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ADMA induces monocyte adhesion via activation of chemokine receptors in cultured THP-1 cells

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ABSTRACT

Asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, is also an important inflammatory factor contributing to the development of atherosclerosis (AS). The present study was to test the effect of ADMA on angiotensin (Ang) II-induced monocytic adhesion. Human monocytoid cells (THP-1) or isolated peripheral blood monocyte cells (PBMCs) were incubated with Ang II (10^{-6} M) or exogenous ADMA (30 μM) for 4 or 24 h in the absence or presence of losartan or antioxidant PDTC. In cultured THP-1 cells, Ang II (10^{-6} M) for 24 h elevated the level of ADMA in the medium, upregulated the protein expression of protein arginine methyltransferase (PRMT) and decreased the activity of dimethylarginine dimethylaminohydrolase (DDAH). Both of Ang II and ADMA increased monocytic adhesion to human umbilical vein endothelial cells (HUVECs), elevated the levels of monocyte chemoattractant protein (MCP)-1, interleukin (IL)-8 and tumor necrosis factor (TNF)-α and upregulated CCR_2 and $CXCR_2$ mRNA expression, concomitantly with increase in reactive oxygen species (ROS) generation and activation of nuclear factor (NF)-κB. Pretreatment with losartan (10 μM) or PDTC (10 μM) abolished the effects mediated by Ang II or ADMA. In isolated PBMCs from healthy individuals, ADMA upregulated the expression of $CXCR_2$ mRNA, which was attenuated by losartan (10 μM), however, ADMA had no effect on surface protein expression of CCR_2. The present results suggest that ADMA may be involved in monocytic adhesion induced by Ang II via activation of chemokine receptors by ROS/NF-κB pathway.

1. 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 member of CC chemokine family, induces the recruitment and accumulation of monocytes to inflammatory sites through its receptor CCR_2. In hypercholesterolemic patients, the expression of CCR_2 in isolated monocytes was markedly elevated and treatment of monocytes with LDL further upregulated CCR_2 expression and enhanced the chemotaxis elicited by MCP-1 [1]. Interleukin-8 (IL-8), a member of CXC chemokine family, is a trigger for firm adhesion of monocytes to vascular endothelium via its receptor $CXCR_2$. It was recently demonstrated that $CXCR_2$ was strongly expressed on monocytes and macrophages in atherosclerotic lesion [2]. $CXCR_2$ deficiency significantly reduced the progression of AS in mice [3] and inhibited macrophage recruitment and leukocyte–endothelial cell interactions induced by angiotensin II (Ang II) [4].

It has been documented that the system of L-arg-NO synthesis occurs in monocytes/macrophages. Under inflammatory conditions, NO is produced from L-arginine by inducible nitric oxide synthase (iNOS) in monocytes and excessive NO can induce superoxide production by uncoupling of iNOS [5]. There is growing evidence that asymmetric dimethylarginine (ADMA), an endogenous NOS inhibitor, is markedly elevated in some cardiovascular diseases and recognized as an important risk factor for cardiovascular events [6]. ADMA, besides inhibiting NO synthesis, may be involved in inflammatory reaction via induction of "NOS uncoupling" to increase intracellular oxygen free radicals generation [7]. Recent work demonstrated that ADMA could induce the apoptosis of endothelial cells and smooth muscle cells via induction of oxidant stress [8–9], elevate the expression of lectin-like ox-LDL receptor (LOX-1) and increase ox-LDL uptake to facilitate foam cell

Abbreviations: ACEI, angiotensin-converting enzyme inhibitor; ADMA, asymmetric dimethylarginine; Ang II, angiotensin II; ARB, angiotensin receptor blocker; AS, atherosclerosis; DDAH, dimethylarginine dimethylaminohydrolase; HPLC, high performance liquid chromatography; THP-1, human monocytoid cells; HUVEC, human umbical vein endothelial cells; ICAM-1, intercellular adhesion molecule; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor kappaB; iNOS, inducible nitric oxide synthase; PRMTs, protein arginine methyltransferases, RAS, renin–angiotensin system; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α, VCAM-1, vascular cell adhesion molecule.

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formation [10], suggesting that ADMA plays an important role in the development of AS. However, the relationship between ADMA and chemokines in monocytes is unclear.

Ang II, the core composition of the renin–angiotensin system (RAS), plays an important role in the development of multiple cardiovascular diseases. The traditional role of RAS has been updated to incorporate the concept that Ang II is a potent pro-inflammatory agent contributing to the progress of AS. In cultured monocytes, Ang II induces the release of chemokines such as MCP-1 and IL-8 and adhesion molecular such as vascular cell adhesion molecule (VCAM-1) and intercellular adhesion molecule (ICAM-1) via activation of nuclear factor-kappa B (NF-κB) [11–12]. There is evidence that Ang II may induce ADMA production via oxidant stress. In the present study, therefore, we tested the role of ADMA in Ang II-induced inflammatory response and the possible mechanisms.

