Downregulation of Neuronal Nitric Oxide Synthase and Interleukin-1β Mediates Angiotensin II–Dependent Stimulation of Sympathetic Nerve Activity

Vito M. Campese, Shaohua Ye, Huiquin Zhong

Abstract—There is substantial evidence that angiotensin II (Ang II) enhances sympathetic nervous system (SNS) activity. We recently observed that nitric oxide and interleukin-1β (IL-1β) exert a tonic inhibitory action on central SNS activity. Moreover, in 2 rat models of neurogenic hypertension, one caused by intrarenal injection of phenol and the other by 5/6 nephrectomy, we observed that losartan, an Ang II type 1 receptor blocker, inhibits SNS activity and increases the abundance of IL-1β and the neuronal isoform of nitric oxide synthase (nNOS) in the posterior hypothalamic nuclei (PH), paraventricular nuclei (PVN), and locus ceruleus (LC). This raises the possibility that the stimulatory effects of Ang II on central SNS activity may be mediated by inhibition of nNOS and IL-1β. To test this hypothesis, we studied the effect of an intracerebroventricular (ICV) infusion of Ang II on blood pressure (BP), norepinephrine (NE) secretion from the PH, renal SNS activity (RSNA), and abundance of IL-1β and nNOS mRNA in the PH, PVN, and LC of normal Sprague-Dawley rats. Finally, we measured the concentration of nitrate/nitrite in the dialysate collected from the PH after Ang II or vehicle. ICV infusion of Ang II (100 ng/kg body wt dissolved in 10 μL of artificial cerebrospinal fluid) raised BP, RSNA, and NE secretion from the PH compared with control rats. Ang II reduced the abundance of IL-1β and nNOS mRNA in the PH, PVN, and LC. Pretreatment with losartan (10 μg/kg body wt dissolved in 10 μL of aCSF) given ICV 20 minutes before Ang II abolished the effects of Ang II on BP, RSNA, and NE secretion from the PH and IL-1β and nNOS mRNA. Ang II also decreased the secretion of NO from the PH. In conclusion, these studies suggest that Ang II inhibits the expression of IL-1β and nNOS in the brain. Because locally produced NO exerts a tonic inhibitory action on SNS activity, the decrease in NO expression caused by Ang II results in greater SNS activity.

Key Words: angiotensin II • sympathetic nervous system • losartan • nitric oxide • interleukins

There is substantial evidence that angiotensin II (Ang II) enhances sympathetic nervous system (SNS) activity via actions on the brain, sympathetic ganglia, and sympathetic nerve endings.1–9 The intracerebroventricular (ICV) administration of Ang II causes a dose-related increase in blood pressure (BP),10 which is probably related to activation of Ang II type 1 (AT1) receptors localized in the median preoptic nucleus and juxtaventricular neurons of the subfornical organ and organum vasculosum laminae terminalis11,12 and the brain stem.13 Ang II of central origin may activate peripheral sympathetic nerve activity through a decrease of the arterial baroreceptor gain14 or activation of preganglionic neurons in the rostral ventrolateral medulla (RVLM) or intermediolateral column.15

Agents that inhibit the renin-angiotensin system (angiotensin-converting enzyme inhibitors and Ang II AT1-receptor antagonists) may provide important information on the effects and site of action of Ang II in the brain. ICV administration of losartan, a nonpeptide-selective AT1-receptor antagonist, to rats consuming a low, high, or normal dietary sodium diet decreased basal renal SNS activity (RSNA) in the low and normal but not high dietary sodium group.16 ICV infusion of losartan fully prevented the sympathoexcitation and development of hypertension in Dahl S rats fed a high-salt diet.17 Microinjection of Ang II into the RVLM increased BP and peripheral SNS activity, and these effects were blocked by AT1-receptor blockers.18,19 In all, these studies provide evidence that Ang II activates specific areas of the brain connected with the SNS system, resulting in hypertension.

