

# Role of Asymmetric Dimethylarginine in Inflammatory Reactions by Angiotensin II

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## Key Words

Angiotensin II · Asymmetric dimethylarginine · CXCR<sub>2</sub> · Dimethylarginine dimethylaminohydrolase · Interleukin-8 · Losartan · Nuclear factor- $\kappa$ B · Protein arginine methyltransferase

## Abstract

Previous investigations have demonstrated that angiotensin (Ang) II induces inflammatory reactions and asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, might be a novel inflammatory factor. Endothelial cell activation was induced by incubation with Ang II or ADMA. Incubation with Ang II ( $10^{-6}$  M) for 24 h elevated the levels of ADMA and decreased the levels of nitrite/nitrate concomitantly with a significant increase in the expression of protein arginine methyltransferase and a decrease in the activity of dimethylarginine dimethylaminohydrolase (DDAH). Exposure to Ang II ( $10^{-6}$  M for 24 h) also enhanced intracellular ROS elaboration and the levels of tumor necrosis factor (TNF)- $\alpha$  and interleukin (IL)-8, upregulated chemokine receptor CXCR<sub>2</sub> mRNA expression, increased adhesion of endothelial cells to monocytes and induced a significant increase in the activity of nuclear factor (NF)- $\kappa$ B, which was attenuated by pretreatment with the Ang II receptor blocker losartan (1, 3 and 10  $\mu$ M). Exogenous ADMA (30  $\mu$ M) also increased ROS generation and the levels of TNF- $\alpha$  and IL-8, decreased the

levels of nitrite/nitrate, upregulated CXCR<sub>2</sub> gene expression, increased endothelial cell binding with monocytes and activated the NF- $\kappa$ B pathway, which was inhibited by pretreatment with losartan or L-arginine. These data suggest that ADMA is a potential proinflammatory factor and may be involved in the inflammatory reaction induced by Ang II.

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## Introduction

Angiotensin II (Ang II), the core composition of the renin-angiotensin system (RAS), may promote atherogenesis by stimulating cell growth and proliferation, oxidative stress, cell adhesion and release of chemotactic factors and cytokines. The traditional role of RAS has been updated by the concept that Ang II is a potent proinflammatory substance. It has been demonstrated that Ang II induces monocyte chemoattractant protein-1 (MCP-1) and interleukin-8 (IL-8) release via activation of the nuclear factor (NF)- $\kappa$ B pathway [1]. A large scale of clinical studies have demonstrated that blockade of Ang II by angiotensin-converting enzyme inhibitor (ACEI) or AT<sub>1</sub> receptor antagonist reduces the incidence of cardiovascular events in patients with hypertension, atherosclerosis (AS) and after myocardial infarction [2–4].

There is growing evidence that a profound inflammatory response exists in the hypertensive arterial wall, e.g. in AS. The initial step of this inflammatory response is characterized by adhesion of monocytes to injured endothelium in the arterial wall. IL-8 is a powerful trigger for firm adhesion of monocytes to vascular endothelium. The CXCR<sub>2</sub> receptor, which is activated by IL-8 and growth-regulated oncogene, is a G-protein-coupled receptor expressed in neutrophils, monocytes, endothelial cells, some fibroblasts and cancer cells [5]. It was reported that CXCR<sub>2</sub> is highly expressed on macrophages in AS lesions, and CXCR<sub>2</sub> deficiency markedly attenuates AS progression in mice [6], suggesting that the IL-8/CXCR<sub>2</sub> pathway appears to be involved in the adhesion of monocytes at inflammatory sites contributing to the development of AS.

Impaired endothelium-dependent vasodilation is the primary trigger of some cardiovascular diseases such as hypertension or AS. Previous investigations have demonstrated that asymmetric dimethylarginine (ADMA), which can competitively inhibit the activity of nitric oxide synthase (NOS), is an important risk factor contributing to endothelial dysfunction. It was reported that in cultured endothelial cells, ADMA upregulates the expression of MCP-1 and increases endothelial adhesion to monocytes via an increase in the activity of NF- $\kappa$ B [7]; in activated macrophages, ADMA elevates the expression of lectin-like ox-LDL receptor (LOX-1) and ox-LDL uptake to facilitate foam cell formation [8]. Thus, besides the decrease in the synthesis of NO, ADMA might be a novel inflammatory factor. Previous studies have shown that RAS may play an important role in the elevation of the ADMA level in hypertensive patients, and blockade of Ang II by ACEI or Ang II receptor blocker (ARB) significantly attenuates the elevated level of ADMA, resulting in endothelial protection [9–11]. The present study was designed to examine whether ADMA plays an important role in inflammatory reactions induced by Ang II and to explore possible molecular mechanisms.

## Materials and Methods

### Reagents

Human umbilical vein endothelial cells (HUVECs, ATCC) were obtained from the Tumor Research Institute of the Beijing Medical University (Beijing, China). Human monocytoid cells (THP-1, ATCC) were purchased from the Cell Culture Center of the Xiang-Ya Medical School (Changsha, China). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640, benzylpenicillin and streptomycin were obtained from Gibco-BRL. Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineer-

ing Materials (Hangzhou, China). ADMA standard, Ang II, L-arginine, trypan blue and monoclonal anti-protein arginine methyltransferase (PRMT) I were purchased from Sigma. Losartan was freely supplied by Merck.  $\gamma$ -[<sup>32</sup>P]ATP was obtained from the Furui Biological Engineering Institute (Beijing, China). NO assay kits were purchased from the Juli Biological Medical Engineering Institute (Nanjing, China). ROS detection kits and BCA protein kits were purchased from Beyotime (Jiangsu, China). ELISA kits for the measurement of tumor necrosis factor (TNF)- $\alpha$  and IL-8 were obtained from Senxiong Biological (Shanghai, China). Western blotting kits and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were purchased from KPL. First-strand cDNA synthesis kit was obtained from Fermentas. The primers of CXCR<sub>2</sub> and  $\beta$ -actin were synthesized by Shanghai Biological and Engineering Technology (Shanghai, China). TRIzol, a gel shift assay system for the determination of NF- $\kappa$ B activity, and the probe of NF- $\kappa$ B were obtained from Promega.

### Cell Culture and Treatment

Endothelial cells were cultured in DMEM containing 10% FBS, 100 U/ml benzylpenicillin and 100  $\mu$ g/ml streptomycin at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. When the cells reached subconfluence, cells were transferred to 6-well culture dishes at an optimal density of 10<sup>5</sup> cells/ml. Before the experiment, the conditioned medium was replaced by the medium containing 1% FBS for 24 h. THP-1 cells were cultured in RPMI-1640 containing 15% FBS, 100 U/ml benzylpenicillin and 100  $\mu$ g/ml streptomycin. The viability of endothelial and monocytic cells was assessed by trypan blue exclusion before the experiment, and only cells with a viability >95% were used in the experiment.

Endothelial cell activation was induced by incubation with different concentrations of Ang II (10<sup>-9</sup> to 10<sup>-6</sup> M) for various times (1–48 h). For losartan, endothelial cells were exposed to losartan (1, 3 or 10  $\mu$ M) for 1 h and then incubated with Ang II (10<sup>-6</sup> M) for 4 or 24 h in the presence of losartan. For L-arginine, cells were exposed to L-arginine (0.5 mM) for 1.5 h, and then incubated with Ang II (10<sup>-6</sup> M) for 4 or 24 h in the presence of L-arginine. In order to clarify whether ADMA triggers inflammatory actions induced by Ang II, exogenous ADMA (30  $\mu$ M) was used in the present study.

