Vasoconstrictor Effect of the Angiotensin-Converting Enzyme–Resistant, Chymase-Specific Substrate \([\text{Pro}^{11}D\text{-Ala}^{12}]\) Angiotensin I in Human Dorsal Hand Veins

In Vivo Demonstration of Non-ACE Production of Angiotensin II in Humans

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**Background**—\([\text{Pro}^{11}D\text{-Ala}^{12}]\) angiotensin I is an ACE-resistant substrate specific for chymase. We used this peptide to determine whether a functionally significant non-ACE angiotensin (Ang) II–generating pathway exists in human dorsal hand veins.

**Methods and Results**—Using a modified Aellig technique, we studied the response to Ang I and \([\text{Pro}^{11}D\text{-Ala}^{12}]\) Ang I in dorsal hand veins in vivo in patients with coronary heart disease. We measured the venoconstrictor effect of each peptide given before and after a 6.25-mg oral dose of the ACE inhibitor captopril or matching placebo. Placebo or captopril was given in a double-blind, randomized fashion. Ang I induced a mean \(\pm SEM\) venoconstrictor response of 45 \(\pm 6\)% before placebo, 40 \(\pm 10\)% after placebo, 55 \(\pm 8\)% before captopril, and 4 \(\pm 4\)% after captopril, respectively. Hence, the response to Ang I was reproducible and was reduced significantly only after treatment with captopril \((P=0.002)\).

\([\text{Pro}^{11}D\text{-Ala}^{12}]\) Ang I induced a mean venoconstrictor response of 42 \(\pm 9\)% before placebo, 49 \(\pm 9\)% after placebo, 48 \(\pm 10\)% before captopril, and 54 \(\pm 11\)% after captopril, respectively. Hence, captopril had no significant effect on the response to \([\text{Pro}^{11}D\text{-Ala}^{12}]\) Ang I.

**Conclusions**—We have demonstrated that \([\text{Pro}^{11}D\text{-Ala}^{12}]\) Ang I is able to induce venoconstriction in humans in vivo. With this specific pharmacological probe, we have shown that a non-ACE pathway capable of generating Ang II exists in human veins in vivo and is potentially functionally important. This pathway is likely to involve the enzyme chymase. (Circulation. 2001;104:1805-1808.)

**Key Words:** angiotensin \(\bullet\) peptides \(\bullet\) veins \(\bullet\) vasoconstriction \(\bullet\) enzymes

**There has been much interest in the possibility that**

angiotensin (Ang) I can be converted to Ang II by enzymes other than ACE. Chymase is the most studied alternative enzyme.\(^1\)\(^-\)\(^7\) The role of chymase in humans has been deduced principally from biochemical and functional studies using the chymase inhibitor chymostatin in tissue homogenates and isolated blood vessels.\(^1\)\(^-\)\(^9\) We and others have confirmed chymostatin-sensitive conversion of Ang I to Ang II that has functional effects in vitro.\(^8\)\(^-\)\(^10\) There are concerns, however, that because chymase has been localized to intracellular sites within mast cells,\(^5\) the techniques used for these studies may overestimate its activity because of tissue handling.\(^4\)\(^,\)\(^11\)\(^,\)\(^12\) Adventitial exposure and mast cell degranulation may exaggerate chymase activity. Furthermore, because chymase requires release into the extracellular space to be active,\(^13\) serine protease inhibitors present in interstitial fluid may normally abolish chymase activity in vivo.\(^14\) Hence, the functional significance of chymase activity needs to be assessed within an in vivo system.

An Ang I isopeptide, \([\text{Pro}^{11}D\text{-Ala}^{12}]\) Ang I, has been developed and is ACE-resistant. This is because of the addition of a penultimate proline amino acid residue.\(^15\) This isopeptide does not directly activate the Ang II type I receptor but rather is cleaved by chymase to liberate Ang II and has been used to study non-ACE pathways in animals and human tissues.\(^16\)\(^-\)\(^20\) We have found that this peptide will induce ACE-resistant, chymostatin-sensitive, vasoconstriction in resistant arteries dissected from patients with coronary heart disease (unpublished observations). \([\text{Pro}^{11}D\text{-Ala}^{12}]\) Ang I has not, however, been used in vivo in humans before.

