Multiple Pathways of Angiotensin I Conversion and Their Functional Role in the Canine Penile Corpus Cavernosum

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ABSTRACT

Multiple pathways of angiotensin (Ang) I conversion and their functional role in the canine penile corpus cavernosum were investigated. Biochemical analysis revealed high activities of angiotensin-converting enzyme (ACE) (6.9 ± 1.7 mU/mg of protein, mean ± S.E.M., n = 8) and chymase-like enzyme (4.0 ± 1.4 mU/mg of protein). Functional recording of isometric tension showed that Ang I (3 × 10⁻⁷ M) induced a tension of 0.17 ± 0.05 g (n = 5), which was reduced to about 60% by pretreatment with an ACE inhibitor, lisinopril (10⁻⁶ M), and almost completely blocked by lisinopril in combination with a chymase inhibitor, chymostatin (10⁻⁴ M). Binding sites for ACE and Ang II receptors were studied by in vitro autoradiography using ¹²⁵I-351A and ¹²⁵I-[Sar¹,Ile⁸]Ang II as ligands, respectively. Dense binding of ACE appeared in the endothelial layer of the corpus cavernosum penis, and Ang II receptors were localized in the trabecular smooth muscle layer. An AT1 receptor antagonist, CV-11974 (10⁻⁶ M), markedly displaced ¹²⁵I-[Sar¹,Ile⁸]Ang II bindings, indicating that the corpus cavernosum penis contains AT1 receptors exclusively. Immunohistochemical studies demonstrated ACE in the endothelium of the corpus cavernosum penis. Mast cells that produce chymase were present mainly in the cavernosal area. These results demonstrate that chymase, in addition to ACE, is involved in the contraction of canine penile corpus cavernosum through local Ang II formation.

The renin angiotensin system (RAS) has been considered to be an endocrine system. In this system, angiotensin (Ang) II, the active principle of RAS, is generated in the circulation by the actions of two important enzymes, renin and angiotensin-converting enzyme (ACE), and it is delivered to its target organs via the blood stream. The physiological responses are vasoconstriction and aldosterone release with facilitation of noradrenergic transmission (Peach, 1977). However, recent evidence has suggested that the components of RAS are present in various local tissues and that Ang II generated in the local tissues may function in both autocrine and paracrine fashions (Dzau, 1988).

The corpus cavernosum penis is a vascular tissue that contains endothelial and smooth muscle cells. Relaxation and contraction of the corporal smooth muscle are regulated by humoral, neural, and local factors, such as norepinephrine, sex hormones, neuropeptide Y, acetylcholine, vasoactive intestinal polypeptide, nitric oxide, prostaglandins, and endothelin (Adams et al., 1997). Ang II is a potent vasoconstrictor in the segments of the extracavernosal vascular beds. Although Ang II is known to induce contraction of the corporal smooth muscle in vitro and in vivo (Kifor et al., 1997; Park et al., 1997), a physiological role of Ang II to RAS in the corpus cavernosum penis has not been elucidated.

ACE was generally considered to be responsible for the local conversion of Ang I to Ang II. However, alternative pathways of Ang II formation, other than via ACE, have been demonstrated in the blood vessels of humans, monkeys, and dogs (Okunishi et al., 1984, 1993). It also occurs in human heart (Urata et al., 1990a) where the alternative Ang II-forming enzyme is mast cell chymase (Urat a et al., 1990b, 1991). Chymase is a chymotrypsin-like serine protease found in the secretory granules of mast cells (Miyazaki and Takai, 2000). In several species, chymases have been shown to have structural homology, but it is known that the physicochemical properties differ considerably with respect to substrate specificity. In human, dog, monkey, and hamster, chymase cleaves the Phe⁴-His⁵ bond of Ang I and produces Ang II, whereas rat or rabbit chymase hydrolyzes the Tyr⁴-Ile⁵ bond of Ang I and yields inactive fragments (Trong et al., 1987).

