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Dual pathway for angiotensin II formation in human internal mammary arteries

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1 Angiotensin converting enzyme (ACE) is thought to be the main enzyme to convert antiotensin I to the vasoactive angiotensin II. Recently, in the human heart, it was found that the majority of angiotensin II formation was due to another enzyme, identified as human heart chymase. In the human vasculature however, the predominance of either ACE or non-ACE conversion of angiotensin I remains unclear.

2 To study the effects of ACE- and chymase-inhibition on angiotensin II formation in human arteries, segments of internal mammary arteries were obtained from 37 patients who underwent coronary bypass surgery.

3 Organ bath experiments showed that 100 μM captopril inhibited slightly the response to angiotensin I (pD_2 from $7.09 \pm 0.11 - 6.79 \pm 0.10$, $P < 0.001$), while 100 μM captopril nearly abolished the response to [pro^{10}] angiotensin I, a selective substrate for ACE, and the maximum contraction was reduced from $83 \pm 19\% - 23 \pm 17\%$ of the control response ($P = 0.01$). A significant decrease of the pD_2 of angiotensin I similar to captopril was observed in the presence of 50 μM chymostatin (pD_2 from $7.36 \pm 0.13 - 6.99 \pm 0.15$, $P < 0.039$), without influencing the maximum response. In the presence of both inhibitors, effects were much more pronounced than either inhibitor alone, and a 300 times higher dose was needed to yield a significant contraction response to angiotensin I.

4 These results indicate the presence of an ACE and a non-ACE angiontensin II forming pathway in human internal mammary arteries.

Keywords: Angiotensin I; angiotensin-converting enzyme inhibitors; human mammary arteries; protease inhibitors; renin-angiotensin system

Introduction

Formation of angiotensin II is traditionally thought to depend on angiotensin-converting enzyme (ACE), and the beneficial effects of ACE-inhibitors on the treatment of various cardiovascular diseases are mainly attributed to a reduced conversion of angiotensin I to angiotensin II. This explanation was challenged by a study which showed that ACE-inhibition only partly reduced angiotensin II forming activity in the human left ventricle (Urata *et al*., 1990a). The majority of ACE-independent angiotensin II formation in the human left ventricle was due to an unknown serine protease. This cardiac serine protease has been purified, characterized and identified as a novel member of the chymase subfamily (Urata *et al*., 1990a, 1990b). Furthermore, a number of studies with ACE-inhibitors in humans provided additional evidence for the existence of an alternative angiotensin II forming pathway other than ACE. For example, angiotensin II levels were not decreased in plasma and tissue despite adequate and longterm ACE-inhibition (Nussberger *et al*., 1986; Rousseau *et al*., 1994). Also, increased plasma angiotensin II formation during dynamic exercise or in ischaemic conditions was not inhibited by the treatment with high doses of ACE-inhibitors (Urabe *et al*., 1993; Miura *et al*., 1994). Based on these observations, it was hypothesized that also in the vasculature, non-ACE-dependent angiotensin I conversion may play a role in local angiotensin II formation (Urata & Ganten, 1993). This hypothesis was supported by data from Mangiapane *et al*. (1994), who presented a potential role for 'angiotensin I-convertase' in the conversion of angiotensin I in the marmoset vasculature. Differences between rodents and humans in non-ACE angiontensin I conversion were reported by Okunishi *et al*. (1993). They revealed that in rodent arteries, angiotensin II formation totally depended on ACE, while in human gastroepiploic arteries, angiotensin I was only partly converted by ACE. The rest of the conversion was ascribed to a chymostatin-sensitive angiotensin II-generating enzyme (CAGE).

In the present study, we tested the hypothesis that in human internal mammary arteries (IMA), angiotensin I can be converted to angiotensin II by enzymes other than ACE.

Methods

Human vessels

Graft material was obtained from 46 patients who underwent coronary-bypass surgery between January, 1994 and August, 1997 in the University hospital of Groningen, The Netherlands. No patient received ACE-inhibition therapy before surgery. Studies with excess human arteries were approved by the Ethics Review Committee.

