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PHARMACODYNAMICS

Losartan inhibits monocytic adhesion induced by ADMA via downregulation of chemokine receptors in monocytes

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Abstract
Objective Asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor, can induce the adhesiveness of monocytes to vascular endothelium, and chemokines play an important role in this process. The present study was carried out to test whether the inhibitory effect of losartan on ADMA-induced monocytic adhesion is mediated by chemokine receptors.
Methods Human monocytoid cells (THP-1) were incubated with exogenous ADMA (30 μM) for 4 or 24 h in the absence or presence of losartan. The monocytic adhesion, the levels of chemokines, and the expression of chemokine receptors were determined. The possible signal pathway was also explored.
Results In cultured monocytes, ADMA (30 μM) markedly increased monocytic adhesion to endothelial cells, elevated the levels of monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8), and upregulated the mRNA expression of chemokine receptors CCR_2 and $CXCR_2$. Exposure to ADMA (30 μM) significantly induced the generation of intracellular reactive oxygen species (ROS) and activation of nuclear factor (NF)-κB. Pretreatment with AT_1 receptor blocker (ARB) losartan (1, 3, 10 μM) attenuated monocytic adhesiveness elicited by ADMA and downregulated the expression of CCR_2 and $CXCR_2$ mRNA, accompanied by a significant decrease in ROS generation and NF-κB activity and expression.
Conclusion The present study suggests that the inhibitory effect of losartan on ADMA-induced monocytic adhesion may be related to downregulation of chemokine receptors by inhibiting the ROS/NF-κB pathway.

Keywords Asymmetric dimethylarginine (ADMA) · Monocyte chemoattractant protein-1 (MCP-1) · Interleukin-8 (IL-8) · Nuclear factor-kappaB (NF-κB) · Losartan

Introduction

The initial step of inflammatory responses in vascular vessels is characterized by the recruitment of monocytes to injured endothelium, and chemokines play a critical role in this process. Monocyte chemoattractant protein-1 (MCP-1), a subfamily of CC chemokines, induces the recruitment and accumulation of monocytes to inflammatory sites through its receptor CCR_2. Previous studies have demonstrated that the expression and immunostaining of MCP-1 and CCR_2 appeared ahead of atherosclerotic lesions [1]. In hypercholesterolemic patients, the expression of CCR_2 in isolated monocytes was markedly increased, and treatment of monocytes with LDL further upregulated CCR_2 expression and enhanced the chemotaxis elicited by MCP-1 [2]. Interleukin-8 (IL-8), a subfamily of CXC chemokines, is a trigger for firm adhesion of monocytes to vascular endothelium via its receptor $CXCR_2$. It was documented

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that $CXCR_2$ was strongly expressed in monocytes of atherosclerotic lesion [3], and $CXCR_2$ deficiency significantly reduced the progression of atherosclerosis (AS) in mice [4] and inhibited monocytic recruitment induced by angiotensin II (Ang II) [5]. These results suggest that MCP-1/CCR_2 and IL-8/$CXCR_2$ systems appear to be involved in the chemotaxis and adhesiveness of monocytes to inflammatory sites, contributing to the progress of AS.

It has been documented that the system of L-arginine-nitric oxide (L-arg-NO) synthesis occurs in monocytes. There is growing evidence that asymmetric dimethylarginine (ADMA), an endogenous nitric oxide synthase (NOS) inhibitor, is markedly elevated in many cardiovascular diseases and recognized as a potential risk factor for cardiovascular events. ADMA, besides inhibiting NO synthesis, may participate in inflammatory reactions via induction of oxidant stress [6]. Our recent work demonstrated that ADMA could induce the apoptosis of endothelial cells and vascular smooth muscle cells (VSMCs) via the intracellular reactive oxygen species (ROS)-p38 mitogen-activated protein kinases (MAPKs) signaling pathway [7–8]. It was reported that in activated monocytes, ADMA could increase the expression of lectin-like ox-LDL receptor (LOX-1) and ox-LDL uptake to facilitate foam cell formation [9].