### Determination of Nitrite/Nitrate Concentrations

The NO level in the medium was indirectly determined by the nitrite and nitrate contents. As previously described [12], nitrate was converted to nitrite with *Aspergillus* nitrite reductase, and the total nitrite was measured with the Griess reagent. Absorbance was determined at 540 nm with a spectrophotometer.

### Determination of ADMA Concentration

The conditioned medium was deproteinized with 5-sulfosalicylic acid. Using high-performance liquid chromatography (HPLC), ADMA content was assessed in the supernatant according to a previous method [13]. HPLC was carried out using a Shimadzu LC-6A (Shimadzu, Kyoto, Japan) liquid chromatograph connected with a Shimadzu SCL-6A system controller and a Shimadzu SIC-6A autosampler. O-Phthaldialdehyde adducts of methylated amino acids and internal standard ADMA produced by precolumn mixing were monitored using a model RF 530 fluorescence detector set at  $\lambda^{ex}$  = 338 nm and  $\lambda^{em}$  = 425 nm on a Re-

solve C<sub>18</sub> column. Samples were eluted from the column using a linear gradient containing mobile phase A composed of 0.05 M (pH 6.8) sodium acetate-methanol-tetrahydrofuran (81:18:1, v:v:v) and mobile phase B composed of 0.05 mM sodium acetate-methanol-tetrahydrofuran (22:77:1, v:v:v) at a flow-rate of 1 ml/min.

#### *Western Blotting to Determine PRMT Protein Expression*

Subsequent to the different treatments, endothelial cells were lysed in SDS sample buffer containing 62.5 mM Tris (pH 6.8), 2% SDS (w/v), and 10% glycerol. Samples were heated at 95°C for 5 min, and proteins of equal concentration (100 µg) 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-PRMT I 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 an enhanced chemiluminescence kit.

#### *Dimethylarginine Dimethylaminohydrolase Activity Assay*

The activity of dimethylarginine dimethylaminohydrolase (DDAH) in endothelial cells was estimated by directly measuring the amount of ADMA metabolized by the enzyme [14]. In an ice bath, cell lysates were divided into two groups, and ADMA was added (final concentration 500 µM). To inactivate DDAH, 30% 5-sulfosalicylic acid was immediately added to one experimental group. This group provided a baseline of 0% DDAH activity. The other lysate was incubated at 37°C for 2 h before the addition of 30% 5-sulfosalicylic acid. The ADMA levels in each group were measured by HPLC as described above. The difference in ADMA concentrations between two groups reflects DDAH activity. For every experiment, DDAH activity of cells in the control group is defined as 100%, and DDAH activity in other conditions was expressed as the percentage of metabolized ADMA compared with the control.

#### *ROS Determination*

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell-permeable 2',7'-dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) using a fluorospectrophotometer (F4000, Japan). Cells in 6-well culture dishes were incubated with Ang II (10<sup>-6</sup> M) or ADMA (30 µM) for various times (1–48 h) in the absence or presence of losartan (1, 3 or 10 µM) or L-arginine (0.5 mM). The cells were washed with DMEM and incubated with DCFH-DA at 37°C for 20 min. DCF fluorescence distribution in 20,000 cells was detected fluorospectrophotometrically at an excitation wavelength of 488 nm and at an emission wavelength of 525 nm.

#### *Reverse Transcription-PCR Analysis*

Total mRNA was extracted from cells in 6-well culture dishes using TRIzol. First-strand cDNA was then synthesized from 4 µg total RNA using reverse transcriptase. Primers were synthesized as previously reported [15]: 5'-CGGAATTCAAATGGAAGATTTTAACATGGAG-3' (CXCR<sub>2</sub>, sense), 5'-CCGCTCGAGTTAGAGAGTAGTGGAAGTGTG-3' (CXCR<sub>2</sub>, antisense), amplification length 417 bp; 5'-CTGTCCCTGTATGCCTCTG-3' (β-actin, sense) and 5'-ATGTCACGCACGATTTCC-3' (β-actin, antisense),

amplification length 218 bp. cDNA was amplified by 38 cycles at 94°C for 60 s (denaturation), 58°C for 60 s (annealing) and an extension step at 72°C for 60 s. PCR products were analyzed by 2% agarose gel electrophoresis. The housekeeping actin gene was used for 28 cycles as a control to normalize relative changes in CXCR<sub>2</sub> mRNA expression in reverse transcription PCR.

#### *Determination of IL-8 and TNF-α*

The levels of IL-8 and TNF-α in the conditioned medium were measured using ELISA kits strictly following the instructions of the manufacturer.

#### *Static Adhesion Assays*

Endothelial cells were plated into 12-well dishes and treated with Ang II (10<sup>-6</sup> M) or ADMA (30 µM) for 24 h in the absence or presence of losartan or L-arginine; 30 min before the adhesion assay, endothelial cells were washed with Hanks balanced salt solution. Then THP-1 cells were diluted to a final concentration of 10<sup>6</sup> cells/ml and added to each well (1 ml/well). The dishes were again incubated for 30 min at 37°C. Non-adherent THP-1 cells were carefully removed by washing twice with Hanks balanced salt solution. Adherent THP-1 cells were counted in six high-power microscopic fields for each well.

#### *Preparation of Nuclear Extracts*

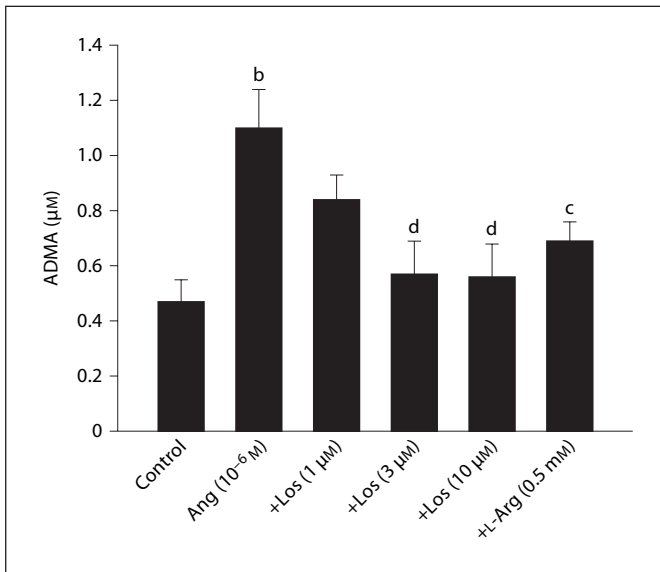
Endothelial cells were seeded into 25-cm<sup>2</sup> culture flasks at an optimal density of 10<sup>5</sup> cells/ml. Cells were washed with PBS twice and incubated with 400 µl buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) for 15 min on ice. Then 25 µl 10% NP-40 was added. After vortexing, cell lysates were centrifuged at 12,000 g for 3 min at 4°C and nuclei were resuspended in 50 µl of buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 25% glycerin, 1 mM DTT and 1 mM PMSF) and vigorously vortexed at 4°C for 15 min. Nuclear lysates were subsequently centrifuged at 12,000 g for 5 min, and the supernatant containing the nuclear proteins was carefully removed. Protein aliquots were either frozen at -70°C or immediately used for electrophoretic mobility shift assays (EMSA).