Functional measurements

The vessels were dissected free, cleaned of surrounding tissues, and cut into several rings (2 mm) with a sharp razor blade,

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while care was taken not to damage the endothelium. Mean internal diameter of the rings was 1.5–2 mm. Rings were mounted in 15 ml organ baths, containing a buffer solution of the following (Krebs) composition (mM): NaCl (120.4), KCl (5.9), $Cacl_2$ (2.5), $MgCl_2$ (1.2), NaH_2PO_4 (1.2), Glucose (11.5) and $NaHCO_3$ (25.0). The medium was aerated continuously with 95% O_2-5% CO_2 and kept at 37°C. The rings were connected to an isotonic displacement transducer by 5–0 braided, uncoated polyester suture, where they received a preload of 1.4 g (Buikema *et al*., 1992). Both the isotonic transducer, the recording system, and the software were custom made and calibrated by the University of Groningen, The Netherlands.

The artery rings were allowed to equilibrate for 60 min, during which regular washing periods were performed. Rings were primed and checked for viability by repeated stimulation (three to four times) with 10 μM phenylephrine and intermediate washing and stabilization periods. The last response to phenylephrine was used as a control response, based on which comparable rings were selected to study the effects of ACE-inhibition, chymase inhibition and angiotensin II-receptor antagonism in parallel experiments. Rings were preincubated (20 min) with either 100 μM captopril, 50 μM chymostatin, 100 nM irbesartan (BMS186295/SR47436, an angiotensin II AT1 receptor antagonist) (Cazubon *et al*., 1993), or none. They were then stimulated with increasing doses (0.1 nM–1 μM) of Ang I or [Pro^{10}] angiotensin I, a selective substrate for ACE (Kinoshita *et al*., 1993). To exclude the possibility that any non-specific decrease in contractility of individual rings during the time course of the experiment would be misinterpreted as being an effect of the above inhibitors, we determined the contractility of all rings to 10 μM phenylephrine at the beginning of each experiment (PE-pre). The results of contraction responses to angiotensin I and [Pro^{10}] angiotensin I were presented as the percentage of the PE-pre. To exclude the possibility that high doses of captopril or chymostatin would influence the response to angiotensin II, we performed control experiments in which the response to angiotensin II (0.1 nM to 1 μM) was determined in the absence or presence of 100 μM captopril or 50 μM chymostatin. As described earlier, the responses to angiotensin I and II were studied in the continuous presence of 100 μM L-N^G-monomethyl arginine acetate hydrate (L-NMMA) to prevent any confounding effects by the basal release of nitric oxide (Buikema *et al*., 1996). Rings which failed to produce a contractile response of 100 μm throughout the experiment were not included for analysis.

Statistical methods

To avoid non-specific differences between subjects (inter-assay variance), we evaluated the effect of the inhibitors by comparing dose-response curves obtained with parallel rings from the same patient. To control for non-specific differences between rings from the same patient (intra-assay variance), contractile responses of individual rings to angiotensin I and angiotensin II were expressed as a percentage of the contraction response to phenylephrine (PE-pre), which was determined before each experiment. Comparisons were made using the mean values of the contractions of one group of rings and the mean values of another group of rings within one patient.

When maximum contraction was reached, the negative log of the concentration at which 50% of the maximum response was reached (pD_2), was calculated. Maximum responses and individual pD_2 values were derived from the fits of each separate curve. Curve fits were made by the curve fitting option of SigmaPlot® for Windows™. Maximum responses and pD_2 values in the presence and absence of an inhibitor were compared using a paired *t*-test (PROC MEANS, SAS Institute Inc, Chicago, IL, U.S.A.). If during an experiment, maximum responses were not reached despite an adequate (>100 μm) response to phenylephrine, the pD_2 value could not be calculated. Therefore, pD_2 values could not be calculated for the effects of captopril to [Pro^{10}] angiotensin I, and for the effects of both catopril and chymostatin to angiotensin I. These computational problems were overcome by establishing the mean first concentration at which a significant contraction response to angiotensin I was observed ('first-response concentration'). In order to do so, mean responses of the rings preincubated with either captopril, chymostatin, irbesartan, or none were compared to the baseline response by a two-tailed *t*-test (PROC TTEST, SAS Institute Inc, Chicago, IL, U.S.A.). Baseline response and standard deviation were calculated from the mean response of the two lowest concentrations (0.1 nM and 0.3 nM) from the same of all experiments performed. On average, contractions to angiotensin I were significant if larger than 10% of PE-pre. No contraction responses were observed during these first three concentrations. Comparisons between these 'first response concentrations' were only done as paired observations within parallel artery rings (i.e. control vs treatment) from the same patients.