Recent studies have provided evidence that neuronal nitric oxide synthase (nNOS) is present in specific areas of the brain involved in the neurogenic control of BP.20,21 and it is an important component of transduction pathways that tonically inhibit the sympathetic outflow from the brain stem.22–25 Administration of interleukin-1β (IL-1β) in the lateral ventricle of control and chronic renal failure (CRF) rats causes a dose-dependent increase in nNOS-mRNA abundance and a...
decrease in BP and norepinephrine (NE) secretion from the posterior hypothalamic nuclei (PH). 26 Moreover, in 2 rat models of neurogenic hypertension, one caused by an intrarenal injection of phenol 27 and the other by 5/6 nephrectomy, 28 losartan reduced SNS activity. 29,30 and raised the abundance of IL-1β and nNOS in the PH, paraventricular nuclei (PVN), and locus ceruleus (LC). These findings suggest that inhibition of nNOS and IL-1β may mediate the stimulatory effects of Ang II on SNS activity.

To test this hypothesis, we studied the effect of ICV infusion of Ang II on BP, the secretion of NE from the PH, RSNA, and abundance of IL-1β and nNOS in the PH, PVN, and LC.

Methods

Animal Preparation

Male Sprague-Dawley rats that weighed 280 to 300 g were used for these studies. Rats received a normal rat diet (ICN Nutritional Biochemical) and tap water. After anesthesia with sodium pentobarbital (35 mg/kg intraperitoneally), we implanted catheters (PE-10) in a femoral artery and vein for subsequent measurements of arterial pressure and for administration of drugs.

Preparation for ICV Infusion

For ICV infusion of drugs, we implanted a cannula (23 gauge) in the right lateral ventricle (coordinates: 1.4 mm lateral, 0.8 mm posterior, and 3.8 mm deep from the bregma).

NE Secretion From the PH by the Microdialysis Technique

To measure NE secretion from the PH, we placed rats in a stereotaxic apparatus and implanted a 2-mm-long Teflon 22-G guide cannula (IV Catheter Placement Unit; Critikon, Inc) using coordinates A-P 0.4 mm and lateral ±0.4 mm (V = 8 mm) and secured the guide in place with dental cement. A 28-G stainless steel stylus was lowered through the guide cannula to a depth of 1.5 mm dorsal to the doroventral coordinate for PH, namely −8.5 mm from the skull surface.

The stylus was removed from the guide cannula, replaced with a microdialysis probe (CMA Microdialysis AB), and secured to the guide with sticky wax. The inlet tubing of the dialysis probe was connected by polyethylene 20 tubing to a 1-mL disposable syringe driven by a microinfusion pump (Razel Model A-99), and infusion of artificial cerebrospinal fluid (aCSF) (in mmol/L: 150 Na+, 155 Cl−, 1.4 Ca2+, 0.8 Mg2+, 1.0 K+, 155 Cl−, pH 7.2) was initiated at the rate of 1.7 μL/min. PE-10 tubing was attached to the outlet side of the probe, and the free end was led to a 0.5-mL vial set in a small box of ice. The vial contained 2 μL of 0.1 N HCl for preservation of NE.

All samples were immediately frozen and stored at −80°C until the time of assay. After 90 minutes of dialysis equilibration, dialysate samples were collected every 5 minutes for the entire duration of the experiment.

Renal Sympathetic Nerve Activity

To directly record RSNA, we prepared rats according to the method of Lundin and Thoren, 34 as modified by DiBona and Sawin. 31 The left kidney, the left renal artery, and the abdominal aorta were exposed retroperitoneally via a flank incision. A renal nerve branch was dissected free from fat and connective tissue for the length of approximately 10 mm and placed on thin bipolar platinum electrodes (Cooner Wire Co) connected to a high-impedance probe Grass H11002 (Grass Instrument Co). RSNA was amplified (×10 000 to 50 000) and filtered with a Grass 11 bandpass amplifier. The voltage-integrated frequency discharge was then displayed on a Grass Polygraph. The quality of RSNA was assessed during operation by examining the magnitude of changes in recorded RSNA during sinoaortic baroreceptor unloading with injection of acetylcholine (1 μg intravenously) and during sinoaortic baroreceptor loading with the injection of NE (5 μg intravenously). When an optimal recording was achieved, the nerve on the electrode was isolated with silicone rubber (Wacker Sil-Gel 604). During this time, the animals were kept warm under heated lamps and received an infusion of 50 μL/min 0.45% saline. Arterial pressure, heart rate, and RSNA were continuously monitored.

