Investigation of the biochemical effects of renin inhibition in normal volunteers treated by an ACE inhibitor

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- 1 In order to investigate accurately the biochemical effects of renin inhibition in man, we have developed a sensitive assay to measure angiotensin I (1-10) decapeptide.
- 2 Angiotensins were extracted from plasma by adsorption to phenylsilylsilica, and angiotensin I (Ang I) was quantified by radioimmunoassay. The detection limit was 0.77 fmol ml⁻¹, and the extraction recovery of [¹²⁵I]-Ang I added to albumin buffer was 83% at the inflection point (10 fmol ml⁻¹) of the standard curve. The overall recovery was 98.5 ± 3.5%. The intra- and inter-assay reproducibility was 10.4% and 9.7% respectively. Cross-reactivity of the antiserum used was low (< 0.3%) with all angiotensin peptides tested except Ang (2-10) nonapeptide.
- 3 A human pharmacological model was subsequently used to assess *in vivo* the biochemical effects of the renin inhibitor CGP 38560A. Six healthy volunteers received 20 mg lisinopril, a long-acting ACE-inhibitor. During the following 24 h, the renin-angiotensin system was reset with typically elevated active plasma renin and Ang I, at respectively 275 and 429% of basal values.
- 4 In a randomized three-way cross-over protocol, the six volunteers received a 30 min infusion of the renin inhibitor CGP 38560A (125 or 250 µg kg⁻¹) or 5% glucose. The fall in plasma Ang I was 92% and 97.5% after the lowest and highest dose of the renin inhibitor, respectively. A concomitant increase in active plasma renin was observed. After the infusion ceased, reappearance of Ang I was dose-related, whereas PRA remained durably inhibited, even when measured by the trapping assay of Poulsen & Jorgensen (1974) in the absence of angiotensinase inhibitors other than ethylenediamine-tetraacetic acid.
- 5 The use of a sensitive method of Ang I measurement applied in this human pharmacological model demonstrates that plasma Ang I investigates *in vivo* the intravascular renin-blockade more reliably than PRA, an *in vitro* index of limited value. The dissociation between the short-term fall in plasma Ang I and the long term inhibition of PRA induced by CGP 38560A may be dependent on Ang I production outside of plasma, the compartment to which the distribution of this renin inhibitor is restricted.

Keywords renin inhibition CGP 38560A angiotensin I radioimmunoassay
angiotensin I-converting enzyme inhibition

Introduction

Blockade of the renin-angiotensin system with angiotensin converting enzyme (ACE) inhibitors has convincingly been demonstrated an effective treatment for essential hypertension and heart failure (William, 1988). As renin inhibition offers a more selective method of blockade, it may provide advantages over ACE inhibition (Haber, 1989). Experimental data on short-term renin inhibition

have demonstrated that 1) the effects of renin inhibitors on blood pressure are closely related to the participation of the renin-angiotensin system (RAS) in maintenance of blood pressure, 2) both magnitude and duration of antihypertensive effects are related to the doses used (see review in Corvol *et al.* (1990)). In clinical studies renin inhibitors have been demonstrated to promote

dissociation between plasma renin activity (PRA) and plasma angiotension II (Ang II) (Jeunemaitre *et al.*, 1989; Nussberger *et al.*, 1989; Van Den Meiracker *et al.*, 1990), and between PRA and blood pressure (Van Den Meiracker *et al.*, 1990; Weber *et al.*, 1990).