Data are presented as the means ± s.e.mean. A probability level of <0.05 was considered statistically significant.

Drugs, hormones and reagents

Irbesartan (2-*n*-butyl-3-[2′-(1H-tetrazol-5-yl)-biphenyl-4-yl)-methyl]-1,3-diaza-spiro[4,4] non-1-en-4-one), and captopril were a kind gift from the Squibb institute for medical research (Princeton, NJ, U.S.A.). Angiotensin II was obtained from Ciba-Geigy, Arnhem, The Netherlands. Angiotensin I was obtained from ICN Biochemicals (Aurora, Ohio, U.S.A.). A selective substrate for ACE, [Pro^{10}] angiotensin I, was synthesized in Bio-Tez Co., Berlin-Buch, Germany. L-N^G-monomethyl arginine acetate hydrate (M-NMMA) was a kind gift of Wellcome Research Laboratories (Beckenham, Kent, U.K.). All other chemicals and reagents were obtained from Sigma (St Louis, MO, U.S.A.).

Results

Baseline characteristics of the patients from which graft material was obtained are presented in Table 1. The patient population was a representative group of patients undergoing coronary bypass surgery: almost 50% previously suffered a myocardial infarction, the majority (62%) of the patients had severe (three-vessel) coronary artery disease, and 38% of the patients had an impaired left ventricular function.

Functional measurements

The contractile effects of (0.1 nM–1 μM) angiotensin I, [Pro^{10}] angiotensin I and angiotensin II were completely mediated through the AT1-receptor since the presence of 100 nM irbesartan completely abolished their contraction responses ($n=5$).

Neither captopril (100 μM) nor chymostatin (50 μM) significantly affected the contraction responses to angiotensin II. In the presence of captopril ($n=5$), the maximum contraction response to angiotensin II was not affected (Table

2) (from 58.1±13.9%–57.0±14.0%, $P=0.85$), and the pD_2 shifted from 7.85±006–7.91±0.14 ($P=0.72$). In the presence of chymostatin ($n=5$), the maximum contraction response to angiotensin II shifted from 103±22.9%–88.5±9.1% ($P=0.44$), and the pD_2 shifted from 7.98±0.08–7.81±0.13 ($P=0.30$) (Table 2).

A representative contraction trace of angiotensin I mediated contraction responses in the human internal mammary artery is shown in Figure 1. Angiotensin I caused a steady vasoconstriction and the inhibitory effect of chymostatin and synergistic inhibitory effects of captopril and chymostatin are also shown. The effects of ACE-inhibition on the conversion of angiotensin I to angiotensin II in human arteries *ex vivo* was studied in rings obtained from 13 patients. The maximum contraction response in rings pretreated with captopril (83.9±15.5%) was not different from the control rings (69.7±10.2%; $P=0.3$). Average contraction responses of both groups to increasing doses of angiotensin I (1 nM–1 μM) are presented in the left panel of Figure 2. In the presence of captopril, the pD_2 shifted from 7.09±0.11–6.79±0.10 ($P<0.001$). The 'first-response concentration' in the rings without captopril was increased with one dose, from 30 nM in the rings with captopril, indicating that in the presence of captopril, a 3 fold higher dose was needed to yield a significant contraction response.

Table 1 Clinical characteristics of the study population

	$n=46$
Mean Age (years)	65.6 (31–82)
Female	24%
History of myocardial infarction	46%
Diabetes mellitus	14%
Positive family history of coronary artery disease	50%
Smoking history	29%
History of hypertension	14%
Left ventricular dysfunction	38%
One vessel diseased	8%
Two vessels diseased	31%
Three vessels diseased	62%
Mean systolic blood pressure (mmHg)	138 (110–180)
Mean diastolic blood pressure (mmHg)	77 (60–105)
Mean cholesterol (mmol/l)	5.9 (4.2–8.4)
Mean body mass index (kg m^2)	27.7 (19.6–37.6)
ACE-inhibitors	0%
Acetylsalicyclic-acids	78%
Beta-blockers	84%
Ca^{2+}-re-entry-blockers	70%
Long-acting nitrates	68%

Data are mean (range) or frequency (% of total).

