sodium intake (Manning *et al.* 2001). This suggested that nNOS might enhance renal sodium excretion and thus help to prevent salt-sensitive hypertension. However, the role of renal nNOS in the natriuresis induced by brain cholinergic stimuli remains to be investigated.

ICV injection of nitro-L-arginine methyl ester (L-NAME), an inhibitor of NOS, antagonized drinking in response to ICV injection of Ang II, and this effect could be abolished by ICV injection of L-arginine, implicating that Ang II interacted with NO in the control of drinking (Mathai *et al.* 1998). The increase of mean arterial blood pressure, renal sodium and potassium excretion in response to ICV injection of hypertonic saline could be completely or partially abolished by AT_1 receptors blocker, losartan (Skott 2003).

Based on these findings, it could be speculated that there is an interaction between central angiotensinergic pathway and central NO-dependent neural pathway in the renal sodium excretion caused by brain cholinergic stimuli. To investigate these, we tested 1) the effect of blocking brain angiotensin AT_1 receptors by losartan (20 μg) on renal sodium excretion after ICV injection of carbachol (0.5 μg), and 2) the effect of blocking brain angiotensin AT_1 receptors by losartan (20 μg) on the changes of nNOS-IR in the LC and kidney induced by ICV injection of carbachol.

Methods

Animals

Healthy male Sprague-Dawley rats (Dalian Medical University Animal Center, China) weighing 250-280 g were kept on a 12 h/12h light/dark schedule with a free access to standard laboratory food and water at room temperature.

Experimental protocols

Forty-eight rats were anesthetized with ether. A 23 gauge guide cannula was implanted in the right lateral ventricle using a stereotaxic apparatus. Coordinates for ICV injection were based on the atlas of Paxinos and Watson with the skull horizontal: posterior 1.0 mm from the bregma, lateral 1.5 mm from the midline, deep 3.0 mm from the skull. The cannula was fixed to the skull with super glue and dental cement. Another stylette (31 gauge) was placed in the guide cannula to prevent occlusion. The rats were housed individually and were allowed to recover for one week after the surgery. During this time, stylettes were checked regularly and rats were handled daily to avoid stress-induced expression on the day of the experiment.

ICV injection protocols

All ICV injections were made between 8:00 and 9:00 a.m. to exclude the interference of circadian rhythms of nNOS expression with the experiment. All injected solutions were made in the volume of 5 μl over a period of 2 min with an additional 2 min allowed to elapse prior to the removal of infusion needles and replacement of the cannulas.

Twenty-four rats for the experiment *in vivo* were divided randomly into four groups (n=6 per group): physiological saline (NS) + carbachol (CBC) group, losartan (Los) + CBC group, Los + NS group and NS + NS group. NS + CBC group received ICV injection of 0.9 % NaCl (5 μl), followed by ICV injection of 0.1 μg/μl carbachol (5 μl) 20 min later. The treatment of other groups was similar to the NS + CBC group. The total injected volume was 10 μl (5 μl the first drug, followed by 5 μl the second drug 20 min later). Another 24 rats for immunohistochemistry were also divided into 4 groups and treated as described in the experiment *in vivo*.

Experiment in vivo

Animals were anesthetized with urethane (1 ml/100 g b.w., i.p.). The right external jugular vein, the left carotid artery and urinary bladder were cannulated with a polyethylene catheter for intravenous (i.v.) drug administration, MAP recording and urine collection, respectively. After surgery, an infusion of 0.9 % NaCl (0.025 $ml \cdot min^{-1} \cdot 100\ g^{-1}$) was started and maintained for one hour to allow the stabilization of hemodynamic and renal parameters. The first 60 min following stabilization was considered as the reference period and the following 120 min were monitored after the final injection.

In all groups, urine samples were collected every 20 min over the experimental period. MAP was recorded with PcLab polygraph. Urine volume was determined gravimetrically and urinary sodium concentration was measured by Easylyte plus Na/K/Cl Analyzers (Medica Corporation, USA).

Immunohistochemistry analysis

Forty minutes after the final injection, 24 rats were deeply anesthetized with 4 % chloral hydrate (400 mg/kg b.w., i.p.) and perfused transcardially with 1 % and 4 % paraformaldehyde for the fixation of the brain and kidney tissue. Brain and kidney tissue were removed, post-fixed in 4 % paraformaldehyde and immersed into a phosphate buffer saline (PBS) containing 30 % sucrose for three days.

