Losartan inhibited expression of matrix metalloproteinases in rat atherosclerotic lesions and angiotensin II-stimulated macrophages[1]

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ABSTRACT

AIM: To explore whether the angiotensin II (Ang II) receptor 1 (AT_1) antagonist, losartan could reduce activity and expression of matrix metalloproteinases (MMPs) in rat atherosclerotic plaques. **METHODS:** Male Wistar-Kyoto rats were ip injected a single dose of vitamin D_3 600 $kU·kg^{-1}·month^{-1}$ and fed an atherogenic diet for 4 months to induce experimental atheroma. Then either placebo or losartan 50 $mg·kg^{-1}·d^{-1}$ was administered in rats for another 2 months. *In vitro*, the effect of losartan 0.1-10 µmol/L on the expression of MMP-2 and MMP-9 was investigated in Ang II-stimulated rat peritoneal macrophages. The expression and activity of MMP-2 and MMP-9 were monitored by Western blot, RT-PCR, and SDS-PAGE zymography analysis. **RESULTS:** High levels of MMP-2 and MMP-9 were expressed in rat atherosclerotic lesions. Losartan significantly reduced the activity and expression of MMP-2 and MMP-9 compared with the placebo group (MMP-2, 5861±539 *vs* 8991±965, *P*<0.05; MMP-9, 10527±1002 *vs* 14623±2462, *P*<0.01). In cultured rat peritoneal macrophages, Ang II 0.1 µmol/L elicited an increase in MMP-2 and MMP-9 activity and expression that were prevented by losartan in a dose-dependent manner (*P*<0.01). But the AT_2 receptor antagonist PD123319 had no effect. **CONCLUSION:** Losartan reduced the expression and activity of MMP-2 and MMP-9 in rat atherosclerotic lesions. The anti-atherogenic effects of losartan were due to the direct inhibition of Ang II bioactivity.

INTRODUCTION

Acute coronary syndromes (unstable angina, acute myocardial infarction, and ischemic sudden cardiac death) resulted from disruption of atherosclerotic plaques that caused coronary thrombosis. The potential cellular mechanism involved in plaque disruption remained to be understood. Macrophages-rich areas were frequently found in coronary plaques of patients with acute coronary syndromes[1]. Lesional macrophages produced proteolytic enzymes including members of the matrix metalloproteinases (MMPs) family. Among which MMP-9 (gelatinase B) and MMP-2 (gelatinase A) had

been shown to be up-regulated in unstable human coronary plaques[2], indicating that they played key roles in extracellular matrix degradation. Macrophage-related proteolysis within atheroma might contribute to the weakness of protective fibrous cap of the plaque and promoted potential of those plaques to rupture and trigger thrombosis.

Angiotensin II (Ang II) had multiple effects that might contribute to the development of atherosclerosis. These actions were most likely to be mediated by Ang II type 1 (AT$_1$) receptor. Clinical trials showed that early-administered angiotensin converting enzyme (ACE) inhibitors improved the prognosis in patients with myocardial infarction and unstable angina[3,4], indirectly suggesting a possible role of renin-angiotensin system (RAS) in acute coronary syndromes. Increased ACE expression had, in fact, been found in hypercellular plaques and in areas of clustered macrophages of patients with unstable angina[5]. Additionally, both Ang II and AT$_1$ receptors were detected in close proximity to the presumed plaque rupture site in patients who died acutely after myocardial infarction[6]. Destabilized atherosclerotic plaques induced by excessive Ang II would result in fatal disorders like myocardial infarction.

Type 1-specific angiotensin receptor blockers were promising anti-hypertensive drugs, but there was little research on their effect on atherosclerosis. In the present experiment we investigated the effect of AT$_1$-antagonist, losartan, on expression of MMPs induced by Ang II *in vitro* and *in vivo*.

MATERIALS AND METHODS

Materials Angiotensin II, phorbol-12 myristate-13 acetate (PMA), PD123319, and gelatin were obtained from Sigma Chemical Co. MMP-9 and MMP-2 mice monoclonal antibody and the peroxidase goat anti-mouse IgG were obtained from Oncogene Science. Antibody detection kit (ECL) was obtained from Amerplacebo. Losartan was kindly donated by Merck Sharp & Dohme.

