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Characterization of angiotensin II formation in human isolated bladder by selective inhibitors of ACE and human chymase: a functional and biochemical study

Kristian Waldeck, B. Fredrik Lindberg, Katarina Persson & [1]Karl-Erik Andersson

Department of Clinical Pharmacology, Lund University, Lund, Sweden

1 Functional recordings of smooth muscle tension and biochemical experiments on membrane fractions were performed to characterize angiotensin II (AII) formation in human isolated bladder smooth muscle.

2 A novel human chymase inhibitor CH 5450 (Z-Ile-Glu-Pro-Phe-CO_2Me) and a recently developed human chymase substrate Pro[11]-,D-Ala[12])-angiotensin I, claimed to be resistant to angiotensin converting enzyme (ACE) and carboxypeptidase, were used.

3 Angiotensin I (AI) (0.3 μM) induced a contractile response amounting to $58 \pm 5\%$ ($n=12$) of the initial K^+ (124 mM)-induced contractions. This response was reduced to $36 \pm 3\%$ ($n=8$) by the ACE-inhibitor enalaprilat (10 μM), while pretreatment with soybean trypsin inhibitor (STI 200 μg ml^{-1}) or CH 5450 (10 μM) had no effect. However, the combination of enalaprilat and STI reduced the AI-induced contractions to $19 \pm 5\%$ ($n=6$), and the combination of enalaprilat and CH 5450 caused an almost complete inhibition of the AI-induced contractions to $1 \pm 1\%$ ($n=6$).

4 The substrate (Pro[11]-,D-Ala[12])-AI (3 μM) produced contractions which amounted to $57 \pm 4\%$ ($n=13$) of the initial K^+ (124 mM) contractions. These contractions were not affected by enalaprilat (10 μM). On the other hand, STI (200 μg ml^{-1}) and CH 5450 (10 μM) added separately, depressed the (Pro[11]-,D-Ala[12])-AI-induced contractions to $34 \pm 5\%$ ($n=6$) and $24 \pm 4\%$ ($n=6$), respectively. The combination of enalaprilat and STI or enalaprilat and CH 5450 did not produce any further inhibition.

5 Experiments with detrusor membrane fractions incubated with AI (50 μM) were performed. In the presence of enalaprilat (100 μM), carboxypeptidase inhibitor CPI (10 μg ml^{-1}) and aprotinin (15 μM), CH 5450 (10 nM – 1 μM) caused a concentration-dependent inhibition of AII formation.

6 The results confirm that AII is a potent contractile agent in the human isolated detrusor muscle. They also indicate that the serine protease responsible for AII formation in the human bladder *in vitro* is human chymase or an enzyme similar to human chymase.

Keywords: Urinary bladder; angiotensin; angiotensin converting enzyme; chymase

Introduction

The traditional view of the renin-angiotensin system is that renin and angiotensin converting enzyme (ACE) act on angiotensinogen and angiotensin I (AI), respectively, to generate angiotensin II (AII). The predominant effects of the renin-angiotensin system have been considered to be regulation of vascular tone and aldosterone secretion (Peach, 1977). However, this view has been revised and it is now believed that AII also may act as a neuromodulator and may be involved in, for example, cell proliferation and smooth muscle hypertrophy (Timmermans *et al*., 1993). Local renin-angiotensin systems have been suggested to occur in several peripheral tissues (Oliver & Sciacca, 1984; Dzau, 1988; Paul *et al*., 1993; Ganong, 1994), even if there is some scepticism whether renin is expressed in extrarenal tissues (Kvist *et al*., 1991; von Lutterotti *et al*., 1994).

A physiological role for the renin-angiotensin system in the lower urinary tract has not been established. However, functional *in vitro* experiments on urinary bladder smooth muscle from several species, including man, have revealed that both AI and AII act as potent contractile agents in this tissue (Falconieri Erspamer *et al*., 1973; Erspamer *et al*., 1981; Andersson *et al*., 1992; Lindberg *et al*., 1994). AII-induced contractions in the human isolated bladder were suggested to be mediated through AT_1 receptors since the contractile effect was blocked by the non-peptide AT_1 receptor antagonist losartan (Waldeck *et al*., 1995). Similar results have also been obtained in the rat bladder (Tanabe *et al*., 1993). Most likely, the AI-induced contractions are mediated through the same receptor after conversion of AI to AII. However, a direct effect of AI cannot be ruled out.

Alternative pathways for AII formation, in addition to ACE, have previously been demonstrated in the human heart (Urata *et al*., 1990a,b; Husain *et al*., 1993). The finding that AI-induced contractions in human isolated bladder smooth muscle are not abolished by the ACE inhibitors captopril or enalaprilat (Andersson, *et al*., 1992; Lindberg *et al*., 1994) indicates that an ACE-independent pathway for AII formation might be present in the bladder as well. These experiments also gave a clue to the mechanism for this ACE-independent pathway, since ACE-inhibition in combination with the serine protease inhibitor soybean trypsin inhibitor (STI) resulted in an enhanced inhibition of the AI-induced contractions (Lindberg *et al*., 1994). It has been proposed that human chymase is the major AII-forming serine protease in the human heart (Urata *et al*., 1990b; 1991), although conflicting results have been obtained (Zisman *et al*., 1995).