Reliable measurement of plasma angiotensins remains a key issue in RAS investigation; Nussberger *et al.* (1985, 1988) have developed a three-step procedure to measure specifically Ang II. Immunoreactive angiotensins were completely extracted on bonded-phase silica; Ang II was then isolated by high performance liquid chromatography (h.p.l.c.) and sensitive quantitation was made by radioimmunoassay (Nussberger *et al.*, 1985). Subsequently, measurement of 'true' plasma Ang II, was demonstrated to be an accurate tool to estimate ACE inhibition *in vivo* (Juillerat *et al.*, 1990; Nussberger *et al.*, 1988). We hypothesize that during renin inhibition, sensitive and specific angiotensin I (Ang I) could evidence blockade of the renin-angiotensin system, more reliably than measurement of PRA and more easily than that of 'true' Ang II. However, concentrations of circulating Ang I are very low in normal man. In order to amplify the drop in plasma Ang I during renin inhibition, we envisioned 'resetting' the renin-angiotensin system by pre-treatment with an ACE inhibitor (Juillerat *et al.*, 1990). Our aim with this ACE inhibitor was to promote a dose-related rise in active renin and Ang I, due to the interruption of the negative feedback loop of Ang II on renin release. Our objectives were 1) to devise accurate ultrasensitive measurement of plasma Ang I and 2) to examine the biochemical effects in plasma of the renin inhibitor, CGP 38560A, in healthy volunteers pretreated with a long-acting ACE-inhibitor.

Methods

Volunteers

Six healthy male volunteers aged 24–30 years participated in the study which was approved by the Broussais Hospital Ethics Committee. All subjects had given written consent and were instructed to keep to their regular diet and to avoid salty foods. In all six volunteers the control values of blood pressure and pulse rate, and the plasma parameters of the renin-angiotensin system were measured in the absence of any medication after 1 h in sitting position, between 08.00 h and 09.00 h. On the morning of the study days, volunteers had a light early breakfast without coffee and presented at 08.00 h to the Pharmacology Study Room. On arrival they were weighed, and then rested in a sitting position throughout the 3 h infusion.

Design (Figure 1)

The day before each study day, all volunteers received lisinopril, 20 mg orally at 12.00 h, i.e. 20 h before the beginning of the experiment. Each subject was randomly allocated to receive two doses of the renin inhibitor, CGP 38560A, and 5% glucose as placebo. The study was conducted on the basis of a double-blind three-way cross-over design. Each study consisted of three periods: during the first (–60 min to 0) and the third (+30 to +120 min), 5% glucose solution was infused at a rate of 100 ml h⁻¹. In the second period (0 to +30 min), the volunteers received either 5% glucose or CGP 38560A. The doses of CGP 38560A were 125 and 250 µg kg⁻¹.

Figure 1 Study protocol. Experiments were performed at random, according to a latin square cross-over design.

The substance was infused through a catheter inserted into a brachial vein, and blood samples were drawn from an indwelling cannula inserted in the other arm. The infusion rate was kept constant at 100 ml h⁻¹ with a pump (type Infusomat from Melsungen AG, Frankfurt, Germany). Blood pressure and heart rate were recorded with a Sentrom automatic blood-pressure recorder (Screening Devices Inc., Costa Mesa, CA, USA) every 5 min between $t - 60$ to $t - 15$ and between $t + 60$ to $t + 120$, and every 2 min between these two periods.

Blood samples were collected in heparinized vacutainers before (t_0) and 15, 30, 45, 60, 90 and 120 min after the infusion of the drug or placebo. For the measurement of plasma Ang I, 10 ml of blood was taken into prechilled glass tubes, and a mixture of inhibitors was immediately added to prevent *in vitro* generation and degradation of angiotensins (Nussberger *et al.*, 1988). The solution included 62.5 mM Na₂ EDTA, 50 mM phenanthroline and 0.1 mM CGP 38560A. The plasma was separated after centrifugation at 4° C and immediately extracted (angiotensin I) or stored at -20° C until analyzed (other samples).

All volunteers were examined 7 days after completing the tests. Blood pressure was recorded after 60 min in the sitting position and blood samples were taken to assess basal values of PRA, active plasma renin, plasma aldosterone and Ang I, and converting-enzyme activity.

Angiotensin I assay

Plasma Ang I was measured after extraction (Nussberger *et al.*, 1985, 1988). For each plasma sample, one cartridge containing 100 mg of phenylsilylsilica (Bond Elut, Analytichem, Harbor City, CA, USA) was pre-washed with methanol (1 ml) followed by water (2 × 1 ml). Cold plasma (2 × 1 ml) was passed through the cartridge, allowing all peptides, including angiotensins, to be adsorbed. After washing with water (2 × 1 ml), angiotensins were eluted with 0.5 ml methanol into previously albumin-coated conical polypropylene tubes. The methanol was evaporated with compressed air at 37° C for 60 min. Dried extract was dissolved with 0.6 ml of buffer (2 g l⁻¹ bovine serum albumin in 0.1 M Tris, pH 7.5), and aliquots were stored at -80° C until analyzed.