#### *EMSA*

EMSA determining NF-κB DNA-binding activity was performed by incubating aliquots of nuclear extracts containing 15 µg of total protein with γ-[<sup>32</sup>P]-labeled double-strand NF-κB-specific oligonucleotide probe (sense: 3'-TCAACTCCCCTGA-AAGGGTCCG-5'; 5'-AGTTGAGGGGACTTCCAGGC-3') by T<sub>4</sub> polynucleotide kinase. The labeled probe was purified through Sephadex G-25. After a 10-min incubation at room temperature, 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.

#### *Statistic Analysis*

Results are expressed as means ± SEM. Data were analyzed by ANOVA followed by the Newmann-Keuls test for multiple comparisons. Cell binding data are expressed as the mean relative number of cells binding per high power field. Statistical significance was tested using analysis of variance followed by Fisher's protected least-significant difference test. Statistical significance was considered if p < 0.05.



**Fig. 1.** Effect of losartan on the elevated level of ADMA by Ang II: endothelial cells were incubated with losartan (+Los; 1, 3 or 10  $\mu\text{M}$  for 1 h) or L-arginine (+L-Arg; 0.5 mM for 1.5 h) and then exposed to Ang II ( $10^{-6}$  M) for 24 h. Data are expressed as means  $\pm$  SEM.  $n = 4-6$ . <sup>b</sup>  $p < 0.01$ , vs. control; <sup>c</sup>  $p < 0.05$ , <sup>d</sup>  $p < 0.01$ , vs. Ang II.

## Results

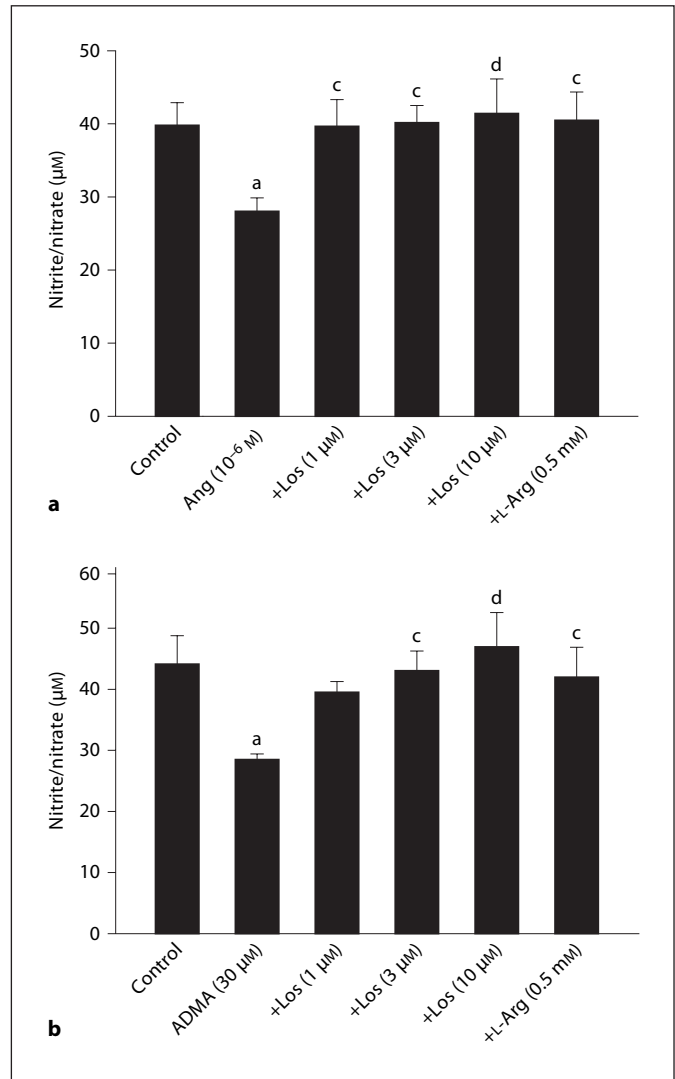
### ADMA Content

Incubation of endothelial cells with Ang II ( $10^{-6}$  M) for 24 h caused a significant increase in the ADMA level in the conditioned medium ( $p < 0.01$ ). Pretreatment with losartan (3 or 10  $\mu\text{M}$ ) significantly reduced the elevated ADMA level by Ang II ( $p < 0.01$ ). L-arginine (0.5 mM) also decreased the Ang II-induced increase in ADMA ( $p < 0.05$ ) (fig. 1).

### NO Level

After incubation of endothelial cells with Ang II ( $10^{-6}$  M) for 24 h, the level of nitrite/nitrate was markedly decreased in the cultured medium ( $p < 0.05$ ). Treatment with losartan (1, 3 or 10  $\mu\text{M}$ ) significantly attenuated the inhibitory effect of Ang II on nitrite/nitrate ( $p < 0.05$  and  $p < 0.01$ ). Adding L-arginine (0.5 mM) also recovered the decreased level of nitrite/nitrate by Ang II (fig. 2a).