In the present study, we performed functional recordings of bladder contractility and biochemical experiments on membrane fractions to characterize the ACE-independent, serine protease-mediated, pathway for AII formation in the human isolated bladder. In this study, we used a recently developed substrate, (Pro[11]-,D-Ala[12])-AI, suggested to be resistant to ACE and carboxypeptidase (Mangiapane *et al*., 1994), and a novel enzyme inhibitor selective for human chymase, CH 5450 with the peptide sequence Z-Ile-Glu-Pro-Phe-CO_2Me (Bastos *et al*., 1995).

[1]Author for correspondence at: Dept. Clinical Pharmacology, Lund University Hospital, S-221 85 Lund, Sweden.

Methods

Tissue preparation

After informed consent and approval of the Ethics Committee (University of Lund, Sweden), urinary bladder specimens were obtained from 15 patients (age 53–89 years; 11 males) undergoing cystectomy because of bladder carcinoma. The patients were not irradiated or treated with ACE-inhibitors. All tissues used in the experiments were taken from areas macroscopically free from tumours and placed in cold Krebs solution (for composition see below). Functional experiments were performed within 24 h after the operation and tissues used in the biochemical studies were frozen and stored at −70°C.

Measurements of mechanical activity

Smooth muscle strips (1 × 1 × 5 mm) were prepared from the detrusor after the mucosa had been removed. The strips were mounted between two hooks in 5 ml tissue baths by means of silk ligatures. One of the hooks was connected to a force transducer (Grass model FT03, Grass Instruments, U.S.A.) and the other was connected to a movable unit, making it possible to adjust the tension. The Krebs solution was continuously bubbled with a mixture of 95% O_2 and 5% CO_2, giving a pH of 7.4, and the solution was exchanged every 20 min. The temperature was maintained at 37°C by a thermoregulated water circuit surrounding the tissue bath. During an equilibration period of one hour, the preparations were repeatedly stretched until a resting tension of 4 mN was obtained.

Each experiment was started by repeated exposures to K^+ Krebs (124 mM) until reproducible contractions were obtained. Enzyme inhibitors and receptor antagonists were added 20 min before the angiotensin receptor agonists. Due to a pronounced tachyphylaxis (Andersson *et al*., 1992), each preparation could only be exposed to one single dose of AI. Therefore, the contractile effect is expressed as % of the initial K^+ induced contraction.

Preparation of membranes from human bladder

The specimens from two human bladders were prepared as previously described (Lindberg *et al*., 1994), and alkaline phosphatase (ALP) (kit 245, Sigma Diagnostics) was assayed as a membrane-bound marker enzyme for each preparation step. The enrichment of ALP-activity was 10 and 11 times, respectively, for the two specimens.

Effect of CH 5450 and enalaprilat on hippuric acid formation from Hip-Gly-Gly

A possible inhibitory effect of CH 5450 on ACE was investigated by incubating membrane fractions from two individuals (final protein concentration 0.66 and 1.22 mg ml^{-1}, respectively) and the ACE-substrate N-Hip-Gly-Gly (1 mM), with or without enalaprilat (1–100 μM) or 10 μM CH 5450 (Lindberg *et al*., 1994).

Measurement of AII formation in membrane fractions

AI or (Pro^{11}-,D-Ala^{12})-AI was dissolved in HEPES buffer to a final concentration of 50 μM and equilibrated at 37°C for 5 min. The reaction, carried out in a water bath (37°C), was initiated by the addition of an aliquot of membranes with the final protein concentration of 4 and 15 μg ml^{-1}, respectively. Incubations were stopped by the addition of ice-cold ethanol and the precipitates were separated by centrifugation at 1000 × *g*, 15 min, 4°C. An aliquot of the supernatant was collected and the products formed were separated from unmetabolized AI by high performance liquid chromatography (h.p.l.c). Further experiments were performed in the presence of enalaprilat (100 μM) and carboxypeptidase inhibitor from potato (CPI; 10 μg ml^{-1}) in order to inhibit AII formation by ACE and sequentially hydrolysing carboxypeptidases. In order to expose AI only to human chymase, all other serine proteases known to catalyse AII formation were inhibited by 15 μM aprotinin (Husain *et al*., 1993; Wintroub *et al*., 1986). The concentration-dependent (10 nM–1 μM) inhibitory effect of the selective chymase inhibitor, CH 5450 (Bastos *et al*., 1995), was tested in the presence of the inhibitor cocktail.