Radioimmunoassay was performed entirely at 4° C: the volume of each trial sample was inferred from presumed concentration of Ang I, and completed to 0.5 ml with 0.1 M Tris buffer (2 g l⁻¹ albumin, pH 7.5). Ang I antiserum (0.5 ml) (final dilution 1:440 000) produced according to Ménard & Catt (1972) and 0.1 ml of a radiolabeled tracer (mono-iodo [¹²⁵I]-angiotensin I, 4000 counts min⁻¹ with a specific activity of 55.5 µCi µg⁻¹, ORIS, Gif-sur-Yvette, France) were added. All determinations were assayed in duplicate. A standard curve was established by serial triplicate dilutions of Ang I (MRC, London, England) in 0.1 M tris buffer (2 g l⁻¹ albumin, pH 7.5). After mixing and incubation for 72 h at 4° C, 0.3 ml 1.25% charcoal-0.25% dextran suspension was added to each sample and incubated for 10 min at room temperature. The tubes were centrifuged for 10 min at 4000 rev min⁻¹, and the radioactivity of

the supernatant containing the bound angiotensins was counted in an automatic gamma spectrometer for 2 min.

Other assays

PRA was measured according to the methods of Sealey (PRA) (Sealey *et al.*, 1972) and Poulsen (trapping assay, PRA-TA) (Poulsen & Jorgensen, 1974), both at pH 7.4 as previously described (Jeunemaitre *et al.*, 1989). Plasma concentrations of CGP 38560 were measured by a radio-inhibitor binding assay which measures the free base (CGP 38560) and any active metabolites. Its detection limit is between 0.5 and 1 pmol ml⁻¹ (Cumin *et al.*, 1989).

Angiotensinogen was determined as the Ang I produced in the presence of an excess of semi-purified human renin. Marketed kits were used to measure active renin in a radioimmunometric assay according to Ménard *et al.* (1985) (Pasteur Diagnostics, Marnes-la-Coquette, France) and plasma aldosterone after extraction of the plasma (ORIS, Gif-sur-Yvette, France). Converting-enzyme activity was determined according to Lieberman as modified by Cushman & Cheung (1971).

Statistical methods

Results are expressed as means ± s.d. in tables and means ± s.e mean in figures. The analysis of variance (ANOVA) considered periods, treatments and interaction as sources of variation. A difference was considered as significant when $P < 0.05$.

Results

Angiotensin I assay system

The assay system for Ang I was sensitive and precise: at a dilution of 1:440 000, the antiserum bound 30% of [¹²⁵I]-Ang I and the detection limit was 0.77 fmol (95% CI, 0.1 to 1.5). 9.9 fmol Ang I displaced half of this tracer amount.

Table 1 shows the assay specificity for angiotensin peptides taking Ang I (1-10) decapeptide as 1.0. The Ang (2-10)-nonapeptide exhibited a high cross-reactivity as it remained very low (< 0.25%) for all other peptides.

The recovery of Ang I was first assessed by adding ¹²⁵I-labelled Ang I to albumin buffer: doses of Ang I (5000 and 1250 counts min⁻¹) were chosen to be near

Table 1 Specificity of the angiotensin I radioimmunoassay

Angiotensin peptide	Cross reactivity* (%)
des-Asp-Ang I (2-10)	100
Ang II (1-8)	0.14
Ang III (2-8)	0.21
Ang 3-8	0.13
Ang 4-8	0.18
Ang 5-8	0.08

*Unlabeled standard peptides (purchased from MRC, London, England) at a final concentration of 10 ng ml⁻¹ were quantified by Ang I radioimmunoassay. Cross reactivity is deduced from apparent concentration on standard curve ($n = 6$).

Table 2 Effects of lisinopril on mean arterial blood pressure, and the renin-angiotensin system, 21 h after drug intake (mean $\pm$ s.d.)

