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The Role of Brain Angiotensinergic AT_1 Receptor in the Carbachol-Induced Natriuresis and Expression of nNOS in the Locus Coeruleus (LC) and Proximal Convoluted Tubule (PCT)

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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 angiotensinergic AT_1 receptors and the natriuresis induced by brain cholinergic stimuli is still unclear. The purpose of the study is to reveal the role of brain angiotensinergic 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 (0.5μg), urinary sodium excretion was significantly increased to (0.548±0.049) μmol/min·100g^{-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 20μg, urinary sodium excretion was reduced to (0.249±0.067) μmol/min·100g^{-1}, and nNOS-IR in the LC and PCT induced by carbachol were also reduced. The present data suggested that ICV cholinergic stimulation could induce the natriuresis and up regulate the activity of nNOS in the LC and PCT. The blockade of AT_1 receptor might down regulate the effects in the LC and PCT induced by carbachol. Consequently, we provided new evidence that brain angiotensinergic pathway and NO-dependent neural pathway contributed to renal changes in the natriuresis to brain cholinergic stimuli and thus played an important role in the regulation of fluid homeostatic. In addition, the final effect of nitric oxide (NO) on proximal tubular sodium reabsorption participated in the natriuresis induced by brain cholinergic stimuli.

Key words

AT_1 receptor • neuronal nitric oxide synthase • carbachol • locus coeruleus • proximal convoluted tubule

Introduction

Injection of cholinergic agonists into lateral cerebral ventricle, medial septal area (MSA), subfornical organ (SFO) was found to produce the natriuresis (Fitts et al. 1986, Colombari et al. 1992, Colombari et al.1992). 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 et al.1986, De Arruda Camargo and Saad 1999, Saad et al. 2002, Camargo and Saad 2001). 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 response induced by Ang II or carbachol (Colombari et al.1992, De Arruda Camargo and Saad 1999, Thunhorst and Johnson 1993, Saad et al.1997, Saad et al.2004). These data implicated that brain angiotensinergic pathway interacted with brain cholinergic pathway in the control of drinking and blood pressure. However, it is not clear whether the angiotensinergic 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 (McCann et al.1997, De Luca et al.1990). Cholinergic stimulation in the SFO, lateral preoptic area (LPO), ventromedial hypothalamus (VMH), LC and septal area resulted in the natriuresis and diuresis, indicating a correlation of cholinergic stimulation in the LC, septal area, hypothalamus with the natriuresis (Fitts et al.1986, Colombari et al.1992, Colombari et al.1992, De Luca et al.1990). Neuronal nitric oxide synthase (NOS) positive neurons were found presence 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 fluid and, in

that way, to regulate the blood pressure. Recent studies indicated that renal nitric oxide was an important controller of urinary sodium excretion. NO might enhance the natriuresis by inhibiting transport along the nephron as well as altering renal hemodynamics (Noonan and Banks 1999, Manning and Hu 1994, Krier and Romero 1998). Renal medullary infusion of 7-nitroindazole (7NI), a specific inhibitor of nNOS, decreased medullary nNOS activity by 37% and increased arterial blood pressure in SD 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-loading hypertension. However, the role of nNOS in kidney in the natriuretisis 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 the effect could be abolished by ICV injection of L-arginine, implicating that Ang II interacted with NOS 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 diminished by AT_1 receptors blocker, losartan (Skott 2003).

Based on these findings, it could be speculated that there was 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 angiotensinergic AT_1 receptors by losartan (20μg) on renal sodium excretion after ICV injection of carbachol (0.5μg). (2) the effect of blocking brain angiotensinergic AT_1 receptors by losartan (20μg) on the changes of nNOS-IR in the LC and kidney induced by ICV injection of carbachol.

Methods

Animals

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

Experimental protocols

48 rats were anesthetized with aether. 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 skull flat: posterior 1.0mm from the bregma, lateral 1.5mm from the midline, deep 3.0mm from the skull. The cannula was fixed to the skull with super glue and the dental crement. Another stylette (31 gauge) was placed in the guide cannula to prevent occlusion. The rats were housed individually and were allowed a one week recovery period after 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 and 9 a.m. to exclude the interference of circadian rhythms of nNOS expression with the experiment. All injected solutions were made in the volume of 5μl over a period of 2 min with an additional 2 min allowed to elapse prior to removal of the infusion needles and replacement of the stylettes.