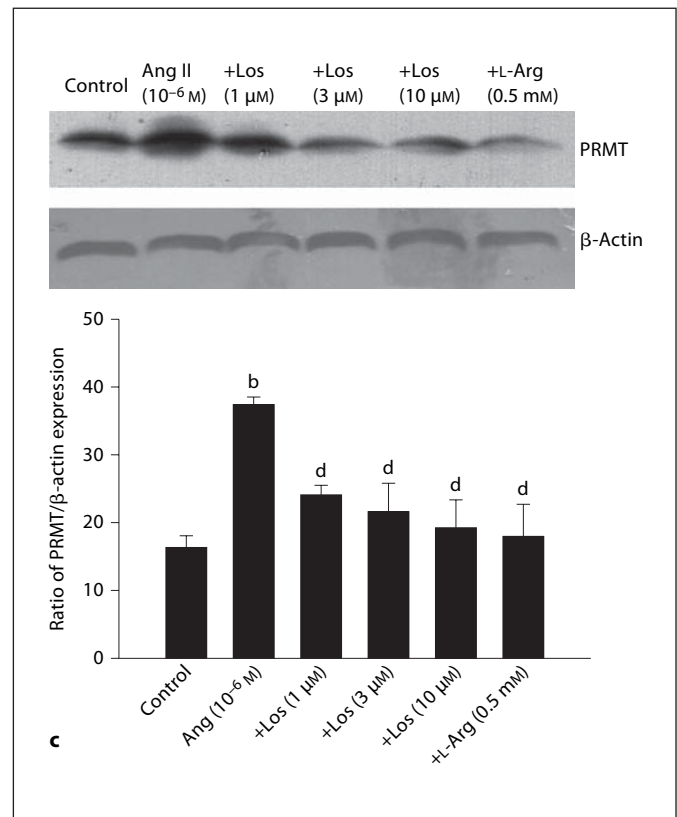
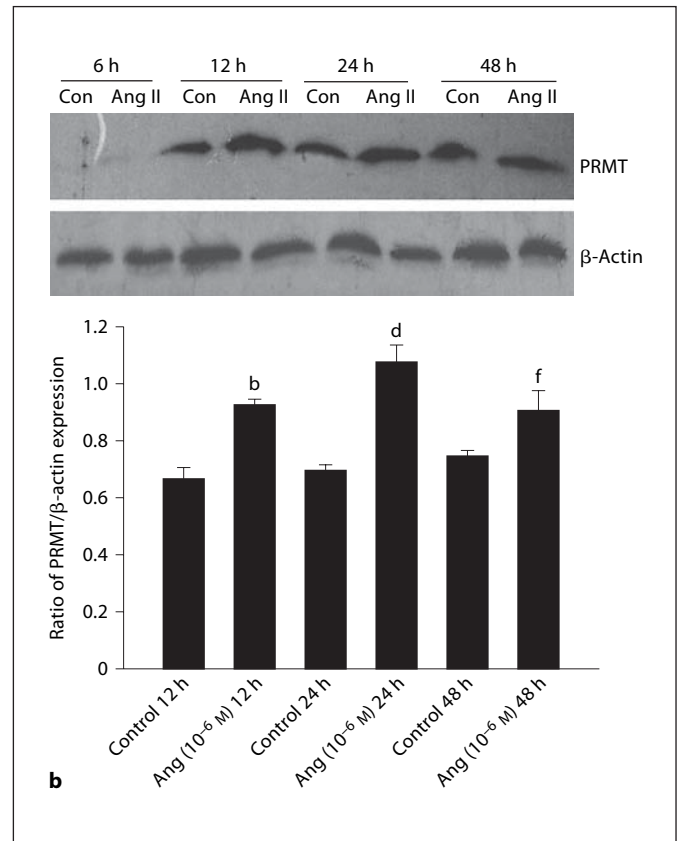
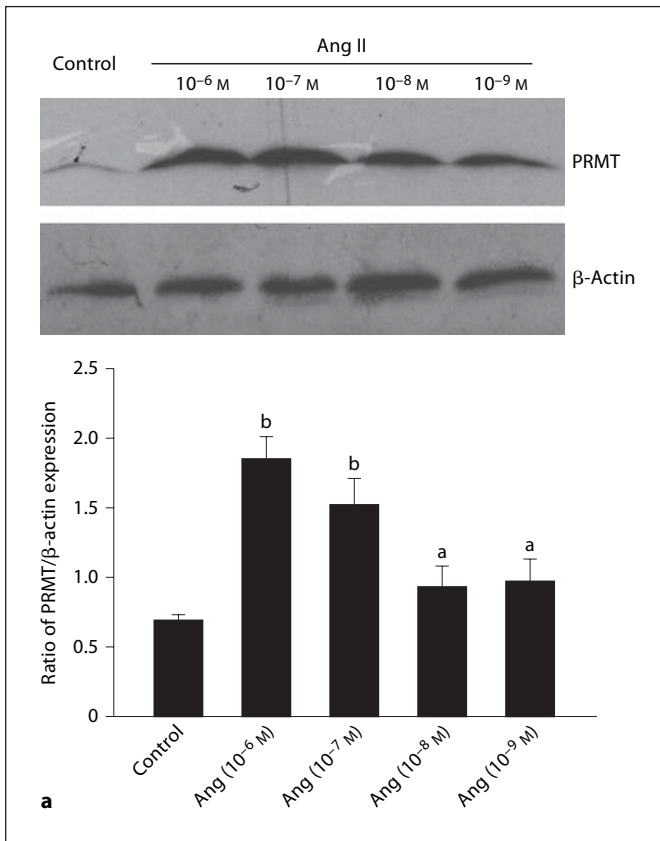
Exogenous ADMA (30  $\mu\text{M}$ ) also significantly decreased the nitrite/nitrate level in cultured endothelial cells. Pretreatment with losartan (3, 10  $\mu\text{M}$ ) attenuated the decreased level of nitrite/nitrate by ADMA ( $p < 0.05$  and  $p < 0.01$ ). A similar effect was observed in the presence of L-arginine (0.5 mM; fig. 2b).



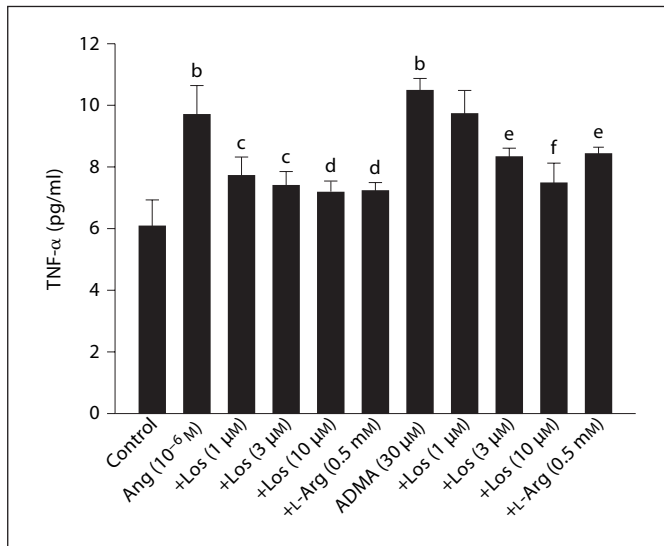
**Fig. 2.** Effect of Ang II (a) or ADMA (b) on the nitrite/nitrate level. Endothelial cells were treated with Ang II ( $10^{-6}$  M) or ADMA (30  $\mu\text{M}$ ) for 24 h. Data are expressed as means  $\pm$  SEM.  $n = 4-6$ . <sup>a</sup>  $p < 0.05$  vs. control; <sup>c</sup>  $p < 0.05$ , <sup>d</sup>  $p < 0.01$ , vs. Ang II or ADMA. Los = Losartan; L-Arg = L-arginine.

### Protein Expression of PRMT I

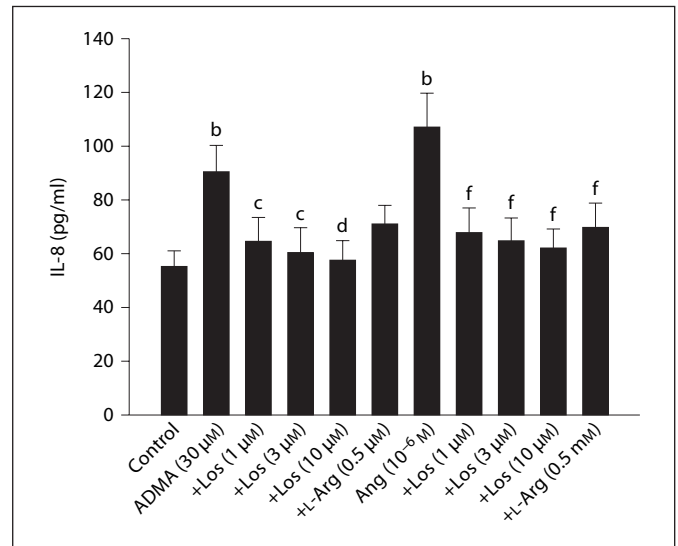
Compared to control, treatment with Ang II for 24 h concentration-dependently increased the protein expression of PRMT I in endothelial cells (fig. 3a). PRMT protein was not expressed in control endothelial cells at 6 h. Incubation with Ang II ( $10^{-6}$  M) for 12, 24 or 48 h caused a significant increase in the expression of PRMT I, peaking at 24 h (fig. 3b), whereas PRMT I expression was low at 6 h.