When the brain tissues were submerged, 50 μm thick coronal brain sections were sliced on a vibratome. The identification of LC (bregma –9.68 mm ~ –10.04 mm) was based on the atlas by Paxinos and Watson. The sections above were rinsed three times in PBS 10 min and then incubated with 0.3 % H_2O_2 for 1 h. The sections were rinsed three times in PBS 10 min and incubated with bovine serum albumin (2 % BSA) (Sigma Co., USA) for 1 h. Thereafter the sections were incubated in the primary antibody (nNOS-Ab, 1:100, Boster Company, P.R. China) overnight at 4 °C. The sections were then rinsed three times in PBS for 10 min and incubated with 2 % BSA for 1 h. Subsequently, the sections were rinsed three times in PBS for 10 min, and further incubated in the biotinylated-second antibody (Boster Company, P.R. China) at room temperature for 2 h. Finally, the sections were again rinsed three times in PBS for 10 min and incubated with the avidin-biotin complex ABC (Boster Company, P.R. China) at room temperature for 2 h. Diaminobenzidine (DAB, Sigma Co., USA) was used for signal detection. The control sections were incubated with PBS instead of primary antibody. The HPIAS series colorful pathology photographic system was used to analyze nNOS-IR positive neurons. The brain sections were observed in a 20x magnification. The number and optical density of nNOS-IR positive neurons were calculated per area and per group.

Kidney tissue was frozen in liquid nitrogen and then mounted in OCT mounting media. Cryostat sections (8 μm thick) were cut at –12 °C and placed onto low-iron clear glass slides. Sections were washed three times in PBS for 10 min, then incubated in 30 % H_2O_2 in methanol for 10 min. Sections were then washed with PBS and incubated in 0.4 % pepsin at 37 °C for 1 h. Following three times 10-min washing with PBS, sections were incubated in the primary antibody (nNOS-Ab, 1:250, Boster Company, P.R. China) overnight at 4 °C. Sections were then washed with PBS and further incubated in the biotinylated-second antibody (Boster Company, P.R. China) at room temperature for 1 h. The sections were then rinsed three times in PBS for 10 min and incubated with avidin-biotin complex ABC (Boster Company, P.R. China) at room temperature for 1 h. Diaminobenzidine (DAB, Sigma Company, USA) and hematoxylin were used to detect the signal. The control sections were incubated with PBS instead of the primary antibody. Image Pro Plus image analysis system was used to analyze nNOS-IR positive granules. Kidney sections were observed in a microscope at 20x magnification. Optical density of nNOS-IR positive granules was calculated per area and per group.

Statistical analysis

All data were expressed as mean ± S.E.M. Statistical evaluation was done using ANOVA with *post hoc* test of LSD in Equal Variances Assumed. In all comparisons, statistical significance was set at $P<0.05$.

Results

Effect of pretreatment with losartan on the natriuresis induced by ICV injection of carbachol

After ICV injection of 0.5 μg carbachol in the NS + CBC group, urinary sodium excretion was immediately increased at 20 min, reached the peak 0.548±0.049 μmol·min^{-1}·100 g^{-1} at 40 min, and the enhancement in sodium excretion lasted for about 100 min (Fig. 1). However, this effect was significantly attenuated after the pretreatment with 20 μg losartan in the Los+CBC group, compared with NS+CBC group, reaching only 0.249 ±0.067 μmol·min^{-1}·100 g^{-1} at the peak response. However, urinary sodium excretion in Los+CBC group was still higher than that of the NS+NS group ($P<0.05$, Fig. 1). Injection of 20 μg losartan into the lateral ventricle (Los+NS group) did not induce a significant change in urinary sodium excretion within 120 min observed, compared with that in the NS+NS group.

The above results indicated that the blockade of brain angiotensin AT_1 receptor partially inhibited the natriuresis induced by carbachol.

Effect of losartan pretreatment on the changes of nNOS-IR in the LC induced by ICV injection of carbachol

In the pons of NS+NS group, nNOS-IR positive neurons were distributed predominantly in the LC (Fig. 2C). The most striking difference in nNOS-IR positive neurons in NS+CBC group compared with the NS + NS group was a significant increase in optical density and number of nNOS-IR staining neurons in the LC. This was illustrated by comparing Figure 2A (NS+CBC group) with Figure 2C (NS+NS group). There was also a significant decrease of optical density and number of nNOS-IR positive neurons in the LC of Los+CBC group (Fig. 2B) when compared with NS+CBC group. nNOS-IR in the LC of Los+NS group was similar to that in NS+NS group. The data on nNOS-IR in the LC were summarized in Figure 3.