Effect of ICV Ang II on BP, NE Secretion From the PH, and RSNA

Ang II (100 ng/kg body wt dissolved in 10 μL of aCSF) was infused ICV over 10 minutes by an infusion pump in 5 rats. A control group of 5 rats received vehicle in the cerebroventricle. A third group of 5 rats received ICV losartan (10 μg/kg body wt dissolved in 10 μL of aCSF) 20 minutes before the infusion of Ang II. BP, NE secretion from the PH, and RSNA were recorded for 20 minutes before the infusion of Ang II and 60 minutes thereafter.

NE Microassay

We used a highly sensitive microradioenzymatic assay. 32 Ten microliters of dialysate was added to 5 μL of reaction mixture containing 1 μL of 3.7 mol/L Tris base (with 0.37 mol/L ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid and 1.8 mol/L MgCl2, pH 8.2), 0.06 μL of 36 mmol/L benzoxylamine, 1.5 μL of S-[methyl-3H]adenosine-5′-monophosphate, and 2.4 μL of partially purified catechol-O-methyltransferase and incubated for 60 minutes at 37°C. The sensitivity of this method is 0.5 pg.

Measurements of IL-1β and nNOS mRNA Abundance in the Brain

At the end of the experiments, rats were euthanized by decapitation, and brains were immediately removed, frozen in dry ice, and stored at −80°C until assay but for no longer than 3 weeks. Brains were cut into consecutive 200-μm sections in a cryostat at −20°C, and bilateral micropunches 0.5 mm in diameter from several brain nuclei were obtained according to the Paxinos and Watson rat atlas. 33,35 The coordinates for the PH were A-P from −3.5 to −4.1 mm, lateral ±0.4 mm (V = 8 mm); for the PVN were A-P from −1.4 to −2.0 mm, lateral ±0.3 mm (V = 7.9 mm); for the LC were A-P from −9.8 to −10.2 mm, lateral ±1.4 mm (V = 7.2 mm). The nucleoli so isolated were used to measure IL-1β and nNOS m-RNA gene expression.

Reverse Transcription–Polymerase Chain Reaction

For total RNA extraction and reverse transcription (RT), we used methods previously described by us 25 Polymerase chain reaction (PCR) was performed on the RT product using specific oligonucleotide primers for either neural-NOS (n-nNOS) or IL-1β derived from cDNAs cloned from rat brain 36 (GenBank Accession No. X59949) or rat liver. 37 A master mix of PCR reagents was made for duplex reactions containing primers for the “house-keeping” gene β-actin (GenBank Accession No. J00691) and primers for either nNOS (GenBank Accession No. X59949) or IL-1β (GenBank Accession No. M98820).

The RT-PCR products were quantified by the method of Higuchi and Doolinger. 36 Fluorescence was measured in a fluorescence spectrofluorometer (F-2000, Hitachi Ltd). Excitation was at 280 nm, and emitted light was selected at 590 nm. Results were expressed as a ratio of the resultant optical densities for the specific gene to β-actin.

Random hexamers, dithiothreitol (DTT), Super Script Super reverse transcripase with reaction buffer (5× [20 mmol/L Tris-HCl, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 0.01% NP 40, and 50% glycerol]), Taq DNA polymerase with reaction buffer (10× [50 mmol/L Tris-HCl, 10 mmol/L NaCl, 0.1 mmol/L EDTA, 5 mmol/L DTT, and 50% glycerol, and 1.0% Triton X-100]), deoxynucleotide mixture and MgCl2 were purchased from GibCO/BRL.
Nitrate/Nitrite Assay

Two subgroups of 5 rats each received ICV Ang II (100 ng/kg in 10 μL of aCSF) or aCSF. Dialysate was collected from the PH for 30 minutes before and 2 periods of 30 minutes each after the infusion of Ang II or vehicle.