Although the study of ADMA and monocytic function is extremely difficult, the possibility of a facilitative effect of ADMA on monocytic adhesion is plausible and worth exploring. Previous studies have demonstrated that treatment with angiotensin-converting enzyme inhibitor (ACEI) or AT_1 receptor blocker (ARB) reduced the elevated levels of ADMA in patients with hypertension, syndrome X, and diabetes [10–12] and inhibited endothelial injury induced by ADMA [13]. In the present study, therefore, we tested the effect of losartan on ADMA-induced monocytic adhesion in cultured monocytes and further explored whether chemokine receptors are involved in this process.

Materials and methods

Reagents

Human monocytoid cells (THP-1, ATCC) were purchased from Cell Culture Center of Xiang-Ya Medical School (Changsha, China), and human umbilical vein endothelial cells (HUVECs, ATCC) were obtained from Tumor Research Institute of Beijing Medical University (Beijing, China). Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials (Hangzhou, China). ADMA standard and trypan blue was purchased from Sigma. Losartan was freely supplied by Merck. [γ-^{32}P] ATP was obtained from Furui Biological Engineering Institute (Beijing, China). ROS detection and BCA protein kits were purchased from Beyotime (Jiangsu, China). ELISA kits for measurement of MCP-1 and IL-8 were obtained from Senxiong Biological (Shanghai, China). First-strand cDNA synthesis kits were obtained from Fermentas. The primers of CCR_2, $CXCR_2$, and β-actin were synthesized by Shanghai Biological and Engineering (Shanghai, China). TRIzol and gel shift assay system were obtained from Promega. NF-κB p65 antibody for Western blot was purchased from Santa Cruz Biotechnology.

Cell culture and treatment

THP-1 cells were cultured in RPMI 1640 medium at a density of up to 10^6 cells/ml containing 15% FBS, and endothelial cells were cultured in DMEM containing 10% FBS. Monocytes were incubated with ADMA (30 μM) for 4 or 24 h. For ARB losartan, monocytes were pre-incubated with losartan (1, 3, 10 μM) for 1 h, and then exposed to ADMA (30 μM) for 4 or 24 h in the presence of losartan.

Determination of MCP-1 and IL-8

The levels of MCP-1 and IL-8 in the cultured medium were measured by ELISA kits strictly following the instructions of the manufacturer.

Reverse transcription-PCR (RT-PCR) analysis

Total mRNA was extracted from monocytes of six-well culture dishes after exposure to ADMA for 4 h using TRIzol, and first-strand cDNA was then synthesized from 4 μg RNA using reverse transcriptase. PCR protocols are summarized in Table 1; PCR products were analyzed by 2% agarose gel electrophoresis.

Static adhesive assays

After the 24-h treatment and 30 min before the adhesive assays, monocytes (10^6 cells/ml) were added to 12-well plates of endothelial cells without any treatment. The plates were incubated for an additional 30 min at 37°C. Non-adherent monocytes were carefully removed, and adherent monocytes were counted (cells/hpf).

Determination of ROS

After the cells that had incubated with ADMA for 4 h in six-well culture dishes were collected, the cell deposit was washed with RPMI 1640 with no FBS, and then incubated with 2′,7′-dichlorofluorescein diacetate (DCFH-DA) at 37°C for 30 min. Dichlorofluorescein (DCF) fluorescence distribution of 20,000 cells was detected by fluorospectropho-

Table 1 Primer sequences and PCR protocols

Gene	PCR primer sequences		Length	PCR protocol
$CXCR_2$	UP	5′-CGGAATTCAAATGGAAGATTTTAACATGGAG-3′	417 bp	94°C/60 s, 58°C/60 s, 72°C/60 s, 38 cycles
	DP	5′-CCGCTCGAGTTAGAGAGTAGTGGAAGTGTG-3′		
CCR_2	UP	5′-ATGCTGTCCACATCTCGTTCTCG-3′	1083 bp	94°C/45 s, 62°C/45 s, 72°C/60 s, 40 cycles
	DP	5′-TTATAAACCAGCCGAGACTTCCTGC-3′		
β-actin	UP	5′-CTGTCCCTGTATGCCTCTG-3′	218 bp	94°C/45 s, 58°C/45 s, 72°C/60 s, 28 cycles
	DP	5′-ATGTCACGCACGATTTCC-3′		

UP Upstream primer, *DP* downstream primer

tometer analysis at an excitation wavelength of 488 nm and an emission wavelength of 525 nm.