Effect of losartan pretreatment on the changes of nNOS-IR in PCT induced by ICV injection of carbachol

Figure 4 shows the results of immunohisto-chemistry study for nNOS-IR in the kidney tissue. In the NS+NS group, nNOS-IR positive granules were detected very weakly in proximal convoluted tubular cells (Fig. 4C). Forty min after ICV injection of carbachol (0.5 μg), it was evident that nNOS-IR positive granules

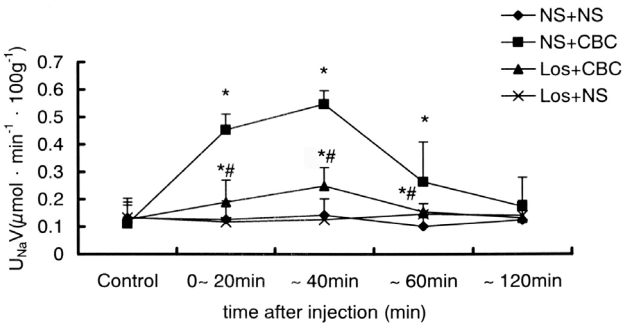

Fig. 1. Effect of pretreatment with losartan (20 μg) on the natriuresis induced by ICV injection of carbachol (0.5 μg). Data are shown as mean ± S.E.M. n=6 (* $P<0.05$ vs. NS+NS group, # $P<0.05$ vs. NS+CBC group).

were detected mainly in proximal convoluted tubular cells (Fig. 4A). Optical density of nNOS-IR positive granules in the NS+CBC group was significantly increased in renal proximal tubular cells compared with that in the NS+NS group ($P<0.05$, Fig. 5). There was a significant decrease in optical density of nNOS-IR positive granules in renal proximal tubular cells in the Los+CBC group (Fig. 4B), compared with that in the NS+CBC group. No significant difference was found in optical density of nNOS-positive granules in the kidney, when the Los+NS group (Fig. 4D) was compared with the NS+NS group. The average data are summarized in Figure 5.

These results indicated that ICV injection of carbachol resulted in an increase of nNOS-IR both in the LC and in proximal convoluted tubular cells. Blockade of brain angiotensin AT_1 receptor partially inhibited the changes in the LC and proximal convoluted tubular cells induced by carbachol.

Discussion

A number of studies have shown that the administration of ICV injection of Ang II produced persistent water drinking and natriuresis (Saad *et al.* 2002), whereas pretreatment with losartan (5 μg), the angiotensin AT_1 receptor antagonist, reduced the natriuretic effect of hypertonic saline (Skott 2003). In the present study, we observed that the natriuresis and diuresis induced by ICV injection of carbachol were blunted by losartan pretreatment, indicating that brain angiotensin system contributed to the natriuretic effect elicited by brain cholinergic stimulation. Furthermore, we also observed that ICV injection of carbachol induced an increase of nNOS-IR in the LC. Our results