24 rats for the experiment in vivo were divided randomly into 4 groups (n=6 per group): NS +CBC group, Los+ CBC group, Los + NS group and NS+ NS group. NS+CBC group received ICV injection of 0.9% NaCl (5 μl), 20 min followed by ICV injection of 0.1 μg/μl carbachol (5 μl). The treatment of other groups was similar to NS+CBC group. The total injected volume was 10 μl (5μl the first drug, 20 min followed by 5μl the second drug). 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 anaesthetized with urethane (1ml/100g BW, 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 ($0.025ml/min \cdot 100g^{-1}$) was started and maintained for 1 h to allow stabilization of haemodynamic and renal

parameters. The first 60 min following stabilization was considered the based period and the following 120 min was observed after the final injection.

In all groups, urine samples were collected every 20 min over the experiment 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 min after the final injection, 24 rats were deeply anesthetized with 4% Chlorali Hydras (400 mg/kg BW, 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 in 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 -9.68mm ~ -10.04mm) was based on the Paxinos and Watson atlas. The sections above were rinsed first in PBS 10 min×3, then incubated with 0.3%H_2O_2 for 1 h. The sections were rinsed in PBS 10 min×3, then incubated with bovine serum albumin (2%BSA) (Sigma Company, USA) for 1 h. The sections were incubated in the primary antibody (nNOS-Ab, 1:100, Boster Company, P.R. China) overnight at 4°C. The sections were rinsed in PBS 10 min×3, then incubated with 2%BSA for 1 h. The sections were rinsed in PBS 10 min×3, and further incubated in the biotinylated-second antibody (Boster Company, P.R. China) at room temperature for 2 h. Then, the sections were rinsed in PBS 10 min×3 and followed by incubating with avidin–biotin complex ABC (Boster Company, P.R. China) at room temperature for 2 h. Diaminobenzidine (DAB; Sigma Company, USA) was used to detect signals. The control sections were incubated with PBS instead of primary antibody. HPIAS series colorful pathology photograph system was used to analyze nNOS-IR positive neurons. The brain sections were observed in 20×microscope. The number and optical density of nNOS-IR positive neurons were calculated per area and per group.

Kidney tissues were frozen in liquid nitrogen and then mounted in OCT mounting media. 8-μm-thick

cryostat sections were cut at -12°C and placed onto low iron clear glass slides. Sections were washed in PBS 10 min×3, then incubated in 30%H_2O_2 in methanol for 10 min. Sections were then washed with PBS and then incubated in 0.4% pepsin at 37°C for 1 h. Following 10 min×3 washed 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. Then, the sections were rinsed in PBS 10 min×3 and followed by incubating with avidin–biotin complex ABC (Boster Company, P.R. China) at room temperature for 1 h. Diaminobenzidine (DAB; Sigma Company, USA) and haematoxylin were used to detect signals. The control sections were incubated with PBS instead of primary antibody. Image Pro Plus image analysis system was used to analyze nNOS-IR positive granules. Kidney sections were observed in 20×microscope. 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 Equar 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 carbachol 0.5 μg in NS+CBC group, urinary sodium excretion was immediately increased at 20 min, reached the peak [(0.548±0.049) μmol/min·100g $^{-1}$] at 40 min, and the enhancement in sodium excretion lasted for 100 min (Fig. 1). However, this effect was significantly attenuated after pretreatment with losartan 20 μg in Los+CBC group, compared with NS+CBC group, reaching only (0.249 ±0.067) μmol/min·100g $^{-1}$ at the highest response level. But the value of urinary sodium excretion in Los+CBC group was still higher than that of the NS+NS group ($P < 0.05$, Fig. 1). Injection of losartan 20 μg 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 NS+NS group.