Electrophoretic mobility shift assay (EMSA)

Subsequent to treatment with ADMA for 4 h, nuclear protein was extracted and frozen at –70°C. The EMSA for determining the NF-κB DNA-binding activity was performed by incubating aliquots of 15 μg nuclear protein extracts with γ-^{32}P-labelled double-stranded NF-κB–specific oligonucleotide probe (sense: 3′-TCA ACT CCC CTG AAA GGG TCC G-5′; antisense: 5′-AGT TGA GGG GAC TTT CCC AGG C-3′) by T4 polynucleotide kinase. After incubation at room temperature for 10 min, the mixture was run on a 4% non-denaturing polyacrylamide gel in 0.5× TBE buffer. After electrophoresis, the gels were dried and the DNA-protein complexes were detected by autoradiography.

Western blotting

Subsequent to the different treatments for 24 h, monocytic THP-1 were washed with PBS and lysed with 100 μl/10^6 cells in SDS sample buffer containing 62.5 mmol/L Tris (pH 6.8), 2% SDS (w/v), 10% glycerol, and 1 mmol/L PMSF. Extracted protein samples were heated at 95°C for 5 min, and proteins of equal concentration (60 μg per lane) were separated by 12% SDS-PAGE. Then proteins were electrophoretically transferred to nitrocellulose membranes and the membranes were blocked for 1 h with 1% blocked milk. After blocking, the membranes were incubated in the primary monoclonal-NF-κB antibody (1:1,000) at 4°C overnight. Membranes were washed in TBST for 1 h before incubation for 1 h in goat anti-rabbit secondary antibody (1:1,000). Then membranes were washed in TBST for 1 h and developed with enhanced chemiluminescence kit.

Statistic analysis

Results are expressed as means ± SEM. The data were analyzed by ANOVA followed by Newman-Keuls-Student test for multiple comparisons. The statistical significance was considered as $P<0.05$.

Results

The effect of losartan on monocyte adhesion induced by ADMA

Exposure to ADMA (30 μM) for 24 h significantly increased the number of monocytes binding to endothelial cells. Treatment with losartan (1, 3, 10 μM) decreased the number of adhesive monocytes elicited by ADMA in a concentration-dependent manner (Table 2).

The effect of losartan on the release of MCP-1 and IL-8 induced by ADMA

After incubation with ADMA (30 μM) for 24 h, the levels of IL-8 and MCP-1 in the medium were markedly elevated.

Table 2 Effect of ADMA on the levels of IL-8 and MCP-1 in the medium and the number of monocytes binding to endothelial cells

Group	Number	Cells/hpf	MCP-1 (pg/ml)	IL-8 (pg/ml)
Control	6	139±20	15.68±2.34	340.9±36.7
ADMA (30 μM)[a]	4	428±19**	28.95±3.58**	485.4±15.9**
+Losartan (1 μM)[b]	4	228±27##	19.26±2.96##	407.0±36.2#
+Losartan (3 μM)[b]	4	211±19##	18.53±2.45##	394.1±16.4 ##
+Losartan (10 μM)[b]	4	183±12##	17.21±2.21##	358.5±31.3##

Data are expressed as means ± SEM

ADMA Asymmetric dimethylarginine, *MCP-1* monocyte chemoattractant protein-1, *IL-8* interleukin-8

**$P<0.01$ vs. control, # $P<0.05$ vs. ADMA (30 μM), ## $P<0.01$ vs. ADMA (30 μM)

[a] ADMA (30 μM): monocytes were incubated with ADMA (30 μM) for 24 h

[b] + Losartan (1, 3 or 10 μM): cells were incubated with losartan at the concentration of 1, 3, or 10 μM for 1 h, and then exposed to ADMA (30 μM) for 24 h

Pretreatment with losartan (1, 3, 10 μM) markedly attenuated the elevated levels of IL-8 and MCP-1 induced by ADMA (Table 2).