The aim of the present study was to determine the presence of multiple pathways of Ang I conversion and their functional role in the canine penile corpus cavernosum. For this purpose, we carried out enzymatic assays of ACE and chymase and measured isometric tension produced by Ang I conversion through the action of both enzymes. We also lo-

ABBREVIATIONS: RAS, renin angiotensin system; Ang, angiotensin; ACE, angiotensin-converting enzyme; BSA, bovine serum albumin.
calized ACE, mast cells, and Ang II receptor subtypes in the canine penile corpus cavernosum.

Materials and Methods

Animals and Tissue Preparations. Eight adult male beagle dogs (Animal Experimental Center, Osaka Medical College, Osaka, Japan), each weighing about 10 kg, were killed by exsanguination from the brachial artery under ketamine anesthesia (50 mg/kg, i.m.). For all animals, the entire penis was rapidly removed, and the body (5 cm in length) was excised and separated into two parts. One part (1 cm in length) was immediately frozen in isopentane-dry ice (−40°C) and stored at −80°C until being used for in vitro autoradiography and immunohistochemistry. The corpus cavernosum penis of another segment (4 cm in length) was carefully dissected from the surrounding tunica albuginea and subdivided into two parts (1 and 3 cm in length). The shorter length was used for the measurement of ACE and chymase-like activities and the longer length for the measurement of isometric tension. During the preparation, special care was taken not to damage or overstretch the tissues. The experimental procedures for animals were planned and conducted in accordance with the guidelines of Osaka Medical College.

Measurement of ACE and Chymase-Like Activity. ACE and chymase-like activities were measured by methods reported previously (Shiota et al., 1993). In brief, the corpus cavernosum was minced and homogenized in 10 volumes (w/v) of 20 mM sodium-phosphate buffer, pH 7.4. The homogenates were centrifuged at 20,000g for 30 min at 4°C, and the supernatants were discarded. The pellets were resuspended and homogenized in 5 volumes (w/v) of 20 mM sodium-phosphate buffer, pH 7.4, containing 2 M KCl and 0.1% Nonidet P-40. The homogenates were stored overnight at 4°C and then centrifuged at 20,000g for 30 min at 4°C. The supernatants were used as tissue extracts for the measurement of ACE and chymase-like activities. Protein concentrations were measured by bichinonic acid protein assay reagents (Pierce Chemical, Rockford, IL) using bovine serum albumin as a standard.

