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 vasocative 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,7 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
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 presumned to mediate the late slow excitatory postsynaptic potential (EPSP).24 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.25 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 surgical procedures were performed principally according to the methods described by Flacke and Gillis26 and Fleisch et al.27 Both preganglionic and postganglionic stimulation of the stellate ganglia were performed in dogs principally according to the methods described previously.15,16 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 5.6, CaCl2 2.2, NaH2PO4 1.2, NaHCO3 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 acid (Plummer’s inhibitor, Calbiochem); and dextran (Clinical grade dextran D, Otsuka Pharmaceutical). Forasartan (5-[(3,5-dibutyl-1H-

Radioimmunoassay

Equal amounts of 1% trifluoroacetic acid (0.7 mL) were added to each sample medium and then centrifuged at 15 000g for 20 minutes at 4°C. The sample solution was loaded onto the pretreated C18 Sep-Column. The column was slowly washed with 1% trifluoroacetic acid (3 mL twice) and then discarded in the wash, and the peptide was eluted slowly with 60% acetonitrile in 1% trifluoroacetic acid (3 mL once). Ang II in the aliquots of incubation medium was quantified routinely by radioimmunoassay (RIA) using the Ang II RIA instructions (Peninsula Laboratories, Inc). The source of the Ang II antibody was the rabbit antiserum specific for the peptide. The Ang II antiserum cross reacts minimally with Ang I (0.5%) but strongly with Ang III. The sensitivity of the RIA kit is 50% displacement at 16 pmol/L or 50 pg/mL. At the end of the experiment, the ganglion without a nerve trunk was frozen on dry ice and stored at −80°C until protein assay. Protein was determined by using a protein assay (Coomassie Plus Protein Assay Reagent, Pierce), which is a quick and ready to use modification of the well-known Bradford method.28 Immunoreactive (IR) Ang II released into the incubation medium was expressed as nanograms per gram of protein of ganglion. The levels of IR Ang II were accurately calculated by subtracting the values of blank solution. Each sample was analyzed in duplicate.

Tissue Preparation for the Northern Blot Analysis

For the preganglionic conditioning stimulation in the in vivo animals under artificial ventilation, a bipolar platinum electrode was placed on the distal end of the right ansa subclavia and repetitive stimulation with an electric stimulator was given (MSE-3R, Nihon Kohden) at supramaximal square-wave impulses with a duration of 1 ms at a frequency 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. 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 Transcripatse–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(AG/CT)TT(C/T)CA(T/C)(T/C) and 5’-TTCAT(T/C)TT(AG/CT)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 pET7-blue plasmid, amplified in transformed Escherichia coli and sequenced directly in an automated system using fluorescence-labeled deoxycytidylate. 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.29,30 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-mercaptoethyl-3-guanidothiopropionic acid (Plummer’s inhibitor, Calbiochem); and dextran (Clinical grade dextran D, Otsuka Pharmaceutical). Forsasartan (5-[(3,5-dibutyl-1H-

Statistical Analysis

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

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Figures 1 and 2. Dose-dependent inhibition by forasartan or captopril of the positive chronotropic response to high-frequency preganglionic stimulation in the in vivo pentrinium- and atropine-treated pithed dogs. Preganglionic stimulation at 20 Hz for 10 seconds in the untreated animals (○) and after forasartan (●) and captopril (▲) treatment in the same pentrinium- and atropine-treated animals. Vertical bars indicate SEM. *Significant difference between the values before and after either forasartan or captopril treatment in the same kidneys. (A) and after 2 mg/kg (●) and 4 mg/kg (▲) of forasartan in the pentrinium- and atropine-treated pithed animals. Vertical bars indicate SEM. *Significant difference between the values before and after forasartan treatment in the same animals. **P<0.05.

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 increases of heart rate and 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 in 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.

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 significantly 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), preganglionic 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 preganglionic 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 preganglionic stimulation and intravenous injection of norepinephrine were unaffected by intravenous captopril at doses of 0.25 and 0.5 mg/kg.

Figure 1. Dose-dependent inhibition by forasartan or captopril of the positive chronotropic response to high-frequency preganglionic stimulation in the in vivo pentrinium- and atropine-treated pithed dogs. Preganglionic stimulation at 20 Hz for 10 seconds in the untreated animals (○) and after forasartan (●) and captopril (▲) treatment in the same pentrinium- and atropine-treated animals. Vertical bars indicate SEM. *Significant difference between the values before and after forasartan treatment in the same animals (P<0.05).

Figure 2. Influence of forasartan on the positive chronotropic responses to preganglionic stimulation of the right stellate ganglion and to intravenous norepinephrine in pithed dogs. Before (○) and after 2 mg/kg (●) and 4 mg/kg (▲) of forasartan in the pentrinium- and atropine-treated pithed animals. Vertical bars indicate SEM. *Significant difference between the values before and after forasartan treatment in the same animals (P<0.05).

Figure 3. Influence of captopril on the positive chronotropic responses to preganglionic stimulation of the right stellate ganglion and to intravenous norepinephrine in pithed dogs. Before (○) and after 0.25 mg/kg (●) and 0.5 mg/kg (▲) of captopril in the pentrinium- and atropine-treated pithed animals. Vertical bars indicate SEM.
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 antagonize by pretreatment with 10^{-2} 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).

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. 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.

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,
Ang II, but the adrenalectomy did not affect the tachycardia.42 Moreover, the increased amount of Ang II output elicited by the conditioning stimulation is formed by 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,47 dog,48,49 and baboon50 hearts but not in the rodent heart.51 Therefore, in the present study, we examined whether the enhanced release of Ang II induced by the conditioning stimulation was inhibited by pretreatment with chymostatin.

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 (10⁻⁴ 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,47,48,49 and baboon50 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.52,53 Furthermore, there are remarkable differences in Ang II–forming pathways among both species and different organs.54 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.43 It has recently been proposed that Ao 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.
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. 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 ganglionic 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. Renin and Ang II are localized in the adrenergic primary cultured fetal pig superior cervical ganglion neurons but not in nonneuronal cells. However, in the present study, we did not elucidate whether the Ang II, present in ganglion, is synthesized in the neuronal or glial cells themselves. In conclusion, the findings obtained from a Northern blot analysis, RIA, and in vivo experiments provide evidence that Ao mRNA is expressed and Ang II is present in the canine cardiac sympathetic ganglia, whereas the sympathetic ganglia may also possess an ACE-dependent and chymostatin-sensitive chymase-independent intrinsic RAS, which may be controlled by the preganglionic neuronal activity.

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References


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