The role of losartan in CCR_2 and $CXCR_2$ mRNA expression mediated by ADMA

Incubation of monocytes with ADMA (30 μM) for 4 h significantly upregulated the expression of CCR_2 and $CXCR_2$ mRNA. Pretreatment with losartan (1, 3, 10 μM) significantly attenuated the upregulated expression of CCR_2 and $CXCR_2$ mRNA caused by ADMA (Fig. 1).

The effect of losartan on intracellular ROS generation induced by ADMA

After incubation with ADMA (30 μM) for 4 h, intracellular ROS generation of monocytes was significantly increased. The facilitative effect of ADMA on ROS generation was markedly attenuated by treatment with losartan (1, 3, 10 μM) (Fig. 2).

The effect of losartan on the activity of NF-κB induced by ADMA

As shown in Fig. 3a, the binding activity of NF-κB was significantly enhanced compared with control after incubation with ADMA (30 μM) for 4 h. Treatment with losartan (1, 3, 10 μM) inhibited the activation of NF-κB induced by ADMA.

The effect of losartan on the protein expression of NF-κB induced by ADMA

Exposure to ADMA (30 μM) for 24 h significantly upregulated the protein expresssion of NF-κB p65 ($P<0.01$).

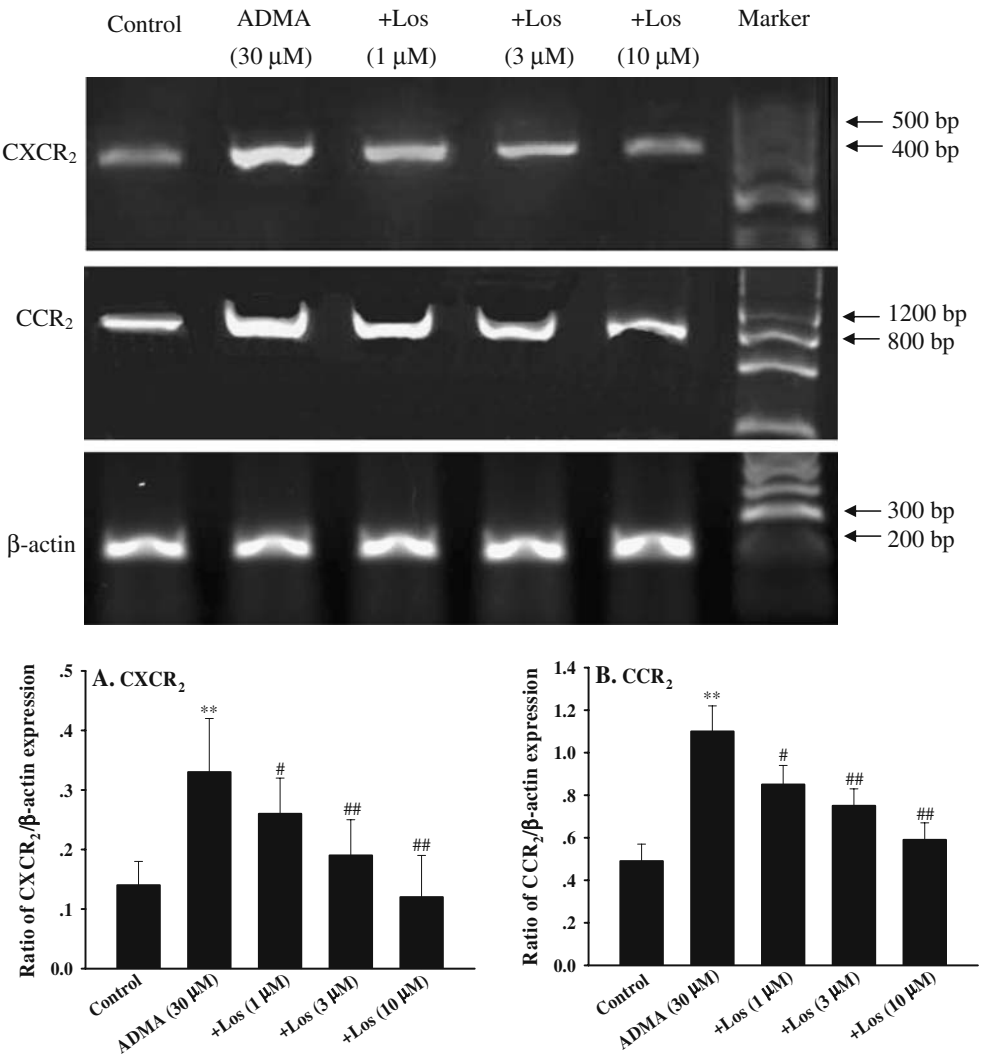


Fig. 1 Effect of losartan (Los) on the expression of $CXCR_2$ (**a**) and CCR_2 (**b**) mRNA of monocytes induced by asymmetric dimethylarginine (ADMA). ADMA (30 μM): monocytes were incubated with ADMA (30 μM) for 4 h. +Los (1, 3 or 10 μM): cells were incubated with Los at a concentration of 1, 3, or 10 μM for 1 h, and then exposed to ADMA (30 μM) for 4 h. Data are expressed as means ± SEM. $n=3$. $^{**}P<0.01$ vs. control, $^{\#}P<0.05$ vs. ADMA (30 μM), $^{\#\#}P<0.01$ vs. ADMA (30 μM)

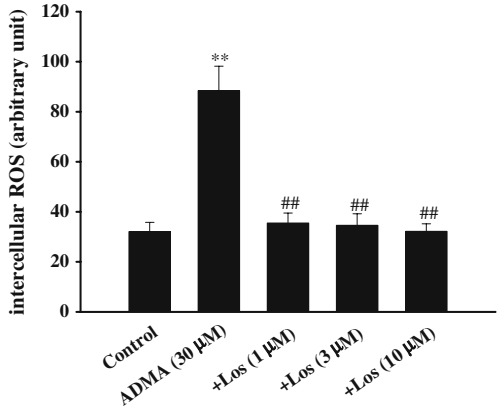


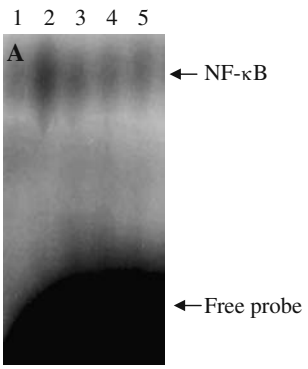
Fig. 2 Effect of losartan (Los) on intracellular reactive oxygen species (ROS) generation by asymmetric dimethylarginine (ADMA) in monocytes. ADMA (30 μM): monocytes were incubated with ADMA (30 μM) for 4 h. +Los (1, 3 or 10 μM): cells were incubated with losartan at the concentration of 1, 3 or 10 μM for 1 h, and then exposed to ADMA (30 μM) for 4 h. Data are expressed as means ± SEM (n=4). **P<0.01 vs. control; $^{\#\#}P$<0.01 vs. ADMA (30 μM)