**Fig. 3.** Effect of losartan (Los) on the expression of PRMTs of endothelial cells treated with Ang II. **a** Effect of Ang II at different concentrations on the protein expression of PRMT: cells were incubated with different concentrations of Ang II ( $10^{-9}$  to  $10^{-6}$  M) for 24 h. <sup>a</sup>  $p < 0.05$ , <sup>b</sup>  $p < 0.01$ , vs. control. **b** Effect of Ang II for different times on the protein expression of PRMT. Con = Control, incubation of endothelial cells with DMEM containing 1% FBS for 6, 12, 24 and 48 h; Ang II = incubation of endothelial cells with Ang II ( $10^{-6}$  M) for 6, 12, 24 and 48 h. <sup>b</sup>  $p < 0.01$  vs. control (12 h); <sup>d</sup>  $p < 0.01$  vs. control (24 h); <sup>f</sup>  $p < 0.01$  vs. control (48 h). **c** Effect of losartan on PRMT protein expression induced by Ang II. Endothelial cells were exposed to losartan (+Los; 1, 3 or 10  $\mu$ M) for 1 h or L-arginine (+L-Arg; 0.5 mM) for 1.5 h and then incubated with Ang II ( $10^{-6}$  M) for 24 h.  $n = 3$ . <sup>b</sup>  $p < 0.01$  vs. control; <sup>d</sup>  $p < 0.01$  vs. Ang II.



**Fig. 4.** Effect of losartan (Los) on the level of TNF- $\alpha$  by Ang II or ADMA. Data are expressed as means  $\pm$  SEM. n = 4–6. <sup>b</sup> p < 0.01 vs. control; <sup>c</sup> p < 0.05, <sup>d</sup> p < 0.01, vs. Ang II; <sup>e</sup> p < 0.05, <sup>f</sup> p < 0.01, vs. ADMA. L-Arg = L-Arginine.



**Fig. 5.** Effect of losartan (Los) on the release of IL-8 from endothelial cells treated with Ang II or ADMA. Data are expressed as means  $\pm$  SEM. n = 4–6. <sup>b</sup> p < 0.01 vs. control; <sup>c</sup> p < 0.05, <sup>d</sup> p < 0.01, vs. ADMA; <sup>f</sup> p < 0.01 vs. Ang II.

**Table 1.** Effect of losartan on the activity of DDAH of endothelial cells treated with Ang II

| Group                       | n | ADMA <sub>0</sub> | ADMA <sub>1</sub> | D = ADMA <sub>0</sub> – ADMA <sub>1</sub> | DDAH activity, %              |
|-----------------------------|---|-------------------|-------------------|---|-------------------------------|
| Control                     | 6 | 388.6 $\pm$ 27.8  | 315.3 $\pm$ 35.5  | 73.3 $\pm$ 12.7                           | 100.0 $\pm$ 16.8              |
| Ang II (10 <sup>-6</sup> M) | 5 | 376.8 $\pm$ 36.6  | 358.8 $\pm$ 34.1  | 18.0 $\pm$ 4.2                            | 24.6 $\pm$ 5.7 <sup>a</sup>   |
| + Losartan (1 $\mu$ M)      | 5 | 441.3 $\pm$ 40.6  | 355.6 $\pm$ 40.4  | 85.7 $\pm$ 5.4                            | 117.0 $\pm$ 7.0 <sup>b</sup>  |
| + Losartan (3 $\mu$ M)      | 5 | 406.4 $\pm$ 33.8  | 306.4 $\pm$ 28.7  | 100.0 $\pm$ 14.3                          | 136.5 $\pm$ 19.5 <sup>b</sup> |
| + Losartan (10 $\mu$ M)     | 5 | 459.4 $\pm$ 33.0  | 372.0 $\pm$ 28.3  | 87.4 $\pm$ 13.5                           | 119.3 $\pm$ 18.4 <sup>b</sup> |
| + L-arginine (0.5 mM)       | 7 | 433.9 $\pm$ 36.6  | 365.7 $\pm$ 34.3  | 68.1 $\pm$ 13.7                           | 93.0 $\pm$ 18.7 <sup>b</sup>  |

Data are expressed as means  $\pm$  SEM.

<sup>a</sup> p < 0.01 vs. control; <sup>b</sup> p < 0.01 vs. Ang II. ADMA<sub>0</sub> = The levels of ADMA in the unincubated lysate; ADMA<sub>1</sub> = the levels of ADMA in the incubated lysate.

Exposure of endothelial cells to Ang II (10<sup>-6</sup> M) for 24 h increased the protein expression of PRMT I (p < 0.01). Pretreatment with losartan dose-dependently inhibited the elevated protein expression of PRMT I by Ang II (p < 0.01). Treatment with L-arginine also attenuated the increased expression of PRMT I by Ang II (fig. 3c).