2. Materials and methods

2.1. Reagents

Human monocytoid cells (THP-1, ATCC) were purchased from Cell Culture Center of Xiang-Ya Medical School (Changsha, China). Human umbilical vein endothelial cells (HUVECs, ATCC) were obtained from Tumor Research Institute of Beijing Medical University (Beijing, China). RPMI 1640, DMEM, benzylpenicillin and streptomycin were obtained from Gibco-BRL. Fetal bovine serum (FBS) was obtained from Sijiqing Biological Engineering Materials (Hangzhou, China). ADMA standard, Ang II, pyrrolidine dithiocarbamate (PDTC), trypan blue and monoclonal anti-PRMT I was purchased from Sigma. Losartan was freely supplied by Merck. [γ-^{32}P]ATP was obtained from Furui Biological Engineering Institute (Beijing, China). ROS (reactive oxygen species) detection and BCA protein kits were purchased from Beyotime Company (Jiangsu, China). Elisa kits for measurement of MCP-1, TNF-α and IL-8 were obtained from Senxiong Biological Limited Corporation (Shanghai, China). Western blotting kits and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were purchased from KPL. 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 Technology Limited Corporation (Shanghai, China). TRIzol and a gel shift assay system for the determination of NF-κB activity and the probe of NF-κB were obtained from Promega. Monoclonal anti-CCR_2 antibody and FITC-conjugated rabbit anti-rat secondary antibody were obtained from Abcam.

2.2. Cell culture and treatment

THP-1, a monocytic cell line, were cultured in RPMI 1640 medium at a density of up to 1×10^6 cells/ml containing 15% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. HUVECs were cultured in DMEM containing 10% FBS. Monocytic THP-1 were incubated with Ang II (10^{-6} M) for 4 or 24 h. For AT_1 receptor blocker (ARB) losartan or antioxidant PDTC, monocytic THP-1 were pre-incubated with losartan (10 μM) for 1 h or PDTC (10 μM) for 1.5 h, and then exposed to Ang II (10^{-6} M) for 4 or 24 h. In order to clarify the role of ADMA in inflammatory actions of THP-1 induced by Ang II, exogenous ADMA (30 μM) was used in the present study in the absence or presence of PDTC (10 μM).

2.3. Determination of ADMA concentration

The conditioned medium was deproteinized with 5-sulfosalicylic acid. ADMA content in the supernatant was assessed by using HPLC. HPLC was carried out using a Shimadzu LC-6A (Shimadzu Corporation 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 λ^{ex} = 338 nm and λ^{em} = 425 nm on a resolve C_{18} 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.

2.4. Western blotting to determine PRMT protein expression

Subsequent to the different treatments, 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 (80 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-PRMT I antibody (1:1000) at 4 °C overnight. Membranes were washed in TBST for 1 h before incubation for 1 h in goat anti-rabbit secondary antibody (1:1000). Then membranes were washed in TBST for 1 h and developed with enhanced chemiluminescence kit.

2.5. DDAH activity assay

The activity of dimethylarginine dimethylaminohydrolase (DDAH) in THP-1 was estimated by directly measuring the amount of ADMA metabolized by the enzyme. 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 high-performance liquid chromatography (HPLC) as described above. The difference in ADMA concentration between two groups reflected the DDAH activity. For every experiment, DDAH activity of cells in control group is defined as 100%, and DDAH activity in other conditions was expressed as percentage of the metabolized ADMA compared with the control.

2.6. Determination of MCP-1, IL-8 and TNF-α

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

2.7. Reverse transcription-PCR analysis

Total mRNA was extracted from monocytic THP-1 of six-well with TRIzol. First strand cDNA was then synthesized from 4 μg total RNA using reverse transcriptase. DNA sequences of upstream primers and downstream primers and PCR protocols are summarized in Table 1. PCR products were analyzed by 2% agarose gel electrophoresis. The housekeeping gene actin was used as a control to normalize relative changes of $CXCR_2$ and CCR_2 mRNA expression in RT-PCR.

2.8. Static adhesion assays

THP-1 cells were diluted to a final concentration of 10^6 cells/ml and planted in 12-well, then treated with ADMA for 24 h in the

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Table 1
Primer sequences and PCR protocols

Gene		PCR primer sequences	Length (bp)	PCR protocol
$CXCR_2$	UP	5′-CGGAATTCAAATGGAAGATTTTAACATGGAG-3′	417	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	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	94 °C/45 s, 58 °C/45 s, 72 °C/60 s, 28 cycles.
	DP	5′-ATGTCACGCACGATTTCC-3′		

UP, upstream primer; DP, downstream primer.

presence or absence of losartan or PDTC. Thirty minutes before the adhesion assay, monocytic THP-1 (1 ml/well) were added to 12-well plates of HUVEC cells without any treatment. The plates were incubated for additional 30 min at 37 °C. Non-adherent THP-1 cells were carefully removed by washing twice with Hanks balance solution. Adherent THP-1 cells were counted in six high power microscopic fields for each well.

2.9. Determination of ROS

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) in fluorospectro-photometer (F4000, Japan). The cells in six-well were collected and centrifuged, then cell deposit was washed with RPMI 1640 with no serum and incubated with DCFH-DA at 37 °C for 30 min. DCF fluorescence distribution of 20,000 cells was detected fluorospectrophotometically at an excitation wave length of 488 nm and at an emission wave length of 525 nm.