The effects of ACE-inhibition on the conversion of angiotensin I were then compared to the effects on [Pro^{10}] angiotensin I, a modified angiotensin I, which is entirely ACE-dependent to be converted to angiotensin II. The right panel of Figure 2 shows that in the presence of captopril, the response

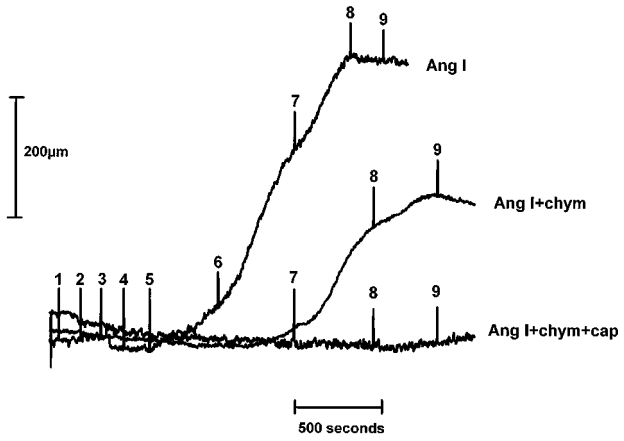

Figure 1 Example of a contraction experiment with an internal mammary artery from one patient, cut into rings and mounted in organ baths. This figure represents the contractile responses of internal mammary artery rings to increasing concentrations of angiotensin (0.1 nM–1 μM, represented by numbers 1–9) with or without chymostatin (chym) (50 μM) and/or captopril (cap) (100 μM). After this experiment, phenylephrine (10 μM) was added to check whether the rings were still able to contract (not shown). All organ baths were pre-incubated with L-NMMA.

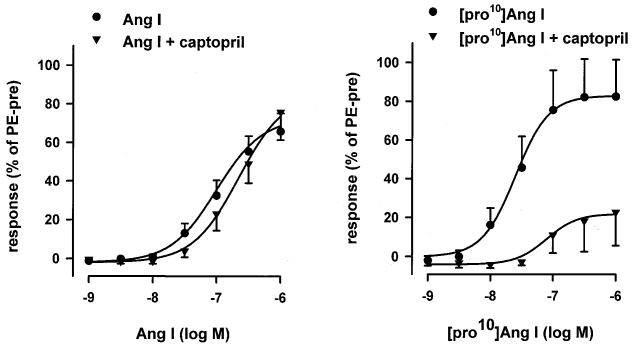

Figure 2 Concentration-response curves for the contractile responses of the human isolated internal mammary artery to angiotensin I (left panel, $n=13$ and [pro^{10}] angiotensin I (right panel, $n=7$) in the presence (solid triangles) or absence (solid circles) of captopril (100 μM). Contractions are presented as percentage (±s.e.mean) of the response to 10 μM phenylephrine (PE-pre) response before the experiment.

Table 2 The effect of 100 μM captopril (cap) and 50 μM chymostatin (chym) on the maximal contraction (E_{max}) and the concentration causing 50% of E_{max} (expressed as the negative log, pD_2) to angiotensin I and II in human isolated internal mammary artery

Angiotensin I	pD_2 (M)	E_{max} (%)	*Angiotensin II*	pD_2 (M)	E_{max} (%)
***n*=13**			***n*=5**		
Control	7.09±0.11	69.7±10.2	control	7.85±0.06	58.1±13.9
+Cap	6.79±0.10	83.9±15.5	+cap	7.91±0.14	57.0±14.0
***n*=7**			***n*=5**		
Control	7.36±0.13	83.9±14.6	control	7.98±0.08	103±22.9
+Chym	6.99±0.15	64.9±13.1	+chym	7.81±0.13	88.5±9.1