We measured the stable metabolites of NO₂ and NO₃ (NOₓ) in the dialysate from the PH using the Microplate Manager Bio-Rad Laboratories kit. This assay is a 2-step process. The first step is the conversion of nitrate to nitrite using nitrate reductase. The second step is the addition of the Griess Reagents, which convert nitrite into a deep purple azo compound that can be measured by photometric method (Shimadzu Corp). Known concentrations of NaNO₂ and NaNO₃ are used as standards in each assay.

Statistical Analyses

Data were analyzed by 1-way analysis of variance, by the Fisher’s test for comparisons among groups using the computer program Statview and Graphics 4.01 (Abacus Concepts). When indicated, repeated measures analysis of variance was performed. Results are expressed as mean±SEM. The accepted level of significance was P<0.05.

Results

ICV infusion of Ang II (100 ng/kg body wt dissolved in 10 μL of aCSF) raised mean BP from 110±1.0 to 127±1.2 mm Hg (P<0.01; Figure 1A) and NE secretion from the PH from 158±2.9 to 209±2.2 pg/mL (P<0.01; Figure 1B), whereas ICV administration of aCSF did not alter BP (109±1.3 versus 108±1.2 mm Hg, respectively) or NE secretion from the PH (164±4.3 versus 164±3.3 pg/mL, respectively). ICV infusion of Ang II also increased RSNA by approximately 21% (Figure 1C). Pretreatment with ICV losartan (10 μg/kg body wt dissolved in 10 μL of aCSF) 20 minutes before Ang II abolished the effects of Ang II on BP (109±1.9 versus 109±1.9 mm Hg) and NE secretion from the PH (163±2.6 versus 161±3.5 pg/mL, respectively; Figures 1A and 1B). In addition, losartan reduced RSNA by 5.2% and abolished the effects of Ang II on RSNA (Figure 2C).

ICV infusion of Ang II reduced the concentration of NOₓ in the dialysate collected from the PH from 8.2±0.64 to 3.77±0.10 μmol/L, whereas administration of aCSF did not alter NOₓ (Figure 2).

Ang II reduced (P<0.01) the abundance of IL-1β in the PH (from 41.8±0.85 to 33.9±0.63), PVN (from 42.2±1.03 to 33.1±0.94), and LC (from 34.3±0.79 to 27.9±0.96; Figure 3A). Ang II also reduced (P<0.01) the abundance of nNOS mRNA in the PH (from 54.6±1.31 to 43.9±0.63), PVN (from 54.0±0.65 to 45.6±0.76), and LC (from 61.4±1.26 to 51.9±1.61; Figure 3B). Pretreatment with losartan com-

Figure 1. The line graphs show the levels of mean BP (A), the concentration of NE in the dialysate collected from the PH (B), and the changes in RSNA (C) after the injection of Ang II (100 ng/kg body wt) or vehicle in the lateral ventricle in rats. The open triangles indicate BP levels in rats pretreated with losartan (10 μg/kg body wt in the lateral ventricle) given 20 minutes before the administration of Ang II. Each group comprised 5 rats. Values are expressed as mean±SEM. The difference among groups was statistically significant with P<0.001.

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pletely abolished the effects of Ang II on IL-1β and nNOS mRNA.

**Discussion**

In these studies, ICV infusion of Ang II raised BP, NE secretion from the PH, and RSNA. Pretreatment with losartan, a selective AT1-receptor antagonist, abolished these effects. Other investigators previously showed that infusion of Ang II into the cerebroventricle of rats or dogs increases RSNA. The mechanisms and regions of activation remain under investigation. We observed that Ang II reduced the abundance of IL-1β and nNOS and decreased NOx secretion from the PH. When these studies are analyzed in the context of previous observations from our laboratory, they suggest that the stimulatory action of Ang II on SNS activity may be mediated by downregulation of IL-1β and nNOS expression.