ACE activities were measured by incubating 50-μl aliquots of the tissue extracts for 30 min at 37°C with 5 mM hippuril-His-Leu, a synthetic substrate designed specifically for ACE, in 100 mM phosphate buffer, at pH 8.0, containing 800 mM NaCl. The enzymatic reaction was terminated by addition of 3% (w/v) metaphosphoric acid, and the reaction mixture was placed in iced water for 10 min. After centrifugation of the mixture for 5 min at 20,000g, the supernatant (50 μl) was applied to an octadodecyl silica reversed-phase column (4.6 × 25 cm; Tohso, Tokyo, Japan) that had been equilibrated beforehand with 10 mM KH2PO4 and CH3OH (1:1, pH 3.0) and stored at 4°C overnight under reduced pressure, and stored at 2°C in sealed boxes with silica gel. For ACE, a previously published procedure using 125I-351A was followed (Mendelsohn, 1984). The sections were preincubated in 10 mM sodium-phosphate buffer containing 150 mM NaCl and 0.2% bovine serum albumin (BSA), pH 7.4, for 15 min at 22°C and then incubated in a fresh volume of the same buffer containing 0.3 μCi/ml of 125I-351A for 1 h at 22°C. Nonspecific binding was determined in parallel incubations containing 1 mM EDTA. After incubation, the sections were transferred through four successive 1-min washes of buffer without BSA at 0°C. The slides were dried under a stream of cool air, loaded into X-ray cassettes, and exposed to Agfa Scopix CR3B film (Agfa-Gevaert Ltd., Morssel, Belgium) for 2 days at room temperature. Ang II receptors were labeled with 125I-[H]-[Sar1, Ile8]Ang II as described elsewhere (Song et al., 1991). The sections were preincubated in 10 mM sodium-phosphate buffer containing 150 mM NaCl, 5 mM Na2EDTA, 0.2% BSA, and 0.4 mM bacitracin, pH 7.4, for 15 min at 22°C and then incubated in a fresh volume of the same buffer containing 0.2 μCi/ml of 125I-[H]-[Sar1, Ile8]Ang II for 1 h at 22°C. Nonspecific binding was determined by parallel incubations containing 0.5 μM unlabeled [Sar1, Ile8]Ang II. To characterize Ang II receptor subtypes, the competitive inhibition of 125I-[H]-[Sar1, Ile8]Ang II binding was examined on consecutive sections by coincubation with 5 μM PD123319, an AT2 antagonist, and 1 μM CV-11974, an AT1 antagonist. AT1 binding was determined by the presence of an excess of PD123319 and AT2 binding by the presence of an excess of CV-11974. After incubation, the sections were transferred through four successive 1-min washes of buffer without BSA and bacitracin at 0°C. The slides were dried under a stream of cool air, loaded into X-ray cassettes, and autoradiographed with Agfa Scopix CR3B film for 14 days at room temperature. 125I-Radioactivity standards were exposed to the same films in parallel with tissue sections. The optical density of the films was quantitated using a microcomputer imaging device with a high-resolution charge-coupled device camera (Imaging Research, St. Catherine’s, ON, Canada). The radioactivity standards were corrected for decay and fitted to calibration curves by a computer to convert optical density values for each pixel into 125I radioactivity (dpm/mm²).

Immunohistochemistry. Serial sections (5-μm thick) were cut in the transverse plane, thaw-mounted onto gelatin-coated slides, dried in a desiccator at 4°C overnight under reduced pressure, and stored at −80°C in

In Vitro Autoradiography for ACE and Ang II Receptors. Serial cryostat sections (20-μm thick) were cut in the transverse plane, thaw-mounted onto gelatin-coated slides, dried in a desiccator at 4°C overnight under reduced pressure, and stored at −80°C in
mM NaCl, 4.7 mM KCl, 1.2 mM NaH₂PO₄, 2.5 mM CaCl₂, 25 mM NaHCO₃, and 11 mM glucose), and the solution was aerated continuously with O₂/CO₂ (95:5) with a pH of 7.4 at 37°C. Before starting the experiments, all strips were allowed to equilibrate for 90 to 120 min in a bath, during which time oxygenated medium was replaced every 15 min. The initial resting tension was set at 0.2 g. Reagents were added at volumes less than 1% of the total volume. All reported concentrations of reagents are bath concentrations exposed for 3 min at least every 30 min, during which time the oxygenated medium was replaced every 15 min. After an equilibration period, the contractile response to 30 mM KCl was first obtained as a reference for the corresponding agonist-induced contraction (Okamura et al., 1999). The strips were repeatedly washed with fresh media, and a control cumulative concentration-response curve for Ang I (1 × 10⁻⁶ – 1 × 10⁻⁶ M) was obtained. As previously reported (Okunishi et al., 1993), responses to Ang I were stabilized with sufficient reproducibility after the second or third dose; therefore, the response after the third or fourth dose was regarded as the control response. After the maximum contractile response was obtained, the strips were rinsed four times over a 1-h period and allowed to relax to the baseline tension. Inhibitory effects of the ACE inhibitor lisinopril (10⁻⁶ M) alone or in combination with the chymase inhibitor chymostatin (10⁻⁴ M) on Ang I-induced (3 × 10⁻⁷ M) contraction were studied. Furthermore, effects of AT1 or AT2 receptor antagonists (10⁻⁶ M CV-11974 and 5 × 10⁻⁶ M PD123319) on Ang I-induced contraction were also examined.

