Upregulation of Immunoreactive Angiotensin II Release and Angiotensinogen mRNA Expression by High-Frequency Preganglionic Stimulation at the Canine Cardiac Sympathetic Ganglia

Kazushi Kushiku, Hiromi Yamada, Kazuhiko Shibata, Ryoko Tokunaga, Takeshi Katsuragi, Tatsuo Furukawa

Abstract—The possible involvement of the local angiotensin system in ganglionic functions was investigated in the canine cardiac sympathetic ganglia. Positive chronotropic responses to preganglionic stellate stimulation at high frequencies, after intravenous administration of pentolinium plus atropine, were inhibited by the nonpeptide angiotensin AT1 receptor antagonist forasartan or the angiotensin I–converting enzyme inhibitor captopril, whereas the rate increases elicited by the postganglionic stellate stimulation and norepinephrine given intravenously failed to be inhibited by these antagonists. The levels of endogenous immunoreactive angiotensin II, as determined by radioimmunoassay in the incubation medium of the stellate and inferior cervical ganglia, were increased after the high-frequency preganglionic stimulation of the isolated ganglia. The increment of the peptide was also antagonized by the pretreatment with captopril but not by a chymase inhibitor, chymostatin. The expression of angiotensinogen mRNA was observed in the stellate ganglion, adrenal, liver, and lung but not in the ovary and spleen. The expression of the mRNA in the stellate and inferior cervical ganglia increased after high-frequency preganglionic stimulation of the in vivo dogs for a period of 1 hour. These results indicate that an intrinsic angiotensin I–converting enzyme–dependent angiotensin system exists in the cardiac sympathetic ganglia, which is activated by high-frequency preganglionic stimulation. (Circ Res. 2001; 88:110-116.)

Key Words: cardiac sympathetic ganglia ■ angiotensin ■ angiotensinogen mRNA ■ chymase ■ ganglionic transmission

Octapeptide angiotensin (Ang) II is well-known to exert a wide range of physiological effects on the cardiovascular, renal, and endocrine systems, including the peripheral and central nervous systems.1 Exogenous Ang II potentiates the sympathetic nervous system by enhancing the sympathetic tone through central sites of action2,3 and stimulating both the sympathetic ganglion cells and adrenal medulla.4 Accordingly, the renin-angiotensin system (RAS) plays a pivotal role in normal blood pressure and fluid and electrolyte homeostasis.

It has also been demonstrated that Ang II is importantly involved in the pathogenesis and pathophysiology of several common clinical syndromes, such as congestive heart failure, coronary insufficiency, and a variety of renal diseases associated with albuminuria.5 Ang II influences the cardiac function because of its actions on myocytes (contraction, growth, and metabolism), conduction tissue, fibroblasts (matrix deposition), coronary artery smooth muscle cells (constriction, dilation, and hypertrophy), coronary artery endothelium (release of vasoactive peptides and altered permeability), and sympathetic nerve endings (norepinephrine release, which could indirectly affect contractile state, conduction, growth, coronary resistance, and metabolic state).6

Recently, the existence of local Ang II–generating systems has been established not only in the kidney but also many other organs, such as the brain, adrenal glands, testes, and arterial wall.7,8 The stimulation or inhibition of the RAS respectively raises or lowers blood pressure.9 Ang II is a potent stimulant of the sympathetic ganglia, which exerts its action directly on the postganglionic cells in the superior cervical4,10 and cardiac sympathetic ganglia.11–16 In addition, specific binding sites for Ang II have also been shown to exist in multiple sites of the sympathetic nervous system, including the sympathetic stellate as well as the superior cervical ganglia,17 brain,18,19 and adrenal medulla.20,21 As for the Ang receptor, the Ang II–induced stimulation of the rat and dog sympathetic ganglia has also been proposed to be mediated via the AT1 subtype of Ang II receptor.6,16,22,23

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A variety of peptides, Ang II and other neuropeptides, have been identified in the ganglia by immunofluorescence. They appear to be localized to particular cell bodies, nerve fibers, or small intensely fluorescent cells and are released on nerve stimulation. They are thus presumed to mediate the late slow excitatory postsynaptic potential (EPSP).\(^1\) It has recently been proposed that angiotensinogen (Ao) mRNA is located in the sensory neural tissues as well as the sympathetic ganglia in the rat.\(^2\) However, the intrinsic biosynthesis and physiological roles of Ang II on the sympathetic ganglia still remain to be determined.