	Control (n = 6)	After lisinopril (21 h) (n = 18)		
Blood pressure (mm Hg)	80.3 $\pm$ 4.4	75.6 $\pm$ 7.1	74.0 $\pm$ 8.3	74.1 $\pm$ 6.7
ACE-activity (μ l ml ⁻¹)	38.2 $\pm$ 15.8	13.3 $\pm$ 3.8	13.3 $\pm$ 2.9	14.0 $\pm$ 3.6
Angiotensinogen (ng Ang I ml ⁻¹)	846 $\pm$ 103	851 $\pm$ 189	809 $\pm$ 175	827 $\pm$ 158
PRA (ng Ang I ml ⁻¹ h ⁻¹)	0.76 $\pm$ 0.41	2.90 $\pm$ 0.96	2.48 $\pm$ 2.2	3.30 $\pm$ 2.4
PRA-TA (ng Ang I ml ⁻¹ h ⁻¹)	1.00 $\pm$ 0.24	3.40 $\pm$ 0.96	3.44 $\pm$ 2.4	4.05 $\pm$ 3.1
AR (pg ml ⁻¹)	44 $\pm$ 12	100 $\pm$ 31	115 $\pm$ 89	148 $\pm$ 165
Aldosterone (pg ml ⁻¹)	100 $\pm$ 43	67 $\pm$ 31	56 $\pm$ 5	75 $\pm$ 38
Plasma Ang I (fmol ml ⁻¹)	17 $\pm$ 4.9	75 $\pm$ 26	58 $\pm$ 43	79 $\pm$ 72

the maximal concentration (50 fmol ml⁻¹) and the usual inflection point (10 fmol ml⁻¹) of the standard curve. Recovery was 89% and 83%, respectively. The overall recovery was then studied by adding 10 and 20 fmol of Ang I to 2 ml of normal human plasma. After processing and assay, recovery was calculated by subtracting the endogenous Ang I concentration. Mean recovery ($\pm$ s.e.) was 98.5 $\pm$ 3.5% (n = 6). No correction was subsequently performed.

Intra-assay reproducibility was calculated on 10 measurements of low Ang I (mean value 1.7 fmol ml⁻¹) and normal Ang I plasma (mean value 7.8 fmol ml⁻¹). The coefficients of variability were 10.4 and 9.8%, respectively. Inter-assay reproducibility was calculated on five determinations of normal Ang I plasma and five determinations of high Ang I plasma. The coefficients of variation were 9.7% and 6.4%, respectively.

Effects of lisinopril on blood pressure and the renin-angiotensin system of normal volunteers 21 h after 20 mg lisinopril intake

Blood pressure and the parameters of the plasma renin-angiotensin system were measured four times in the same conditions, once in the absence of lisinopril, and three times, 21 h after lisinopril intake. Results are shown in Table 2. Mean arterial blood pressure was slightly lower after lisinopril, but the difference was not statistically significant. Twenty-one hours after 20 mg lisinopril intake, ACE activity remained durably reduced (35% of baseline), whereas PRA, plasma active renin and plasma Ang I were increased by three to four fold. Plasma aldosterone was slightly lower than its control values after lisinopril treatment, but the difference was not statistically significant, plasma angiotensinogen was unaffected by the single dose of lisinopril. Data show the highly reproducible results of this protocol of lisinopril administration to normotensive volunteers. Before the infusion of CGP 38560A or 5% glucose, plasma Ang I

and PRA were significantly correlated ($y = 24.7x + 437$, $r = 0.90$) as were plasma Ang I and plasma active renin ($y = 1.4x - 3.4$, $r = 0.92$).

Effects of the renin inhibitor CGP 38560A on blood pressure and the renin-angiotensin system of normal volunteers, 21 h after 20 mg lisinopril intake

Pulse rate and blood pressure were not affected by infusion of the renin inhibitor.