We have used this peptide and a dorsal hand vein technique to determine whether or not non-ACE production of biologically active Ang II occurs in vivo in humans.
Methods

Patients

Patients with chronic stable angina attending outpatient clinics were studied. All patients had preserved left ventricular systolic function, determined as an echocardiographic left ventricular ejection fraction of \( \geq 40\% \) (Simpson’s biplane method), and none were treated with an ACE inhibitor. Patients with renal failure (creatinine \( > 200 \mu \text{mol/L} \)) and diabetes mellitus were not recruited. Written informed consent was obtained from each patient, and all protocols were approved by the local Committee on Medical Ethics. After consent, suitable patients underwent a series of randomized double-blind dorsal hand vein studies, as described below. Patients were asked to refrain from caffeine and alcohol for \( \geq 12 \) hours before each study. Patients were also asked to attend for up to 5 such studies, with \( \geq 1 \) week between studies.

Materials

Human Ang I and [Pro\(^{11}\)-Ala\(^{12}\)] Ang I were purchased from Clinalfa.

Dorsal Hand Vein Technique

The dorsal hand vein technique, as modified by Aellig, was used as described previously.\(^{21}\) Subjects attended the Clinical Research Center at 9 AM on the study day after a light breakfast containing no caffeine, and they remained supine at a room temperature from 21°C to 23°C during the entire experimental session. One arm of each subject was placed on a support sloping upward at an angle of 30° from horizontal to ensure complete emptying of the superficial hand veins. A 23-gauge butterfly needle was inserted into a suitable dorsal hand vein, and a continuous infusion of physiological saline solution (at a rate of 0.26 mL/min) was started. After 30 minutes, a linear variable differential transducer (model 250 MHR, Schaevitz Engineering) was mounted on the back of the hand by means of a tripod.

The linear variable transducer’s freely movable core was placed on top of the vein under study \( \approx 5 \) to 10 mm downstream from the tip of the needle. When the core was properly centered within the transformer and placed on top of the vein, there was a linear relationship over the range used between the vertical movement of the core and the voltage output; the voltage output was recorded with a MacLab recorder. Recordings of the position of the core were made before and after inflation of a sphygmomanometer cuff on the same arm to 40 mm Hg. The baseline venous caliber (maximum venuoconstriction) during saline infusion under this congestion pressure was defined as 0% venuoconstriction. The recording obtained with the cuff not inflated (and the vein emptied) was defined as 100% venuoconstriction. The venuoconstriction induced by Ang I or [Pro\(^{11}\)-Ala\(^{12}\)] Ang I was calculated as a percentage of the range between 0% and 100%.

Infusion Protocol

Dose of Ang I

The rapid development of tachyphylaxis to Ang I prevents the construction of full dose-response curves. Therefore, a single dose of Ang I (infusion rate 400 ng/min) was used. This infusion rate of Ang I has been shown to cause a mean venuoconstriction of 50% in dorsal hand veins, without systematic effects.\(^{22}\)

Determination of Dose of [Pro\(^{11}\)-Ala\(^{12}\)] Ang I

During initial studies with [Pro\(^{11}\)-Ala\(^{12}\)] Ang I (which has not been given to humans in vivo previously), we found that a 6-fold higher dose, ie, 2.4 \( \mu \text{g/min} \), was necessary to produce similar venuoconstrictor response. This is in keeping with the finding that the specificity constant for the conversion of [Pro\(^{11}\)-Ala\(^{12}\)] Ang I to Ang II by human chymase is \( \approx 7 \) - 10-fold higher than that for the conversion of Ang I to Ang II by ACE.\(^{23}\)