In the present study, we investigated whether the intrinsic angiotensin system is present in the dog cardiac sympathetic ganglia and whether it plays a role in the ganglionic transmission.

**Materials and Methods**

**Animal Care**
The experimental procedures were carried out under protocols approved by the Experimental Animal Care and Use Committee of Fukuoka University.

**In Vivo Experiment on the Ganglia**
All dogs used in the present investigation weighing between 6 and 10 kg were anesthetized with 30 mg/kg of pentobarbital sodium administered intravenously. The trachea was cannulated, and ventilation was maintained by a Harvard animal respirator (model 613). The experimental procedures were performed principally according to the methods described by Flacke and Gillis\(^2\) and Fleisch et al.\(^3\)

Both preganglionic and postganglionic stimulation of the stellate ganglia were performed in dogs principally according to the methods described previously.\(^4,5\) Ten minutes was allowed to elapse after the intravenous administration of the agents before another response to preganglionic stimulation was begun.

**In Vitro Experiments on the Ganglia: Incubation Medium Preparation to Determine the Ang II Immunoreactivity**

Both sides of the stellate and inferior cervical ganglia, together with about 2 cm of preganglionic sympathetic trunk, were removed for nerve stimulation. The composition of Locke’s solution was (in mmol/L) NaCl 136, KCl 2.2, NaHPO\(_4\) 1.2, NaHCO\(_3\) 20, and glucose 11. To prevent a degradation of the released endogenous Ang II, Locke’s solution also contained 50 mmol/L of amastatin and bestatin, aminopeptidase inhibitors, and 1 mmol/L of Plummer’s inhibitor, carboxypeptidase inhibitor. The ganglion was transferred to a micro test tube containing 0.7 mL of fresh medium at 37°C gassed with a mixture of 95% O\(_2\) and 5% CO\(_2\). The preganglionic nerve was laid across a bipolar platinum electrode suspended just above the surface of the solution and was stimulated by square wave pulses lasting 1 ms in duration at frequencies of 40 Hz at a strength of 60 V for 30 minutes with an electric stimulator (SEN-3201, Nihon Kohden) at frequencies of 40 Hz for 1 hour. The left ganglia without preganglionic stimulation were used as the control group. The ganglion specimens of 4 ganglia were then used for each sample and subjected to a Northern blot analysis.

In the inferior cervical and stellate ganglia, adrenal, liver, lung, ovary, and spleen were immediately removed. The tissue specimens were chilled with dry ice and then stored at −80°C until an assay was performed. Both of the ganglia were used after the removal of the sheath.

**Preparation of cDNA Probes by Reverse Transcriptase–Polymerase Chain Reaction**
The dog Ao cDNA was amplified from the total dog liver cDNA by polymerase chain reaction (PCR). We made two oligonucleotide mixtures corresponding to the highly conserved amino acid sequence in the Ao of human, sheep, and rat. These primers were as follows: 5’-TA(T/C)ATACA(T/C)CC(AT/CG)TT(T/C)CA(T/C)(T/C) and 5’-TTCTAT(T/C)TTAT(AT/CG)CC(T/C)TG(A/G)AA(A/G)IG. The PCR was carried out by Taq DNA polymerase under the following conditions: 92°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, and 35 cycles in a DNA thermal cycler (QTP-1, Nippon Genetics). PCR product was 758 bp. The amplified band was ligated into PT7-blue plasmid, amplified in transformed Escherichia coli and sequenced directly on an automated system using fluorescently labeled deoxyxynucleotides. The dog Ao cDNA clone sequence reported in this study has been deposited in the DNA Data Bank of Japan (accession No. AB023422).

**Northern Blot Analysis**
Total RNA was extracted using guanidine isothiocyanate according to a previous reported method, fractionated on agarose-formaldehyde gel, and transferred onto nylon membranes.\(^6,7\) The blots were hybridized with 32P-labeled cDNA probe. The blots were exposed to radiograph film (Kodak, XAR-5). The density of the bands was quantified with a densitometer and standardized with G3PDH.