**Statistical Analysis.** The results shown in the text and figures were expressed as mean values ± S.E.M. Statistical analyses were made by Student's unpaired t test for two groups and Fisher's protected least significant difference test after one way analysis of variance for three groups.

**Results**

**ACE and Chymase-Like Activities.** The ACE activity in the corpus cavernosum was 6.9 ± 1.7 mU/mg of protein (n = 8), and the chymase-like activity was 4.0 ± 1.4 mU/mg of protein. The ACE and chymase-like activities were 30- and 160-fold higher, respectively, than those reported for the canine common carotid artery (Miyazaki et al., 1999).

**Autoradiographic Localization of ACE and Ang II Receptors.** Nonspecific binding determined in the presence of 1 mM EDTA or 0.5 μM [Sar¹,Ile⁸]Ang II was very weak and produced no visible images on the X-ray films, confirming that the results in this study represent specific binding. The histology of the canine penis is demonstrated in Fig. 1, A and B. Very dense binding of ¹²⁵I-351A was observed in the corpus cavernosum penis and urethra (Fig. 1C). Punctate binding was also observed in the tunica albuginea. Microscopic comparison between the autoradiograms and stained sections revealed that the punctate binding corresponded with the vasculature in the tunica albuginea. Fibrous frameworks of trabeculae and tunica albuginea were devoid of specific ACE binding. The binding pattern of ¹²⁵I-[Sar¹,Ile⁸]Ang II was similar to that of ¹²⁵I-351A. Ang II receptors were localized in the smooth muscle layer of the

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**Fig. 1.** Autoradiographic localization of ACE and Ang II receptor subtypes in the dog penis. ACE was labeled with ¹²⁵I-351A and Ang II receptors by ¹²⁵I-[Sar¹,Ile⁸]Ang II. A, the histology of a hematoxylin- and eosin-stained section; B, a schematic drawing of the corpus cavernosum penis (ccp), trabecula (tr), urethra (u), corpus cavernosum urethra (ccu), and vessels (v) in the tunica albuginea (ta); C, ¹²⁵I-351A binding; D, ¹²⁵I-[Sar¹,Ile⁸]Ang II binding; E, residual ¹²⁵I-[Sar¹,Ile⁸]Ang II binding in the presence of 5 μM PD123319 indicating AT1 receptor binding; F, residual ¹²⁵I-[Sar¹,Ile⁸]Ang II binding in the presence of 1 μM CV-11974 indicating AT2 receptor binding. White in the autoradiograms indicates a high level of binding against the black background.
corpus cavernosum penis and urethra, and the vessels in the tunica albuginea (Fig. 1D). The AT1 receptor antagonist CV-11974 potently inhibited radioligand binding, while the AT2 receptor antagonist PD123319 did not affect radioligand binding (Fig. 1, E and F).

**Immunolocalization of ACE.** ACE-like immunoreactivity was localized in the endothelium lining the lacunar space (Fig. 2, A and B) and also in the urethra and emissary veins in the tunica albuginea (Fig. 2C).

**Localization of Mast Cells.** Mast cells visualized by toluidine blue staining were present both in the cavernosal area and stroma (Fig. 2D). Quantitation of cell numbers revealed the predominant occurrence of mast cells in the cavernosal area (43.1 ± 11.3 cells/mm²) rather than in the stroma (8.1 ± 2.1 cells/mm², p < 0.01, n = 3).