2.10. Electrophoretic mobility shift assay (EMSA)

Monocytic THP-1 were seeded into culture flasks at an optimal density of 10^5 cells/ml (5 ml). After incubation for about 2–3 days, cells were collected 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 and 0.5 mM PMSF) for 15 min on ice. After vortexing, cell lysates were centrifuged by 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 EMSA.

EMSA determining the NF-κB DNA-binding activity was performed by incubating aliquots of nuclear extracts containing 15 μg total protein with γ-^{32}P-labelled double-strand NF-κB specific oligonucleotide probe (sense: 3′-TCAACTCCCCT-GAAAGGGTCCG-5′; antisense: 5′-AGTTGAGGGGACTTTCCCAGGC-3′) by T4 polynucleotide kinase. The labelled probe was purified through sephadex G-25. After 10 min of incubation at room temperature, the mixture was run on a 4% non-denaturing polyacrylamide gel in 0.5× TBE buffers. After electrophoresis, the gels were dried and the DNA–protein complexes were detected by autoradiography.

2.11. Isolation and culture of peripheral blood mononuclear cells and treatment

Venous blood samples (15 ml) were collected in sterile EDTA tubes from clinic healthy subjects. Peripheral blood mononuclear cells (PBMC) were isolated by using Ficoll-Hypaque (1.077) density gradient centrifugation. Finally, cells were suspended to a final concentration of 2×10^6/ml and cultured in RPMI 1640 containing 15% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a humidified atmosphere of 5% CO_2. After culture for 24 h, non-adherent cells were carefully removed. Adherent cells were monocytes and then incubated with exogenous ADMA (30 μM) for 4 h in the presence or absence of losartan (10 μM).

2.12. Flow cytometry

The expression of CCR_2 protein was determined by using a flow cytometer (FACScan, Becton Dickinson, Inc.). Adherent monocytes were collected and resuspended in 100 μl PBS (10^5/ml). Cells were incubated with rat anti-human monoclonal antibody for 1.5 h at a 1:50 dilution and then incubated with FITC rabbit anti-rat secondary antibody for 1 h at a 1:20 dilution on ice.

Table 2
Effect of Ang II or ADMA on the release of IL-8, MCP-1 and TNF-α from monocyte cells and monocytic binding to HUVEC cells

Group	*n*	Cells/hpf	MCP-1 (pg/ml)	IL-8 (pg/ml)	TNF-α (pg/ml)
Control	6	139 ± 20	15.68 ± 2.34	340.9 ± 36.7	58.0 ± 10.3
Ang (10^{-6} M)	4	449 ± 38**	31.12 ± 3.92**	502.6 ± 37.9**	102.3 ± 10.4**
Ang + Los (10 μM)	4	192 ± 15$^{\#\#}$	18.58 ± 3.26$^{\#\#}$	362.5 ± 29.3$^{\#\#}$	54.0 ± 12.0$^{\#\#}$
Ang + PDTC (10 μM)	4	242 ± 23$^{\#\#}$	21.37 ± 2.66$^{\#}$	383.3 ± 29.2$^{\#\#}$	67.7 ± 13.2$^{\#}$
ADMA (30 μM)	4	428 ± 27**	28.95 ± 3.58**	485.4 ± 15.9**	99.5 ± 11.0**
ADMA + PDTC (10 μM)	4	202 ± 25^{++}	19.10 ± 3.20^{+}	388.1 ± 32.2^{++}	65.1 ± 14.1^{++}

Ang (10^{-6} M): incubation of monocytes with Ang II (10^{-6} M) for 24 h. Ang + Los (10 μM): cells were incubated with losartan (10 μM) for 1 h, and then exposed to Ang II (10^{-6} M) for 24 h. Ang + PDTC (10 μM): cells were incubated with PDTC (10 μM) for 1.5 h, and then exposed to Ang II (10^{-6} M) for 24 h. ADMA (30 μM): cells were incubated with ADMA (30 μM) for 24 h. ADMA + PDTC (10 μM): cells were incubated with PDTC (10 μM) for 1.5 h, and then exposed to ADMA (30 μM) for 24 h. Data are expressed as means ± SEM.

** $P < 0.01$ vs. control.
$^{\#}$ $P < 0.05$ vs. Ang (10^{-6} M).
$^{\#\#}$ $P < 0.01$ vs. Ang (10^{-6} M).
$^{+}$ $P < 0.05$ vs. ADMA (30 μM).
$^{++}$ $P < 0.01$ vs. ADMA (30 μM).