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

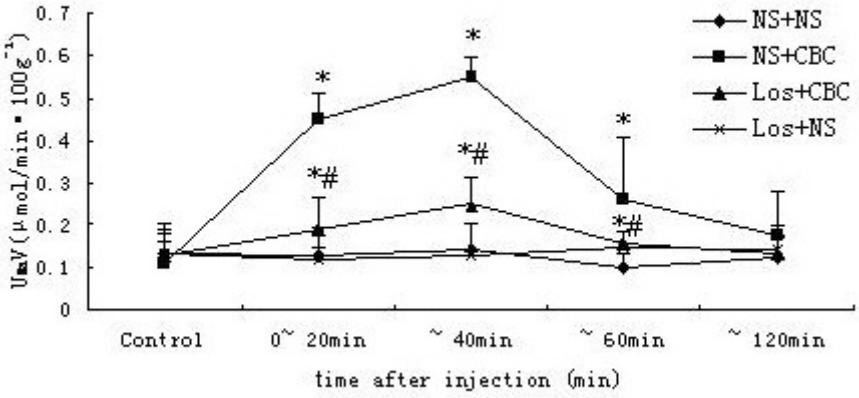

Fig. 1. Effect of pretreatment with losartan (20 μg) on the natriuresis induced by ICV injection of carbachol (0.5 μg). Data are reported as mean ± SEM. N= 6 (*P < 0.05 vs. NS+NS group, $^{\#}P$ < 0.05 vs. NS+CBC group)

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 differences in nNOS-IR positive neurons in NS+CBC group compared with NS+NS group were a significant increase in optical density and number of nNOS-IR staining neurons in the LC. This was illustrated by comparing Fig.2A (NS+CBC group) with Fig. 2C (NS+NS group). There also appeared to be a significant decrease of optical density and number of nNOS-IR positive neurons in the LC in Los+CBC group (Fig. 2B) when compared with NS+CBC group. nNOS-IR in the LC in Los+NS group was similar to that in NS+NS group. The data in the LC were summarized in Fig. 3.

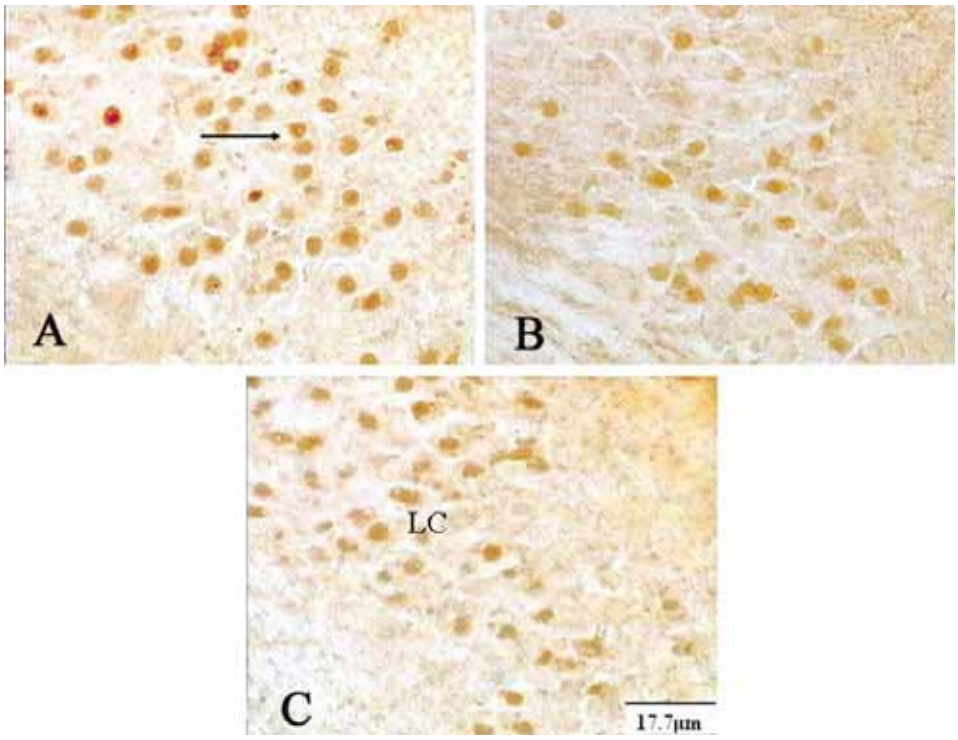

Fig. 2. Effect of pretreatment with losartan (20 μg) on the changes of nNOS-IR in the LC induced by ICV injection of carbachol (0.5 μg). A: NS+CBC group; B: Los+CBC group; C: NS+NS group. Bar= 17.7μm