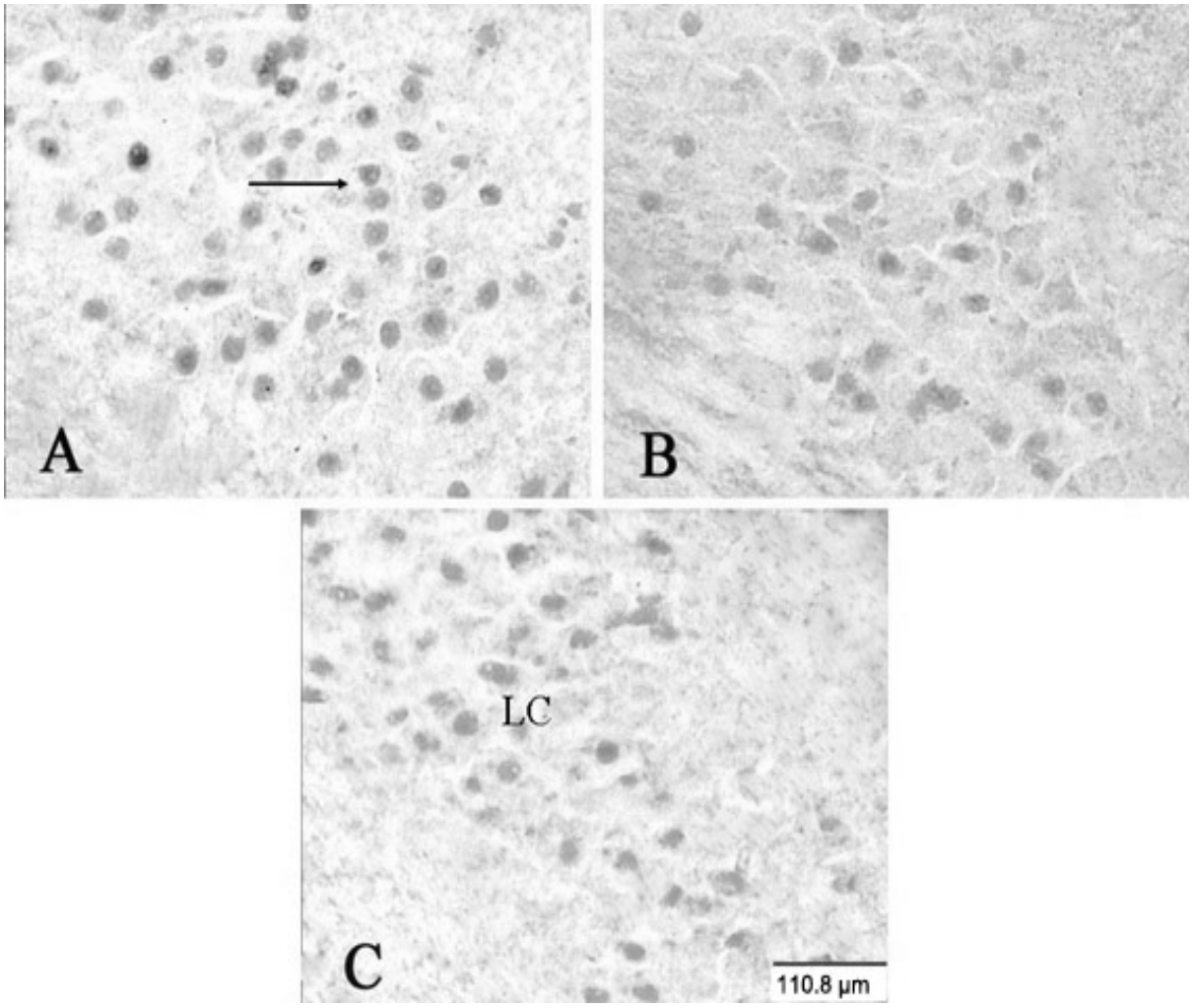

Fig. 2. Effect of pretreatment with losartan (20 μg) on the changes of nNOS-IR in the LC induced by ICV injection of carbachol (0.5 μg). A: NS+CBC group; B: Los+CBC group; C: NS+NS group. Bar indicates 110.8 μm, arrow points to nNOS-IR positive neurons.