Responses have been expressed as a percentage of the concentration to 10 μM phenylephrine (%PE-pre) and values are the mean±s.e.mean of the indicated number (n) of paired observations.

to [Pro^{10}] angiotensin I was nearly abolished. Since in some patients, no contractions could be evoked, even in the presence of the highest concentration of angiotensin I, the mean pD_2 could not be calculated. In the presence of captopril (100 μM) no statistically significant contraction response to [Pro^{10}] angiotensin I could be evoked, and the maximum response to [Pro^{10}] angiotensin I was reduced from $83 \pm 19\% - 23 \pm 17\%$ of the PE-pre ($P = 0.01$).

In a set of experiments with material from seven other patients, we examined the inhibitory effects of 50 μM chymostatin on the contraction response to angiotensin I. The maximum contraction response in rings pretreated with chymostatin ($64.9 \pm 13.1\%$) was not significantly different from the control rings ($83.9 \pm 14.6\%$, $P = 0.2$). Figure 3 shows that the presence of chymostatin resulted in an inhibition of the response to angiotensin I, which was comparable to the inhibition with captopril. In the presence of chymostatin, the pD_2 was shifted significantly from $7.36 \pm 0.13 - 6.99 \pm 0.15$ ($P < 0.039$) (Table 2). The mean 'first-response concentration' in rings with chymostatin was also shifted with one dose, from 10 nM to 30 nM, indicating that in the presence of chymostatin a 3 fold higher dose was needed to yield a significant contraction response.

The inhibitory effects of the combination of 100 μM captopril and 50 μM chymostatin are presented in Figure 3. In the presence of both captopril and chymstatin, the contraction response to angiotensin I was nearly abolished. Since in some patients, no contractions could be evoked, even in the presence of the highest concentration of angiotensin I, the mean pD_2 could not be calculated. The 'first-response concentration' was shifted from 10 nM in the control rings to 3 μM in the rings with both captopril and chymostatin, indicating that in the presence of both captopril and chymostatin, a 300 times higher dose was needed to yield a significant contraction response.

Discussion

These results confirm that the human internal mammary artery contains a dual pathway for angiotensin formation. ACE-dependent angiontensin II formation was clearly shown by the effect of the ACE selective substrate, [Pro^{10}] angiotensin I (Kinoshita *et al*., 1993) and its complete inhibition with captopril, while chymase-dependent angiotensin II formation was evident by a synergistic inhibitory action with both captopril and chymostatin on the angiotensin I-induced vasoconstriction.

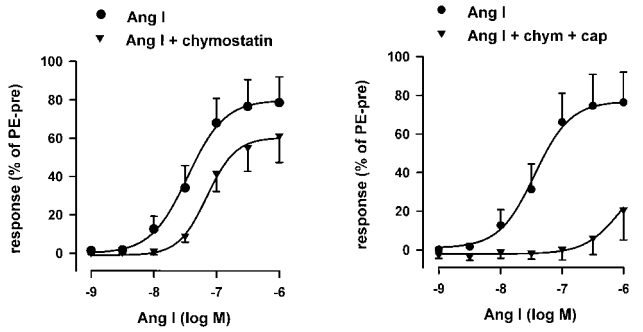

Figure 3 Concentration-response curves for the contractile response of the human isolated internal mammary artery to angiotensin I in the presence (solid triangles) or absence (solid circles) of chymostatin (50 μM) (left panel, $n = 7$) or in the presence (solid triangles) or absence (solid circles) of the combination of both chymostatin (chym, 50 μM) and captopril (cap, 100 μM (right panel, $n = 6$). Contractions are presented as percentage ($\pm$s.e.mean) of the response to 10 μM phenylephrine (PE-pre) before the experiment.

In the heart, a dual pathway for angiotensin II formation was first shown by Cornish *et al*. (1979) in hamster hearts and by Trachte & Lefer (1979) in cat hearts. In the human heart, Urata *et al*. (1990a) showed similar non-ACE-dependent angiotensin II formation *in vitro*, which was due to a serine proteinase that contributed to 80% of the angiotensin II formation, compared to 11% by ACE. As mentioned earlier, this unknown cardiac serine protease was isolated and identified as chymase.

