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ORIGINAL ARTICLE

Role of angiotensin II in plasma PAI-1 changes induced by imidapril or candesartan in hypertensive patients with metabolic syndrome

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To evaluate the relationship between plasma plasminogen activator inhibitor-1 (PAI-1) and angiotensin II (Ang II) changes during treatment with imidapril and candesartan in hypertensive patients with metabolic syndrome. A total of 84 hypertensive patients with metabolic syndrome were randomized to imidapril 10 mg or candesartan 16 mg for 16 weeks. At weeks 4 and 8, there was a dose titration to imidapril 20 mg and candesartan 32 mg in nonresponders (systolic blood pressure (SBP) >140 and/or diastolic blood pressure (DBP) >90 mm Hg). We evaluated, at baseline and after 2, 4, 8, 12 and 16 weeks, clinic blood pressure, Ang II and PAI-1 antigen. Both imidapril and candesartan induced a similar SBP/DBP reduction (−19.4/16.8 and −19.5/16.3 mm Hg, respectively, $P<0.001$ *vs.* baseline). Both drugs decreased PAI-1 antigen after 4 weeks of treatment, but only the PAI-1 lowering effect of imidapril was sustained throughout the 16 weeks (−9.3 ng ml^{-1}, $P<0.01$ *vs.* baseline), whereas candesartan increased PAI-1 (+6.5 ng ml^{-1}, $P<0.05$ *vs.* baseline and $P<0.01$ *vs.* imidapril). Imidapril significantly decreased Ang II levels (−14.6 pg ml^{-1} at week 16, $P<0.05$ *vs.* baseline), whereas candesartan increased them (+24.2 pg ml^{-1}, $P<0.01$ *vs.* baseline and *vs.* imidapril). In both groups there was a positive correlation between Ang II and PAI-1 changes ($r=0.61$, $P<0.001$ at week 16 for imidapril, and $r=0.37$, $P<0.005$ at week 16 for candesartan). Imidapril reduced plasma PAI-1 and Ang II levels, whereas candesartan increased them. This suggests that the different effect of angiotensin-converting enzyme inhibitors and Ang II blockers on Ang II production has a role in their different influence on fibrinolysis.

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INTRODUCTION

Considerable evidence has accumulated demonstrating that the renin–angiotensin system (RAS) is a key regulator of the fibrinolytic balance, mainly by inducing the expression of plasminogen activator inhibitor-1 (PAI-1), a major physiological inhibitor of fibrinolysis.[1–3] The main effector peptide of the RAS, angiotensin II (Ang II), stimulates PAI-1 expression *in vitro* in a variety of cell types, including smooth muscle cells, endothelial cells, adipocytes, astrocytes, proximal tubular epithelial cells and mesangial cells.[4–7] Similarly, also *in vivo* studies conducted both in animal models and in humans demonstrated that Ang II induces PAI-1 expression.[8–10]

Blockade of the RAS by angiotensin-converting enzyme inhibitors (ACE-Is) has been generally demonstrated to reduce PAI-1 levels in both experimental and clinical studies.[11–16] Contrasting results have been reported about the effects on fibrinolysis of Ang II receptor blockers (ARBs), which inhibit the RAS throughout the block of the effects of Ang II at the AT1 receptor level.[17–22] Most studies showed no influence,[17–20] whereas only a few studies showed a reduction in PAI-1 plasma levels with ARBs.[21,22] Accordingly, most, although not all,[23,24] direct comparative studies on the effects of ACE-Is and ARBs on the fibrinolytic balance observed a decrease in PAI-1 plasma levels with ACE-Is, but not with ARBs.[25–29] One possible reason for such a dissimilar effect might be the different effect of the two drug classes on plasma Ang II. Unlike ACE-Is, which inhibit ACE-dependent production of Ang II, thus decreasing its plasma levels, treatment with ARBs is known to elevate markedly circulating Ang II.[30–34] These effects, which occur in response to changes in physiological cascades and feedback regulations in the RAS, might partly account for the dissimilar influence of ACE-Is and ARBs on the fibrinolytic balance.