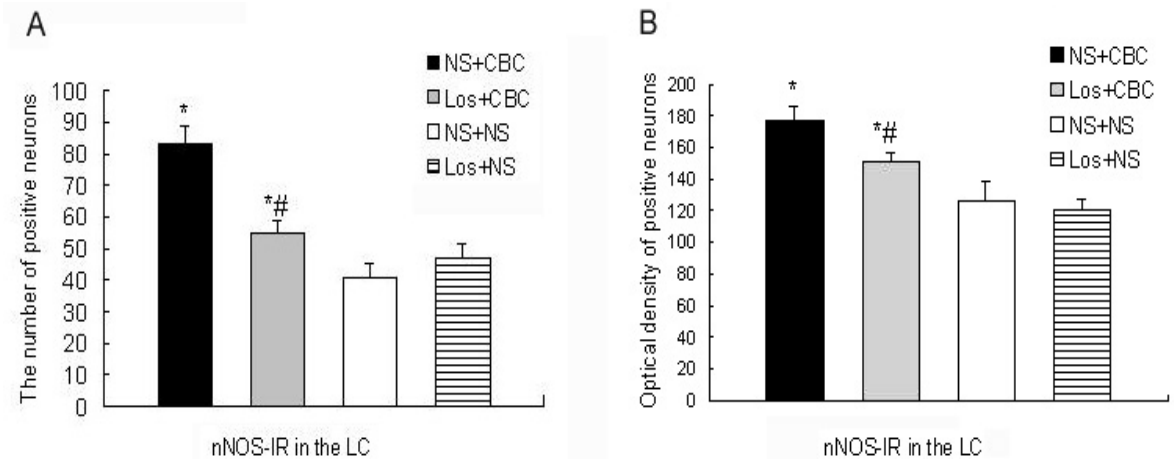

Fig. 3. Quantitative analysis of the effect of pretreatment with losartan (20 μg) on the changes of nNOS-IR in the LC induced by ICV injection of carbachol (0.5 μg). A: the number of nNOS-IR positive neurons. B: optical density of nNOS-IR positive neurons. Data are mean ± S.E.M. n=6 (* $P<0.05$ vs. NS+NS group, $^{\#}$ $P<0.05$ vs. NS+CBC group).

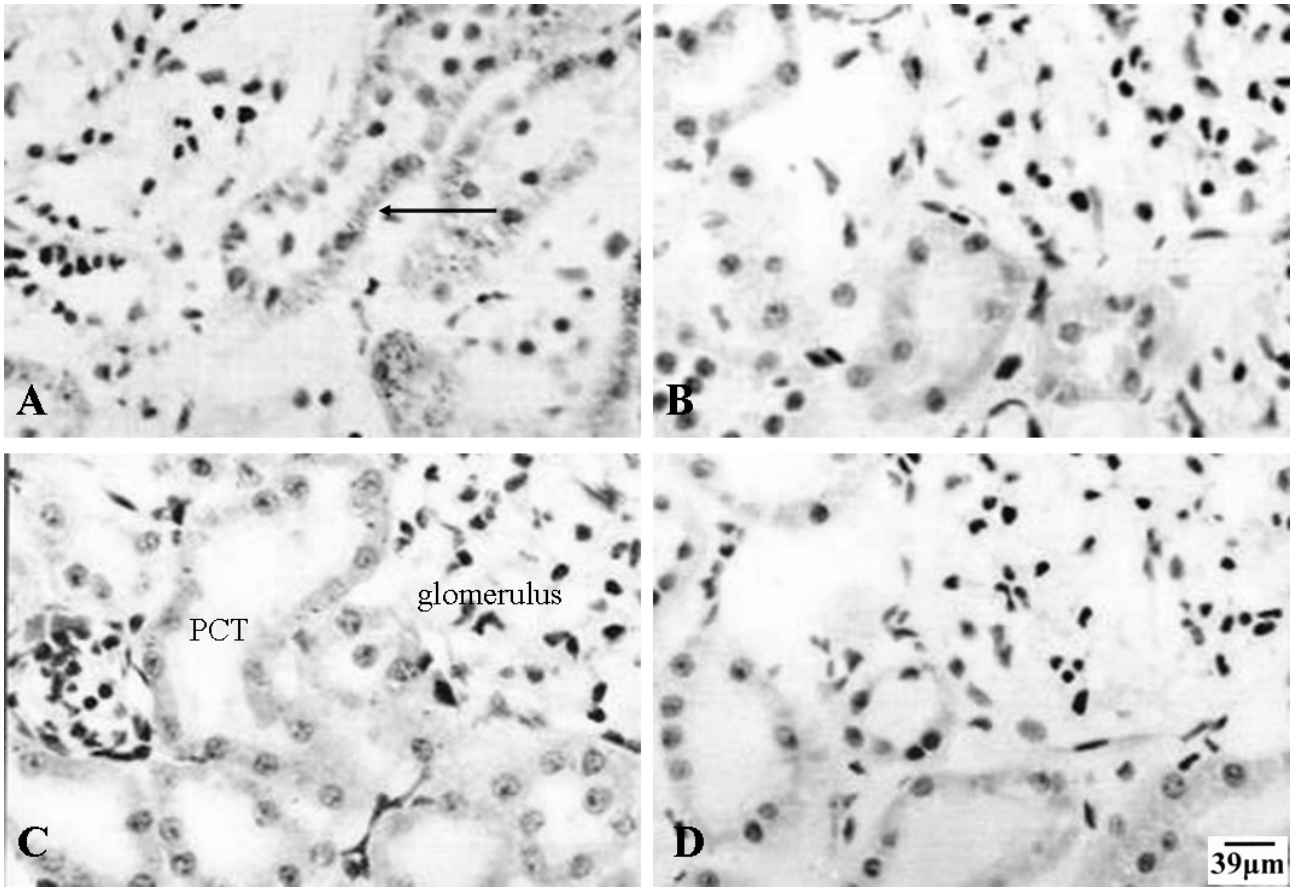

Fig. 4. Effect of pretreatment with losartan (20 μg) on the changes of nNOS-IR in PCT induced by ICV injection of carbachol (0.5 μg). A: NS+CBC group; B: Los+CBC group; C: NS+NS group; D: Los+NS group. Bar indicates 39 μm, arrow points to nNOS-IR positive granules.