H.p.l.c. was performed on a Spectra-Physics system, equipped with a SP 8700 XR pump, an autosampler model SP 8780 XR and a Waters LC spectrophotometer model 481. Separation was performed on a μBondapak C_{18} reverse phase column (300 × 3.9, Millipore). Angiotensins were separated with a linear gradient of 20–32% acetonitrile in 0.1% trifluoroacetic acid (TFA)/water over 19 min, and detected at 215 nm. Formed angiotensin products were identified by comparing the retention times on the chromatograms with those of synthetic angiotensins.

Drugs and solutions

Human synthetic AI, AII, A(1–9), His-Leu, phosphoramidon, carboxypeptidase inhibitor (potato; CPI), soybean trypsin inhibitor (STI) and aprotinin were obtained from Sigma Chemical Company (St Louis, MO, U.S.A.). Enalaprilat was purchased from Merck-Sharp & Dohme (Sweden), CH 5450 (Z-Ile-Glu-Pro-Phe-CO_2Me) was a gift from Ferring Research Institute (U.K.) and (Pro^{11}-,D-Ala^{12})-AI was a gift from Pfizer Incorporated Central Research Division, Groton, U.S.A.). TFA (h.p.l.c.-grade) was purchased from Pierce Chemical Co, and acetonitrile (h.p.l.c.-grade) from Merck (Germany). All other chemicals were of analytical reagent grade.

A Krebs solution of the following composition was used in the functional experiments (mM): NaCl 119, KCl 4.6, $CaCl_2$ 1.5, $MgCl_2$, 1.2, $NaHCO_3$ 15, NaH_2PO_4 1.2 and glucose 5.5. K^+ Krebs solution (124 mM) prepared by replacing NaCl with equimolar amounts of KCl. The membrane incubations were carried out in 50 mM HEPES buffer, pH 8.0, containing 300 mM NaCl and 400 mM Na_2SO_4 (final concentrations).

Analysis of data

The contractile effects of the agonists are expressed as % of the initial 124 mM K^+-induced contractions. Results are given as mean values ± s.e.mean and *n* denotes the number of individuals. Statistical significance was calculated by using analysis of variance, followed by a Dunnett's *post hoc* test. A general linear model (proc GLM in the SAS software) was used for the analysis of variance, since we did not have observations from all treatments from all subjects. A probability level $P < 0.05$ was accepted as significant. IC_{50} values denote the concentrations which inhibit the maximally obtained agonist-induced contractions by 50%, and the pIC_{50} value represents the negative logarithm of the IC_{50} value.

Results

Contractions induced by AI in isolated bladder smooth muscle strips

Recordings of tension induced by AI on isolated bladder smooth muscle showed that AI is a potent contractile agent in this tissue. Preliminary experiments were performed to determine the concentration of AI which contracted the tissue to 75% of maximal AI-induced contraction. This AI concentration, 0.3 μM, produced contractions amounting to 58 ± 5% ($n = 12$) of the initial K^+ (124 mM)-induced contractions (44 ± 7 mN; $n = 12$). The AI-induced contractions were depressed ($P < 0.001$) to 36 ± 3% ($n = 8$) by pretreatment with 10 μM enalaprilat.

STI (200 μg ml^{-1}) or CH 5450 (10 μM) used to inhibit AII formation through the proposed serine protease pathway. Pretreatment with these inhibitors did not cause any inhibition of the contractions induced by AI (0.3 μM), the responses being 56$\pm$8% (n=6) with STI and 58$\pm$9% (n=7) with CH 5450. However, a combination of enalaprilat and STI reduced (P<0.001) the contractions to 19$\pm$5% (n=6), and a combination of enalaprilat and CH 5450 blocked (P<0.001) the contractions almost completely (1$\pm$1%; n=6) (Figure 1). In the presence of enalaprilat (10 μM), the inhibitory effect of CH 5450 (10 nM – 10 μM) was concentration-dependent and resulted in a pIC_{50} value of 7.0$\pm$0.4 (n=6) (Figure 2).

Contractions induced by (Pro^{11}-,D-Ala^{12})-AI in isolated bladder smooth muscle strips

Initial experiments with (Pro^{11}-,D-Ala^{12})-AI were performed in order to determine a concentration which had the same contractile effect as 0.3 μM AI. This resulted in a concentration of 3 μM, which caused a contraction amounting to 57$\pm$4% (n=13) of the initial K^+ (124 mM)-induced contraction (40$\pm$8 mN; n=13). Pretreatment with enalaprilat (10 μM) had no depressant effect on these contractions (58$\pm$5%; n=10). On the other hand, inhibition of the serine protease pathway by STI (200 μg ml^{-1}) resulted in a significant (P<0.01) inhibition to 34$\pm$5% (n=6), and serine protease inhibition by CH 5450 (10 μM) reduced (P<0.001) the contractile effect to 24$\pm$4% (n=6) (Figure 3). A lower concentration of CH 5450 (1 μM) had no effect when added separately (63$\pm$6%; n=7), but tended to decrease the contractions to 37$\pm$4% (n=6) when in combination with enalaprilat (10 μM). The inhibitory effects of CH 5450 (10 μM) or STI (200 μg ml^{-1}) on (Pro^{11}-,D-Ala^{12})-AI-induced contractions were not enhanced by pretreatment with enalaprilat (10 μM), the responses being 30$\pm$6% (n=7) and 42$\pm$8% (n=5), respectively. Pretreatment with losartan (10 μM) blocked the (Pro^{11}-,D-Ala^{12})-AI-induced contractions completely.