As shown in Table 3, PRA (both Sealey's assay and Poulsen's assay), active renin and plasma Ang I slightly decreased with time during 5% glucose infusion. However, changes were not statistically significant, and may reflect circadian variations, or the waning effects of lisinopril. By contrast, 15 min after beginning infusion of the renin inhibitor, Ang I decreased significantly and reached a minimum at the end of the perfusion. The fall in Ang I was greater with the higher dose (92% after 0.125 mg kg⁻¹ vs 97.5 after 250 μ g kg⁻¹). Although very low, individual values remained in the range of the assay sensitivity. After the low dose infusion was stopped, the plasma concentration of Ang I reverted rapidly (within 90 min) to initial values. With the highest dose (0.250 μ g kg⁻¹) Ang I remained low until the end of the recovery period (35% of basal values) whereas after infusion of the low dose of CGP 38560A it did not differ from the control experiment (69% vs 67% of basal values). These results suggest a dose-response relationship on the duration of renin blockade, although it did not reach statistical significance (Figure 2). Whatever the assay used, PRA decreased below the limit of detection between 15 and 30 min and rose progressively thereafter. By the end of the experiment a prolonged inhibition of PRA was demonstrable by the classical assay at pH 7.4 (9 and 5% of basal values with 125 μ g kg⁻¹ and 250 μ g kg⁻¹ doses, respectively). PRA-TA was also profoundly depressed (37 and 13% of basal values, Figure 3).

Active immunoreactive renin already stimulated by ACE inhibition rose within the first 15 min of the renin

Table 3 Effects of the renin inhibitor CGP 38560A (infused from 0 to 30 min) on plasma angiotensin I (Ang I), plasma renin activity (PRA and PRA-TA) and plasma active renin (AR). Results are expressed as mean $\pm$ s.d.

Time (min)	Placebo 5% glucose	CGP 38560A	
		125 $\mu\text{g kg}^{-1}$	250 $\mu\text{g kg}^{-1}$
<i>Ang I (fmol ml⁻¹)</i>			
0	75 $\pm$ 26	58 $\pm$ 43	79 $\pm$ 72
15	68 $\pm$ 24	8 $\pm$ 7	5 $\pm$ 4
30	73 $\pm$ 33	5 $\pm$ 4	2 $\pm$ 2
45	52 $\pm$ 24	12 $\pm$ 10	8 $\pm$ 5
60	62 $\pm$ 14	38 $\pm$ 41	12 $\pm$ 5
90	52 $\pm$ 14	38 $\pm$ 33	25 $\pm$ 21
120	50 $\pm$ 14	40 $\pm$ 38	28 $\pm$ 19
<i>PRA (ng Ang I ml⁻¹ h⁻¹)</i>			
0	2.9 $\pm$ 0.9	2.5 $\pm$ 2.1	3.3 $\pm$ 2.4
15	2.4 $\pm$ 0.7	0	0
30	2.7 $\pm$ 1.0	0	0
45	2.5 $\pm$ 1.0	0.01	0.01
60	2.5 $\pm$ 0.5	0.1	0.03
90	2.3 $\pm$ 0.7	0.2 $\pm$ 0.2	0.1
120	2.1 $\pm$ 0.5	0.2 $\pm$ 0.2	0.2 $\pm$ 0.1
<i>PRA-TA (ng Ang I ml⁻¹ h⁻¹)</i>			
0	3.4 $\pm$ 0.9	3.4 $\pm$ 2.4	4.0 $\pm$ 3.1
15	3.8 $\pm$ 1.4	0	0
30	3.3 $\pm$ 0.1	0	0
45	2.8 $\pm$ 0.7	0.03 $\pm$ 0.02	0
60	3.1 $\pm$ 0.7	0.2 $\pm$ 0.2	0.01
90	3.0 $\pm$ 1.0	0.6 $\pm$ 0.7	0.3 $\pm$ 0.02
120	2.7 $\pm$ 1.0	1.3 $\pm$ 1.4	0.5 $\pm$ 0.2
<i>AR (pg ml⁻¹)</i>			
0	100 $\pm$ 31	115 $\pm$ 89	148 $\pm$ 166
15	103 $\pm$ 24	236 $\pm$ 206	291 $\pm$ 295
30	95 $\pm$ 24	312 $\pm$ 305	357 $\pm$ 333
45	96 $\pm$ 24	326 $\pm$ 307	376 $\pm$ 329
60	87 $\pm$ 22	325 $\pm$ 300	340 $\pm$ 281
90	82 $\pm$ 17	296 $\pm$ 240	307 $\pm$ 271
120	44 $\pm$ 12	265 $\pm$ 221	259 $\pm$ 223