Pretreatment with losartan (1, 3, 10 μM) attenuated the enhanced protein expression of NF-κB p65 induced by ADMA (P<0.05, P<0.01) (Fig. 3b).

Discussion

The major findings of this study are that (1) ADMA induces monocytic adhesiveness via activation of chemokines and their receptors and (2) losartan may attenuate ADMA-induced monocytic adhesion by inhibiting the activation of chemokines via the ROS/NF-κB pathway.

ADMA, an endogenous inhibitor of NOS, can induce cell apoptosis [7], cell senescence [14], and inhibition of cell motility [15] by decreasing the production of NO. There is growing evidence to support that ADMA is a novel independent cardiovascular risk factor. Previous studies have demonstrated that ADMA level was positively correlated with carotid artery intima-media thickness (IMT) [16–17] and the strongest predictor of the degree of coronary artery calcification (CAC) [18]. Recently, it was reported that ADMA was associated with major adverse cardiovascular events after percutaneous coronary intervention (PCI) [19] and with cardiovascular morbidity and mortality in middle-aged men [20], patients with coronary artery disease (CAD) [21], and patients on an intensive care unit (ICU) [22]. Taken together, these clinical studies unanimously showed that ADMA does act as a novel cardiovascular risk factor. However, with the progress of the research on ADMA, the traditional role of ADMA has been updated by the concept that ADMA acts as a novel proinflammatory factor contributing to the development of AS. It was reported that in cultured endothelial cells, ADMA increased the levels of MCP-1 and induced the adhesion of endothelial cells to monocytes via activation of NF-κB, and in cultured monocytes, ADMA could facilitate foam cell formation via upregulation of the expression of scavenger receptors [9, 23]. In hypercholesterolemic patients, the levels of ADMA were correlated with the adhesiveness of mononuclear cells [24]. The present study