Animals and experimental protocol Male Wistar-Kyoto (WKY) rats (280-300 g, n=40) were fed an atherogenic diet (4 % cholesterol, 10 % coconut oil, and 1 % cholic acid) and ip injected a single dose of vitamin D$_3$ 600 kU·kg^{-1}·month^{-1} for 4 months to induce atheroma formation[7]. Plasma total cholesterol (TC) concentrations were measured at week 16. Rats were randomized into placebo group (n=16) or losartan 50 mg·kg^{-1}·d^{-1} group (n=18). Placebo or losartan were

administered for another 2 months. Control rats (n=5) were fed standard chows.

Harvesting of vessels The rats were sacrificed by iv injection of sodium pentobarbital. Each artery was harvested from its origin at the aortic valve to 2 cm beyond the bifurcation of the internal iliac artery. Adventitial tissue was carefully dissected and any adipose tissue was cleaned. The vessels were flushed with ice-cold saline, and cut into 2 segments. Arterial segments were immediately frozen in liquid nitrogen and stored at -80 ℃. The proximal and distal segments were used for reversed transcriptase polymerase chain reaction (RT-PCR) and gelatin zymography, respectively.

Cell isolation and culture Cells in rat peritoneal exudates were harvested by peritoneal lavage using cold Hanks' solution containing 5 % fetal calf serum. Cells were washed twice and re-suspended in RPMI-1640 medium containing 10 % fetal calf serum (FCS). Monolayers of peritoneal macrophages were prepared by plating cell suspensions (2×10^9 L^{-1}) in 6-well plate and incubated at 37 ℃ in a humidified atmosphere with 5 % CO$_2$ for 2 h to allow for macrophages adherence. The non-adherent cells were removed by washing with warm RPMI-1640 medium thrice. About 85 %-95 % adherent cells were macrophages as determined by morphology and Giemsa staining. The cell viability was evaluated by Trypan blue staining.

Rat peritoneal macrophages were growth-arrested by incubation in 0.5 % FCS medium for 24 h and then incubated with the corresponding stimuli. The effect of Ang II was tested at the concentration of 0.1 µmol/L. The AT$_1$-antagonist losartan was added at the concentrations of 0.1, 1, and 10 µmol/L. For determination of MMP-9and MMP-2 mRNA expression, cells were cultured for 6 h, and then RNA was extracted for RT-PCR analysis. For measurement of MMP-2 and MMP-9 protein expression and activity, cells were cultured for 24 h, and then media were collected for Western blot analysis and zymography.

SDS-PAGE zymography analysis[8] A gelatin substrate was included in the composition of the polyacrylamide/SDS gels (10 % gelatin gel), and samples were separated according to their apparent molecular weight by electrophoresis. After electrophoresis, the gel was washed in 2.5 % Triton X-100 solution with gentle agitation for 6 h at room temperature, followed by replacement with developing buffer (in g/L) containing dH$_2$O: Tris base 12.1, Tris-HCl 63, NaCl 117,

CaCl$_2$ 7.4, and Brij-35 0.2). The gel was agitated at room temperature for 30 min, placed into fresh developing buffer, and incubated at 37 ℃ overnight. The gel was stained with 0.5 % Coomassie brilliant blue R-250 and de-stained in 5 % methanol and 7 % acetic acid. To verify the metalloproteinase nature of the detected enzymes, identical gels were incubated in the presence of edetic acid 30 mmol/L, an inhibitor of MMPs. Results were expressed in densitometric units/mg wet wt. Gelatinolytic bands were quantified by scanning densitometry with NIH Image 1.57 software.

Protein extraction and Western blot analysis Samples were electrophoresed on 8 % SDS-PAGE. Proteins were transferred onto nitrocellulose membranes and incubated overnight at 4 ℃ with blocking solution (1 % nonfat dried milk in PBS containing 0.1 % Tween-20). Membranes were incubated with anti-MMP-2 or anti-MMP-9 monoclonal antibodies (1 mg/L, Oncogene Science) followed by incubation with secondary antibodies coupled to horseradish peroxidase and development of a chemiluminescent reaction (ECL kit from Amerplacebo International). Signals (positive bands) were quantified as described above.