Figure 2 Effects of CGP 38560A (125 $\mu\text{g kg}^{-1}$ (◆) and 250 $\mu\text{g kg}^{-1}$ (▲)) and 5% glucose (○) infusions on plasma Ang I (mean $\pm$ s.e. mean). Shaded areas represent control values.

inhibitor infusions, reaching a maximum at 45 min, then decreased slowly and was still above pretreatment values 90 min after the end of the infusion. The rise in plasma active renin was similar with the two doses of the CGP 38560A which were infused (Table 3).

Table 4 shows the plasma concentrations of CGP 38560 and its active metabolites as measured by a radio-inhibitor binding assay, and their strikingly fast decrease

Figure 3 Comparison of the percentage changes in PRA (Sealey's method, pH 7.4), PRA-TA (Poulsen's method, pH 7.4), plasma Ang I and plasma active renin, 90 min after the end of the infusion of placebo (□) and CGP 38560A (125 $\mu\text{g kg}^{-1}$ (▨) and 250 $\mu\text{g kg}^{-1}$ (▩)) (mean $\pm$ s.e. mean).

Table 4 Effects of the renin inhibitor CGP 38560A (infused from 0 to 30 min) on CGP 38560 plasma concentrations, plasma aldosterone and ACE-activity. Results are expressed as mean $\pm$ s.d.

Time (min)	CGP 38560A		Placebo 5% glucose
	125 $\mu\text{g kg}^{-1}$	250 $\mu\text{g kg}^{-1}$	
Plasma CGP 38560 (pmol ml ⁻¹)			
0	1.2 $\pm$ 0.7	1.2 $\pm$ 0.5	59 $\pm$ 31
15	1107 $\pm$ 725	1333 $\pm$ 614	54 $\pm$ 16
30	1360 $\pm$ 1044	1733 $\pm$ 801	60 $\pm$ 22
45	315 $\pm$ 350	569 $\pm$ 506	50 $\pm$ 17
60	128 $\pm$ 151	298 $\pm$ 415	57 $\pm$ 24
90	34 $\pm$ 31	80 $\pm$ 115	70 $\pm$ 31
120	18 $\pm$ 14	31 $\pm$ 33	73 $\pm$ 19
Plasma aldosterone (pg ml ⁻¹)			
0	56 $\pm$ 5	52 $\pm$ 38	13.3 $\pm$ 3.8
15	55 $\pm$ 12	70 $\pm$ 17	ND
30	50 $\pm$ 19	65 $\pm$ 16	ND
45	48 $\pm$ 20	60 $\pm$ 20	ND
60	56 $\pm$ 7	57 $\pm$ 12	ND
90	73 $\pm$ 15	59 $\pm$ 8	ND
120	75 $\pm$ 26	60 $\pm$ 12	13.8 $\pm$ 3.1
Plasma ACE-activity (mu ml ⁻¹)			
0	13.3 $\pm$ 2.9	14.0 $\pm$ 3.6	
15	ND	ND	
30	ND	ND	
45	ND	ND	
60	ND	ND	
90	ND	ND	
120	12.7 $\pm$ 2.2	14.3 $\pm$ 3.6	

ND: not determined.

at the end of the infusions. Plasma aldosterone and plasma ACE were not affected by renin inhibition.

Discussion

In addition to its potential importance in clinical investigation of the circulating renin-angiotensin system in hypertension, measurement of plasma angiotensins provides a unique tool for the biochemical evaluation of ACE inhibitors (Nussberger *et al.*, 1988). More attention has been paid to the measurement of Ang II than to that of Ang I (Waite, 1973), for two main reasons. Ang II is the active peptide of the RAS, but the presence in the circulation of various metabolites which cross-react *in vitro* with the antibodies and have different physiological roles *in vivo* requires a specific measurement of 'true' Ang II (Lawrence *et al.*, 1990). Secondly, anti-Ang II antibodies cross-react with Ang I, the precursor of Ang II which is increased during ACE inhibition, and may cause an overassessment of Ang II. Ang I measurement, even if it measures both Ang I and des-Asp-Ang I, does not share these drawbacks. As previously shown (Nussberger *et al.*, 1988), the method used to extract Ang II from plasma can be applied to Ang I, and this may provide a more reliable assay than previous methods, which required blood precipitation, with incomplete and variable recovery (Waite, 1973). This paper describes in detail a reliable and sensitive method for measuring Ang I in human plasma. It associates solid phase extraction of angiotensins, followed by ultrasensitive radioimmunoassay due to the high Ang I affinity of the antiserum