⟶ nNOS-IR positive neurons

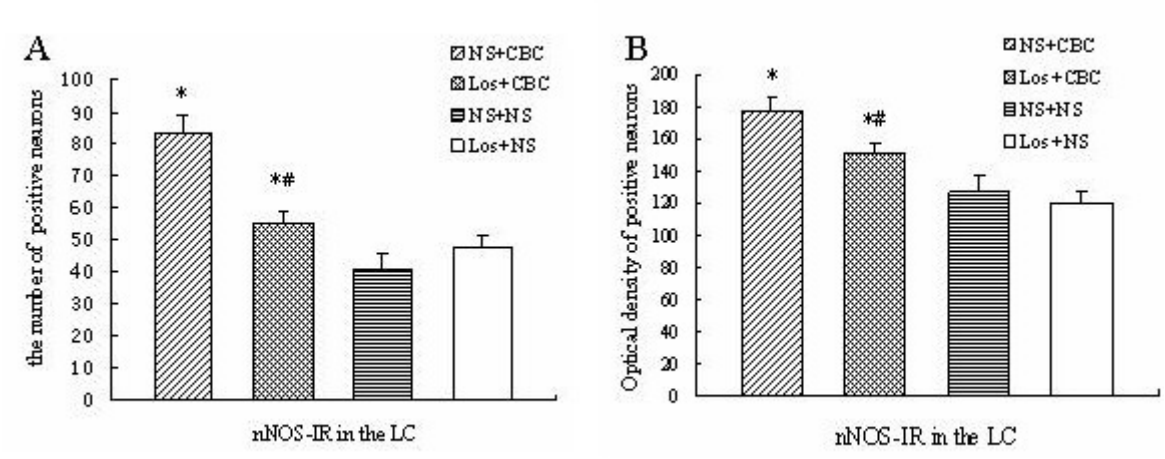

Fig. 3. Quantitative analysis of effect of pretreatment with losartan (20 μg) on the changes of nNOS-IR in the LC induced by ICV injection of carbachol (0.5 μg). A: the number of nNOS-IR positive neurons. B: optical density of nNOS-IR positive neurons. Data are reported as mean ± SEM. N = 6 (*P < 0.05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

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

Fig. 4 showed the results of immunohistochemistry studies for nNOS-IR in the kidney tissue. In NS+NS group, nNOS-IR positive granules were detected very weekly in proximal convoluted tubular cells (Fig. 4C). Forty min after ICV injection of carbachol (0.5μg), Fig. 4A showed that nNOS-IR positive granules were detected mainly in proximal convoluted tubular cells. Optical density of nNOS-IR positive granules in NS+CBC group revealed a statistically significant increase in renal proximal tubular cells, compared with that in 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 Los+CBC group (Fig. 4B), compared with that in NS+CBC group. No significant difference was found in optical density of nNOS-positive granules in kidney, when Los+NS group (Fig. 4D) was compared with NS+NS group. The data above were summarized in Fig. 5.

The results above 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 angiotensinergic AT_1 receptor partially inhibited the changes in the LC and proximal convoluted tubular cells induced by carbachol.