However, to the best of our knowledge, no study has specifically evaluated the possible relationship between PAI-1 and Ang II changes under treatment with ACE-Is and ARBs. With this background, the present study was undertaken in order to assess the role of Ang II in plasma PAI-1 changes induced by the ACE-I imidapril and the ARB candesartan in the treatment of hypertensive patients with metabolic syndrome. We chose this type of population as hypertension and metabolic syndrome are both characterized by impaired fibrinolysis, mainly expressed by elevated PAI-1 plasma levels[35,36] and therefore

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these patients were more likely to present detectable changes in PAI-1 values under drug treatment.

METHODS

Study design

This was a 16-week prospective, randomized, open label, blinded end-point (PROBE),[37] parallel group study, with two treatments arms.

Study population

Both sex consecutive outpatients, aged 18–65 years, with stage I essential hypertension (defined as sitting systolic blood pressure (SBP) $\geqslant$140 mm Hg and $<$160 mm Hg and sitting diastolic blood pressure (DBP) $\geqslant$90 mm Hg and $<$100 mm Hg after a 2-weeks washout placebo period) and metabolic syndrome (AHA/NHLBI criteria)[38] were considered eligible to be enrolled in the study. Exclusion criteria were as follows: secondary hypertension, creatinine clearance $<$80 ml min^{-1}, smoking habits, history of myocardial infarction or stroke within 6 months before the study, congestive heart failure or any severe disease likely to interfere with the conduction of the study, known contraindications or intolerance to ACE-Is or ARBs.

Ethics

The study protocol was approved by the local Ethical Committee and all eligible candidates had to provide signed informed consent before enrolling in the study.

Study protocol

Following a 2 weeks placebo washout period, patients who fulfilled the inclusion criteria were randomized in a 1:1 manner to receive either imidapril 10 mg or candesartan 16 mg both given once daily (o.d.) in the morning (at approximately 0800 hours) for 16 weeks. After 2, 4 and 8 weeks a dose titration to imidapril 20 mg or candesartan 32 mg was permitted in nonresponder patients (SBP $\geqslant$140 mm Hg and/or DBP $\geqslant$90 mm Hg).

All participants maintained their usual diet and level of physical activity and avoided changes in body weight throughout the study. No concomitant medication was allowed. Compliance to trial medications was evaluated by counting the number of pills returned at the time of specified clinic visits.

Clinic blood pressure (BP), PAI-1 antigen and Ang II plasma levels were evaluated at the end of the washout period and after 2, 4, 8, 12 and 16 weeks of treatment, whereas fasting plasma glucose, fasting plasma insulin, total cholesterol, high-density lipoprotein cholesterol and triglycerides were assessed at the end of the washout period and of each treatment period.

BP was measured with the patient in the seated position by using a standard mercury sphygmomanometer (Korotkoff I and V) with a cuff of appropriate size. Measurements were taken in the morning after the subject had rested 10 min in a quiet room and before daily drug intake (that is, 24-h after dosing, at trough). The average of three successive BP readings obtained at 1-min interval was recorded.

After BP measurements, venous blood was drawn from an antecubital vein for blood sampling at the same hour in the morning (approximately between 0800 and 0900 hours), as PAI-1 concentration is known to be at its peak during this period. Blood samples were collected on ice and centrifuged immediately at 0 °C for 20 min. All plasma or serum samples were separated and stored at −70 °C until assay. Blood for measurements of PAI-1 antigen was collected in Vacutainer tubes (Becton Dickinson, Meylan, France) containing 0.105 nmol l^{-1} acidified sodium citrate and antigen level was determined by using a 2-site enzyme-linked immunosorbent assay (Biopool AB, Umea, Sweden).

Blood samples for the determination of plasma Ang II concentration were drawn from patients in supine position after 1 h of complete rest and were collected into prechilled 10-ml syringes prepared with 125 mmol EDTA and 26 mmol phenanthrolin (Merck KGaA, Darmstadt, Germany) to inhibit ACE. The samples were centrifuged for 10 min at 4 °C immediately after collection and plasma was stored rapidly after centrifugation at −21 °C and analyzed within 3 months. Plasma samples (1 ml) were extracted with Bond Elut PH cartridges (PK 100, ICT-ASS-Chem, Analytichem, Harbor City, CA, USA) and bound angiotensin was eluted with 0.5 ml methanol. Subsequently, plasma extracts were evaporated in a SpeedVac (SVC 100, Savant Instruments, Pvt Ltd, Hyderabad, India) for 1 h under reduced pressure. Immediately after purification of the samples, immunoreactive Ang II was measured by radioimmunoassay with antiserum and labelled Ang II (NEX 105, Du Pont).[39] Cross-reactivity of this method is 100% for Ang II and 1.2% for Ang I. The quantification of radioactivity was performed with a gamma counter. Measurements made by this method are accurate within a range from 1.9 to 32 pg Ang II ml^{-1}. All determinations of immunoreactive Ang II were made in duplicate and the mean values are given. The coefficient of variation was $<$5%.