Duration and Order of Infusions

Each local infusion lasted 6 minutes, and the cuff was inflated for 2 minutes at the end of each infusion. The baseline venuoconstrictor response to Ang I (days 1 and 2) or [Pro\(^{11}\)-Ala\(^{12}\)] Ang I (days 3 and 4) was measured 45 minutes after the insertion of the needle. Captopril as a 6.25-mg tablet or placebo was administered orally (in a double-blind fashion) with 200 mL of tap water immediately after this baseline infusion, and the effect of this oral treatment on the venuoconstrictor response to the peptide was reassessed 1 hour later. This low dose of captopril has been shown to substantially attenuate the pressor response to intravenous Ang I in healthy volunteers.\(^{22}\)

Irbesartan Studies

Patients were asked to return for a fifth visit to assess their venuoconstrictor response to [Pro\(^{11}\)-Ala\(^{12}\)] Ang I after pretreatment with the selective Ang II type I receptor antagonist irbesartan. Irbesartan 150 mg was taken orally 2 hours before infusion of [Pro\(^{11}\)-Ala\(^{12}\)] Ang I. This time was chosen to coincide with peak plasma concentrations of irbesartan.

Statistical Analysis

All data are given as mean\( \pm \)SEM unless otherwise indicated. Analyses of the response to Ang I and [Pro\(^{11}\)-Ala\(^{12}\)] Ang I were based on the percentage of change from baseline. Responses before and after treatment with either captopril or placebo, within an individual, were compared by paired Student’s \( t \) test. The response to [Pro\(^{11}\)-Ala\(^{12}\)] Ang I after pretreatment with irbesartan was also compared with the response to [Pro\(^{11}\)-Ala\(^{12}\)] Ang I after placebo within an individual by the paired \( t \) test. Differences were considered significant at a value of \( P<0.05 \).

Results

The clinical characteristics of the subjects studied are summarized in the Table. Seven men were studied on 4 occasions each, receiving infusions of Ang I and [Pro\(^{11}\)-Ala\(^{12}\)] Ang I before and after placebo and captopril. Five were able to return for a fifth visit (the irbesartan study). These 5 did not differ in their characteristics from the overall group of 7. The responses to Ang I and [Pro\(^{11}\)-Ala\(^{12}\)] Ang I are shown in the Figure.

Ang I Placebo/Captopril Studies

The mean\( \pm \)SEM venuoconstriction observed during infusion of Ang I before placebo was \( 44.6 \pm 7.5\% \). The mean response during reinfusion of Ang I, after placebo, was \( 39.6 \pm 9.8\% \).
The mean vasoconstrictor responses to Ang I before and after captopril 6.25 mg were 54.5±7.5% and 3.9±3.9%, respectively. In other words, Ang I–mediated vasoconstriction was almost completely blocked by pretreatment with a small oral dose of captopril (P=0.002).

**[Pro1°-D-Ala12]** Ang I Placebo/Captopril Studies

The mean±SEM vasoconstrictor observed during infusion of [Pro1°-D-Ala12] Ang I before placebo was 42.0±9.9%. The mean response during reinfusion of [Pro1°-D-Ala12] Ang I after placebo was 49.0±9.1%. The responses to [Pro1°-D-Ala12] Ang I before and after captopril 6.25 mg were 47.6±10.2% and 53.8±10.7%, respectively. In other words, captopril did not attenuate the vasoconstrictor response to [Pro1°-D-Ala12] Ang I (P=0.6).

**[Pro1°-D-Ala12]** Ang I Irbesartan Studies

Five of the 7 subjects who took part in the 4 study days described above returned for a fifth study, in which [Pro1°-D-Ala12] Ang I was infused 3 hours after pretreatment with 150 mg of oral irbesartan. The mean±SEM vasoconstrictor response during infusion of [Pro1°-D-Ala12] Ang I after pretreatment with irbesartan was 2.7±2.0%. In other words, irbesartan completely abolished the vasoconstrictor response to [Pro1°-D-Ala12] Ang I (P=0.0007).

**Discussion**

In our study, the ACE-resistant isopeptide [Pro1°-D-Ala12] Ang I, which is specific for chymase, caused vasoconstriction. This response was not attenuated by the ACE inhibitor captopril but was completely blocked by irbesartan. Because this peptide does not directly activate the Ang II type 1 receptor,23 we conclude that non-ACE conversion of Ang I to Ang II does occur in human dorsal hand veins and that this pathway is functionally important. We believe that this is the first demonstration of such a pathway in vivo in humans.