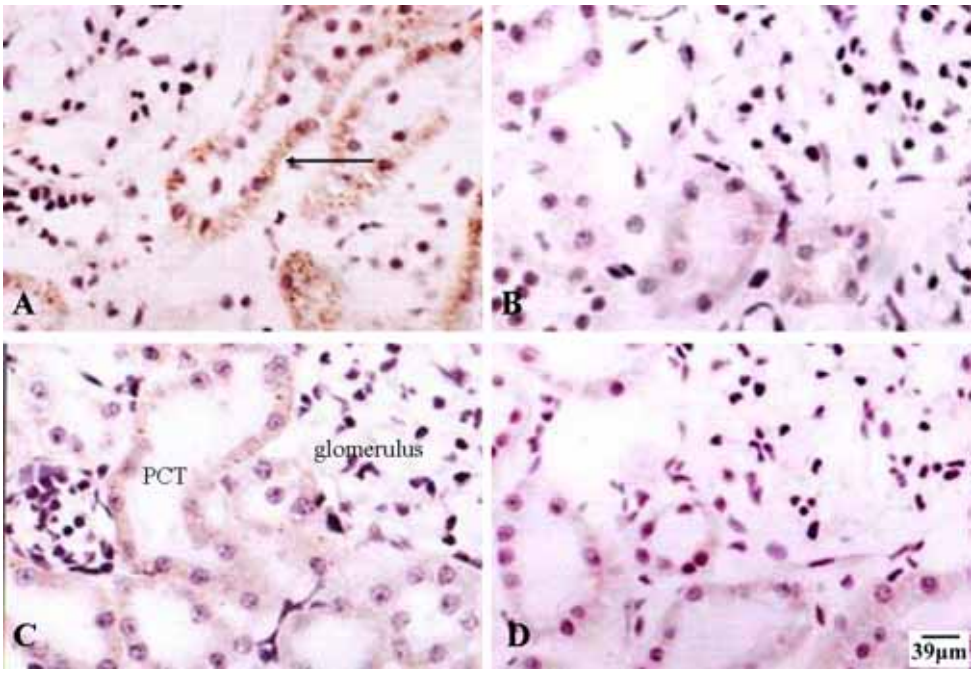

Fig. 4. Effect of pretreatment with losartan (20 μg) on the changes of nNOS-IR in PCT induced by ICV injection of carbachol (0.5 μg).A: NS+CBC group; B: Los +CBC group; C: NS+NS group; D: Los+NS group. Bar= 39μm ⟵ nNOS-IR positive granules

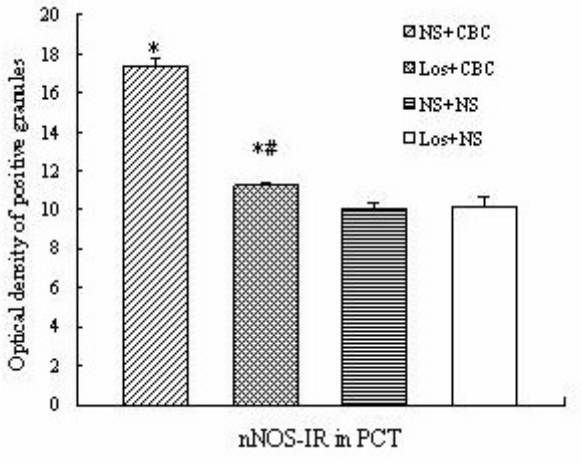

Fig. 5. Quantitative analysis of effect of pretreatment with losartan (20 μg) on the changes of nNOS-IR in PCT induced by ICV injection of carbachol (0.5 μg). Data are reported as mean ± SEM. N = 6 (*P < 0.05 vs. NS+NS group, $^{\#}P$ < 0.05 vs. NS+CBC group)

Discussion

Lots of studies have shown that the administration of ICV injection of Ang II produced persistent water drinking and the natriuresis (Saad et al. 2002); pretreatment with losartan (5μg), the Ang II AT_1 antagnoist, 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 brain cholinergic-induced natriuretic effect. Furthermore, we also observed that ICV injection of carbachol induced an increase of nNOS-IR in the LC. Our results demonstrated that NO-dependent neurons in the LC were excited after ICV injection of carbachol. Consistent with this, in one experiment, activation of the L-arginine-NO pathway by injection of L-arginine into the lateral cerebral ventricle of water-deprived rats stimulated the drinking (Mathai et al.1998). Acute volume expansion produced a significant increase in urine flow and sodium excretion, which was diminished in rats treated with L-NAME

on volume expansion-induced diuresis and natriuresis (Li et al. 2003). These evidences suggested that NO-dependent neural pathway in the LC was involved in the natriuresis induced by brain cholinergic stimuli. Experiments in vitro have demonstrated that in rat neuronal cultures, 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 stimulation of muscarinic receptor 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 studies in vivo 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 RT-PCR technique revealed a similar distribution of nNOS mRNA levels and AT_1 receptor mRNA levels in the rat brain (Krizanova 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 at the level of AT_1 receptors. Ang II besides its peripheral actions, also exerted central effects. The immediate response induced an increased release of arginine vasopressin (AVP), the 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). Our careful examination of the data represented in Fig.2 has revealed that the inhibition of brain angiotensinergic AT_1 receptors abolished the changes of nNOS-IR in the LC induced by ICV injection of carbachol. The finding indicated that the blockade of brain angiotensinergic AT_1 receptor might down regulate the effects in the LC induced by carbachol. Recently it has been reported that losartan prevented the effect of endogenous Ang II for stimulating 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 via activation AT_1 receptors in the LC mediated the expression of nNOS.