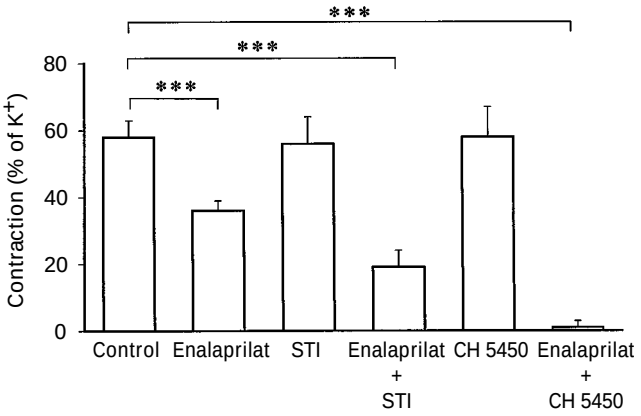

Figure 1 The effect of enalaprilat (10 μM), soybean trypsin inhibitor (STI, 200 μg ml^{-1}) and CH 5450 (10 μM) on angiotensin I (AI)-induced (0.3 μM) contraction of the human isolated bladder. Responses have been calculated as a percentage of the response to 124 mM K^+ Krebs solution and are shown as the mean$\pm$s.e.mean of observations in preparations from 6–12 individuals. ***Denotes a statistically significant difference (P<0.001) from control responses.

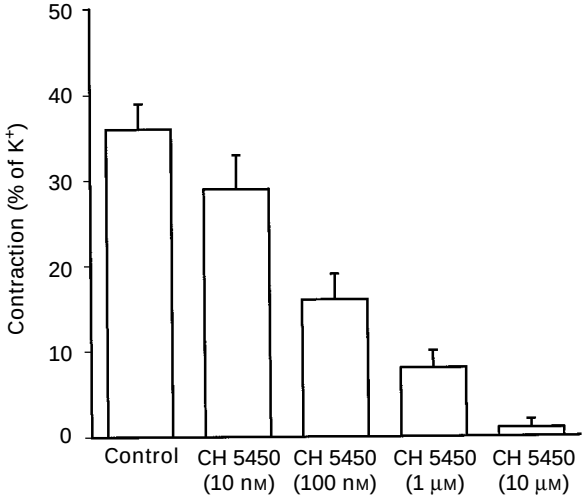

Figure 2 The effect of CH 5450 (10 nM – 10 μM) on angiotensin 1 (AI)-induced (0.3 μM) contraction of the human isolated bladder. Responses have been calculated as a percentage of the response to 124 mM K^+ Krebs solution and are shown as the mean$\pm$s.e.mean of observations in preparations from 6 individuals. The experiments were performed in presence of enalaprilat (10 μM).

Angiotensin II formation in membrane fractions

The selectivity of CH 5450 was verified by using the synthetic ACE-substrate Hip-Gly-Gly. In control experiments, where membrane fractions from two individuals were incubated with Hip-Gly-Gly, 22% and 42%, respectively, of the Hip-Gly-Gly was hydrolyzed to hippuric acid. Enalaprilat at all concentrations used (1 – 100 μM) completely inhibited hippuric acid formation, while CH 5450 (10 μM) had no effects on the ACE activity.

Chromatographic evaluation of membrane fractions incubated with AI (50 μM) resulted in four chromatographic peaks with retention times (RT) corresponding to those obtained with synthetic His-Leu (RT 3.40 min), AI (1 – 9) (5.82 min), AII (9.00 min) and AI (12.26 min), respectively (Figure 4a). In the presence of CPI (10 μg ml^{-1}), no formation of AI (1 – 9) was observed. All further experiments were performed in presence of CPI (10 μg ml^{-1}) in order to inhibit potential AII formation from AI (1 – 9). Inhibition of ACE by enalaprilat (100 μM) reduced the AII formation by 11 and 17% (n=2). In contrast, simultaneous inhibition of ACE and serin proteases by enalaprilat (100 μM) and STI (100 μg ml^{-1}) inhibited AII formation completely. Replacement of STI by aprotinin (15 μM), an inhibitor of serine proteases except human chymases (Husain *et al*., 1993), did not inhibit the AII formation more than enalaprilat added separately (12 and 19%; n=2). During concomitant pretreatment with enalaprilat (100 μM) and aprotinin (15 μM), the human chymase inhibitor CH 5450 (10 nM – 1 μM) caused a concentration-dependent inhibition of the AII for-