**Measurement of Isometric Tension.** The contractile response to 30 mM KCl was 0.21 ± 0.05 g (n = 5). Exposure to Ang I, a precursor of Ang II, caused a dose-dependent increase in tension. The threshold concentration of Ang I causing contraction was 10⁻⁶ M, and maximal contractile responses occurred at 10⁻⁶ M (Fig. 3). Administration of 3 × 10⁻⁷ M Ang I solution yielded the most reproducible contractile response, averaging 92.9 ± 9.4% of the contractions caused by 30 mM KCl. Pretreatment with lisinopril (10⁻⁶ M) suppressed the responses to Ang I (3 × 10⁻⁷ M) by about 40%. The combination of lisinopril (10⁻⁶ M) and chymostatin (10⁻⁴ M) almost completely abolished the responses to Ang I (Fig. 4, A and B). The contractile responses of corporal smooth muscle to Ang I were completely blocked by CV-11974 but not by PD123319. By the end of the equilibration period, none of the canine corpus cavernosal strips displayed spontaneous oscillating activity.

**Discussion**

The present study indicated that Ang II is generated from Ang I by at least two independent pathways, ACE and chymase, with contraction of the corporal smooth muscle via AT1 receptors in the canine penile corpus cavernosum. The biochemical experiments demonstrated considerably higher ACE and chymase-like activities in the corpus cavernosum penis than in the contracting vessels, which is the main target of Ang II. The Ang I-induced contraction of the corporal smooth muscle was only slightly inhibited by the pretreatment of 10⁻⁶ M lisinopril, which was sufficient to completely block the Ang I-induced contraction in various isolated blood vessels from monkeys, dogs, rats, and rabbits (Okunishi et al., 1993; Jin et al., 2000). Pretreatment of the

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**Fig. 2.** ACE localized by immunohistochemistry and mast cells visualized by toluidine blue staining. A, ACE is localized in the endothelium lining the lacunar space of the dog corpus cavernosum penis (original magnification, 100×); B, high-power magnification of the corpus cavernosum penis (original magnification, 200×); C, ACE in the emissary veins in the tunica albuginea (original magnification, 200×); D, mast cells (arrow heads) in the dog corpus cavernosum penis (original magnification, 100×). Scale bar = 50 μm.
corporal strips by combination of lisinopril and chymostatin almost completely inhibited the Ang I-induced contraction, indicating that Ang II is generated in situ by chymase in addition to ACE. Morphological studies revealed that ACE was present in the endothelium lining the lacunar spaces of the corpus cavernosum penis, and mast cells that produce chymase were present in the cavernosal area. AT1 receptors were also localized in the corporal smooth muscle area. These data obtained from morphological studies also support the conclusion that Ang I is converted in situ to Ang II to affect corporal smooth muscle.

Corporal smooth muscle tone is important in the regulation of penile erection (Christ et al., 1992). Decreased tone of corporal smooth muscle leads to an increase of blood pooling in the lacunar spaces and reduced cavernosal venous outflow by compression of emissary veins against the tunica albuginea, the surrounding fibrous structure (Lue, 2000). On the other hand, contraction of corporal smooth muscle and penile arteries is required for penile detumescence. This contraction is probably mediated mainly by the release of noradrenaline acting on post junctional α-adrenoreceptors. However, it is suggested that additional mechanisms are involved in the long-term maintenance of the high penile smooth muscle tone necessary for keeping the penis in a flaccid state (Andersson and Holmquist, 1990). Other contractile factors such as endothelin and endotheliun-derived contracting factors may also contribute to the flaccid state (Holmquist et al., 1992).

Ang II, a major modulator of regional blood flow in the extracavernosal segments of the vascular beds, has been reported to cause contraction of the canine corporal smooth muscle via AT1 receptors (Comiter et al., 1997). Ang I also causes contraction of the rabbit corporal smooth muscle in an ACE-dependent fashion (Park et al., 1997). Kifor et al. (1997) demonstrated that the human corpus cavernosum contained Ang I and Ang II at levels that were 30- and 200-fold greater, respectively, than in human plasma. Furthermore, they showed that intracavernosal injection of the AT1 receptor antagonist losartan increased intracavernosal pressure dose dependently up to mean arterial pressure, and also intracavernosal injection of Ang II decreased intracavernosal pressure and terminated spontaneous erection in anesthetized dogs (Kifor et al., 1997). These data suggest that large amounts of Ang II are locally generated in the corpus cavernosum penis and regulate penile blood flow.