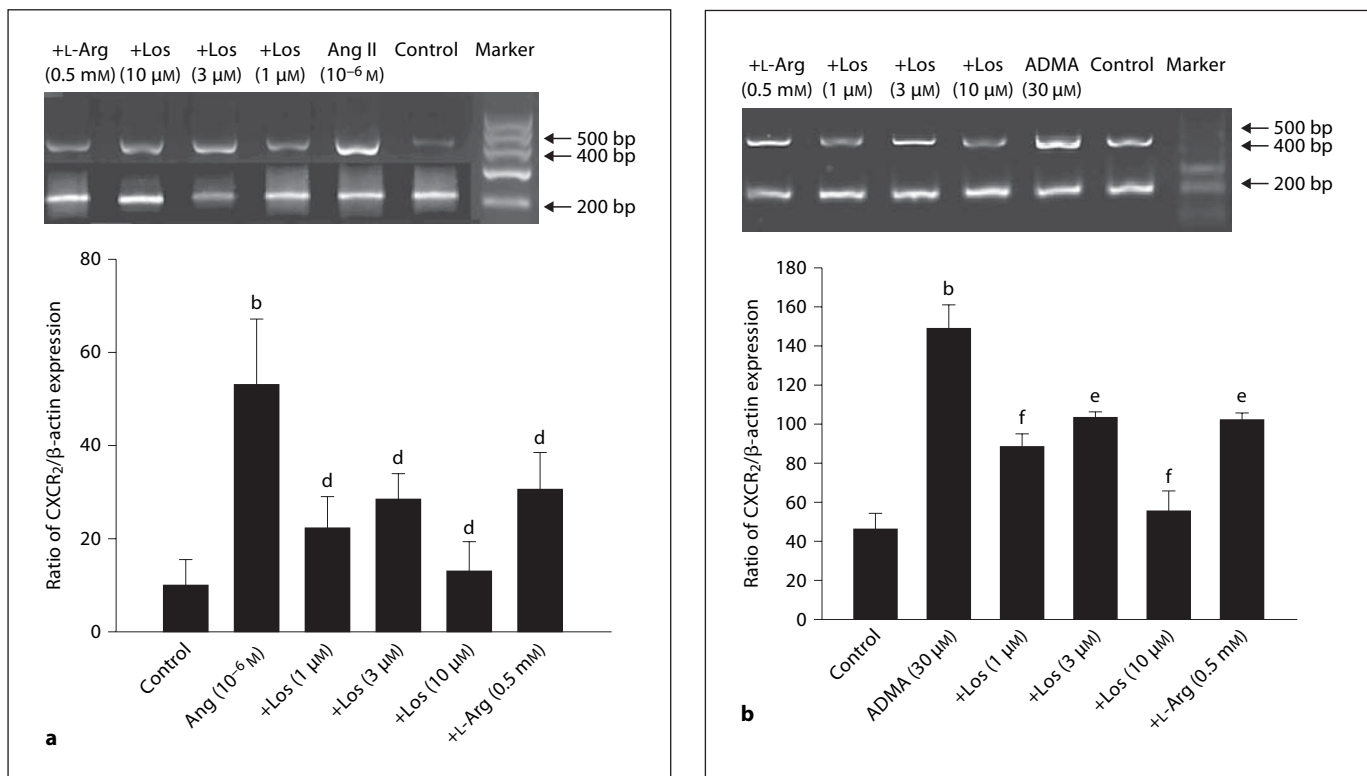
#### DDAH Activity

Compared to control, DDAH activity was significantly reduced in the endothelial cells treated with Ang II (10<sup>-6</sup> M) for 24 h (p < 0.01). Incubation with losartan (1, 3 or 10  $\mu$ M) attenuated the decreased activity of DDAH

by Ang II in a dose-dependent manner (p < 0.01). Treatment with L-arginine had a similar effect on DDAH activity (table 1).

#### IL-8 and TNF- $\alpha$ Content

After incubation of endothelial cells with Ang II (10<sup>-6</sup> M) or ADMA (30  $\mu$ M) for 24 h, the levels of IL-8 and TNF- $\alpha$  were markedly increased (p < 0.01). Pretreatment with losartan (1, 3 or 10  $\mu$ M) significantly inhibited the increased concentration of IL-8 or TNF- $\alpha$  by Ang II or ADMA. L-arginine attenuated the elevated level of IL-8 and TNF- $\alpha$  by Ang II (fig. 4, 5).



**Fig. 6.** Effect of losartan on the expression of CXCR<sub>2</sub> mRNA of endothelial cells treated with Ang II (a) or ADMA (b). n = 3. <sup>b</sup> p < 0.01 vs. control; <sup>d</sup> p < 0.01 vs. Ang II; <sup>e</sup> p < 0.05, <sup>f</sup> p < 0.01, vs. ADMA.

**Table 2.** Effect of Ang II or ADMA on monocytic binding to endothelial cells

| Group                       | n | Cells/hpf                 | Group                 | n | Cells/hpf                 |
|-----------------------------|---|---------------------------|-----------------------|---|---------------------------|
| Control                     | 6 | 163.5 ± 9.6               |                       |   |                           |
| Ang II (10 <sup>-6</sup> M) | 4 | 473.0 ± 17.3 <sup>a</sup> | ADMA (30 μM)          | 4 | 316.7 ± 10.3 <sup>a</sup> |
| + Losartan (1 μM)           | 4 | 241.8 ± 24.1 <sup>b</sup> | + Losartan (1 μM)     | 4 | 247.3 ± 6.9 <sup>c</sup>  |
| + Losartan (3 μM)           | 4 | 213.8 ± 12.9 <sup>b</sup> | + Losartan (3 μM)     | 4 | 198.7 ± 7.3 <sup>c</sup>  |
| + Losartan (10 μM)          | 4 | 200.5 ± 18.6 <sup>b</sup> | + Losartan (10 μM)    | 4 | 186.0 ± 5.6 <sup>c</sup>  |
| + L-arginine (0.5 mM)       | 4 | 229.3 ± 16.2 <sup>b</sup> | + L-arginine (0.5 mM) | 4 | 200.7 ± 4.9 <sup>c</sup>  |

<sup>a</sup> p < 0.01 vs. control; <sup>b</sup> p < 0.01 vs. Ang II; <sup>c</sup> p < 0.01 vs. ADMA.

#### CXCR<sub>2</sub> mRNA Expression

As shown in figure 6, treatment of endothelial cells with Ang II (10<sup>-6</sup> M) or ADMA (30 μM) for 4 h significantly upregulated the expression of CXCR<sub>2</sub> mRNA (p < 0.01). Pretreatment with losartan (1, 3 or 10 μM) markedly inhibited the increased expression of CXCR<sub>2</sub> mRNA by Ang II or ADMA. Treatment with L-arginine (0.5 mM) also attenuated the elevated expression of CXCR<sub>2</sub> mRNA by Ang II or ADMA (p < 0.01 and p < 0.01).

#### Monocyte Adhesion

Compared to control, exposure to Ang II (10<sup>-6</sup> M) or ADMA (30 μM) for 24 h significantly increased the number of monocytes binding to endothelial cells (p < 0.01). Pretreatment with losartan or L-arginine markedly decreased the increased binding of monocytes to endothelial cells by Ang II or ADMA (p < 0.01; table 2).

### Intracellular ROS

Treatment of endothelial cells with Ang II ( $10^{-6}$  M) or ADMA ( $30 \mu\text{M}$ ) increased intracellular ROS generation in a time-dependent manner. ROS generation began to increase at 4 h and reached a maximum at 24 h (fig. 7a).

Supplementation with Ang II ( $10^{-6}$  M) or ADMA ( $30 \mu\text{M}$ ) for 24 h significantly increased intracellular ROS generation ( $p < 0.05$  and  $p < 0.01$ ). The effect of Ang II or ADMA on the ROS level was reversed by treatment with losartan or L-arginine. Losartan or L-arginine itself had no effect on the ROS level (fig. 7b, c).