There is substantial evidence that Ang II may increase SNS activity via actions on sympathetic nerve endings, sympathetic ganglia, and direct actions on the brain. Both circulating and Ang II of central nervous system origin may increase peripheral SNS activity and shift the arterial baroreflex regulation of peripheral SNS activity to a higher level of BP. In mice, infusion of Ang II activated RSNA, whereas infusion of NE essentially abolished RSNA despite similar increase in BP. Hexamethonium eliminated baseline high-amplitude bursts of RSNA but did not blunt the Ang II–induced RSNA, suggesting that Ang II may directly activate postganglionic sympathetic neurons through AT1 receptors.

Circulating Ang II may have access to the circumventricular area of the brain, where the normal blood brain barrier is lacking. These regions include the subfornical organ and the area postrema. From the subfornical area, projections to the PVN may modulate peripheral SNS activity. Evidence also indicates that Ang II of central nervous system origin, particularly in the PVN and RVLM, may increase peripheral SNS activity. Ang II receptor antagonists decrease BP and SNS activity when injected in the RVLM. Some evidence, however, indicates that Ang II may act within the central nervous system to attenuate acutely the maximum RSNA observed at low BP.

In these studies, we evaluated the effects of Ang II on NE secretion from PH as an indirect marker of brain pathways regulating SNS activity, well aware that neurons that release NE may not necessarily be sympathetic neurons. In this and previous studies, we selected the PH because this is an important brain region in the noradrenergic regulation of the cardiovascular system. Previous investigators used NE secretion from this region as a marker of activation of pathways regulating SNS activity. This approach is supported by evidence that electrical stimulation of PH increases BP, whereas destruction of PH decreases BP in the rat. Local perfusion of hypertonic saline or phenylephrine in PH elicited an increase in BP, NE release, and tachycardia. Blockade of α-receptors with phenoxybenzamine prevented the cardiovascular effects caused by NaCl or phenylephrine in this region.

In 5/6 nephrectomized rats, we showed a rise in both NE turnover rate and NE secretion from PH, suggesting that NE secretion from this nucleus can be used as a marker of...
activity of noradrenergic neurons. In the phenol renal-injury model, we also showed that changes in NE secretion from PH correlate with changes in RSNA.\(^{21}\) One could speculate that the increase in NE secretion from the PH is secondary to the rise in BP, rather than being its cause. This, however, is unlikely because NE turnover in this region decreases when BP rises and increases when BP falls.\(^{47}\) We previously showed that when Ang II is infused intravenously, the rise in BP is associated with reduced NE secretion from PH and increased IL-1β and nNOS abundance,\(^{22}\) effects opposite those seen when we infused Ang II in the lateral ventricle.

Recent studies have provided evidence that nNOS modulates SNS activity.\(^{19–24}\) The hypertensive response to N\(^\_\)nitro-L-arginine methyl ester in normal rats is greatly attenuated by sympathectomy\(^{51}\) or by renal denervation.\(^{52}\) In normal and 5/6 nephrectomized rats, NE turnover rate in several brain nuclei was modulated by NO production.\(^{22}\)

In the current studies, ICV Ang II reduced the expression of nNOS in the PH, PVN, and LC and the production of NO from the PH. In vitro studies support an inhibitory action of Ang II on NO production. After a transitory initial rise, Ang II decreased in a dose-dependent manner NO production in cultured sympathetic neurons from 12- to 14-day-old chick embryos.\(^{55}\)

Administration of IL-1β in the lateral ventricle reduced BP and NE secretion from PH and raised the abundance of nNOS-mRNA.\(^{23}\) ICV infusion of a specific anti-rat IL-1β antibody decreased NOS-mRNA expression in the PH, PVN, and LC of 5/6 nephrectomized and control rats while raising BP and NE secretion from the PH.\(^{23}\) In all, these studies suggest that IL-1β modulates NE secretion from the PH, and this modulation is mediated by local production of NO. The existence of complex interrelationships among cytokines, nitric oxide, and SNS activity is well established.\(^{54–58}\)

In conclusion, these studies suggest that Ang II in the brain inhibits the expression of IL-1β and nNOS. Because locally produced NO exerts a tonic inhibitory action on SNS activity, the decrease in NO expression caused by Ang II results in greater SNS activity.

References


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