used. Sensitivity of this assay is characterized by a limit of detection of 0.77 fmol and a 50% displacement of the iodinated tracer on standard curve by 9.9 fmol Ang I. This constitutes a 10 fold improvement by comparison with commercially available kits for renin activity measurement. Being more sensitive and having better accuracy due to the steeper slope of the standard curve, this method overcomes the problems of sensitivity and precision. Supplementation of plasma with CGP 38560A on the one hand and phenantroline and EDTA on the other blocked renin and converting enzyme and angiotensinases respectively. Rapid deep freezing (-80°C) of aliquots allowed conservation up to 30 days (data not shown), and avoided immediate extraction and assay as initially performed in our laboratory (Jeunemaitre *et al.*, 1989).

To estimate renin inhibition accurately *in vivo*, the use of plasma Ang I rather than plasma Ang II or PRA assay is justified for theoretical and practical reasons. Renin is the only enzyme which generates Ang I. When there is no short-term change in plasma and endothelial ACE, the production of Ang II is directly dependent on the changes in Ang I. The accurate measurement of the active peptide, plasma Ang II, requires isolation by h.p.l.c. and is still a difficult and time-consuming procedure. During renin inhibition, the direct measurement of the enzymatic reaction product Ang I can provide useful information, since dissociations have been reported between blood pressure and PRA (Nussberger *et al.*, 1988; Weber *et al.*, 1990), and between PRA and plasma Ang II (Jeunemaitre *et al.*, 1989; Nussberger *et al.*, 1989; Van Den Meiracker *et al.*, 1990).

The main advantage of inducing ACE inhibition before testing the plasma effects of a renin inhibitor is that the renin-angiotensin system does not participate in maintaining sitting blood-pressure at a time when high values of renin and Ang I are obtained. The use of diuretics or vasodilators such as hydralazine could also stimulate the RAS for testing the haemodynamics and/or biochemical properties of renin inhibitors, but too rapid lowering of blood pressure during renin inhibition may cause hypotension, when blood pressure is renin-dependent. The design used in this study obviates this risk, although it does not aim at providing data on blood pressure during renin inhibition which should be preferentially collected in hypertensive patients. In ACE-inhibitor pretreated volunteers, initial values of plasma Ang I (t_0) were considerably higher than in previous experiments in normotensive volunteers and hypertensive patients ($71.2 \pm 20 \text{ fmol ml}^{-1}$ vs $22.2 \pm 6.1 \text{ fmol ml}^{-1}$) (Jeunemaitre *et al.*, 1989). This facilitates precise biochemical quantification of the magnitude of renin inhibition. The infusion of CGP 38560A caused a very rapid and profound decrease in plasma Ang I, confirming the remarkable affinity ($\text{IC}_{50}\%: 0.7 \times 10^{-9} \text{ M}$) of this inhibitor for human renin. Even if plasma Ang I is a sensitive index of renin inhibition, the lowest level of plasma Ang I during renin inhibition by infusion of these doses of CGP 38560A were never unmeasurably low, as already reported for plasma Ang II with this renin inhibitor in hypertensive patients (Jeunemaitre *et al.*, 1989). Two hypotheses may explain this result: 1) the use of the highest doses of CGP 38560A known to suppress plasma Ang I completely would have been more effective

but was prohibited because of toxicity demonstrated in animals. 2) the antiserum used disclosed a 100% cross-reactivity between Ang I (1-10, decapeptide) and des-Asp-Ang I (2-10 nonapeptide), and therefore this assay detects the nonapeptide as immunoreactive 'Ang I'. Persistence of the nonapeptide in the absence of Ang I is very unlikely, but further separation with h.p.l.c. could clarify this possibility.