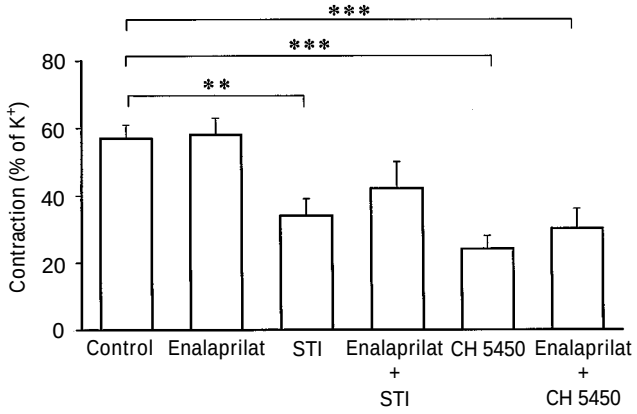

Figure 3 The effect of enalaprilat (10 μM), soybean trypsin inhibitor (STI, 200 μg/ml) and CH 5450 (10 μM) on (Pro^{11}-,D-Ala^{12})-AI-induced (3 μM) contraction of the isolated human bladder. Responses have been calculated as a percentage of the response to 124 mM K^+ Krebs solution and are shown as the mean$\pm$s.e.mean of observations in preparations from 6–13 individuals. *** and ** denotes a statistically significant difference (P<0.001) and (P<0.01), respectively, from control responses.

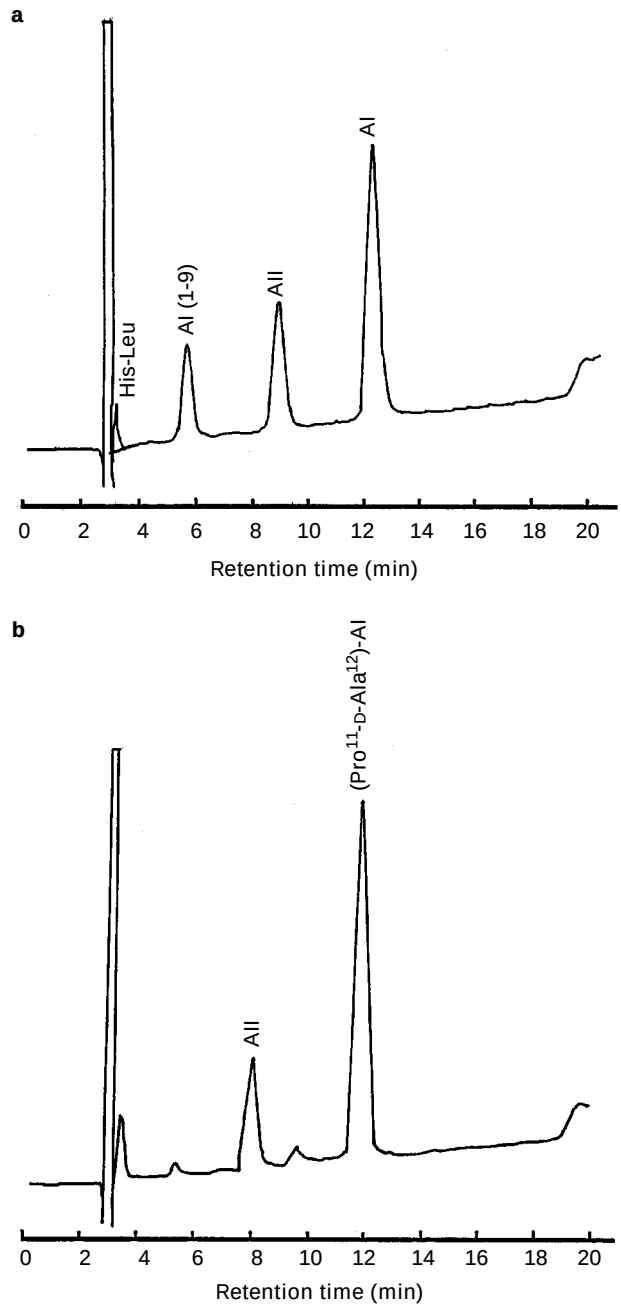

Figure 4 Original chromatogram from incubation of (a) angiotensin I (AI) and (b) (Pro^{11}-,D-Ala^{12})-AI with bladder smooth muscle membranes. The products were identified by comparing their retention times with those of synthetic His-Leu, AII, angiotensin I 1–9, AI and (Pro^{11}-,D-Ala^{12})-AI. Retention times in chromatogram (a) after incubation with AI were: 3.40 min (His-Leu), 5.82 min (AI 1–9), 9.00 min (AII) and 12.26 min (AI). Retention times in chromatogram (b) after incubations with (Pro^{11}-,D-Ala^{12})-AI were: 8.29 min (AII) and 12.06 min (Pro^{11}-,D-Ala^{12})-AI.

mation and resulted in a complete inhibition at the highest concentration used (see Table 1)

Chromatographic evaluation of membrane fractions incubated with (Pro^{11}-,D-Ala^{12})-AI (50 μM) resulted in two major peaks, identified as AII (8.29 min) and (Pro^{11}-,D-Ala^{12})-AI (12.06 min) (Figure 4b). Several minor peaks were not identified. Pretreatment with the ACE inhibitor enalaprilat (100 μM) reduced the AII formation by 12 and 32% ($n=2$). In the presence of enalaprilat (100 μM), CPI (10 μg ml^{-1}) and aprotinin (15 μM), CH 5450 (10 nM – 1 μM) caused a concentration-dependent inhibition of AII formation from (Pro^{11}-,D-Ala^{12})-AI, with a final inhibition of 68 and 88% ($n=2$) at the highest concentration used (see Table 1).

