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ORIGINAL ARTICLE

Antithrombotic effects of losartan in patients with hypertension complicated by atrial fibrillation: 4A (Angiotensin II Antagonist of platelet Aggregation in patients with Atrial fibrillation), a pilot study

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Angiotensin receptor blockers (ARBs) are widely used for the treatment of hypertension. It has been reported that the ARB losartan has antiplatelet, anticoagulant and profibrinolytic effects experimentally. These properties could be desirable to treat hypertensive patients with high atherothrombotic and/or thromboembolic risk. To examine the antithrombotic effects of losartan in hypertension, 20 consecutive patients with hypertension complicated by atrial fibrillation (AF) were enrolled in this study. The patients were treated with losartan 50 mg for 8 weeks followed by 100 mg for 4 weeks. Blood samples were obtained from each patient at 0 (pretreatment), 8 and 12 weeks after initiating treatment. Platelet aggregability, plasma levels of tissue factor (TF) and type 1 plasminogen activator inhibitor (PAI-1) activity levels were measured. The area under the curve for small platelet aggregability decreased from 100 to 42.8% at 12 weeks ($P<0.0001$). TF levels (ng ml^{-1}) and PAI-1 activity (IU ml^{-1}; mean ± s.d.) also changed from 14.2 ± 3.6 to 10.9 ± 4.5 at 12 weeks ($P=0.0299$) and from 11.7 ± 3.6 to 8.5 ± 3.1 at 12 weeks ($P=0.0122$), respectively. Losartan inhibited platelet activity and coagulation factors in a dose- and time-dependent manner in patients with hypertension complicated by AF, whereas the fibrinolytic capacity was increased. The use of losartan could be advantageous in the treatment of hypertensive patients with high atherothrombotic risk.
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Keywords: losartan; platelet aggregation; type 1 plasminogen activator inhibitor

INTRODUCTION

Hypertension is a major risk factor for ischemic heart disease.[1] Furthermore, hypertension is also the most prevalent independent risk factor for atrial fibrillation (AF).[2] Therefore, ischemic heart disease and AF frequently occur together in patients with hypertension. It is well known that intracoronary thrombus formation is common in ischemic heart disease, particularly in acute coronary syndromes.[3] Similarly, patients with AF are compromised by a thromboembolic risk because of intra-atrial thrombus formation.[4] Taken together, patients with hypertension complicated by AF are highly predisposed to experience enhanced platelet activity and coagulation cascades.

The renin–angiotensin system (RAS), through the pleiotropic biological actions of angiotensin II, has a key role in the pathophysiology of hypertension and, moreover, of various other cardiovascular and renal diseases.[5] Therefore, the use of angiotensin receptor blockers (ARBs) is one possible pharmacological intervention. Because losartan, an ARB, was shown to be effective in lowering cardiovascular morbidity and mortality in patients with high-risk hypertension in the Losartan Intervention for Endpoint Reduction in Hypertension (LIFE) trial,[6] it could be a promising agent. One of the LIFE substudies showed that patients with left-ventricular hypertrophy and AF benefited from losartan compared with atenolol, a β-blocker, in preventing cardiovascular morbidity and mortality as well as stroke and cardiovascular death, even though equivalent blood pressure reductions were provided by both agents.[7] These results suggested that losartan could have antithrombotic effects in this patient cohort. In this vein, several articles regarding the antiplatelet,[8] anticoagulant[9,10] and profibrinolytic[10] effects of losartan have been published that may explain the better prognosis of losartan in the LIFE study.

In the present pilot study, we examined dose- and time-dependent antithrombotic effects of losartan in patients with hypertension complicated by AF.

METHODS

Study patients

This study was performed on 20 consecutive patients with untreated essential hypertension (systolic blood pressure ⩾140 mm Hg and/or diastolic blood

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pressure $\geqslant$90 mm Hg) complicated by permanent non-valvular AF who presented at the outpatient clinics of our institutes. All the patients were treated with a vitamin K antagonist first. Written informed consent was obtained from all the subjects.