RNA extraction and RT-PCR Expression of MMP-9 and MMP-2 mRNA was determined by RT-PCR analysis as previously described[9]. RNA was extracted from rat peritoneal macrophages or vessels by the TRIzol reagent (Life Technologies). Total RNA quantify was assessed by resolving in denatured 1 % agarose gels and measuring absorbance ratios at 260/280 nm. The cDNA synthesis was performed using the superscript II Preamplification System from Life Technologies. For each reaction, 2 µg of total RNA were reversely transcribed using random hexamer according to the protocol supplied by Life Technologies with 40 U of RNase inhibitor. The cDNA was amplified using 100 pmol of each of the forward MMP-9 primer (5′-AGA TTC CAA ACC TTT GAG-3′) and reverse MMP-9 primer (5′-GGC CTT GGA AGA TGA ATG-3′). Samples were amplified for 30 cycles in a DNA thermal cycles using the following conditions: 94 ℃ for 1 min, 52 ℃ for 2 min, and 72 ℃ for 3 min. MMP-2 primer (5′-CAA TAC CTG AA C ACC TT-3′) and reverse primer (5′-CTG TAT GTG ATC TGG TT-3′) resulted in a 269-bp PCR product. The primers of GAPDH were 5′-CTG GAG AAA CCT GCC AAG TAT GAT 3′ and 5′-TTC TTA CTC CTT GGA GGC CAT GTA 3′. The PCR mixture was heated to 94 ℃ for 5 min and then run for 30 cycles at 94 ℃ for 30 s, 55 ℃

for 1 min, and 72 ℃ for 1 min, followed by 10-min elongation at 72 ℃.

Statistical analysis Results were expressed as mean±SD. One-way analysis of variance (ANOVA) and *t*-test were used for statistical evaluation. $P<0.05$ was considered statistically significant.

RESULTS

Atherosclerosis Atherosclerosis was established in 34 of 40 rats: 16 rats were randomized to placebo treatment and 18 rats to losartan treatment. Experiments were interrupted in 3 animals because of aversion to the diet. Two additional animals were excluded from study because their plasma TC concentrations fell outside the 95 % confidence limits at week 16 during the dietary period. One rat died during losartan treatment on d 32.

Difference in body weights between placebo (*n*=16) and losartan (*n*=18) groups were not different (391±20 g *vs* 403±19 g, $P>0.05$) at baseline and during the treatment. Plasma TC concentrations at week 16 during the dietary period averaged 11.6±0.6 mmol/L and 10.0±1.6 mmol/L in animals randomized to placebo and losartan treatment, respectively ($P>0.05$). Compared with placebo group, losartan treatment had no effect on TC levels (10.1±0.7 mmol/L *vs* 9.1±1.3 mmol/L, $P>0.05$). After rats were given cholesterol-enriched diet together with vitamin D$_3$ for 16 weeks, advanced atherosclerotic lesions in arterial wall were induced, represented by formation of intimal foam cells, deposition of calcium, proliferation of smooth muscle cells, and appearance of lipid-calcic core.

Effect of losartan on activity and mRNA expression of MMP-9 and MMP-2 in experimental atherosclerotic lesions In rat normal arteries, only few zymogen forms of MMP-2 (pro-MMP-2, 72 kDa) and MMP-9 (pro-MMP-9, 92 kDa) were observed (Fig 1). By comparison with non-atherosclerotic arteries (control group), the activities of both the pro-enzyme and activated forms of MMP-2 and MMP-9 were greatly higher in atherosclerotic lesion extracts (placebo group) (Fig 1, $P<0.01$). Notably, losartan treatment markedly decreased the total MMP-2 and MMP-9 activity compared with the placebo group (Fig 1, $P<0.05$).

