Functional Evidence for a Role of Vascular Chymase in the Production of Angiotensin II in Isolated Human Arteries

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Background—In human arteries, angiotensin-converting enzyme (ACE) inhibitors incompletely block the production of angiotensin (Ang) II from Ang I. This ACE-independent production of Ang II appears to be caused by serine proteases, one of which presumably is chymase. However, several serine proteases may produce Ang II, and the exact role of chymase in the vascular production of Ang II has never been directly evaluated using selective chymase inhibitors.

Methods and Results—Rings of human mammary arteries were subjected to either Ang I or the chymase-selective substrate [pro,11 D-Ala12] Ang I in the absence or presence of the ACE inhibitor captopril, the serine protease inhibitor chymostatin, or the selective chymase inhibitor C41. Captopril only partially inhibited (by 33%) the response to Ang I. In the absence of captopril, C41 markedly reduced (by 44%) the response to Ang I, and this effect was identical to that of chymostatin. C41 also significantly reduced the response to Ang I in the presence of captopril, although this inhibitory effect was slightly less than that of captopril in combination with chymostatin. [Pro,11 D-Ala12] Ang I induced potent contractions that were not affected by captopril but were abolished by chymostatin and markedly reduced by C41. In addition, we found that prior treatment of the patients with an ACE inhibitor did not affect the response to Ang I or Pro–Ang I (in the absence or the presence of captopril) or to [Pro,11 D-Ala12] Ang I.

Conclusions—Our results reinforce the hypothesis that chymase is a major serine protease implicated in the ACE-independent production of Ang II in human arteries. (Circulation. 2001;104:750-752.)

Key Words: angiotensin ■ arteries ■ vasoconstriction

In isolated human arteries, the production of angiotensin (Ang) II from Ang I is only partially blocked by Ang I–converting enzyme (ACE) inhibitors. These ACE-independent responses are blocked by inhibitors of serine proteases,1–3 suggesting that they are mediated by one or more serine proteases, presumably including chymase.4 However, many serine proteases other than chymase have been shown to produce Ang II from Ang I,5 and the exact functional role of chymase has never been demonstrated directly because of the lack of chymase inhibitors that do not also inhibit other serine proteases.

Recently, a series of nonpeptide inhibitors of human heart chymase has been described.6 One compound tested in this series (3-[(3,4-Dimethoxyphenyl)sulfonyl]-1-(3,4-dimethylphenyl)imidazoline-2,4-dione; compound #41) is a potent chymase inhibitor showing no inhibition of the other major serine proteases chymotrypsin and cathepsin G. Thus, using the selective chymase inhibitor C41 and the selective chymase substrate [Pro,11 D-Ala12] Ang I (Pro–Ang I), we assessed the functional role of chymase in the formation of Ang II in isolated human mammary arteries (IMAs).6–9

Methods
Distal segments of the IMA, not used for surgical implantation, were obtained from 70 patients and mounted in organ chambers. All handling of human tissue was performed in such a manner as to avoid possible contamination by the investigators. Cumulative concentration responses to Ang I or Pro–Ang I (3 × 10–4–10–5 mol/L) were assessed at baseline and repeated in the presence of the ACE inhibitor captopril (10–4 mol/L), the serine protease inhibitor chymostatin (5 × 10–5 mol/L), or the chymase inhibitor C41 (10–5 mol/L). Chymostatin and C41 were dissolved in DMSO and were administered in the 20-mL chamber in a volume of 100 μL. DMSO alone did not affect the responses to Ang I in these conditions. All the experiments were performed in the presence of the NO synthase inhibitor Nω-nitro-L-arginine (10–4 mol/L). The presence of functional endothelium was assessed in 14 patients, in whom the relaxations in response to 10–6 mol/L acetylcholine were found to average 60±6%.

Expressed human prochymase was purified from the culture medium of transfected African green monkey kidney (COS-1) cells (a gift from Dr H. Urata, Fukuoka University, Fukuoka, Japan) using gel filtration (4.4 × 96 cm; Ultrogel AcA54, IBF) and heparin-5PW chromatography (TosoHaas).10 Prochymase was activated by cathepsin C (Roche Diagnostics).11 Activated chymase was separated from cathepsin C using the same heparin chromatography. Purity was 90%, as determined by densitometry scanning of 10% SDS-polyacrylamide gel electrophoresis stained with Coomassie Blue.

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Results

In isolated human recombinant chymase, C41 was a potent chymase inhibitor, with an IC$_{50}$ of 41±8 nmol/L and 53±16 nmol/L when Ang I and Pro–Ang I, respectively, were used as substrates, without detectable inhibition of ACE at concentrations up to 10 µmol/L. The affinity of Ang I and Pro–Ang I for chymase were similar ($K_a$: Ang I, 57±2 µmol/L; Pro–Ang I, 47±2 µmol/L).

Responses to Ang I (Figure 1) reached 74±2% of the contractile response to phenylephrine. Captopril only partially inhibited (by 33%) this response, which was also reduced 44% by chymostatin and 76% by the combination of captopril and chymostatin. In the absence of captopril, the chymase inhibitor C41 markedly reduced (by 44%) the response to Ang I. C41 also significantly reduced the response to Ang I in the presence of captopril (maximal responses: captopril, 49±4%; captopril + C41, 32±5%; P<0.05). This inhibitory effect was less than that of captopril in combination with chymostatin (18±3%), but the difference was not statistically significant. At baseline, Pro–Ang I induced potent contractions that were not affected by captopril but were markedly reduced by C41 (Figure 2; maximal responses: baseline, 57±2%; C41, 27±2%; P<0.05), although the inhibitory effect of C41 was less than that induced by chymostatin (5±2%; P<0.05 versus C41).