A large amount of data 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 begun to reveal a close relationship between nitric oxide and the proximal tubule. The proximal tubule, reabsorbing approximately two-thirds of the Na^+ and water filtered by the glomerulus, is quantitatively the most important nephron site for Na^+ and water reabsorption. A recent microdialysis study showed that there was detectable NO, measured by hemoglobin trapping in the microdialysis from the normal rat renal cortex and medulla (Liang and Knox 2000). The data indicated that proximal tubules in vivo were constantly exposed to NO that could potentially affect the function of the proximal tubule. In vivo experiment, infusion of acetylcholine, which caused NO release, into the renal artery resulted in the increase of urinary volume and sodium excretion. And the decrease of water and sodium excretion was observed when the inhibitor of endogenous NO production was infused into 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 renal tubule, but not renal hemodynamics. The results of the present study also indicated that ICV injection of cholinergic agonist carbachol could up regulate the activity of nNOS in proximal convoluted tubule cells. The blocker of AT_1 receptor might down regulate the actions above. So the above evidences suggested that nNOS in kidney and brain angiotensinergic pathway might participate in the natriuresis induced by ICV injection of cholinergic agonist.

Other investigators have reported that incubation of human proximal tubular cells with Ang II**,** atrial natriuretic factor produced a dose and time-dependent increase in NO production, which was possibly through cGMP pathway (Eitle et al. 1998). Ang II increased the cytosolic calcium concentration $[Ca2+]_i$ 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 AT_2 antagonist did not block on either side. 7-NI totally inhibited the NO production caused by Ang II (Liu and Persson 2004).These data suggested that increase Ca^{2+} in macula densa cell by Ang

II activated the nNOS to increase the production rate 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 in kidney has been studied to some extent. One possibility was that nNOS in renal proximal tubular cells might go downstream to reach the proximal tubule and synthesize NO. NO could activate guanylate cyclase (GC) and cGMP (Mathai et al. 1998, Eitle et al. 1998), which exerted its effect through inhibition Na/K ATPase (Ortiz and Garvin 2002, Wongmekiat and Johns 2002), located on the basolateral membrane of renal proximal tubule, to decrease NaCl reabsorption, and lead to the natriuresis. The second possibility was that renal nerve might play a role in the natriuresis and diuresis. It has been reported that nerve bundles were associated with nNOS expressed in kidney and cortical tubules in the rat kidney, especially the proximal tubule received nerve terminals. Stimulation of renal sympathetic nerves enhanced water and Na^+ reabsorption in PCT (Wongmekiat and John 2001). A low dose of L-NAME (3.7nmol/kg per min) reduced urinary sodium excretion only in rats with innervated kidneys, where it had no effects in rats with denervated kidneys (Liang and Knox 2000, Wongmekiat and John 2001). The results indicated that NO through renal sympathetic nerve regulated tubular water and Na^+ reabsorption. On the basis of the results above, we hypothesized that after carbachol administered into the lateral cerebral ventricle, the release of endogenous Ang II activated AT_1 receptors predominant in the LC or other brain nuclei known to subserve cardiovascular and fluid homeostasis. Then excited the NO-dependent neurons in the LC and employed NO as neurotransmitter to result in the natriuresis. Meanwhile, the release of endogenous Ang II, which activated via AT_1 receptors, in some pathway might excite nNOS in kidney, and then inhibit the Na/K ATPase in PCT, decrease the reabsorption of Na^+ and HCO_3^- in PCT, result in the natriuresis.

In conclusion, this study demonstrated that the brain angiotensinergic pathway and NO-dependent neural pathway contributed to renal changes in the natriuresis to brain cholinergic stimuli and thus played an important role in the regulation of fluid homeostatic. In addition, the final effect of NO on proximal tubular sodium reabsorption participated in the natriureisis induced by brain cholinergic stimuli. Further studies are required to

elucidate cellular and molecular physiological mechanism involved in the effects above.

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