Study protocol

All the patients were treated with losartan 50 mg daily for 8 weeks first. Then, the dose was increased to 100 mg daily, and the patients were followed for 4 more weeks (12 weeks in total). No medications other than losartan were changed during the study period. Venous blood sampling was performed in each patient at 0 (pretreatment), 8 and 12 weeks after initiating treatment. Blood was collected from the median cubital vein using a 21-G needle attached to a vacuum blood collection tube containing sodium citrate at the outpatient clinics in the early morning. Sample tubes were centrifuged at 150 *g* at 4 °C for 15 min, and platelet-rich plasma was obtained. The samples were then centrifuged at 3000 *g* at 4 °C for 10 min, and platelet-poor plasma was obtained. Platelet-rich plasma was used for platelet aggregometry within 1 h after collection. Platelet-poor plasma was stored at −80 °C until analyzed. The study protocol was approved by the institutional review board for clinical studies.

Analysis of platelets, coagulation and fibrinolytic markers in blood

Platelet aggregometry. Platelet-rich plasma aggregation was simultaneously determined by evaluating the maximum percent decrease in optical density and by assessing laser-light scattering intensity using an aggregometer, PA-200 (Kowa, Tokyo, Japan). Adenosine diphospate 1.0 μM was used as an agonist for platelet aggregation and was added to platelet-rich plasma samples 60 s after the starting the measurements. The principles of the laser-light scattering method have been described previously.[11,12] This method is based on the fact that the intensity of scattered light emitted from a particle increases in proportion to the square of its diameter. With this method, small aggregates containing approximately 70–1400 platelets are detectable. Generally, aggregates smaller than 10 μM are formed in the early phase of aggregation, and larger aggregates are formed in the following phase. Quantitative estimation of platelet aggregation was performed by determining both the peak intensity and the area under the curve (AUC) for 5 min of laser-light scattering produced by small aggregates.

Flow cytometric analysis of activated platelets. For detection of activated platelets by flow cytometry, an anti-CD42b (GP Ibα) monoclonal antibody that targets all platelets and megakaryocytes and an anti-CD62P (P-selectin) monoclonal antibody that is found on the surface of activated platelets were used. Whole blood containing sodium citrate (5 μl) was added to a 5-ml polystyrene tube containing 20 μl each of fluorescein isothiocyanate-labeled CD62P and phycoerythrin-labeled CD42b (Becton Dickinson, San Jose, CA, USA). After reacting at room temperature for 15 min, the sample was fixed by adding 500 μl of 1% formaldehyde. A fluorescence-activated cell sorting analysis system, FACSCalibur (Becton Dickinson, Franklin Lakes, NJ, USA), was used for flow cytometric analysis. The platelets were gated on CD42b, and the percentage of CD62P-positive platelets per 10 000 gated events was calculated.

Plasma markers of thrombus formation. Platelet-poor plasma was used to examine plasma levels of tissue factor (TF), soluble P-selectin (sP-selectin) and von Willebrand factor antigens as well as type 1 plasminogen activator inhibitor (PAI-1) activity. Each parameter was analyzed using commercially available assay kits. TF levels were measured with an ELISA kit by Chemo Sero Therapeutic Research Institute (Kumamoto, Japan), sP-selectin levels by R&D SYSTEMS (Oxford, UK) and von Willebrand factor levels by Santa Cruz Biotechnology (Santa Cruz, CA, USA). The PAI-1 activity was measured using a chromogenic single-point poly-D-lysine stimulation assay kit by Biopool (Umeå, Sweden).

Statistical analysis

All the data are expressed as the mean ± s.d. except the history of AF, which is expressed as the median and ranges. The time courses of the measured parameters were analyzed by one-way factorial repeated-measures analysis of variance followed by the Tukey–Kramer HSD (honestly significant difference) test. Probability levels less than 0.05 were considered significant. The statistical package used was JMP for Macintosh ver.8.0.1 (SAS Institute, Cary, NC, USA).

RESULTS

Demographic data for the study patients are listed in Table 1. The mean age was 67 years old. The patients had been diagnosed with AF for an average of 7 years, and were being treated with 2.5 mg of vitamin K antagonist. The control level of the drug was appropriate as indicated by an average prothrombin time-international normalization ratio of 1.91. The patients tolerated losartan dosing up to 100 mg well. The average blood pressure decreased from 156/97 mm Hg to 147/89 mm Hg with losartan 50 mg for 8 weeks and to 140/84 mm Hg with losartan 100 mg for 4 more weeks (Figure 1). During the study period, there were no significant changes in other variables affecting the thrombotic state such as body weight or control status of diabetes or dyslipidemia in the study patients.