Table 1 Inhibitory effects of CH 54550 on the conversion of angiotensin I (AI) and (Pro^{11}-, D-Ala^{12})-AI (50 μM) to AII in human bladder membranes

CH 5450 (μM)	*AI*		*(Pro^{11}-,D-Ala^{12})-AI*	
	Patient 1	*Patient 2*	*Patient 1*	*Patient 2*
0.01	62	85	93	77
0.03	42	56	76	60
0.1	21	25	65	31
0.3	9	12	52	18
1	3	0	32	12

The results are expressed as % of AII-formation in control experiments with CH 5450 replaced by its vehicle. The experiments were performed in the presence of enalaprilat (10 μM), CPI (10 μg ml^{-1}) and aprotinin (15 μM).

Discussion

A physiological role for the renin-angiotensin system in the bladder has not yet been established. However, the findings of Cheng *et al.* (1996) suggest a possible growth-stimulating effect in this organ, since ACE-inhibition reduced obstructive changes due to outlet obstruction in the neonatal rabbit bladder. No effect on bladder hypertrophy development was found on treatment of rats with outlet obstruction, with the AT_1-receptor antagonist, losartan (Persson *et al.*, 1996). However, a growth stimulating effect cannot be ruled out since species differences must be considered. Interestingly, increased prostatic ACE-activity has been demonstrated in benign prostatic hypertrophy (van Sande *et al.*, 1985). Whether increased ACE-activity can be demonstrated in the human hypertrophic bladder remains to be established. It has been shown that AII has a potent contractile effect in the human isolated bladder (Andersson *et al.*, 1992) mediated by AT_1-receptors (Waldeck *et al.*, 1995), and that the human bladder has a capacity to produce AII by more than one pathway (Lindberg *et al.*, 1994).

In the present study the inhibitory effect of enalaprilat was studied in concentrations known to inhibit AI-induced contractions in other tissues, e.g. the rabbit mesenteric artery (Andersson *et al.*, 1992). However, in our study enalaprilat failed to block the AI-induced bladder contractions completely, an observation which is in accord with previous investigations in this tissue (Andersson *et al.*, 1992; Lindberg *et al.*, 1994). Thus, the remaining contractile effect is probably due to an additional mechanism for AII formation. ACE-independent AII formation has previously been suggested by *in vitro* experiments on the hamster cheek pouch (Cornish *et al.*, 1979), vascular segments from man, monkey and dog (Okunishi *et al.*, 1984; 1993; Okamura *et al.*, 1990), and the human heart (Urata *et al.*, 1990a,b). Furthermore, *in vivo* experiments have indicated that the haemodynamic variables in conscious baboons are in part determined by ACE-independent AII formation (Hoit *et al.*, 1995).

Previous studies have suggested a role for serine proteases as AII forming enzymes (Urata *et al.*, 1990a). Experiments with serine protease inhibitors, such as chymostatin and soybean trypsin inhibitor (STI), have demonstrated that the concomitant inhibition of ACE and serine proteases results in inhibition of AI-induced contractions in the human bladder (Lindberg *et al.*, 1994), findings which were confirmed in our study. A major AII forming serine protease has been identified as chymase in the human heart (Urata *et al.*, 1990b; 1991), and by using the novel inhibitor of this chymase, CH 5450 (Bastos *et al.*, 1995), we found that the concomitant inhibition of human chymase and ACE resulted in a concentration-dependent inhibition of AI-induced contractions. These results demonstrate that human chymase, or an enzyme resembling human chymase, is present in the human bladder and has the ability to mediate AI-induced contractions. Interestingly, neither the ACE-inhibitor, nor the chymase-inhibitor were able to inhibit the AI-induced contractions completely when added sepa-

rately. It may be speculated that this is due to an overcapacity among the AII generating enzymes, implicating that the supply of substrate is the rate limiting step in AII formation.

The inability of enalaprilat to block AII formation in human bladder was confirmed by chromatographic evaluation of membrane fractions incubated with AI. These experiments also strengthen the hypothesis that a chymase-like enzyme is involved in AII formation, since CH 5450 produced a concentration-dependent inhibition of AII formation in presence of enalaprilat. Aprotinin, on the other hand, which is known to inhibit serine proteases, except human chymase (Wintroub *et al*., 1986), had no inhibitory effect.