**Chemicals**
The drugs used in this study were atropine sulfate (E. Merck, Darmstadt, FRG); pentolinium di-tartrate, bestatin, and captopril (Sigma Chemical Co); chymostatin and amastatin (Protein Research Foundation); DL-2-mercaptopentyl-3-guanidinoethylthiopropionic acid (Plummer’s inhibitor, Calbiochem); and dextran (Clinical grade dextran D, Otsuka Pharmaceutical). Forasartan (5-[(3,5-dibutyl-1H-1,2,4-triazol-1-yl)methyl]-2-[2-(1H-tetrazol-5-yl)phenyl]-pyridine) was a generous gift from Searle (Skokie, Ill).

**Statistical Analysis**
Each value represents the mean±SEM. Statistical analysis was performed using ANOVA followed by Dennett’s and Bonferroni multiple comparison tests in in vivo experiments. Comparisons of...
Results

Effects of Captopril and Forasartan on the Positive Chronotropic Responses to High-Frequency Preganglionic Stimulation After Treatment With Pentolinium and Atropine

In two groups of 7 and 8 pithed dogs (mean basal heart rate 128±3.2 and 126±5.5 bpm), preganglionic stellate stimulation at 20 Hz for 10 seconds immediately increased the heart rate by about 83±6 and 85±6.4 bpm, respectively, as shown in Figure 1. These positive chronotropic responses to stimulation were completely abolished by the intravenous administration of pentolinium (0.5 mg/kg) plus atropine (1 mg/kg) (data not shown). After treatment with both the cholinergic antagonists, a high-frequency preganglionic stellate stimulation at 20 and 40 Hz for a period of 1 minute elicited positive chronotropic responses with a latency of 30 seconds, reaching a maximum within 50 seconds and lasting for 2 to 3 minutes. These increases in the heart rate elicited by high-frequency preganglionic stimulation were markedly inhibited in a dose-dependent manner by forasartan (2 and 4 mg/kg) or a converting enzyme inhibitor, captopril (0.25 and 0.5 mg/kg), respectively, as shown in Figure 1. Intravenous forasartan or captopril given alone at doses of 2 and 4 mg/kg and 0.25 and 0.5 mg/kg, respectively, did not elicit any significant changes in the heart rate.

In two groups of 5 and 4 pithed dogs (mean basal heart rate 123±3.8 and 141±2.7 bpm), postganglionic stellate stimulation and intravenous injection of norepinephrine produced frequency- or dose-dependent increases of heart rate. As shown in the left panel of Figure 2, the rate increase elicited by postganglionic stimulation was not significantly altered 10 minutes after the administration of forasartan at the same doses. The dose-dependent increase in heart rate caused by norepinephrine (0.25 to 2 µg/kg) was significantly enhanced to some extent other than inhibition after the same doses of intravenous forasartan were administered (Figure 2, right). In another two groups of 4 pithed dogs each (mean basal heart rate 148±2.3 and 138±3.5 bpm), as shown in Figure 3, the frequency- and dose-dependent rate increases caused by the postganglionic stimulation and intravenous injection of norepinephrine were unaffected by intravenous captopril at doses of 0.25 and 0.5 mg/kg.

IR Ang II Release From the Stellate and Inferior Cervical Ganglia

In all 8 isolated stellate and inferior cervical ganglia of 4 dogs, Ang II formation was measured as the accumulation of IR Ang II released into the incubation medium. The mean basal amount of the peptide released from the stellate and inferior cervical ganglia in the absence of captopril before the preganglionic stimulation was 37.1±6.96 and 47.8±5.43 ng/g protein per 30 minutes, respectively. As shown in Figure 4, the IR Ang II levels in the medium of the stellate and inferior cervical ganglia increased to 1.6- and 2.4-fold, respectively, above the resting levels when incubated with repetitive high-frequency preganglionic stimulation in both the isolated ganglia at 40 Hz for a period of 30 minutes. These increases of the peptide released were antagonized by incubation with 10⁻⁴ mol/L captopril for 10 minutes. The application of captopril alone in the absence of the conditioning stimulation did not significantly affect the resting release of the peptide.
In the 10 isolated stellate and inferior cervical ganglia of 5 dogs, the mean basal amount of IR Ang II from the two kinds of ganglia in the absence of chymostatin, a chymase inhibitor, and the conditioning stimulation was 15.8 ± 2.43 and 30.7 ± 4.99 ng/g protein per 30 minutes, respectively. As shown in Figure 5, the enhanced amount of the IR Ang II levels in the medium of the stellate and inferior cervical ganglia in the presence of the conditioning stimulation failed to antagonized by pretreatment with 10⁻¹⁴ mol/L chymostatin for 10 minutes. The amount of the peptide release in the absence and presence of preganglionic stimulation increased after the exposure of chymostatin rather than decreased when compared with the results of captopril (Figure 5).