Figure 2 shows representative platelet aggregation analysis results with PA-200. Blue line plots indicate the speed of generation of small-size platelet aggregations (SPAs). Both the peak and AUC levels were decreased in a time- and dose-dependent manner. Furthermore, the percentage of CD62P-positive platelets also decreased in the same manner (Figure 3). Changes in SPA formation were not significant at 8 weeks (100% *vs.* 70.9% at SPA peak; 100% *vs.* 78.7% for the SPA

Table 1 Demographic data of study patients

Characteristics	*Data (n = 20)*
Age, years, mean (s.d.)	67 (8)
Male/female	12/8
Systolic blood pressure, mm Hg, mean (s.d.)	156 (12)
Diastolic blood pressure, mm Hg, mean (s.d.)	97 (8)
History of AF, years, median (ranges)	7 (0–13)
Dose of VKA, mg, mean (s.d.)	2.5 (0.6)
INR, mean (s.d.)	1.91 (0.41)
Smoking, *n* (%)	6 (30)
Dyslipidemia, *n* (%)	4 (20)
Diabetes, *n* (%)	5 (25)
Body mass index, kg m^{-2}, mean (s.d.)	24.1 (3.1)

Abbreviations: AF, atrial fibrillation; INR, international normalization ratio; VKA, vitamin K antagonist.

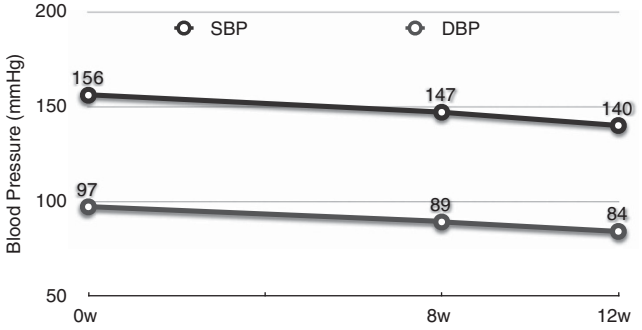

Figure 1 Changes in blood pressure after losartan administration. The systolic (SPB) and diastolic blood pressures (DBP) decreased by 16 and 13 mm Hg, respectively, on 100 mg of losartan. W, weeks. A full color version of this figure is available at the *Hypertension Research* journal online.