Previous in vitro studies in human medium-sized arteries and veins and in small arteries have suggested the existence of such pathways by virtue of the fact that chymostatin, a relatively specific inhibitor of chymase, attenuates the functional response to Ang I. As outlined in the introduction, these studies have been criticized on the basis that the trauma involved in removing and mounting vessels in organ chambers and myographs may activate mast cells.4,5,11–14

Furthermore, in vitro experiments are not physiological, because exogenous angiotensin introduced abuminally can diffuse as freely into the adventitia (which is particularly rich in mast cells) as into the endothelium. In vivo, however, Ang I introduced directly to the lumen will encounter endothelial ACE and be converted to Ang II. Thus, adventitial concentrations of Ang I may be so low as to render the chymase pathway inconsequential. Furthermore, the effect of vessel washing and mounting on the activity of interstitial factors that physiologically bind and inactivate chymase is unclear. These concerns have led some to suggest that in vitro studies are misleading in apportioning a greater significance to the chymase contribution to Ang I to II conversion than actually is the case in vivo or even that such conversion is completely artifactual.5,11–14

Our findings seem to refute these criticisms, because they clearly show that human veins have the capacity to convert Ang I to Ang II through a non-ACE pathway in vivo. On the basis of previous studies with this substrate, we believe that the alternative pathway is likely to be chymase.16–20,23

Our findings gain indirect support from other in vivo studies in humans. The addition of Ang II type 1 receptor (AT1) antagonists to background therapy with ACE inhibitors for heart failure brings about further hemodynamic and neurohumoral changes and improvements in symptom class and functional capacity, suggesting that continuing Ang I to Ang II conversion is occurring.24–26 Similarly, renin inhibitors and AT1 receptor antagonists cause greater renal vasodilatation than the maximum vasodilation obtainable with conventional, full-dose ACE inhibition in humans.27 Even full conventional doses of ACE inhibitors, however, may not completely block ACE.28

What might the clinical significance of our results be? Clearly, if physiological Ang II generation does occur in vivo through a non-ACE pathway, this could become a therapeutic target in disease states in which inhibition of the renin-angiotensin-aldosterone system is thought to be beneficial. These include hypertension, heart failure, and atherosclerotic disease. Indeed, chymase seems to be induced by hypercholesterolemia29 and may be involved, perhaps partly through Ang II generation, in atherosclerotic plaque formation.30

Our work does have a number of limitations. First, only veins were studied. Because [Pro1°-D-Ala12] Ang I had never been administered to humans before, we could not ethically infuse it into the arterial circulation. Second, we had to use a dose of [Pro1°-D-Ala12] Ang I 6 times that of Ang I to obtain the same degree of vasoconstriction. As pointed out in the Methods, this is in keeping with the finding that the specificity constant for the conversion of [Pro1°-D-Ala12] Ang I to Ang II by human chymase is \( \approx 7-10 \) fold higher than that for the conversion of Ang I to Ang II by ACE.23 Although this might mean that the 2 pathways are of comparable capacity, it remains difficult to know for certain how important the non-ACE conversion we found is physiologically. Certainly, in vivo, the full concentration-response curve for [Pro1°-D-Ala12] Ang I is shifted markedly to the right, compared with that for Ang I, in small human resistance arteries; the
maximum contraction obtained is also reduced (unpublished observations). Furthermore, the relative importance of the non-ACE pathway could change during ACE inhibition, for example if it were upregulated during this treatment. Finally, it is not clear why captopril completely inhibits the response to Ang I if there is an alternative non-ACE functional pathway capable of generating Ang II directly. The net response will obviously depend on the dose of captopril used versus the dose of Ang I given (and the degree of contraction it causes). Activation of this alternative pathway may occur only when higher concentrations of Ang I are present.

In conclusion, using a chymase-specific, ACE-resistant, substrate, we have shown that non-ACE generation of Ang II, with a biological effect, can occur in vivo in humans. This finding has therapeutic implications for many cardiovascular disease states.

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