**Increment of Ao mRNA Expression by the Preganglionic Stimulation**

As shown in Figure 7, in 5 of the ganglion specimens, the expression of the Ao mRNA levels in the stellate and inferior cervical ganglia increased after high-frequency preganglionic stimulation for 1 hour. The ratio of Ao/G3PDH mRNA in both ganglia increased to 2.3- and 3.6-fold, respectively, above the resting levels (Figure 8). There was no difference in the expression of the Ao mRNA in these ganglia between both side of ganglia in the sham-operated animals without preganglionic stimulation (data not shown).

**Discussion**

In functional studies using electrophysiological experiments, in addition to the muscarinic slow EPSP, nicotinic fast EPSP, sheep, and rat Ao cDNA sequences, respectively. On the basis of the extensive homology with the human, sheep, and rat cDNA sequences and the pattern of tissue expression, this cDNA was concluded to encode a portion of the dog Ao. As shown in Figure 6, the strongest signal for Ao mRNA was in the liver, followed successively by the stellate ganglion, adrenal, and lung. The ovary and spleen had no signals.

**Representative Autoradiogram of a Northern Blot Analysis of Ao mRNA in Several Types of Tissue**

The dog Ao cDNA clone was obtained by reverse transcription of dog liver mRNA followed by PCR amplification with primers derived from human, sheep, and rat Ao sequences. The dog Ao nucleotides sequence exhibited 76.2%, 80.9%, and 75% nucleic acid identity with the reported human, sheep, and rat cDNA sequences, respectively. On the basis of the extensive homology with the human, sheep, and rat cDNA sequences and the pattern of tissue expression, this cDNA was concluded to encode a portion of the dog Ao.
and catecholaminergic slow inhibitory postsynaptic potential, ganglionic responses include a long-lasting noncholinergic (presumably peptidergic) late slow EPSP, which can be observed after the muscarinic slow EPSP. The positive chronotropic response to high-frequency preganglionic stimulation after treatment with antinicotinic and muscarinic agents was inhibited by intravenous forasartan, the potent nonpeptide competitive AT₁-receptor antagonist, or by captopril, the angiotensin-converting enzyme (ACE) inhibitor, whereas the rate increases elicited by the postganglionic nerve stimulation and intravenous norepinephrine in the same animals were not inhibited by the administration of forasartan and captopril.

However, the direct effect of the AT₁-receptor antagonist on cardiac receptors cannot be ruled out. In the pithed normotensive rat, the positive chronotropic response to the intravenous administration of Ang II was strongly inhibited by pretreatment with reserpine, the Ang II analog saralasin, and the β₁-adrenoceptor antagonist atenolol. Moreover, the ganglionectomy greatly reduced the tachycardiac response to Ang II, but the adrenalectomy did not affect the tachycardia. The heart rate increase caused by the intravenous injection of Ang II was also greatly reduced by crushing the caudal cervical and stellate ganglia and cutting their postganglionic fibers in the dogs. In the pithed dogs, the dose-response curve for Ang II of tachycardia administered via the subclavian artery to the cardiac sympathetic ganglia shifted to the right by the AT₁-receptor antagonist forasartan given intravenously. These facts indicate that positive chronotropic response to the exogenous Ang II in the pithed animals is primarily mediated by acting on the postganglionic sites of AT₁ receptor in cardiac sympathetic ganglia but not by directly acting on the same receptor in cardiomyocytes. In line with these results, Ang II had a positive chronotropic effect on cultured adult guinea pig cardiomyocytes innervated with adult extrinsic or intrinsic cardiac neurons but not those cultured without neurons. Therefore, the results of the present study indicate that ganglionic stimulation elicited by the high-frequency preganglionic stimulation after pretreatment with cholinergic antagonists seems to involve endogenous Ang II release, which acts on AT₁ receptor in the postganglionic cell body.