MMP-9 mRNA was undetectable in rat normal artery. In atherosclerotic arteries (placebo group), MMP-9 mRNA levels increased dramatically and were 33-fold higher in placebo group than that in losartan-
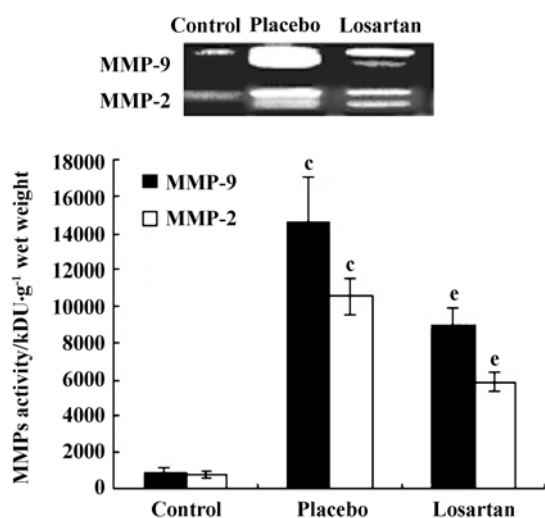
Fig 1. Detection of MMPs activity in the aortic lesions by SDS-PAGE zymography. Control group (*n*=5), placebo group (*n*=16), and 2-month losartan 50 mg·kg^{-1}·d^{-1}-treated group (*n*=18). ^{c}*P*<0.01 *vs* control group. ^{e}*P*<0.05 *vs* placebo group. **Results were expressed in densitometric unit/mg wet weight.**
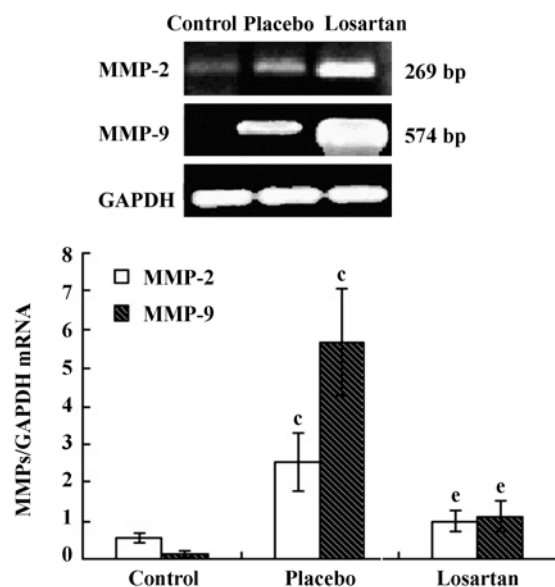

Fig 2. MMPs mRNA levels in rat atherosclerotic arteries determined by RT-PCR. Control group (*n*=5), placebo group (*n*=16), and 2-month losartan 50 mg·kg^{-1}·d^{-1} treated group (*n*=18). ^{c}*P*<0.01 *vs* control group. ^{e}*P*<0.05 *vs* placebo group. **Results were expressed in arbitrary units and adjusted for GAPDH mRNA levels.**

treated group (Fig 2, *P*<0.05). Constitutive MMP-2 mRNA expression was found in rat normal artery, and increased significantly in atherosclerotic arteries (placebo group). Compared with placebo group, losartan treatment decreased MMP-2 mRNA levels (Fig 2, *P*<0.05).

Effect of losartan on MMP-2 and MMP-9 expression in Ang II-induced rat peritoneal macrophages Unstimulated macrophages constitutively secreted MMP-2 but not MMP-9. Significant gelatinolytic activity and protein expression of MMP-9 was found after 24-h stimulation with Ang II 0.1 μmol/L. Pre-incubation of macrophages with losartan 6 h prior to the stimulation with Ang II 0.1 μmol/L resulted in a dose-dependent inhibition of the gelatinolytic activity, as well as protein expression of MMP-9 in a concentration range between 0.1 and 10 μmol/L (Fig 3A). Both Western

blotting and zymography demonstrated a partial inhibition of MMP-2 synthesis and activity after pre-incubation with losartan 10 μmol/L (Fig 3B).

Ang II caused an increase in MMP-9 and MMP-2 mRNA expression up to 5 and 3-fold. Losartan (10 μmol/L), a selective nonpeptide AT$_1$ antagonist, completely abolished MMP-9 mRNA increase, but partially inhibited MMP-2 mRNA increase (Fig 3).