demonstrated that NO-dependent neurons in the LC were excited after ICV injection of carbachol. In accordance with this, the activation of the L-arginine-NO pathway by injection of L-arginine into the lateral cerebral ventricle of water-deprived rats stimulated drinking (Mathai *et al.* 1998). Acute volume expansion produced a significant increase in urine flow and sodium excretion, which was diminished in L-NAME-treated rats subjected to volume expansion (Li *et al.* 2003). These results suggested that NO-dependent neural pathway in the LC was involved in the natriuresis induced by brain cholinergic stimuli. Experiments with rat neuronal cultures have demonstrated that carbachol caused a time- and concentration-dependent increase in cyclic guanidine monophosphate (cGMP) levels, which was antagonized by atropine. This response was depressed by NOS inhibitors (De Wardener 2001), which suggested that the stimulation of muscarinic receptors increased NO production in the cerebral cortex. Therefore, our finding indicated that muscarinic receptor might participate in the expression of nNOS in the LC induced by ICV injection of cholinergic agonist.

Previous *in vivo* studies have indicated that Ang II administered peripherally or volume expansion evoked the activity of Fos and nNOS in PVN (Dawson *et al.* 1998). Studies using the RT-PCR technique revealed a similar distribution of nNOS mRNA levels and AT_1 receptor mRNA levels in the rat brain (Križanová *et al.* 2001). The expression of nNOS and AT_1 receptors in the same brain regions might represent a potential basis of NO and Ang II interaction at the level of AT_1 receptors. Besides its peripheral actions, Ang II also exerts central effects. The immediate response to central Ang II administration involves an increased release of arginine vasopressin (AVP), modulation of sympathetic nerve activity and drinking behavior. It has been established that Ang II acting through AT_1 receptors localized in the SFO, SON and PVN induced the response linked with the central control of blood pressure, salt and water homeostasis (Blume *et al.* 2002). The careful examination of our data presented in Figure 2 has revealed that the inhibition of brain angiotensin AT_1 receptors abolished the changes of nNOS-IR in the LC induced by ICV injection of carbachol. This finding indicated that the

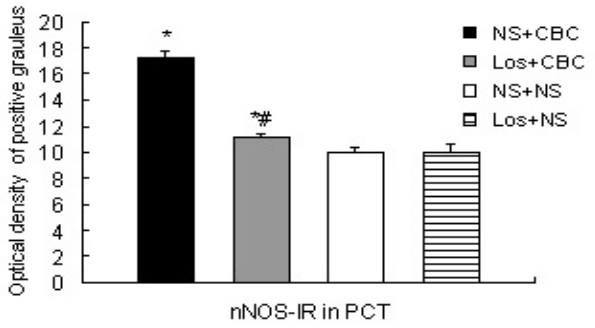

Fig. 5. Quantitative analysis of effect of pretreatment with losartan (20 μg) on the changes of nNOS-IR in PCT induced by ICV injection of carbachol (0.5 μg). Data are mean ± S.E.M. n=6 (* P<0.05 vs. NS+NS group, # P<0.05 vs. NS+CBC group).

blockade of brain angiotensin AT_1 receptor might downregulate the effects in the LC induced by carbachol. It has recently been reported that losartan prevented the stimulating effect of endogenous Ang II on NO synthesis during the development of hypertension, in part by binding selectively to AT_1 receptors (Qadri *et al.* 2003). Combining with our results, we hypothesized that after ICV injection of carbachol, the release of endogenous Ang II enhanced the expression of nNOS *via* activation of AT_1 receptors in the LC.

A large number of results describing the effects of nNOS on renal sodium excretion has been reported (Mattson and Bellehumeur 1996, Liang and Knox 2000, Ortiz and Garvin 2002). In the last few years, a series of *in vivo* and *in vitro* studies have indicated a close relationship between nitric oxide and the proximal tubule.