To study further the alternative AII-forming pathway, we used a substrate, (Pro^{11}-,D-Ala^{12})-AI, suggested to be resistant to ACE and carboxypeptidase (Mangiapane *et al*., 1994). This substrate has previously been shown to induce contractions in the canine mesenteric artery *in vitro*, and pressor effects in marmosets *in vivo*, predominantly by an ACE-independent pathway. Moreover, i.v. administration of (Pro^{11}-,D-Ala^{12})-AI to conscious baboons resulted in changed cardiovascular parameters in both control subjects and in animals receiving ACE-inhibitors (Hoit *et al*., 1995). In these studies, (Pro^{11}-,D-Ala^{12})-AI-induced responses were always inhibited by the AII receptor antagonist losartan, indicating that they were dependent on the formation of AII. These properties made it interesting to use the drug in the characterization of ACE-independent AII formation in the human bladder. Our results demonstrate that contractions induced by this substrate are mediated through a mechanism sensitive to human chymase-inhibition rather than ACE-inhibition. However, almost 50% of the contraction remained after chymase-inhibition. This might be due to additional mechanisms for degradation of (Pro^{11}-,D-Ala^{12})-AI, compared to AI. This is supported by the finding that ACE and chymase-inhibition did not abolish AII formation in experiments on membrane fractions. Furthermore, a direct agonistic effect of (Pro^{11}-,D-Ala^{12}) AI itself can not be excluded. However, the contractions are most likely mediated through the AT_1 receptors since the contractile effect was abolished by losartan. Similar results have been obtained in the canine mesenteric artery (Mangiapane *et al*., 1994).

Surprisingly, enalaprilat was able to reduce AII formation from the substrate in the membrane fraction experiments. The mechanism behind these inhibitory effects is unclear. The possibility that ACE is able to degrade (Pro^{11}-,D-Ala^{12})-AI, or if high concentrations of enalaprilat can inhibit serine proteases (human chymase) cannot be determined from these results. Further experiments are required to characterize the properties of (Pro^{11}-,D-Ala^{12})-AI.

In conclusion, our results indicate that the serine protease responsible for part of the AII formed in the human bladder *in vitro* is human chymase or an enzyme similar to human chymase.

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References

ANDERSSON, K.-E., HEDLUND, H. & STAHL, M. (1992). Contractions induced by angiotensin I, angiotensin II and bradykinin in isolated smooth muscle from human detrusor. *Acta Physiol. Scand.*, **145,** 253–259.

BASTOS, M., MAEJI, N.J. & ABELES, R.H. (1995). Inhibitors of human heart chymase based on a peptide library. *Proc. Natl. Acad. Sci. U.S.A.*, **92,** 6738–6742.

CHENG, E., LEE, C., DECKER, R., SENSIBAR, J., LANG, S., KAPLAN, W., MAIZELS, M. & FIRLIT, C. (1996). Captopril inhibits obstructive changes in the neonatal rabbit bladder. *J. Urol.*, **155,** 599A.

CORNISH, K.G., JOYNER, W.L. & GILMORE, J.P. (1979). Direct evidence for the presence of a different enzyme in the hamster cheek pouch. *Circ. Res.*, **44,** 540–544.

DZAU, V.J. (1988). Circulating versus local renin-angiotensin system in cardiovascular homeostasis. *Circulation*, **77 (Suppl. I),** I4–I13.

ERSPAMER, V., RONZONI, G. & FALCONIERI-ERSPAMER, G. (1981). Effects of active peptides on the isolated muscle of the human urinary bladder. *Invest. Urol.*, **18,** 302–304.

FALCONIERI ERSPAMER, G., NEGRI, L., & PICCINELLI, D. (1973). The use of preparations of urinary bladder smooth muscle for bioassay of and discrimination between polypeptides. *Naunyn-Schmiedeberg's Arch. Pharmacol.*, **279,** 61–74.

GANONG, W.F. (1994). Origin of the angiotensin II secreted by cells. *Proc. Soc. Exp. Biol. Med.*, **205,** 213–219.

HOIT, B.D., SHAO, Y., KINOSHITA, A., GABEL, M., HUSAIN, A. & WALSH, R.A. (1995). Effects of angiotensin II generated by an angiotensin converting enzyme-independent pathway on left ventricular performance in the conscious baboon. *J. Clin. Invest.*, **95,** 1519–1527.

HUSAIN, A. (1993). The chymas-angiotensin system in humans. *J. Hypertens.*, **11,** 1155–1159.

KVIST, S., MULVANY, M.J. & AALKAER, C. (1991). Studies of the renin-angiotensin system in the wall of the rat femoral resistance vessels. *Eur. J. Pharmacol.*, **198,** 77–83.

LINDBERG, B.F., NILSSON, L.-G., HEDLUND, H., STAHL, M. & ANDERSSON, K.-E. (1994). Angiotensin I is converted to angiotensin II by a serine protease in human detrusor smooth muscle. *Am. J. Physiol.*, **266,** R1861–R1867.