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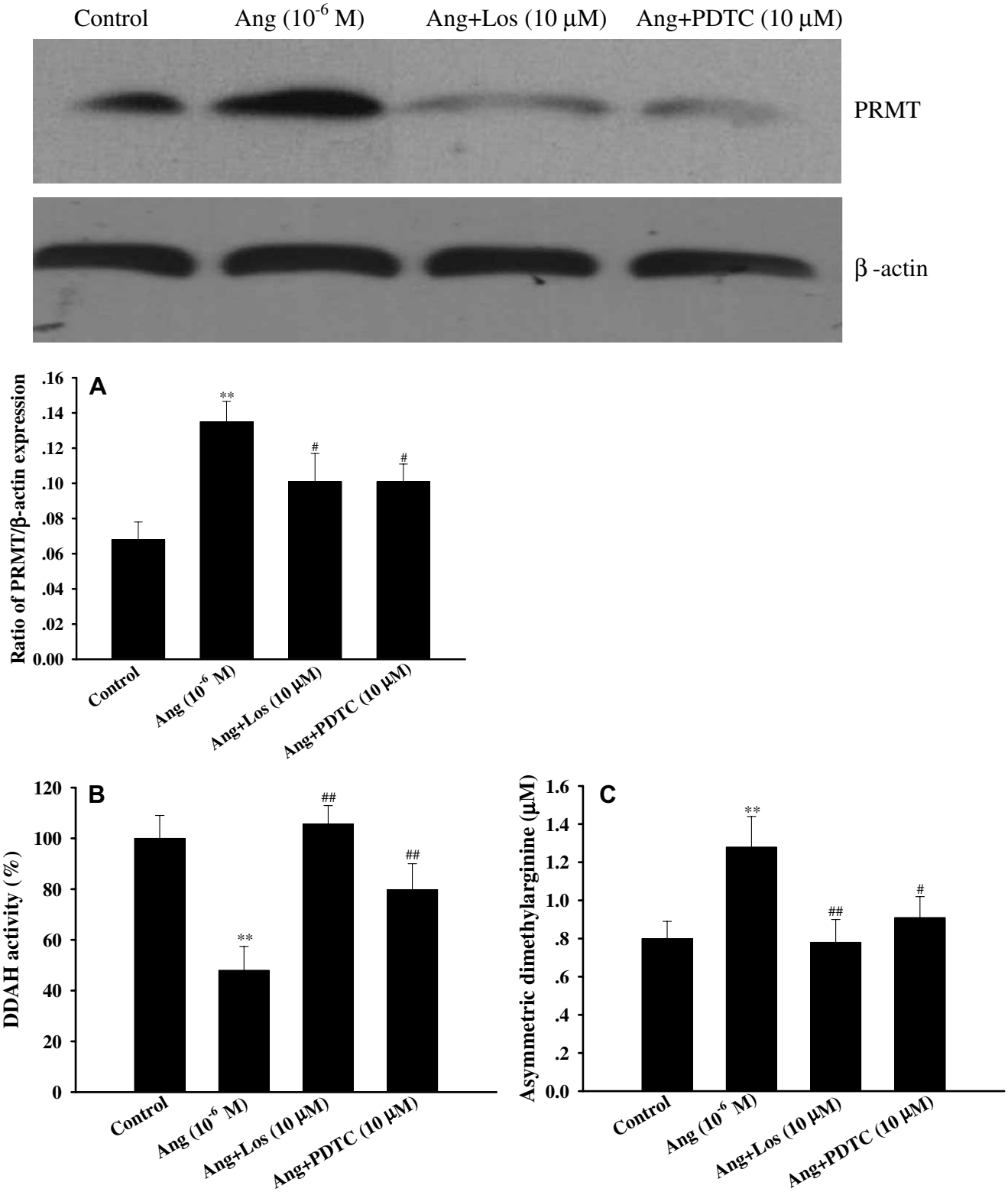

Fig. 1. Effect of Ang II on the system of PRMT/ADMA/DDAH. (A) The effect of ARB losartan or antioxidant PDTC on the expression of PRMT of monocytes treated with Ang II. Ang (10^{-6} M): monocytes were incubated with Ang II (10^{-6} M) for 24 h; Ang + Los (10 μM), Ang + PDTC (10 μM): monocytes were incubated with losartan (10 μM) for 1 h or PDTC (10 μM) for 1.5 h, and then exposed to Ang II (10^{-6} M) for 24 h. (B) The effect of losartan or PDTC on the decreased activity of DDAH induced by Ang II. (C) The effect of losartan or PDTC on the elevated levels of ADMA in the supernatant induced by Ang II. Data are expressed as means ± SEM. $n = 4–6$. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. Ang (10^{-6} M); $^{\#\#}P < 0.01$ vs. Ang (10^{-6} M).

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2.13. 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 if $P < 0.05$.

3. Results

3.1. Monocyte adhesion

Exposure to Ang II (10^{-6} M) for 24 h significantly increased the numbers of monocytic THP-1 binding to HUVEC cells ($P < 0.01$). Pretreatment with losartan or PDTC markedly decreased the numbers of THP-1 cells binding to HUVECs ($P < 0.01$) (Table 2).

Incubation with exogenous ADMA (30 μM) for 24 h also significantly increased adhesion of THP-1 cells to HUVECs ($P < 0.01$). PDTC can markedly attenuated the increased binding numbers of THP-1 cells to HUVECs by ADMA ($P < 0.01$) (Table 2).