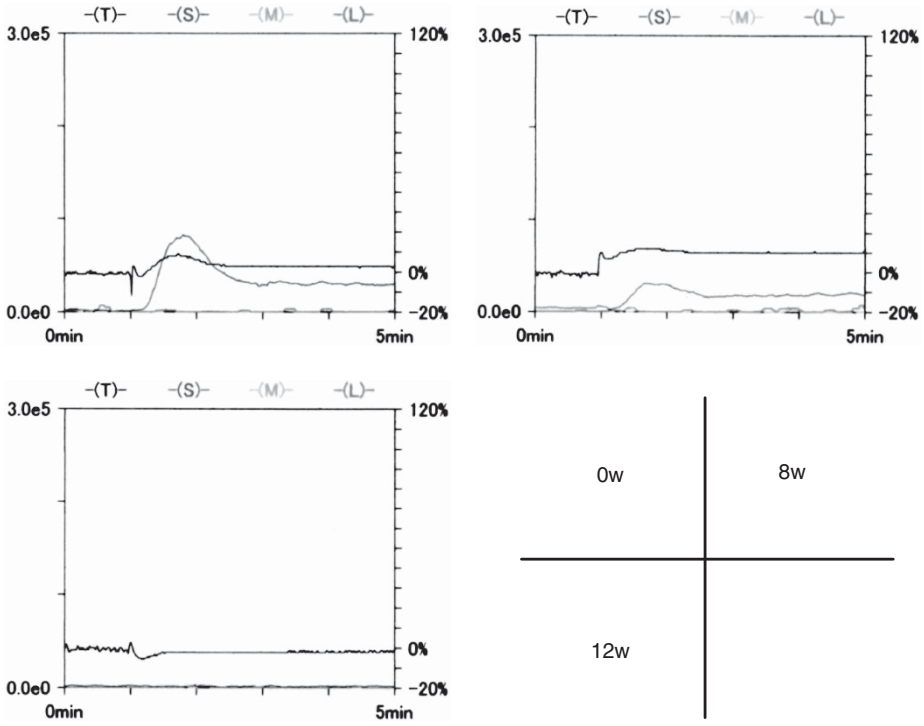

Figure 2 A representative platelet aggregability result measured by PA-200 aggregometry before and after losartan administration. The speed of small platelet aggregate (SPA) generation is indicated by blue lines with T. Both the peak levels and areas under the curve of the SPAs were decreased proportionally to the losartan dose. The left upper panel shows the pre-treatment (0 week (W)) results, the right upper panel shows the results at 8 W after initiating losartan therapy at 50 mg and the left lower panel shows the results at 12 W after initiating losartan therapy and increasing the dose to 100 mg after W 8. Black lines with T indicate conventional platelet aggregometry curves for light transmission with scales on the right-sided *y* axes. The other three colored lines with capital letters indicate the speed of platelet aggregate generation for each platelet aggregate size with scales on the left-sided *y* axes: blue lines with S indicate small platelet aggregates (70–1400 platelets); green lines with M indicate medium platelet aggregates (1400–11 000 platelets) and red lines with L indicate large platelet aggregates (11 000–31 000 platelets). A full color version of this figure is available at the *Hypertension Research* journal online.

AUC), but at 12 weeks, the changes were significant for both the peak and AUC levels (100% *vs.* 57.2% at SPA peak, $P=0.0040$; 100% *vs.* 42.8% for the SPA AUC, $P<0.0001$). Similarly, the percentage of CD62P-positive platelets was significantly decreased from 5.8 to 3.4% at 8 weeks ($P=0.0461$) and to 2.8% at 12 weeks ($P=0.0122$; Table 2). In addition to platelet function, thrombus-related plasma parameters changed during the treatment as listed in Table 2. von Willebrand factor levels (%) did not change throughout the entire treatment period (170.3 at 0 week, 166.9 at 8 weeks and 177.4 at 12 weeks). Similarly, the levels of TF (ng ml^{-1}), PAI-1 (IU ml^{-1}) activity and soluble P-selectin (ng ml^{-1}) were not changed at 8 weeks (14.2 ± 3.6 *vs.* 12.9 ± 3.5, 11.7 ± 3.6 *vs.* 12.3 ± 3.6 and 72.1 ± 20.7 *vs.* 55.2 ± 20.0, respectively). However, the levels were significantly decreased by 100 mg of losartan at 12 weeks (14.2 ± 3.6 *vs.* 10.9 ± 4.5, $P=0.0299$, 11.7 ± 3.6 *vs.* 8.5 ± 3.1, $P=0.0122$ and 72.1 ± 20.7 *vs.* 48.4 ± 34.4, $P=0.0145$, respectively).

DISCUSSION

In this study, we demonstrated that ordinary clinical administration dosages of losartan significantly decreased platelet activity in a time- and dose-dependent manner. These results are consistent with previous preclinical data with a smaller number of study subjects.[13,14] Furthermore, we showed for the first time that losartan also reduced PAI-1 activity in addition to its antiplatelet effects.

The prothrombotic state in hypertension is induced by activation of the RAS, leading to abnormalities in endothelial and platelet function, coagulation and fibrinolysis. Vascular inflammation caused by angiotensin II is one of the possible mechanisms underlying the prothrombotic state.[15] In this way, it is possible that RAS-targeting agents are effective at inhibiting the prothrombotic condition in patients with hypertension. However, mixed results have been reported in previous studies.[15] At the same time, AF is involved in systemic inflammation, resulting in the induction of PAI-1 and TF in endothelial cells and the activation of platelets.[16] Furthermore, the interaction between platelets and inflammatory cells exacerbates this prothrombotic tendency through increased TF expression on platelets.[17] In this way, in patients with hypertension complicated by AF, the prothrombotic state can be synergistically promoted, and in such situations, inhibition of the activated RAS by angiotensin-converting enzyme inhibitors or angiotensin II receptor blockers appears to be equally more effective than in patients without AF.