Plasma renin activity was deeply inhibited during the infusion of CGP 38560A. As compared with plasma Ang I, recovery was delayed even if a less prolonged inhibition was discernible when the trapping assay was used instead of a more widely used method of PRA measurement (Sealey *et al.*, 1972). Previous careful studies in humans have shown dissociation between PRA measurement, plasma angiotensins and blood pressure (De Gasparo *et al.*, 1989; Delabays *et al.*, 1989; Nussberger *et al.*, 1989). Such dissociation may be explained by an artefactual *in vitro* displacement of the protein-bound renin inhibitor by the use of various angiotensinase inhibitors in PRA assays (Derx *et al.*, 1991) that are not present in the trapping assay. However the results obtained with CGP 38560A in this experiment, as well as in two previous investigations, show that even results of the PRA trapping assay are not exactly parallel to those of plasma Ang II (Jeunemaitre *et al.*, 1989; Nussberger *et al.*, 1989) or plasma Ang I (Jeunemaitre *et al.*, 1989) when this renin inhibitor is infused. In dialysis experiments CGP 38560A was strongly bound to plasma proteins (99.7% at pH 7.4) and its volume of distribution, calculated in marmosets, corresponded to plasma volume (Cumin *et al.*, 1990). These results suggest that renin blockade by the slow infusion of both doses was tightly restricted to circulating plasma. Recent evidence confirms that peripheral tissues are major sites of formation of angiotensin peptides by active renin released by the kidney to plasma and interstitial compartments (Admiraal *et al.*, 1990). Continuous release of Ang I from the interstitial space to plasma could explain both very low but still detectable plasma concentrations despite complete PRA inhibition, and the quick rise of Ang I in plasma after the infusion ceased, despite the persistence of plasma renin inhibition. CGP 38560A probably did not obtain an easy access to interstitial renin where a major part of angiotensin I is generated (Admiraal *et al.*, 1990). This may explain why, in hypertensive patients, it did not decrease blood pressure as much as captopril, and why blood pressure, plasma

Ang I and plasma Ang II returned towards their initial values, despite a persistent and probably not artefactual inhibition of plasma renin activity (Jeunemaitre *et al.*, 1989).

Although plasma Ang II determinations were not performed in this study, the rise in active renin during the infusion of the renin inhibitor is an indirect argument to postulate that Ang II did fall, which disrupted the negative feedback that Ang II exerts on renin release. When the infusion of the renin inhibitor was stopped, active renin started declining but remained high until the end of the experiment. The reappearance of Ang I was certainly promoted by this hyperreninism, renin being the limiting step of the enzymatic cascade, both in plasma and in interstitial fluid. This result also brings new information on the mechanisms of action of ACE inhibitors. The reactive rise in plasma renin and Ang I which follows ACE inhibition is the most likely explanation for the normal circulating Ang II levels observed 24 h after repeated doses of an ACE inhibitor (Juillerat *et al.*, 1990; Nussberger *et al.*, 1985). The major fall in plasma Ang I observed during the infusion of a renin inhibitor 21 h after ingestion of a single oral dose of lisinopril is a supplementary argument to eliminate or to minimize the role of Ang II-forming enzymes independent of the renin-angiotensin system (Okunishi *et al.*, 1987) in determining the circulating levels of Ang II in these circumstances. It also suggests that the relatively modest pre-dosing antihypertensive effect of ACE inhibitors when administered once daily (Meredith *et al.*, 1990) may be renin-dependent at a time where the rise in plasma Ang I occurs concomitantly to the disappearance of the ACE inhibitor.

In conclusion, pretreatment of normal volunteers with an ACE-inhibitor produces resetting of the circulating RAS which increases plasma Ang I and renin. This situation provides a safe and useful way to investigate the biochemical effects of a renin inhibitor without the risk of hypotension, as when the stimulation of the RAS is induced by previous salt depletion. Plasma Ang I measurement by a sensitive radioimmunoassay provides an assessment of the *in vivo* degree of renin blockade which is different from both conventional PRA, and trapping assay.

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