3.2. ADMA content in the cultured medium

Incubation of monocytic THP-1 with Ang II (10^{-6} M) for 24 h markedly elevated the level of ADMA in the cultured medium ($P < 0.01$). Pretreatment with losartan (10 μM) or PDTC (10 μM)

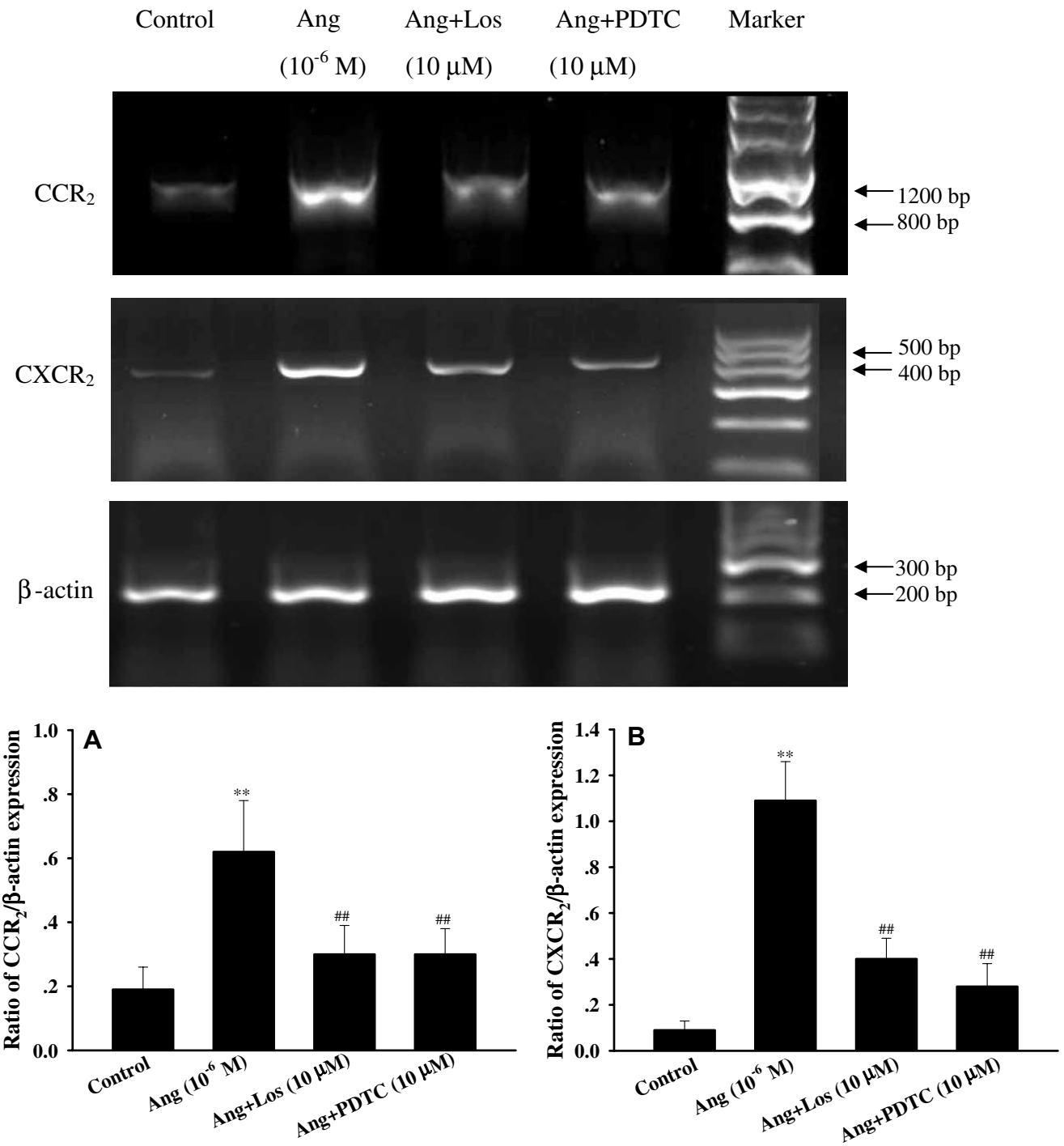

Fig. 2. Effect of Ang II on the expression of CCR_2 (A) and $CXCR_2$ (B) mRNA of monocytes. Monocytes were incubated with Ang II (10^{-6} M) for 4 h in the absence or presence of losartan (10 μM) or PDTC (10 μM). $n = 3$. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. Ang (10^{-6} M).

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significantly decreased the elevated level of ADMA by Ang II ($P < 0.05$, $P < 0.01$) (Fig. 1C).

3.3. Protein expression of PRMT and DDAH activity of THP-1

Exposure to Ang II (10^{-6} M) for 24 h markedly upregulated the protein expression of PRMT and reduced the activity of DDAH of THP-1 ($P < 0.01$). Pretreatment with losartan attenuated the upregulated protein expression of PRMT and decreased activity of DDAH induced by Ang II. Antioxidant PDTC also downregulated the expression of PRMT and improved the decreased DDAH activity by Ang II ($P < 0.01$) (Fig. 1A and B).