Blood glucose in the fasting state was measured by the glucose oxidase method (Beckman Auto-Analyzer, Fullerton, CA, USA). Plasma insulin concentrations were determined by radioimmunoassay.

Statistical analysis

All results are expressed as means ± s.d. All data were analyzed by a split-plot method for analysis of variance; the data for PAI-1 were also analyzed for multiple comparisons by the Dunn-Sidak method for split-plot analysis of variance. For all statistical analyses a *P*-value of $<$0.05 was considered statistically significant.

RESULTS

A total of 100 patients were screened between November 2009 and September 2010. At the end of the 2 weeks placebo washout period, 84 patients were randomized to receive imidapril 10 mg (n=42) or candesartan 16 mg (n=42). Their main demographic and clinic characteristics are shown in Table 1. The two treatment groups were comparable in terms of age, sex, body mass index, baseline sitting BP, fasting plasma glucose, fasting plasma insulin, total cholesterol, high-density lipoprotein cholesterol, triglycerides, creatinine clearance and duration of hypertension. Five patients withdrew after randomization, two in the imidapril group (one withdrew the informed consent, one because of hypotension) and three in the candesartan group (one withdrew the informed consent, one because of excessively high BP values and one because of hypotension). A total of 79 patients, 40 in the imidapril group and 39 in the candesartan group completed the study.

The BP results are reported in Table 2. After 16 weeks of treatment, both imidapril and candesartan significantly decreased sitting SBP (−19.4 and −19.5 mm Hg, respectively, both $P<0.001$ *vs.* baseline) and DBP (−16.8 and −16.3 mm Hg, respectively, both $P<0.001$ *vs.* baseline), with no significant differences between them. The BP

Table 1 Baseline characteristics of the patients at the beginning of the study

Parameters	*Imidapril* (n=*42*)	*Candesartan* (n=*42*)
Age (years)	56.8 ± 9.6	56.6 ± 10.1
Gender (M/F)	19/23	20/22
BMI (kg m^{-2})	27.4 ± 1.49	27.6 ± 1.51
SBP (mm Hg)	150.2 ± 8.4	150.7 ± 8.2
DBP (mm Hg)	98.6 ± 5.2	98.9 ± 4.9
FPG (mg dl^{-1})	92.3 ± 8.4	91.8 ± 8.1
FPI (μIU ml^{-1})	14.3 ± 3.7	14.1 ± 3.8
TG (mg dl^{-1})	168.5 ± 32.4	172.2 ± 33.9
HDL-C (mg dl^{-1})	42.4 ± 5.6	42.1 ± 5.3
TC (mg dl^{-1})	191.1 ± 13.7	190.8 ± 13.5
Creatinine clearance (ml min^{-1})	92.2 ± 11.9	98.9 ± 12.1
Duration of hypertension (years)	8.5 ± 2.8	8.4 ± 3.1

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; F, female; FPG, fasting plasma glucose; FPI, fasting plasma insulin; HDL-C: high density lipoprotein cholesterol; M, male; SBP, systolic blood pressure; TC: total cholesterol; TG, triglycerides.
P not significant.