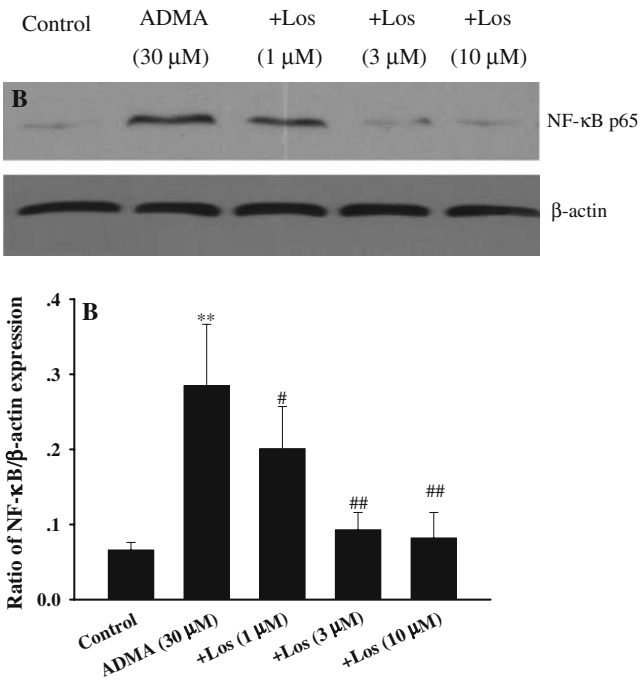


Fig. 3 Effect of losartan on NF-κB DNA-binding activity (**a**) and the protein expression of NF-κB p65 (**b**) induced by ADMA in cultured monocytes. *1* Control; *2* ADMA (30 μM): monocytes were incubated with ADMA (30 μM) for 4 h; *3* +Los (1 μM): cells were incubated with losartan (1 μM) for 1 h, and then exposed to ADMA (30 μM) for 4 h; *4* +Los (3 μM): cells were incubated with losartan (3 μM) for 1 h, and then exposed to ADMA (30 μM) for 4 h; *5* +Los (10 μM): cells were incubated with losartan (10 μM) for 1 h, and then exposed to ADMA (30 μM) for 4 h. In **b**, monocytes were incubated with ADMA (30 μM) for 24 h. Data are expressed as means ± SEM (n=3). **P<0.01 vs. control, $^{\#}P$<0.05 vs. ADMA (30 μM), $^{\#\#}P$<0.01 vs. ADMA (30 μM)

showed that ADMA can induce the activation and adhesion of THP-1 monocytes, suggesting that ADMA can directly mediate activation of monocytes.

Monocytes are important inflammatory cells and also a necessary component of atherosclerotic plaque. Chemokines such as MCP-1 and IL-8 have been demonstrated to be critically involved in the recruitment of monocytes to the site of AS. Recently, it was reported that MCP-1 and CCR_2 expression increased with age in rats [25], and the expression of CCR_2 mRNA in circulating monocytes was upregulated in hypertensive animals [26]. Additionally, blockade of MCP-1/CCR_2 pathway could abolish MCP-1-induced VSMC invasiveness [25] and vascular inflammation in hypertensive mice [27]. IL-8 and $CXCR_2$ profoundly increased stable and firm adhesiveness of monocytes to endothelium [28]. Previous studies have demonstrated that $CXCR_2$ was strongly expressed on monocytes in atherosclerotic lesions [3], and oxidative low-density lipoprotein (ox-LDL) could increase the ad hesion of monocytes via upregulation of the $CXCR_2$ expression [29]. Blockade of $CXCR_2$ could inhibit the recruitment of inflammatory cells [5] and delay the progression of AS in mice [4]. These studies suggest that the recruitment and adhesiveness of monocytes to inflammatory sites is mainly mediated by MCP-1/CCR_2 and IL-8/$CXCR_2$ systems. However, the effects of ADMA on chemokines and receptors in monocytes have not been elucidated. In the present study, incubation of monocytes with exogenous ADMA markedly increased the levels of MCP-1 and IL-8 and upregulated the expression of CCR_2 and $CXCR_2$ mRNA in cultured monocytes, accompanied by enhanced monocytic adhesiveness, suggesting that ADMA-induced monocytic adhesion is related to increases in the release of chemokines and upregulated expression of chemokine receptors.