The proximal tubule, reabsorbing approximately two-thirds of Na^+ and water filtered by the glomerulus, is quantitatively the most important nephron site for Na^+ and water reabsorption. A recent microdialysis study has shown that there was detectable NO, measured by hemoglobin trapping in the microdialysis from the normal rat renal cortex and medulla (Liang and Knox 2000). These data indicated that proximal tubules *in vivo* are constantly exposed to NO that can affect the function of the proximal tubule. *In vivo* infusion of acetylcholine, which caused NO release into the renal circulation resulted in an increase of urinary volume and sodium excretion. The decrease of water and sodium excretion was observed when the inhibitor of endogenous NO production was infused into the kidney (Ortiz and Garvin 2002). The natriuretic and diuretic effects of NO were not accompanied by proportional changes in glomerular filtration rate or renal blood flow, indicating that NO regulated Na^+ transport through the renal tubule, but not *via* renal hemodynamics. The results of the present study also indicated that ICV injection of cholinergic agonist carbachol could upregulate the activity of nNOS in proximal convoluted tubule cells. The blocker of AT_1 receptor might downregulate the actions mentioned above. Thus the above evidence suggested that nNOS in the kidney and brain angiotensinergic pathway might participate in the natriuresis induced by ICV injection of cholinergic agonist.

Other investigators have reported that the incubation of human proximal tubular cells with atrial natriuretic factor produced a dose- and time-dependent increase in NO production (Eitle *et al.* 1998). Ang II increased the cytosolic calcium concentration and the NO production in rabbit macula densa cells. AT_1 antagonist CV-11974 blocked the Ang II-induced calcium response and NO response, but the AT_2 antagonist did not block it on either side. 7-nitroindazole totally inhibited the increase of NO production caused by Ang II (Liu and Persson 2004). These data suggested that the increase of cytosolic calcium in macula densa cells by Ang II activated the nNOS to increase the production of NO in a dose-dependent way.

Nitric oxide has been shown to play an important role in various physiological processes in the kidney, including salt and fluid reabsorption. The mechanism of NO action in the kidney has been studied to some extent. One possibility is that NO in renal proximal tubular cells could activate guanylate cyclase (GC) (Mathai *et al.* 1998, Eitle *et al.* 1998) and cGMP exerts its effect through the inhibition of Na/K-ATPase (Ortiz and Garvin 2002, Wongmekiat and Johns 2002), located on the basolateral membrane of renal proximal tubule cells, to decrease NaCl absorption, and lead to the natriuresis. The second possibility is that renal nerves might play a role in the natriuresis and diuresis. It has been reported that nerve bundles were associated with nNOS expressed in cortical tubules of the rat kidney, especially the proximal tubule received nerve terminals. Stimulation of renal sympathetic nerves enhanced water and Na^+ reabsorption in PCT (Wongmekiat and Johns 2001). A low dose of L-NAME (3.7 $nmol \cdot kg^{-1} \cdot min^{-1}$) reduced urinary sodium excretion in rats with innervated kidneys, whereas it had no effects in rats with denervated kidneys (Liang and Knox 2000, Wongmekiat and Johns 2001). These results indicated that NO together with renal sympathetic nerve participate in the regulation of tubular water and Na^+ reabsorption. On the basis of the above

results, we hypothesized that after carbachol administered into the lateral cerebral ventricle, the release of endogenous Ang II activated AT_1 receptors predominantly in the LC or other brain nuclei known to subserve cardiovascular and fluid homeostasis. Then the excited NO-dependent neurons in the LC in some pathway, might contribute to the natriuresis. Meanwhile, the release of endogenous Ang II, which stimulated AT_1 receptors, might activate nNOS in the kidney, and then inhibit the Na/K-ATPase in PCT, decrease the reabsorption of Na^+ and HCO_3^- in PCT, and result in the natriuresis.

In conclusion, this study demonstrated that the brain angiotensinergic pathway and NO-dependent neural pathway contributed to the natriuresis elicited by brain cholinergic stimulation and thus play an important role in the regulation of fluid homeostasis. In addition, the final effect of NO on proximal tubular sodium reabsorption participated in the natriuresis induced by brain cholinergic stimulation. Further studies are required to elucidate cellular and molecular physiological mechanisms involved in the above effects.

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