### NF- $\kappa$ B Activity

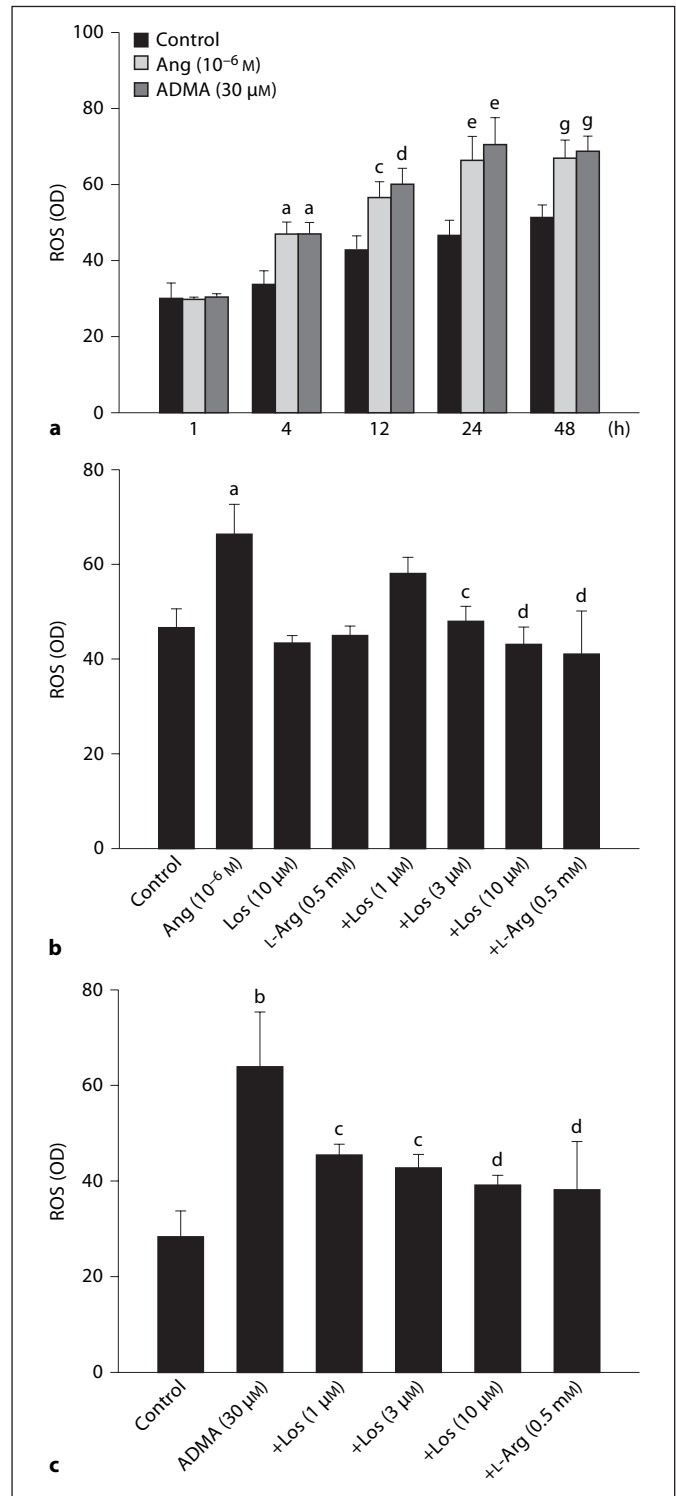
As shown in figure 8, there is no signal in negative controls without adding nuclear protein. In cultured endothelial cells, NF- $\kappa$ B was significantly activated by treatment with Ang II ( $10^{-6}$  M) for 4 h. The effect of Ang II on NF- $\kappa$ B activation was blocked by treatment with losartan. A similar effect was observed in the presence of L-arginine.

Incubation with ADMA ( $30 \mu\text{M}$ ) for 4 h markedly enhanced the activity of NF- $\kappa$ B in endothelial cells. Pre-treatment with losartan or L-arginine significantly inhibited the increased activity of NF- $\kappa$ B by ADMA (fig. 9).

### Discussion

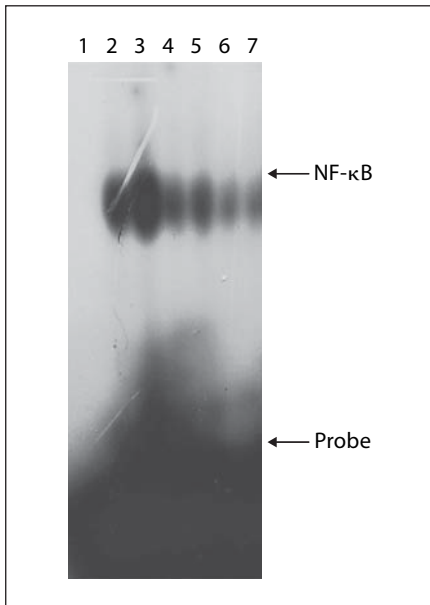
The major findings of the present study are: (1) Ang II increased the level of ADMA in the culture media via increasing PRMT I protein expression and decreasing DDAH activity; (2) ADMA (endogenous or exogenous) induced inflammatory reactions and increased endothelial adhesion to monocytes by increasing CXCR<sub>2</sub> mRNA expression, which was mediated via activation of the NF- $\kappa$ B signaling pathway.

Previous investigations have demonstrated that ADMA, an endogenous NOS inhibitor, is a novel risk factor for cardiovascular disease which contributes to endothelial dysfunction. Many clinical studies have shown that plasma ADMA levels are significantly elevated in-

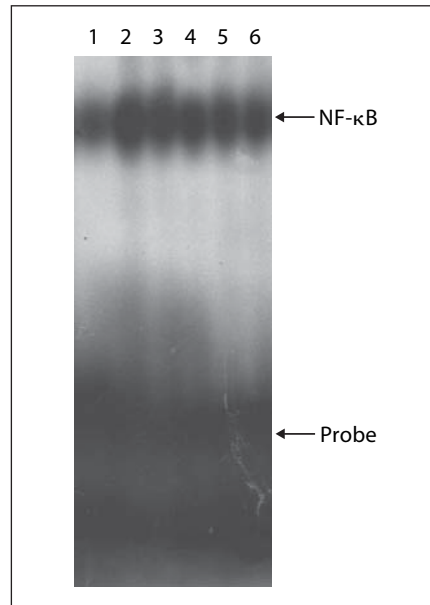


**Fig. 7.** Effect of losartan (Los) on intracellular ROS generation by Ang II or ADMA. **a** Cells were treated with Ang II or ADMA for 1, 4, 12, 24 and 48 h. <sup>a</sup>  $p < 0.05$  vs. control (4 h); <sup>c</sup>  $p < 0.05$ , <sup>d</sup>  $p < 0.01$ , vs. control (12 h); <sup>e</sup>  $p < 0.05$  vs. control (24 h); <sup>g</sup>  $p < 0.05$  vs. control (48 h). **b** Cells were treated with Ang II in the absence or presence of losartan or L-arginine (L-Arg): cells were cultured

with DMEM containing losartan ( $10 \mu\text{M}$ ) or L-arginine ( $0.5 \text{ mM}$ ). <sup>a</sup>  $p < 0.05$ , vs. control; <sup>c</sup>  $p < 0.05$ , <sup>d</sup>  $p < 0.01$ , vs. Ang II. **c** Cells were treated with ADMA for 24 h in the absence or presence of losartan (Los) or L-arginine (L-Arg).  $n = 4-6$ . <sup>b</sup>  $p < 0.01$ , vs. control; <sup>c</sup>  $p < 0.05$ , <sup>d</sup>  $p < 0.01$ , vs. ADMA.



**Fig. 8.** Effect of losartan on NF- $\kappa$ B DNA-binding activity in cultured endothelial cells treated with Ang II. 1 = Negative control; 2 = control; 3 = Ang II; 4 = + losartan (1  $\mu$ M); 5 = + losartan (3  $\mu$ M); 6 = + losartan (10  $\mu$ M); 7 = + L-arginine (0.5 mM). n = 3.