Previous investigations have demonstrated that the elevation of ADMA levels is related to oxidative stress, and in turn ADMA can induce the generation of more oxygen-free radicals via uncoupling of NOS activity. Recently, it was reported that ADMA induced senescence and cell apoptosis via increasing ROS generation in cultured VSMCs, vascular endothelial cells, and lung epithelial cells [7–8, 14, 30]. It is known that oxidant stress can sustain activation of NF-κB, which plays a pivotal role in atherogenesis and inflammatory reactions by regulating expression of downstream genes. It was reported that ADMA could increase the adhesion of endothelial cells to monocytes via activation of NF-κB [23] and upregulate the expression of $CXCR_2$ via induction of ROS generation in cultured endothelial cells [13]. It is known that MCP-1 or IL-8 contains NF-κB binding sites to the promoter region. When cells are stimulated by some stimulants, related signals are activated (e.g., IKK) and phosphorylate IκB-α and free NF-κB are translocated into the nucleus. Then, NF-κB binds to the promoter region of genes and starts transcription of genes. In the present study, incubation of THP-1 with ADMA markedly increased ROS generation and activated NF-κB, associated with a switch to the release of chemokines and the expression of their receptors involved in inflammatory process, in support of the hypothesis that the ROS/NF-κB pathway may be involved in ADMA-induced monocytic adhesion.

Losartan, an AT_1 receptor blocker, is widely used to treat hypertension and AS by inhibiting production of many inflammatory mediators. It was reported that in wild-type mice, Ang II increased CCR_2 intensity in circulating monocytes, which was prevented by olmesartan or blunted in AT_1 receptor-deficient mice [27]. Recently, it was reported that in wild-type mice, long-term treatment with Ang II increased plasma ADMA levels and cardiac oxidative stress to induce a vascular injury similar to ADMA [31], and in cultured endothelial cells, Ang II induced an endothelial injury similar to ADMA. Pretreatment with losartan could block these effects mediated by Ang II and ADMA [13]. Treatment with ADMA for 4 weeks upregulated ACE gene expression, activated the local RAS, and increased superoxide production, which was abolished by temocapril (ACEI) or olmesartan (ARB) [32]. These findings provide the first direct evidence that the long-term vascular effects of ADMA are not solely mediated by simple inhibition of endothelial NO synthesis, but direct upregulation of ACE gene and increased oxidative stress appears to be involved in the long-term vascular effects of ADMA. Some small clinical studies have demonstrated that treatment with ACEI or ARB decreased the elevated levels of ADMA in patients with hypertension, syndrome X, diabetes, and chronic kidney disease [10–12, 33]. The mechanisms responsible for the inhibitory effects of ACEI or ARB on ADMA production or ADMA-mediated vascular effects focus on their anti-inflammatory and antioxidant actions [34–35]. It is interesting that simvastatin had no effect on the levels of ADMA, but it could inhibit ADMA-induced inflammatory reaction via MAPK pathways in endothelial cells [36]. In the present study, losartan almost completely abolished monocytic adhesiveness, decreased the levels of MCP-1 and IL-8, and downregulated the expression of CCR_2 and $CXCR_2$ elicited by exogenous ADMA, while inhibiting ROS generation and NF-κB activity. Thus, it is likely that to inhibit the effect of ADMA on the chemokines and ROS/NF-κB pathway is a new anti-atherosclerotic mechanism of losartan. Unfortunately the ACE gene and AT_1 receptor in monocytes were not determined in this study.

In conclusion, the present results suggest that losartan attenuates ADMA-induced monocytic adhesion via inhibition of chemokines and their receptors and that the ROS/

NF-κB pathway may be involved in this mechanism. Thus, the blockade of chemokines and inhibition of oxidant stress may be a new target for anti-atherosclerotic therapy.

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