3.4. MCP-1, IL-8 and TNF-α content in the cultured medium

After incubation of monocytic THP-1 with Ang II (10^{-6} M) for 24 h, the levels of IL-8, MCP-1 and TNF-α were markedly elevated ($P < 0.01$). Treatment with losartan (10 μM) or PDTC (10 μM) attenuated the elevated levels of IL-8, MCP-1 and TNF-α by Ang II ($P < 0.05$, $P < 0.01$) (Table 2).

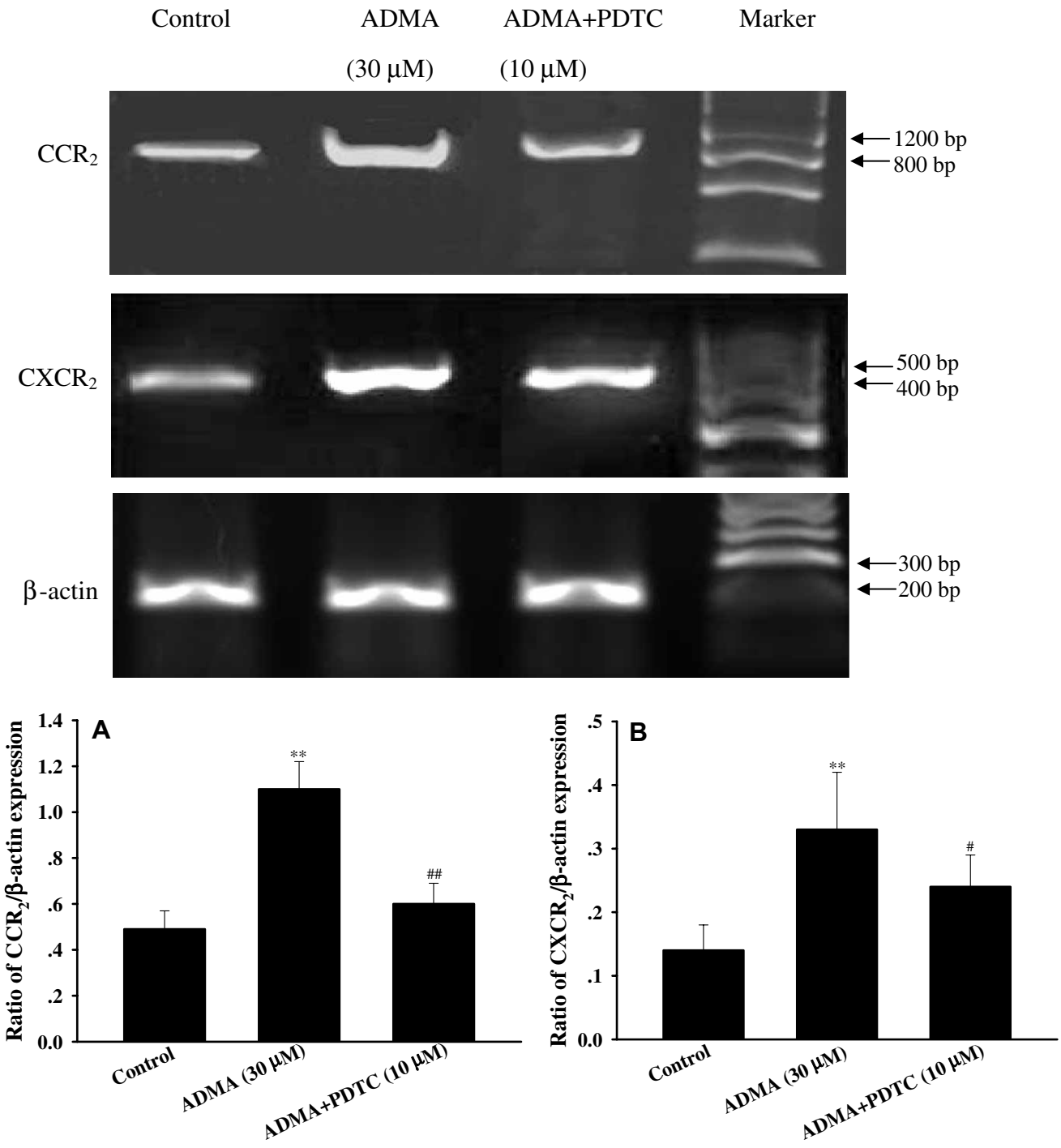

Fig. 3. Effect of ADMA on the expression of CCR_2 (A) and $CXCR_2$ (B) mRNA of monocytes. Monocytes were incubated with ADMA (30 μM) for 4 h in the absence or presence of PDTC (10 μM). $n = 3$. $^{**}P < 0.01$ vs. control; $^{\#}P < 0.05$ vs. ADMA (30 μM); $^{\#\#}P < 0.01$ vs. ADMA (30 μM).