MANGIAPANE, M.L., RAUCH, A.L., MACANDREW, J.T., ELLERY, S.S., HOOVER, K.W., KNIGHT, D.R., JOHNSON, H .A., MAGEE, W.P., CUSHING, D.J. & BUCHHOLZ, R.A. (1994). Vasoconstrictor action of angiotensin I-convertase and the synthetic substrate (Pro^{11},D-Ala^{12})-AI-angiotensin I. *Hypertension*, **23,** 857–860.

OKAMURA, T., OKUNISHI, H., AYAJIKI, K. & TODA, N. (1990). Conversion of angiotensin I to angiotensin II in dog isolated renal artery: Role of two different angiotensin II generating enzymes. *J. Cardiovasc. Pharmacol.*, **15,** 353–359.

OKUNISHI, H., MIYAZAKI, M. & TODA, N. (1984). Evidence for a putatively new angiotensin II-generating enzyme in the vascular wall. *J. Hypertens.*, **5,** 155–160.

OKUNISHI, H., OKA, Y., SHIOTA, N., KAWAMOTO, T., SONG, K. & MIYAZAKI, M. (1993). Marked species-difference in the vascular angiotensin II-forming pathways: Humans versus rodents. *Jpn. J. Pharmacol.*, **62,** 207–210.

OLIVER, J.A. & SCIACCA, R.R. (1984). Local generation of angiotensin II as a mechanism of regulation of peripheral vascular tone in the rat. *J. Clin. Invest.*, **74,** 1247–1251.

PAUL, M., WAGNER, J. & DZAU, V.J. (1993). Gene expression of the renin-angiotensin system in human tissues. *J. Clin. Invest.*, **91,** 2058–2064.

PEACH, M.J. (1977). Renin-angiotensin system: Biochemistry and mechanisms of action. *Physiol. Rev.*, **57,** 313–370.

PERSSON, K., PANDITA, R.K., WALDECK, K. & ANDERSSON, K.-E. (1996). Angiotensin II and bladder obstruction in the rat: Influence on hypertrophic growth and contractility. *Am. J. Physiol.*, **271,** R1186–R1192.

TANABE, N., UENO, A. & TSUJIMOTO, G. (1993). Angiotensin II receptors in the rat urinary bladder smooth muscle: Type 1 subtype receptors mediate contractile responses. *J. Urol.*, **150,** 1056–1059.

TIMMERMANS, P.B.M.W.M., WONG, P.C., CHIU, A.T., HERBLIN, W.F., BENFIELD, P., CARINI, D.J., LEE, R.J., WEXLER, R.R., SAYE, J.A.M. & SMITH, R.D. (1993). Angiotensin II receptors and angiotensin II receptor antagonists. *Pharmacol. Rev.*, **45,** 205–251.

URATA, H., HEALY, B., STEWART, R.W., BUMPUS, F.M. & HUSAIN, A. (1990a). Angiotensin II-forming pathways in normal and failing human hearts. *Circ. Res.*, **66,** 883 – 890.

URATA, H., KINOSHITA, A., MISONO, K.S., BUMPUS, F.M. & HUSAIN, A. (1990b). Identification of a highly specific chymase as the major angiotensin II-forming enzyme in the human heart. *J. Biol. Chem.*, **265,** 22348 – 22357.

URATA, H., KINOSHITA, A., PEREZ, D.M., MISONO, K.S., BUMPUS, F.M., GRAHAM, R.M. & HUSAIN, A. (1991). Cloning of the gene and cDNA for human heart chymase. *J. Biol. Chem.*, **266,** 17173 – 17179.

VAN SANDE, M.E., SHARPÉ, S.L., NEELS, H.M. & VAN CAMP, K.O. (1985). Distribution of angiotensin converting enzyme in human tissues. *Clin. Chim. Acta*, **147,** 255 – 260.

VON LUTTEROTTI, N., CATANZARO, D.F., SEALEY, J.E. & LARAGH, J.H. (1994). Renin is not synthesized by cardiac and extrarenal vascular tissues. *Circulation*, **89,** 458 – 470.

WALDECK, K., PERSSON, K. & ANDERSSON, K.-E. (1995). Angiotensin II receptors in human bladder smooth muscle. *Pharmacol. Res.*, **31 (Supplement),** 371.

WINTROUB, B.U., KAEMPFER, C.E., SCHECHTER, N.M. & PROUD, D. (1986). A human lung mast cell chymotrypsin-like enzyme. *J. Clin. Invest.*, **77,** 196 – 201.

ZISMAN, L.S., ABRAHAM, W.T., MEIXELL, G.E., VAMVAKIAS, B.N., QUAIGE, R.A., LOWES, B.D., RODEN, R.L., PEACOCK, S.J., GROVES, B.M., RAYNOLDS, M.V., BRISTOW, M.R. & PERRYMAN, M.B. (1995). Angiotensin II formation in the intact human heart: Predominance of the angiotensin-converting enzyme. *J. Clin. Invest.*, **96,** 1490 – 1498.

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