**Fig. 9.** Effect of losartan on NF- $\kappa$ B DNA-binding activity in cultured endothelial cells treated with ADMA. 1 = Control; 2 = ADMA; 3 = + losartan (1  $\mu$ M); 4 = + losartan (3  $\mu$ M); 5 = + losartan (10  $\mu$ M); 6 = + L-arginine (0.5 mM). n = 3.

dividuals with endothelial dysfunction, e.g. hypercholesterolemia, hyperglycemia, hyperhomocysteinemia, heart failure, hypertension and diabetes mellitus, to name a few [16–21]. Recently, it has been reported that the ADMA level is positively correlated with carotid artery intima-media thickness and major cardiovascular events in patients with percutaneous transluminal coronary angioplasty and in patients in the intensive care unit [22–24]. All these findings suggest that ADMA may be an important target for pharmacotherapeutic interventions [25]. It is known that ADMA is synthesized by PRMT, which utilizes an S-adenosylmethionine methyl group donor, and hydrolyzed by DDAH, which accounts for most of the clearance of ADMA to L-citrulline and dimethylamine. It was demonstrated that the increase in the level of ADMA by LDL, ox-LDL or TNF- $\alpha$  is due to increased PRMT expression and decreased DDAH activity [26, 27]. Some clinical studies have also reported that RAS plays an important role in the elevation of ADMA in hypertensive patients, and blockade of Ang II by ACEI or ARB significantly attenuates the elevated level of ADMA [9–11]. In the present study in cultured endothelial cells, treatment with Ang II increased ADMA production, accompanied with an increase in the protein expression of

PRMT I and a decrease in the activity of DDAH. These findings provide direct evidence that Ang II is an important factor increasing the ADMA level.

Previous studies have shown that endothelial dysfunction is closely related to oxidative stress induced by stimulation of ROS production. There is evidence that an increase in ADMA is related to oxidative stress, and treatment with antioxidants, e.g. vitamin E, probucol, daviditin A and pyrrolidine dithiocarbamate, decreases both lipid peroxide and ADMA levels [26–31]. It was reported that Ang II can enhance the activity of NADPH oxidase and lead to marked ROS generation in cultured endothelial cells [32] and L6 myotubes, which is blocked by ARB losartan or the NADPH oxidase inhibitor apocynin [33]. In the present study, incubation of endothelial cells with Ang II increased the production of ADMA, concomitantly with an increase in ROS levels, and the effects of Ang II were blocked in the presence of losartan, suggesting that Ang II elevated ADMA levels via induction of oxidant stress mediated by AT<sub>1</sub> receptor. L-arginine is a substrate of NOS for NO synthesis. Previous studies have proven that L-arginine improves endothelial injury against endogenous or exogenous oxygen free radicals [34]. Recently, it was reported that in cultured endothe-

lial cells, L-arginine inhibits ADMA-induced cell apoptosis via a reduction of intracellular ROS levels [35], suggesting that L-arginine possesses antioxidant actions. The results of the present study showed that L-arginine supplementation decreased the elevated ADMA level and ROS generation induced by Ang II. It is likely that L-arginine decreased the ADMA levels via a reduction of ROS production induced by Ang II.

It is known that NF- $\kappa$ B is an important link between Ang II and inflammation, and plays a pivotal role in atherogenesis by regulating proinflammatory gene expression. Recent work has shown that Ang II, besides regulating blood pressure, is a potent activator of inflammatory pathways. ADMA, besides inhibiting NO synthesis, also participates in inflammatory reactions. Previous studies have demonstrated that Ang II-induced activation of NF- $\kappa$ B in monocytes/macrophages, endothelial cells and vascular smooth muscle cells results in a significant increase in the expression of proinflammatory cytokines, e.g. MCP-1, IL-6, IL-8 and growth factors [36], which can be inhibited by the ACEI ramiprilat [1]. There is evidence that inhibition of NO production by ADMA upregulates the expression of redox-sensitive genes and MCP-1, increases the adhesiveness of endothelial cells and activates the NF- $\kappa$ B pathway [7]. In cultured endothelial cells, ox-LDL induced elevation of ADMA and TNF- $\alpha$  levels via activation of the NF- $\kappa$ B pathway [37]. In the present study, stimulation of endothelial cells with both Ang II and ADMA increased ROS generation and activated NF- $\kappa$ B to stimulate inflammatory mediator (TNF- $\alpha$  and IL-8) production, suggesting that Ang II or ADMA induced oxidant stress to mediate inflammatory reactions via activation of the NF- $\kappa$ B signal pathway.

Chemokines, which are classified into four distinct subfamilies (CXC, CC, C and CX<sub>3</sub>C) according to the arrangement of the di-cysteine motif, have been demonstrated to be critically involved in the recruitment of monocytes to vascular endothelium. The CXCR<sub>2</sub> receptor, which is activated by IL-8 and gro- $\alpha$ , is normally expressed by neutrophils, monocytes and endothelial cells, for example. It was demonstrated that IL-8 profoundly increased the migration of monocytes across endothelial cells, and blockade of the CXCR<sub>2</sub> receptor markedly inhibited macrophage recruitment [38]. More recently, it was reported that ox-LDL could upregulate the expression of CXCR<sub>2</sub> and increase chemotaxis of monocytes to IL-8 and adhesion of monocytes to endothelial cells via the p38 MAPK pathway in cultured monocytes, which was attenuated by the scavenger receptor inhibitor polyinosinic acid [15]. The results of the present study showed

that Ang II or ADMA increased the adhesion of endothelial cells to monocytes via increasing the release of IL-8 and upregulating the expression of CXCR<sub>2</sub>. It is likely that chemokine receptors play an important role in endothelial adhesion induced by Ang II or ADMA.

Losartan, an Ang II type 1 receptor antagonist, is widely used in the treatment of hypertension. Large-scale studies have demonstrated that losartan possesses anti-atherosclerotic properties by inhibiting production of many inflammatory mediators such as cICAM-1, cVCAM-1, MCP-1, TNF- $\alpha$  and E-selectin, independent of their effects on blood pressure [39, 40]. Previous studies have shown that Ang II may activate NF- $\kappa$ B and activated protein (AP-1) via a redox-sensitive mechanism involving the NADPH oxidase pathway, which was blocked by losartan treatment [41]. In the present study, ADMA or Ang II induced oxidative stress and inflammatory reactions and increased endothelial adhesion to monocytes, which was blocked by losartan, suggesting that anti-inflammatory and antioxidant effects of losartan may be related to reduced ADMA production via the AT<sub>1</sub> receptor. However, the relationship between ADMA and Ang II remains unclear. Some clinical studies have shown that blockade of Ang II by ACEI or ARB significantly attenuated the elevated level of ADMA in patients with hypertension [9–11]. In the present study, Ang II directly stimulated ADMA production, and endogenous or exogenous ADMA induced an increase in IL-8 and TNF- $\alpha$  levels and activated the NF- $\kappa$ B pathway, which was blocked by losartan, in support of the hypothesis that elevated ADMA plays a facilitative role in inflammatory reactions induced by Ang II. Recently, Suda et al. [42] have reported that in wild-type and eNOS-knockout mice, treatment with ADMA for 4 weeks upregulates ACE gene expression and increased superoxide production, which is abolished by simultaneous treatment with temocapril (ACEI) or olmesartan (ARB), suggesting that ACEI or ARB inhibits the effect of ADMA via their antioxidant actions. These findings suggest that Ang II is an important factor in ADMA production, and the long-term vascular effects of ADMA are performed by a possible Ang-dependent mechanism. Thus it is hypothesized that a mutual and complex relationship exists between Ang II and ADMA. However, future studies have to confirm this hypothesis.

In conclusion, the present study suggests that ADMA is a potential proinflammatory factor and Ang II induces inflammatory reactions via the NF- $\kappa$ B pathway by stimulation of ADMA production.

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