Table 2 Effect of treatment on blood pressure

Time	*Imidapril* (n=*40*)	*Candesartan* (n=*39*)
Seated SBP mm Hg		
Baseline	149.8 ± 8.4	150.1 ± 8.3
Week 2	141.2 ± 7.2**	142.1 ± 7.5**
Week 4	135.3 ± 6.7***	134.9 ± 6.6***
Week 8	133.2 ± 6.5***	132.4 ± 6.4***
Week 12	132.1 ± 6.3***	131.7 ± 6.2***
Week 16	130.4 ± 6.1***	130.6 ± 6.3***
Seated DBP mm Hg		
Baseline	99.1 ± 5.1	98.9 ± 4.9
Week 2	92.3 ± 4.4**	91.8 ± 4.2**
Week 4	88.6 ± 4.1***	88.2 ± 3.8***
Week 8	85.2 ± 3.6***	85.5 ± 3.4***
Week 12	84.3 ± 3.1***	84.5 ± 3.0***
Week 16	82.3 ± 2.7***	82.6 ± 2.8***

Abbreviations: DBP, diastolic blood pressure; SBP, systolic blood pressure.
**$P<0.01$ *vs.* baseline; ***$P<0.001$ *vs.* baseline.

Table 3 Effect of treatment on metabolic parameters

Parameters	*Imidapril* (n=*40*)	*Candesartan* (n=*39*)
FPG (mg dl^{-1})		
Baseline	92.2 ± 8.3	91.9 ± 8.1
Week 16	90.8 ± 8.2	90.9 ± 7.9
FPI (μIU ml^{-1})		
Baseline	14.4 ± 3.8	14.2 ± 3.9
Week 16	13.8 ± 3.5	13.9 ± 3.6
TG (mg dl^{-1})		
Baseline	169.5 ± 33.1	171.4 ± 33.7
Week 16	159.5 ± 31.8	165.3 ± 32.5
HDL-C (mg dl^{-1})		
Baseline	42.6 ± 5.5	42.4 ± 5.3
Week 16	42.9 ± 5.4	42.5 ± 5.5
TC (mg dl^{-1})		
Baseline	189.8 ± 13.6	191.2 ± 13.6
Week 16	186.2 ± 13.5	189.9 ± 13.7

Abbreviations: FPG, fasting plasma glucose; FPI, fasting plasma insulin; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; TG, triglycerides.
P not significant.

Table 4 Effect of treatment on plasma PAI-1 antigen and Ang II

Time	*Imidapril*	*Candesartan*
PAI-1 antigen (ng ml^{-1})		
Baseline	26.6 ± 13.1	26.2 ± 13.3
Week 2	19.2 ± 9.8*	21.1 ± 11.1*
Week 4	16.8 ± 7.6**	25.3 ± 13.7
Week 8	18.1 ± 8.1*	29.5 ± 13.1
Week 12	17.5 ± 7.4**	31.9 ± 12.6* °
Week 16	17.3 ± 7.5**	32.7 ± 13.8** +
Ang II (pg ml^{-1})		
Baseline	29.8 ± 7.8	28.9 ± 7.2
Week 2	17.2 ± 4.1**	32.2 ± 10.5°
Week 4	15.5 ± 3.7**	38.4 ± 12.3* +
Week 8	14.7 ± 3.5**	46.7 ± 16.4** +
Week 12	14.8 ± 3.4**	52.8 ± 19.1** +
Week 16	15.2 ± 3.6**	53.1 ± 20.5** +

Abbreviations: Ang II, angiotensin II; PAI-1, plasminogen activator inhibitor-1.
*$P<0.05$ *vs.* baseline; **$P<0.01$ *vs.* baseline; °$P<0.05$; +$P<0.01$ *vs.* imidapril.

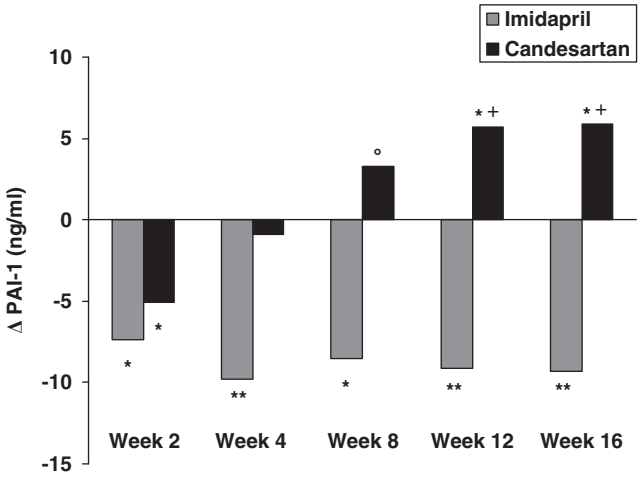

Figure 1 Plasma PAI-1 changes induced by imidapril or candesartan in hypertensive patients with metabolic syndrome. *$P<0.05$ *vs.* baseline; **$P<0.01$ *vs.* baseline; °$P<0.05$ *vs.* imidapril; +$P<0.01$ *vs.* imidapril. PAI-1, plasminogen activator inhibitor.

reduction was already evident after 2 weeks and sustained throughout the study with both treatments.