Losartan had no effect on PMA-stimulated MMP-9 and MMP-2 mRNA expression (data not shown). Furthermore, the AT$_2$ receptor antagonist PD123319 had no effect on Ang II-stimulated up-regulation of MMP-9 and MMP-2 mRNA (Fig 4).
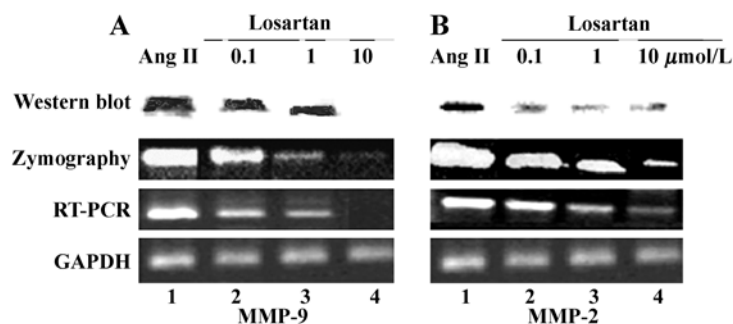

Fig 3. Effect of losartan on MMP-9 (A) and MMP-2 (B) activity and expression in Ang II -induced rat peritoneal macrophages. Macrophages were treated with Ang II 0.1 μmol/L (1), Ang II 0.1 μmol/L+losartan 0.1 μmol/L (2), Ang II 0.1 μmol/L+losartan 1 μmol/L (3), and Ang II 0.1 μmol/L+losartan 10 μmol/L (4).
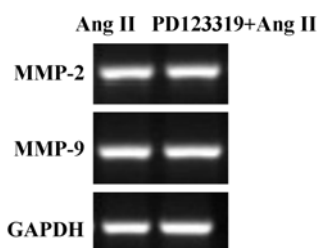
Ang II   PD123319+Ang II

MMP-2

MMP-9

GAPDH

Fig 4. Effects of PD123319 on MMP-2 and MMP-9 mRNA expression in Ang II-stimulated rat peritoneal macrophages. Macrophages were treated with Ang II 0.1 μmol/L and Ang II 0.1 μmol/L+PD123319 10 μmol/L.

DISCUSSION

The present study demonstrated that losartan effectively inhibited the activity and expression of MMP-2 and MMP-9 in rat atherosclerotic lesions. In cultured rat peritoneal macrophages, Ang II elicited an increase in MMP-2 and MMP-9 activation and expression that was abrogated by AT_1-antagonists losartan, but not by AT_2 receptor antagonist PD123319. These findings suggested that Ang II-upregulated MMP-2 and MMP-9 expression was partially mediated by AT_1 receptor in membranes of macrophages. AT_1-antagonists effectively inhibited Ang II-induced stimulation of the MMPs. This mechanism might contribute to stabilization of atherosclerotic plaques and reduction of vascular remodeling during therapy with AT_1-antagonists which might be of importance for prevention of myocardial infarction and stroke.

RAS has been suggested to play an important role in the progression of atherosclerosis and the development of acute coronary syndromes. Experimental studies revealed that ACE inhibitors might exert anti-atherogenic and anti-proliferative effects on vascular walls[10,11]. Clinical trials further reported that administration of ACE inhibitors after myocardial infarction reduced not only the cumulative incidence of heart failure but also the incidence of reoccurrence of myocardial infarction [3,4]. These observations supported the hypothesis that Ang II, generated by ACE, might contribute to the progression of atherosclerosis and potentially to the disruption of coronary plaques.

Macrophage-rich areas were more abundant in human atherosclerotic coronary arteries of patients with unstable angina and non-Q-wave infarction as compared with stable atherosclerotic plaques[1]. Recent observations indicated that the Ang II and AT_1 receptor were

detected in unstable and ruptured human coronary plaques. Importantly, ACE was expressed in human atherosclerotic plaques in areas of clustered macrophages[5]. Therefore, Ang II might be involved in the development of an acute coronary syndrome, based on the observations that Ang II increased biomechanical stress at the shoulder of atherosclerotic lesions [12] and an inflammatory process and an accumulation of macrophages were observed at the site of ruptured plaques[13,14]. Furthermore, Ang II might contribute to the development of an acute coronary syndrome through the migration of macrophages into a neointimal area[15] or by producing reactive oxygen species and thereby increasing oxidative stress[16].