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Similarly, incubation of THP-1 with ADMA (30 μM) for 24 h also increased the levels of IL-8, MCP-1 and TNF-α in the cultured medium ($P < 0.01$). Treatment with PDTC (10 μM) attenuated the elevated levels of IL-8, MCP-1 and TNF-α by ADMA ($P < 0.05$, $P < 0.01$) (Table 2).

3.5. Expression of CCR_2 and $CXCR_2$ mRNA in THP-1

As shown in Fig. 2, incubation of THP-1 cells with Ang II (10^{-6} M) for 4 h upregulated the expression of MCP-1 receptor CCR_2 and IL-8 receptor $CXCR_2$ mRNA. Pretreatment with losartan (10 μM) or PDTC (10 μM) significantly decreased the upregulated expression of CCR_2 and $CXCR_2$ by Ang II.

Similarly, ADMA (30 μM) for 4 h increased the expression of CCR_2 and $CXCR_2$ mRNA. Pretreatment with PDTC (10 μM) markedly downregulated the expression of CCR_2 and $CXCR_2$ mRNA by ADMA (Fig. 3).

3.6. Intracellular ROS generation

After incubation of monocytic THP-1 with Ang II (10^{-6} M) for 4 h, intracellular ROS generation was significantly increased ($P < 0.01$). The facilitative effect of Ang II on ROS production was

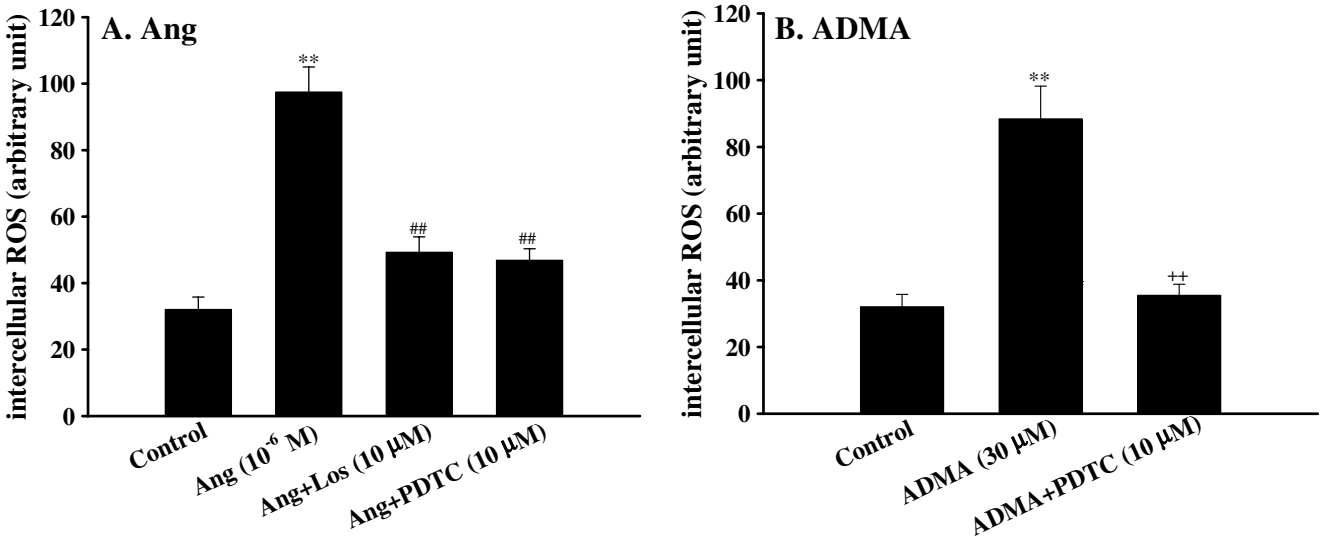

Fig. 4. Effect of Ang II (A) or ADMA (B) on intracellular ROS generation in monocytes. Monocytes were incubated with Ang II (10^{-6} M) or ADMA (30 μM) for 4 h in the absence or presence of losartan (10 μM) or PDTC (10 μM). Data are expressed as means ± SEM. $n = 4–6$. $^{**}P < 0.01$ vs. control; $^{\#\#}P < 0.01$ vs. Ang (10^{-6} M); $^{++}P < 0.01$ vs. ADMA (30 μM).