In neuronal cultures, glial cells and neurons have been shown to produce renin, Ao, Ang I, and Ang II. Various peptides, including Ang II, have also been identified in the glia by immunofluorescence, and the release on nerve stimulation was thus determined. Furthermore, the local RAS in the primary cultured fetal pig superior cervical ganglion has been provided by showing the expression of Ao mRNA and the localization of renin and Ang II determined by in situ hybridization and immunocytochemical approaches. The local Ang II system in the rabbit retina was also demonstrated by examining the Ang II contents with a RIA and Ang II synthesis by detecting the ACE mRNA. In the present study, the resting amount of the IR Ang II content in the incubation medium released from the isolated inferior cervical or stellate ganglia was determined, and the amount was augmented by the repetitive high-frequency preganglionic stimulation. The present results thus indicate Ang II to be present in these ganglia.

The increased amount of Ang II output elicited by the preganglionic conditioning stimulation was inhibited by pretreatment with captopril but was not completely abolished even at higher concentrations (10⁻⁸ mol/L) of the drug. The conversion of Ang I to Ang II has been thought to be mainly catalyzed by ACE. However, recent studies have demonstrated the existence of alternative Ang II–generating pathways independent of ACE. In particular, serine protease with an extremely high affinity for Ang I chymase has been identified in human, dog, and baboon hearts but not in the rodent heart. Therefore, in the present study, we examined whether the enhanced release of Ang II induced by the conditioning stimulation is formed by alternative Ang II–generating enzyme chymase. However, the increased amount of the peptide was not antagonized by the pretreatment with a chymase inhibitor, chymostatin. Although Ang I has been reported to serve as a favorite substrate for chymase, there is a marked species difference in the Ang II–forming activity. In humans, dogs, monkeys, or hamsters, chymase produces Ang II from Ang I, whereas it cleaves Ang I into inactive fragments in rabbits, rats, or mice. Furthermore, there are remarkable differences in Ang II–forming pathways among both species and different organs. Therefore, it is assumed that Ang II formation from Ang I is mediated via an ACE-dependent and chymostatin-sensitive chymase-independent pathway in the canine sympathetic ganglion. Besides, in the present investigation, the pretreatment with chymostatin enhanced rather than inhibited the increased Ang II output elicited by preganglionic-conditioning stimulation. However, this mechanism for the enhanced response to chymostatin still remains to be elucidated.

Ao mRNA is found in the following tissues in a descending order of abundance: liver, fat cells, brain (glial cells), kidney, ovary, adrenal gland, heart, lung, large intestine, and stomach in mouse and rat. It has recently been proposed that Ao
mRNA is located in the sensory neural tissues and sympathetic ganglia in the spontaneously hypertensive rat and also in primary cultured fetal pig superior cervical ganglion neurons.\(^4\)

In this experiment, a dog Ao cDNA was cloned and used as a probe to quantitatively measure the Ao mRNA in the dog inferior cervical and stellate ganglia and also in various other tissues. Thus we found Ao mRNA to be present not only in the liver, adrenal, and lung but also in the inferior cervical and stellate ganglia, whereas no Ao mRNA was present in the ovary or spleen. Furthermore, in the present investigation, the upregulation in efflux of IR Ang II and in signals for Ao mRNA from the dog cardiac sympathetic ganglia appeared after high-frequency preganglionic nerve stimulation to the inferior cervical and stellate ganglia for a period of 1 hour. Although the localization of renin in the ganglia was not determined in the present investigation, it is presumed that Ang II may not be taken up from the extracellular blood serum but instead is synthesized by these gangionic cells themselves, because the existence of Ao mRNA in these ganglia has been demonstrated. Furthermore, neuropeptides seemed to be localized to particular cell bodies, nerve fibers, or small intensely fluorescent cells in the autonomic ganglia.\(^2\)

Renin and Ang II are localized in the adrenergic primary cultured fetal pig superior cervical ganglion neurons but not in nonneuronal cells.\(^4\) However, in the present study, primary cultured fetal pig superior cervical ganglion neurons and Searle for kindly providing the forasartan.

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References


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