The metabolic effects are shown in Table 3. Neither imidapril nor candesartan significantly affected fasting plasma glucose, fasting plasma insulin, total cholesterol, high-density lipoprotein cholesterol and triglycerides plasma levels.

The effects of treatments on plasma PAI-1 antigen and Ang II are reported in Table 4 and Figures 1 and 2. Both imidapril and candesartan significantly decreased PAI-1 antigen after the first 2 weeks of treatment (-7.4 and -5.1 ng ml^{-1}, respectively, both $P<0.05$ *vs.* baseline). Thereafter, only the PAI-1 lowering effect of imidapril was sustained throughout the 16 weeks period (-9.3 ng ml^{-1}, $P<0.01$ *vs.* baseline), whereas in the candesartan group PAI-1 antigen returned to baseline values at week 4 and significantly increased at week 16 ($+6.5$ ng ml^{-1}, $P<0.05$ *vs.* baseline and $P<0.01$ *vs.* imidapril). As shown in Figure 1, the change in PAI-1 antigen over time in response to treatment was significantly less marked during candesartan than during imidapril treatment.

Imidapril significantly decreased Ang II plasma levels; the reduction was already evident after 2 weeks (-12.6 pg ml^{-1}, $P<0.05$ *vs.* baseline) and persisted substantially unchanged throughout the study (-14.6 pg ml^{-1} at week 16, $P<0.05$ *vs.* baseline). By contrast, candesartan progressively increased Ang II values, the rise being statistically significant from week 4 ($+9.5$ pg ml^{-1}, $P<0.05$ *vs.* baseline) onwards ($+24.2$ pg ml^{-1} at week 16, $P<0.01$ *vs.* baseline and *vs.* imidapril; Figure 2). As shown in Figure 3, in the imidapril-treated patients, the Ang II changes strictly paralleled the PAI-1 changes and correlation analysis showed a highly significant relationship between the PAI-1 and the Ang II imidapril-induced decrease for the entire duration of the study ($r=0.48$, $P<0.01$ at week 2; $r=0.61$, $P<0.001$ at week 16). As shown in Figure 4, in the candesartan-treated patients, the change in PAI-1 levels was accompanied by a parallel change in Ang II only from week 8 onwards; also in this group, a positive relationship

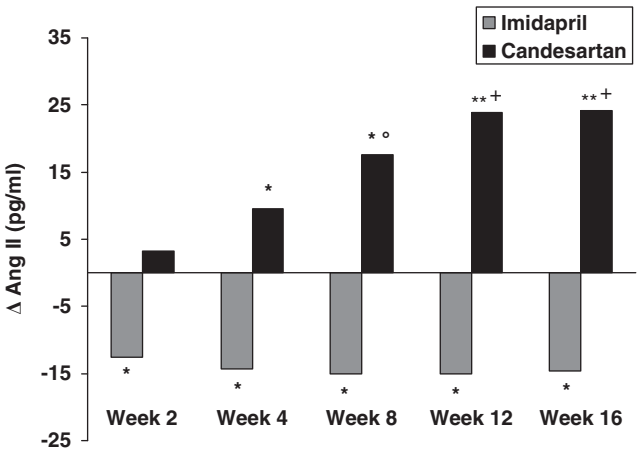

Figure 2 Plasma Ang II changes induced by imidapril or candesartan in hypertensive patients with metabolic syndrome. $^{*}P<0.05$ *vs.* baseline; $^{**}P<0.01$ *vs.* baseline; $^{\circ}P<0.05$ *vs.* imidapril; $^{+}P<0.01$ *vs.* imidapril. Ang II, angiotensin II.