Kranzhoeter *et al* reported that Ang II induced release of MMPs from human vascular smooth muscle cells via NF-kappa B, losartan blocked this release[17]. Chase *et al*[18] demonstrated an essential role for NF-kappa B in mediating the inflammatory stimuli-induced MMPs (MMP-1, -3, and -9) expression by both human vascular smooth muscle cells and monocyte-derived macrophages. Inhibition of NF-kappa B was therefore an attractive candidate strategy to inhibit MMPs induction and promote plaque stability. Ang II exerted its biological effects through the stimulation of specific receptors, namely AT_1 and AT_2, on cell membranes, because activated monocytes and macrophages expressed Ang II, as well as AT_1 receptors[19,20]. Activition of NF-kappa B by AT_1 was demonstrated previously[21]. Yu *et al*[22] showed that losartan markedly inhibited the proliferation of intra-pulmonary artery smooth muscle cells induced by hypoxia, while PD123319 showed no significant effect. Our data showed that losartan blocked Ang II-induced MMP-2 and MMP-9 mRNA expression, but had no effect on PMA-stimulated MMP-9 and MMP-2 expression. Furthermore, PD123319 had no effect on Ang II -stimulated MMP-9 and MMP-2 mRNA expression, suggesting that this effect was mediated via AT_1 receptor, and the AT_2 receptor might not be involved in Ang II-induced MMP-2 and MMP-9 mRNA expression. Previous study showed that expression of MMPs (MMP-1, -3, and -9) was controlled at the transcriptional level through NF-kappa B[23]. Ruiz-Ortega *et al* showed that Ang II via AT_1 receptors activated NF-kappaB in vascular cells both *in vivo* and *in vitro* [24]. A potential explanation was that Ang II stimulated NF-kappa B activation and MMP-2 and MMP-9 gene expression by AT_1 receptor on the macrophages. Up-regulation of Ang II receptors might be involved in the

initiation and progression of atherosclerosis.

Losartan treatment prevented both coronary vascular injury and myocyte damage induced by continuous Ang II infusion in the rat [25]. There were only a few studies on losartan effects in animal models of atherosclerosis. The Ang II type 1 antagonist SC-51316 was shown to have no effect on the progression of atherosclerosis in cholesterol-fed rabbits over a 3-month period[26]. In contrast, losartan treatment for 3 months reduced atherosclerotic lesions in apolioprotein E-deficient mice[27]. Valsartan also reduced endothelial dysfunction and intimal thickening in atherosclerotic rabbits[28]. Strawn *et al* showed that losartan inhibited early atherogenesis in monkeys with diet-induced hypercholesterolemia, the mechanisms might include both protection of LDL from oxidation and suppression of vascular monocyte activation and recruitment factors[29]. Losartan treatment was associated with a modest reduction of cardiac 4-hydroxyproline concentration, and a significant reduction of both MMP-1 and MMP-2 activities in male cardiomyopathic Syrian hamsters [30]. Clinic study showed that treatment with irbesartan in patients with coronary artery disease significantly reduced inflammatory molecules (such as soluble VCAM-1 and tumor necrosis factor-alpha) levels indicating that irbesartan might retard the inflammatory process in premature forms of atherosclerosis[31]. Chen H *et al* showed that losartan reduced lipid deposition, decreased the expression of MMP-1, and inhibited NF-kappa B activation in hypercholesterolemic rabbits[32]. In rat atherosclerotic lesions, we found the similar results that losartan effectively inhibited the activity and expression of MMP-2 and MMP-9 by direct inhibition of Ang II-dependent effect. This effect suggested that losartan might reduce atherogenesis and plaque remodeling via inhibiting MMPs synthesis and secretion in vascular wall.

In summary, the anti-atherogenic effects of losartan are due to the direct inhibition of Ang II activity that were probably associated with the inhibition of MMPs expression and activity. We suggested that attenuation of MMPs activity and expression by losartan might be a cardioprotective mechanism of this agent.

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