In the vasculature, ACE is located in the endothelium (Soffer, 1976), whereas chymase or the chymase-like enzyme (CAGE) was found not only in the endothelium but also in the adventitia of the dog aorta (Okunishi *et al*., 1987) and the human coronary artery (Urata *et al*., 1993). The difference in localization of ACE and chymase may imply that ACE-dependent angiotensin II formation is dominant on the endothelial surface in normal physiological conditions, whereas chymase-dependent angiotensin II formation may be more prevalent in the adventitial regions of the vessel (Urata & Ganten, 1993).

Similar dual pathways of angiontensin II formation have been reported both by biochemical measurements (Urata *et al*., 1990a) and functionally (Chen *et al*., 1991) in the human heart, and by functional measurements in both the gastroepiploic artery (Okunishi *et al*., 1993) and the detrusor smooth muscle (Lindberg *et al*., 1994). However, it can be argued that the biochemical demonstration for a dual pathway fails to establish that non-ACE-dependent angiotensin II formation plays a role *in vivo* (Johnston, 1994). This is relevant because the finding of non-ACE pathways in homogenized tissue preparations could be explained by release of intracellular enzymes that may generate angiotensin II. Although we agree with the possibility for an overestimation of non-ACE dependent angiotensin II formation in these studies, our functional measurements in intact vessel rings additionally demonstrated an important role for non-ACE angiotensin II formation. It has to be noted that our experiments were performed in the presence of L-NMMA to prevent the effects of basal release of nitric oxide, since Yang *et al*. (1991) showed that in human internal mammary arteries, basal release of nitric oxide masked the effects of endothelium-dependent contractions by 30%. In the current study, baseline vascular tone prior to exposure to angiotensin I did not change after exposure to L-NMMA.

We demonstrated that single treatment with captopril or chymostatin only partly inhibited the angiotensin I-induced contraction. In contrast, biochemical experiments indicated that chymase-dependent angiotensin II forming activity was dominant over the ACE-dependent angiotensin II forming activity (Urata *et al*., 1990a). In fact, Lindberg *et al*. (1994) reported a similar discrepancy between functional and biochemical results. In the human detrusor muscle, a positive inotropic effect of angiotensin I was not inhibited by the pretreatment with a broad serine protease inhibitor, soybean trypsin inhibitor, and was partially (approximately 40%) inhibited by an ACE-inhibitor, whereas in the biochemical analysis, angiotensin II formation in the muscle homogenate was completely inhibited by soybean trypsin inhibitor but minimally inhibited by an ACE-inhibitor. So, there is evidence that non-ACE-dependent angiotensin II formation *in vitro* does not directly indicate its physiological function *in vivo*. Nevertheless, in tissues containing a dual pathway for angiotensin II formation, ACE-inhibition always failed to

substantially inhibit angiotensin I-induced contractions. (Kinoshita *et al*., 1993; Hirakata *et al*., 1990; Okunishi *et al*., 1984; Chen *et al*., 1991; Lindberg *et al*., 1994; Trachte & Lefer, 1979; Cornish *et al*., 1979), but completely inhibited angiotensin I-induced contractions in the tissue without non-ACE-dependent angiotensin II formation (Okunishi *et al*., 1993).

Our findings that the 'first-response concentration' of angiotensin I was only three times higher with either captopril or chymostatin, while the presence of both inhibitors almost completely inhibited the contraction response, indicates an increased activity of one pathway when the other is blocked. This 'escape phenomenon' may explain some of the previous findings that plasma angiotensin II concentration was not decreased significantly during adequate and longterm ACE-inhibition (Rousseau *et al*., 1994; Nussberger *et al*., 1986), and that the increase of plasma angiotensin II concentration during 30 min of graded exercise could not be inhibited by pretreatment of high doses of captopril (Miura *et al*., 1994).

In conclusion, our present findings indicate that in human internal mammary arteries, angiotensin I is not only converted by ACE, but also by a non-ACE enzyme. Furthermore, the combination of captopril and chymostatin gave a synergistic inhibition of the angiotensin I contraction response, which suggests an escape phenomenon to the other pathway when one pathway is blocked.

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