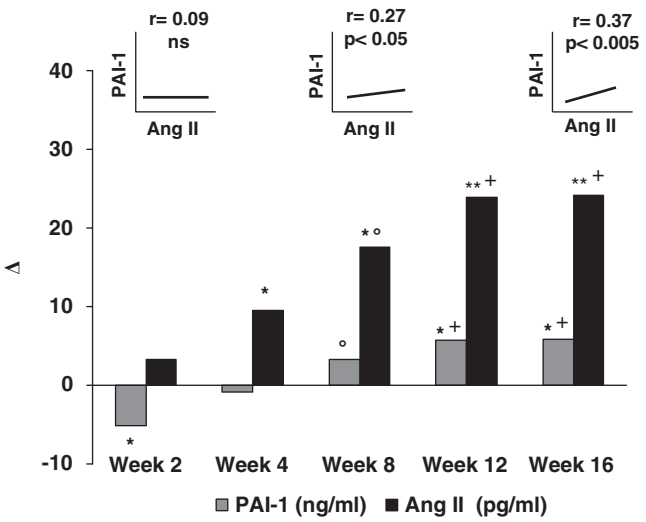

Figure 4 Relationships between plasma PAI-1 and Ang II changes induced by candesartan in hypertensive patients with metabolic syndrome. $^{*}P<0.05$ *vs.* baseline; $^{**}P<0.01$ *vs.* baseline; $^{\circ}P<0.05$ *vs.* imidapril; $^{+}P<0.01$ *vs.* imidapril. Ang II, angiotensin II; PAI-1, plasminogen activator inhibitor.

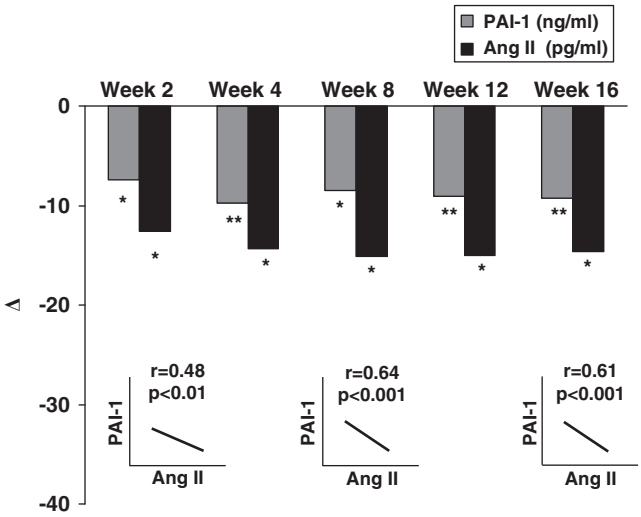

Figure 3 Relationships between plasma PAI-1 and Ang II changes during imidapril in hypertensive patients with metabolic syndrome. $^{*}P<0.05$ *vs.* baseline; $^{**}P<0.01$ *vs.* baseline. Ang II, angiotensin II; PAI-1, plasminogen activator inhibitor.

between PAI-1 and Ang II candesartan-induced changes was found, although the correlation analysis showed a lower level of statistical significance compared with that observed with imidapril ($r=0.27$, $P<0.05$ at week 8; $r=0.37$, $P<0.005$ at week 16).

DISCUSSION

The findings from the present study indicated that in stage I hypertensive patients with associated metabolic syndrome, 16-week antihypertensive treatment with imidapril and candesartan, in front of equivalent antihypertensive efficacy, exerted dissimilar effects on PAI-1 antigen, whose plasma levels were significantly reduced by the ACE-I and increased by the ARB. Besides, when the effects of the two drugs on PAI-1 antigen over time were considered, a different time course of effects was observed. In fact, in agreement with some previous observations by ourselves and other authors,[29,40,41] short-term treatment with both imidapril and candesartan decreased PAI-1 antigen plasma levels, whereas in the long-term only the ACE-I reduced PAI-1, while the ARB progressively enhanced it.

The dissimilar effect of imidapril and candesartan on PAI-1 antigen observed in this study cannot be related to a difference in BP decrease, as the BP lowering effect was similar in the two treatment groups. Possible influence of body weight changes on the fibrinolytic system[42,43] can also be excluded, as all participants in this study maintained their usual diet and level of physical activity and no significant change in their body mass index was observed.