Concerning our present data, there are papers supporting an alternative Ang II-forming pathway attributable to chymase (Urata et al., 1991; Shiota et al., 1993). Chymase is mast cell-derived serine protease, but there is species specificity. Human, monkey, hamster, and dog chymases convert Ang I to Ang II, while rat and rabbit chymases do not. The ratios of chymase/ACE dependence of Ang I-induced vascular contraction in human, monkey, hamster, and dog are about 70:30, 50:50, 35:65, and 30:70%, respectively (Miyazaki and Takai, 2000). In human heart and vessels, chymase contributes considerably to generation of Ang II from Ang I (Urata et al., 1990a; Takai et al., 1997). Therefore, chymase probably plays a more important role than ACE in Ang II formation in the human cardiovascular systems. Interestingly, Arakawa and Urata (2000) indicated a close association between cholesterol level and arterial chymase up-regulation together
with development of atherosclerosis, which is involved in the pathophysiology of erectile dysfunction.

Many antihypertensive drugs, such as methyldopa, thiazides, spiranoltacne, guanethidene, clonidine, and certain β-blockers, have long been implicated in the induction of erectile failure (Jensen et al., 1999). The development of erectile problems is one of the most common causes for non-compliance in the treatment of hypertension (Moraes et al., 1994). Although the mechanism of erectile dysfunction in hypertensive men has not been clearly elucidated, insufficient penile blood flow following a decrease in systemic blood pressure seems to be involved (Jensen et al., 1999). It is therefore important to use an agent with less effect on penile blood flow during antihypertensive therapy. Generally, ACE inhibitors are known to have fewer deteriorating effects on male erectile function. Since Ang II causes contraction of the corporal smooth muscle (Comiter et al., 1997; Park et al., 1997) and decreases penile blood flow (Kifor et al., 1997), blockade of the RAS reduces corporal smooth muscle tone and may overcome the decrease in systemic blood pressure by improving local blood flow. On the other hand, it has recently been suggested that Ang II is in part responsible for endothelial dysfunction because it reduces the action of nitric oxide (Mombouli and Vanhoutte, 1999), which is established as a physiologic mediator of penile erection (Burnett et al., 1992). Moreover, Ang II is thought to be involved in vascular hypertrophy in hypertension and atherosclerosis (Miyazaki et al., 1999). Consequently, ACE inhibitors and AT1 receptor antagonists that prevent the effects of Ang II probably have an advantage over the other antihypertensive drugs in erectile function.

It is unclear at present which is more advantageous in terms of the conservation of penile blood flow, ACE inhibitors or AT1 receptor antagonists. The merit of ACE inhibitors over AT1 receptor antagonists is the associated accumulation of bradykinin, which causes smooth muscle relaxation (Kimoto et al., 1990), because ACE is critical for the metabolism of bradykinin in addition to Ang I. However, ACE inhibitors cannot inhibit all Ang II generation, so the effect of Ang II generated from the alternative pathways may remain. On the other hand, AT1 receptor antagonists block the actions of almost all Ang II generated by both ACE and chymase, which might be dominant in Ang II generation in the penis. If chymase plays a more important role than ACE in Ang II formation, AT1 receptor antagonists may be more advantageous than ACE inhibitors. In this study, we have also demonstrated the presence of ACE and AT1 receptors in emissary veins in the tunica albuginea. This finding should be investigated further.

In conclusion, the present study demonstrated that Ang II is generated by chymase as well as ACE during contraction of canine corporal smooth muscle. The data presented in this study suggesting the role of chymase in Ang II-forming pathways in the corpus cavernosum penis should be a focus for future research.

References


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