Prior treatment of the patients with an ACE inhibitor did not affect the response to Ang I or the inhibitory effect of captopril on this response. Indeed, the response to Ang I in the presence of captopril was 52±3% (n=9) in patients not treated with an ACE inhibitor versus 49±4% (n=12) in patients who were taking an ACE inhibitor. Moreover, prior treatment with an ACE inhibitor did not affect the in vitro response to Pro–Ang I. Indeed, the maximal response to this substrate was 59±4% (n=14) in patients not treated with an ACE inhibitor versus 52±4% (n=11) in patients who were receiving ACE inhibitor treatment at the time of surgery (data not shown).

Finally, immunohistochemical analysis using a mouse anti-human chymase antibody demonstrated the existence of chymase immunoreactivity in IMAs, which was localized exclusively within the adventitia. This immunoreactivity appeared in cells demonstrating a granular structure, which presumably were mast cells (data not shown).

Discussion

Our results obtained in IMAs show that (1) the selective chymase inhibitor C41 significantly reduced the contractile response to Ang I in both the absence or the presence of the ACE inhibitor captopril; (2) Pro–Ang I, which is converted to Ang II by chymase but not by ACE, induced potent vascular contractions which were inhibited by the chymase inhibitor; and (3) prior treatment of patients with an ACE inhibitor did not affect the response to Ang I, Pro–Ang I, or the inhibitory effect of captopril on the responses to Ang I.

In accordance with previous data, we observed that captopril only partially inhibited the response to Ang I in human arteries and that this response was also reduced by the serine protease inhibitor chymostatin. However, although this chymostatin-sensitive pathway usually has been attributed to chymase, many other serine proteases are known to produce Ang II. In this context, our study, using a selective chymase inhibitor, reinforces the hypothesis of a role for chymase in the alternate production of Ang II. This is also supported by the fact that Pro–Ang I, which is cleaved to Ang II by chymase but not by ACE, induced potent contractions that were reduced by the chymase inhibitor.

Although the inhibitory effect of C41 on the response to Ang I was identical to that of chymostatin in the absence of captopril, the effect of C41 in the presence of the ACE inhibitor was slightly less than that of chymostatin. Moreover, we observed that the contraction induced by Pro–Ang I was more potently inhibited by chymostatin than by C41. Thus, from these experiments, one cannot completely exclude the existence of chymostatin-sensitive Ang II–generating enzymes that are different from chymase. However, our data suggest that chymase is indeed a major enzyme for ACE-independent production of Ang II in human IMAs.

We found that the responses to Ang I were not affected by prior ACE inhibition. In the human heart, the effect of long-term ACE inhibition on the in vitro activity of ACE is controversial. Indeed, Wolny et al showed that...
no significant inhibition. Interestingly, Kinoshita et al\textsuperscript{13} found a rapid dissociation of the ACE-inhibitor complex in isolated hamster papillary muscles when incubated at 37°C, although the dissociation rate varied depending on the inhibitor used. Thus, it is possible that a similar dissociation occurs in IMAs because these arteries were kept for \footnotesize{$\approx$}2 hours in organ chambers before the functional responses were assessed. Whether incubation of the arteries for less time would have led to a significant inhibition of ACE in ACE inhibitor–treated patients is unknown. Moreover, given the relatively low number of ACE inhibitor–treated patients used in our study,\textsuperscript{13} it is not possible to assess whether the responses to Ang I differed significantly according to which ACE inhibitor was given to patients.

We also found that neither the response to Pro–Ang I nor the inhibitory effect of captopril on the response to Ang I was affected by prior treatment of the patients with an ACE inhibitor, suggesting that such long-term ACE inhibition did not affect chymase activity. Thus, the fact that Ang II levels return to baseline after long-term ACE inhibition is most likely caused by an increased availability of Ang I for chymase-dependent conversion (secondary to the increased renin secretion in the absence of cleavage of Ang I by ACE) rather than to changes in the expression or activity of chymase.

Using immunohistochemistry, we could detect chymase in the adventitia, most likely within mast cells but not in endothelial cells. Moreover, endothelium removal did not affect the responses to Pro–Ang I (data not shown). This adventitial localization may have important functional consequences. First, manipulation of the arteries might induce mast cell degranulation, leading to increased chymase activity. Second, the fact that, in isolated arteries, Ang I has equal access to the adventitia and the endothelium might result in an overestimation of the chymase-dependent pathway, compared with the in vivo situation. This might explain why the effect of ACE inhibitors is more pronounced in human circulation in vivo\textsuperscript{14} than in isolated human arteries.

**Conclusions**

Our results, obtained using a selective chymase inhibitor, demonstrate for the first time that chymase is the major enzyme responsible for ACE-independent production of Ang II in IMAs. Chymase inhibitors may be useful in the treatment of diseases in which a deleterious effect of the renin-angiotensin system has been implicated, including hypertension and heart failure.

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**References**

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