These relationships explain why losartan had beneficial effects on prothrombotic biomarkers in the present study. These effects have also been observed in RAS-inhibiting drugs in general in special conditions such as acute myocardial infarction.[10] In this way, these effects could be due to the class effects of losartan (the effects of ARBs in general). However, there are different effects between the two RAS-inhibiting drug classes such as the effects on bradykinin.[18] It has been reported that bradykinin decreases PAI-1 production[19] and platelet activity through nitric oxide.[20] Therefore, the profibrinolytic and antiplatelet effects of losartan may not be associated with bradykinin. Furthermore, in previous large-scale clinical trials such as ACTIVE-I

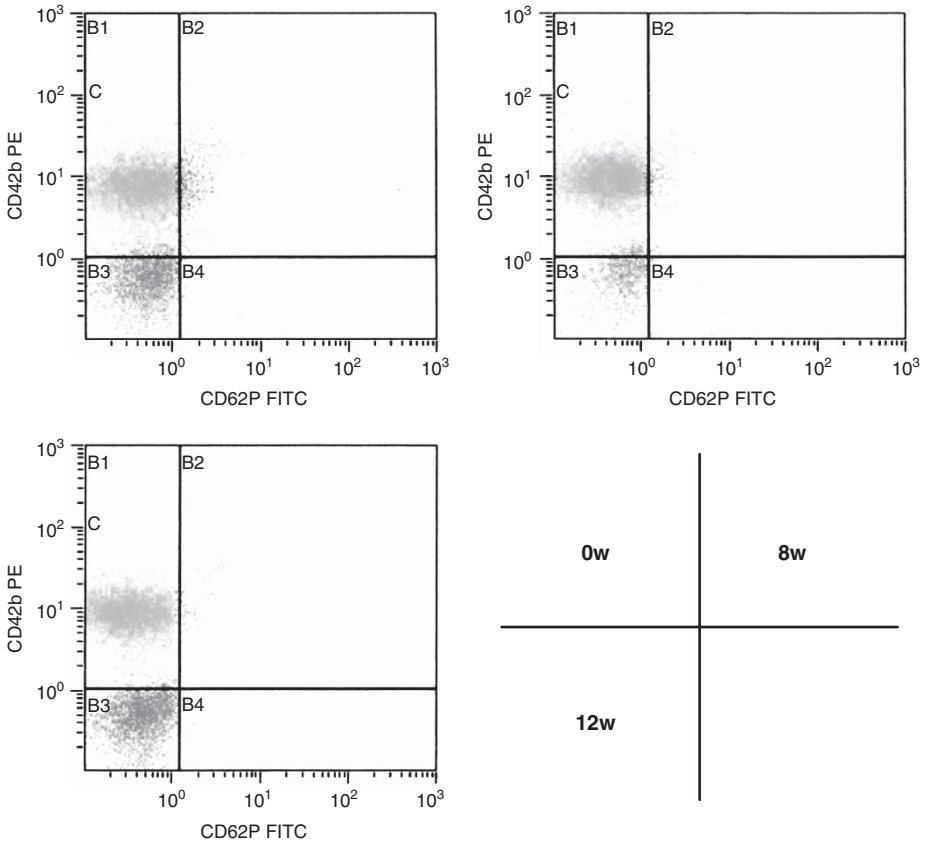

Figure 3 A representative fluorescence-activated cell sorting analysis of CD62-positive platelets before and after losartan administration. The B2 region indicates CD62-positive platelets. The percent positive platelet results were calculated using B2/(B1 + B2) × 100%, which yielded 7.35%, 2.36% and 0.96% before and at 8 and 12 weeks (W) after initiating losartan use, respectively. The left upper panel shows the pre-treatment (0 W) results, the right upper panel shows the results 8 W after initiating losartan therapy at 50 mg and the left lower panel shows the results 12 W after initiating losartan therapy and increasing the dose to 100 mg after W 8. Blue dots represent CD42b-positive/CD62P-positive particles, indicating active platelets; green dots represent CD42b-positive/CD62P-negative particles, indicating inactive platelets; and red dots represent CD42b-negative/CD62P-negative particles, indicating particles other than platelets. FITC, fluorescein isothiocyanate; PE, phycoerythrin. A full color version of this figure is available at the *Hypertension Research* journal online.

Table 2 Serial changes of the platelets, coagulation and fibrinolytic markers in blood

Parameters	*0w*	*8w*	*12w*	P-*value (0w* vs. *8w)*	P-*value (0w* vs. *12W)*
TF (ng ml^{-1}), mean (s.d.)	14.2 (3.6)	12.9 (3.5)	10.9 (4.5)	0.5438	0.0299
vWF (%), mean (s.d.)	170.3 (51.6)	166.9 (41.7)	177.4 (45.1)	0.9702	0.8806
PAI-1 (IU ml^{-1}), mean (s.d.)	11.7 (3.6)	12.3 (3.6)	8.5 (3.1)	0.8442	0.0122
Soluble P-selectin (ng ml^{-1}), mean (s.d.)	72.1 (20.7)	55.2 (20.0)	48.4 (34.4)	0.1054	0.0145
SPA peak (%), mean (s.d.)	100.0 (0)	70.9 (53.4)	57.2 (41.5)	0.0711	0.0040
SPA AUC (%), mean (s.d.)	100.0 (0)	78.7 (48.0)	42.8 (33.4)	0.0947	<0.0001
CD62P (+) platelets (%), mean (s.d.)	5.8 (4.6)	3.4 (2.1)	2.8 (2.1)	0.0461	0.0122