The main element of novelty in the present study was the significant relationship we found between the PAI-1 antigen and the plasma Ang II changes in the two treatment groups. As expected, imidapril treatment significantly reduced Ang II plasma levels, whereas candesartan enhanced them, which confirmed previously described effects of ACE-I and ARB on Ang II.[30–34] Both ACE-I and ARB reduce feedback inhibition of renin release, which triggers a reactive increase in plasma renin activity. With an ACE-I such an increase results in a compensatory rise in Ang I and a reduction of Ang II plasma concentrations, due to the block of ACE-mediated conversion of Ang I to Ang II; in contrast, with an ARB the reactive rise in renin activity causes increases in Ang I, Ang II and its metabolites.[30–34]

Interestingly, in the imidapril group the changes in Ang II values strictly paralleled the PAI-1 antigen changes and a highly significant correlation between the Ang II and the PAI-1 decrease was observed for the entire duration of the study. This suggests that the reduction of circulating Ang II has a major role in the imidapril-induced PAI-1 antigen decrease. In the candesartan group, a positive correlation between Ang II and PAI-1 changes was observed from 8 weeks onwards, when both PAI-1 and much more Ang II plasma levels increased, although the degree of statistical significance of such a correlation was lower as compared with that found in the imidapril-treated patients. These findings are in agreement with the hypothesis

that Ang II may increase PAI-1 synthesis not only through AT1 receptors, but also through its hexapeptide catabolite, Ang IV, which binds to specific AT4 receptors.[44,45] Removal of the aminoterminal amino acid from Ang II by an aspartate amino peptidase yields Ang III (called also angiotensin(2–8); removal of the terminal arginine from Ang III by ACE2 yields Ang IV (called also angiotensin-2(3–8)). Although the reduction of Ang II levels by the ACE-I, limiting the conversion of Ang II to smaller peptides, may prevent the endothelial synthesis of PAI-1, the reactive rise of Ang II because of AT1 blockade might lead to increased Ang IV production and thus favor PAI-1 expression via endothelial AT4 receptor stimulation.[44,45]

Upregulation of the AT4 receptors after 2 weeks of AT1 blockade might be one possible explanation for the reduction of PAI-1 observed with short-term therapy with candesartan.[46] Our observation that AT1 receptor blockade requires some weeks to produce the maximal Ang II increase also suggests that in this period of time the effect of AT1 blockade prevails on that of AT4 stimulation. The different time courses of imidapril and candesartan on PAI-1 levels might also have depended on the different duration of suppression of tissue Ang II, which is suppressed by ACE-I and not suppressed by ARB.[47]

Whatever the mechanisms, the different influence of ACE-I and ARB on the fibrinolytic balance might at least partly explain the different coronary heart disease preventive effect between the two drug classes.[48] Analyses by the Blood Pressure Lowering Treatment Trialists' Collaboration have shown that for ACE-I, but not for ARB, there is evidence of BP independent effect on the risk of major coronary events.[48] ACE-I, besides decreasing the release of Ang II-mediated PAI-1, may favorably alter the fibrinolytic balance also by increasing the endothelial release of bradykinin-induced tissue plasminogen activator (tPA).[2,49,50] Although in the present study we did not evaluate changes in tPA levels, data from previous studies by ourselves and other authors indicated that ARB, which do not affect the metabolism of bradykinin, do not influence or even decrease plasma tPA.[1,25,28,29] Coronary release of tPA from the endothelium is an important defense against coronary heart disease. The recently published results of the Shiga Plasminogen Activator In Coronary Circulation study[51] have demonstrated that ACE inhibition increased coronary release of tPA, although only in women. Such a positive effect of ACE-I on the coronary fibrinolytic balance might contribute to the reduction in coronary events observed with these drugs.

Differently from these authors, we did not observe any differences between sex regarding our data.

CONCLUSIONS

The results of the present study indicated that in the treatment of hypertensive patients with metabolic syndrome, the ACE-I imidapril and the ARB candesartan, despite equivalent antihypertensive efficacy, exerted dissimilar effects on PAI-1 plasma levels, which were reduced by imidapril and increased by candesartan. Such changes appeared to be related to plasma Ang II changes, which suggests that the different effect that ACE-I and ARB exert on Ang II production has a role in their different influence on fibrinolysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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We certify that we have no affiliation with, or financial involvement in, any organization or entity with a direct financial interest in the subject matter or materials discussed in the manuscript.

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