Abbreviations: AUC, area under the curve; CD 62P (+), CD62P positive; PAI-1, type 1 plasminogen activator inhibitor; SPA, small platelet aggregates; TF, tissue factor; vWF, von Willebrand factor; W, week.

with irbesartan[21] and GISSI-AF with valsartan,[22] the investigators failed to prove the positive effects of ARBs in preventing thrombotic events in patients with AF. These data might be contrary to expectations given the results of our study. One possible explanation for the lack of efficacy is the level of hypertension. In our study cohort, the mean pre-treatment blood pressure was 156/97, whereas the mean levels in these prior studies were 138/97 in ACTIVE and 138/82 in GISSI-AF. It has been reported that patients with hypertension have higher platelet adhesion and aggregation activity compared with normotensive subjects.[23] Therefore, clinical trials including subjects with relatively lower blood pressures could fail to show the effects associated with platelet function modulation.

In an *ex-vivo* analysis, the addition of losartan to human blood samples reduced platelet aggregation.[8] Although this effect has also been observed using other ARBs such as irbesartan, only losartan has been shown to reduce platelet activity at plasma drug concentrations that can be reached by normal oral drug intake.[24] In that article, the authors noted that the specific structures of losartan and irbesartan

contributed to the antiplatelet activity of the drugs. Moreover, the level platelet activation inhibition by losartan was shown to be as high as aspirin.[24] It is well known that aspirin is effective for primary and/or secondary prevention of cardiovascular diseases overall.[25] This result suggests that losartan could inhibit the occurrence of cardiovascular events through standard antihypertensive treatment. The results obtained from the LIFE study could be partly explained by the antiplatelet effects of losartan.

We have previously reported that increased platelet activity as detected by a PA-200 aggregometer is observed in acute coronary syndromes[26] as well as in advanced atherosclerotic disease such as peripheral artery disease.[27] Furthermore, data from PA-200 aggregometry are useful to monitor antiplatelet treatment levels for stabilizing such diseases. Therefore, the evidence for the inhibition of platelets by losartan observed in the present study using PA-200 aggregometry might be important for effective cardiovascular prevention in daily practice. However, there is concern that bleeding side effects could be induced by losartan use. But, in the LIFE study, such complications were not reported in the losartan arm.[6,28] Furthermore, in an *ex vivo* analysis, losartan did not increase platelet inhibitory effects when added to aspirin.[24] In this way, this concern would not apply in cases with losartan administration as it does in cases with dual antiplatelet therapy such as aspirin with thienopyridines.

PAI-1 is well known to be the key component regulating fibrinolytic function. In fact, plasma levels of PAI-1 activity are synchronized to intracoronary thrombus formation.[29,30] Furthermore, plasma PAI-1 activity levels can predict long-term prognosis in ischemic heart disease.[31] Therefore, the inhibition of PAI-1 activity could successfully prevent intracoronary thrombus formation and cardiovascular events. In the present study, we showed that PAI-1 activity decreased with losartan use. This effect was consistent with previous reports.[10,32,33] Therefore, it is possible that losartan's profibrinolytic and antiplatelet effects act via a two-pronged strategy to prevent fatal cardiovascular events in relation to thrombus formation.

In conclusion, the antihypertensive drug losartan also has substantial antiplatelet and profibrinolytic effects. In addition to the original blood pressure-lowering capacity of the drug, these pleiotropic effects make it an attractive option to prevent cardiovascular events.

Study limitations

There were a few limitations in the present study. First, the number of patients was small. Therefore, the statistical power of the present study was low. Although important, significant results were obtained in spite of the low power, which could be derived from the effects of background factors or selection bias in the cases. Second, there were no control subjects. Therefore, this study should be a pilot, and the results should be confirmed by large-scale clinical trials with an appropriate control component in the future.

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