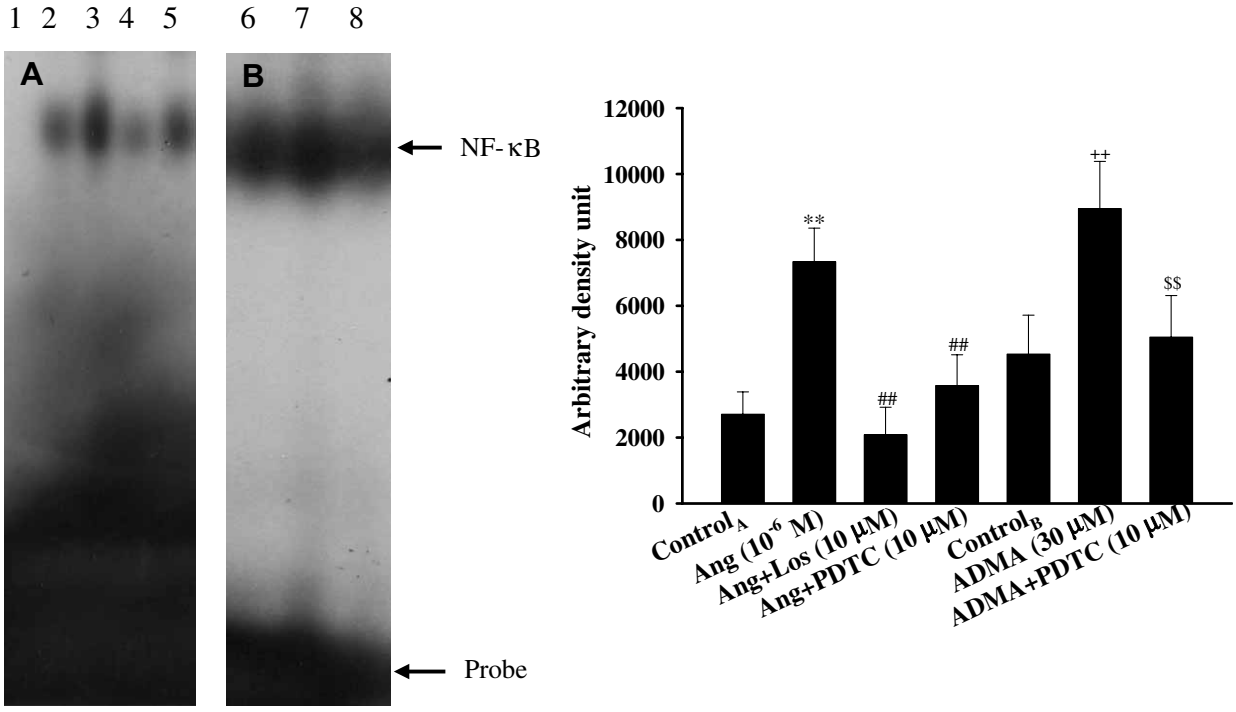

Fig. 5. Effect of Ang II (A) or ADMA (B) on NF-κB DNA-binding activity in cultured monocytes. (A) The effect of losartan (10 μM) or PDTC (10 μM) on the activity of NF-κB in monocyted treated with Ang II (10^{-6} M) for 4 h. (B) The effect of PDTC (10 μM) on the activity of NF-κB in monocyted treated with ADMA (30 μM) for 4 h. 1, negative control; 2, $control_A$; 3, Ang (10^{-6} M); 4, Ang + Los (10 μM); 5, Ang + PDTC (10 μM); 6, $control_B$; 7, ADMA (30 μM); 8, ADMA + PDTC (10 μM). $n = 3$. $^{**}P < 0.01$ vs. $control_A$; $^{\#\#}P < 0.01$ vs. Ang (10^{-6} M); $^{++}P < 0.01$ vs. $control_B$; $^{\$\$}P < 0.01$ vs. ADMA (30 μM).

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markedly inhibited by treatment with losartan or PDTC ($P < 0.01$) (Fig. 4A).

Similarly, exogenous ADMA (30 μM, 4 h) also increased intracellular ROS generation ($P < 0.01$), an effect which was abolished in the presence of antioxidant PDTC ($P < 0.01$) (Fig. 4B).

3.7. The activity of NF-κB inTHP-1

As shown in Fig. 5A, there is no signal in negative control without adding any nuclear proteins. NF-κB was significantly activated by treatment with Ang II (10^{-6} M) for 4 h. The effect of Ang II on NF-κB activity was attenuated by treatment with losartan or PDTC.

In cultured THP-1 cells, NF-κB was also significantly activated by treatment with exogenous ADMA (30 μM) for 4 h. The effect of ADMA on NF-κB activity was inhibited by treatment with PDTC (Fig. 5B).

3.8. The expression of chemokine receptor in isolated human PBMCs

As shown in Fig. 6, the expression of $CXCR_2$ mRNA in isolated human PBMCs was upregulated by treatment with exogenous ADMA (30 μM, 4 h), which was attenuated by incubation with losartan for 1 h ($P < 0.05$). However, due to small number of cells and low content of RNA, the expression of CCR_2 mRNA in PBMCs could not be detected by RT-PCR. Thus, Flow cytometry was used in the present study to determine the protein expression of CCR_2. The results have shown that there is an increasing trend in the protein expression of CCR_2, but there is no significant difference (Fig. 7, $P > 0.05$).

4. Discussion

The major findings in this study are that: (1) there is PRMT/ADMA/DDAH system in THP-1 cells; (2) incubation of monocytic THP-1 with Ang II elevated the levels of ADMA via increasing PRMT expression and decreasing DDAH activity; and (3) ADMA, endogenous or exogenous, increased monocytic adhesiveness via upregulation of chemokines expression by activation of NF-κB pathway.

ADMA, an endogenous inhibitor of NOS, can decrease NO production both in vivo and in vitro. It is known that ADMA is synthesized by PRMTs, which utilize *S*-adenosylmethionine methyl group donor, and hydrolyzed by DDAH, which accounts for most of the

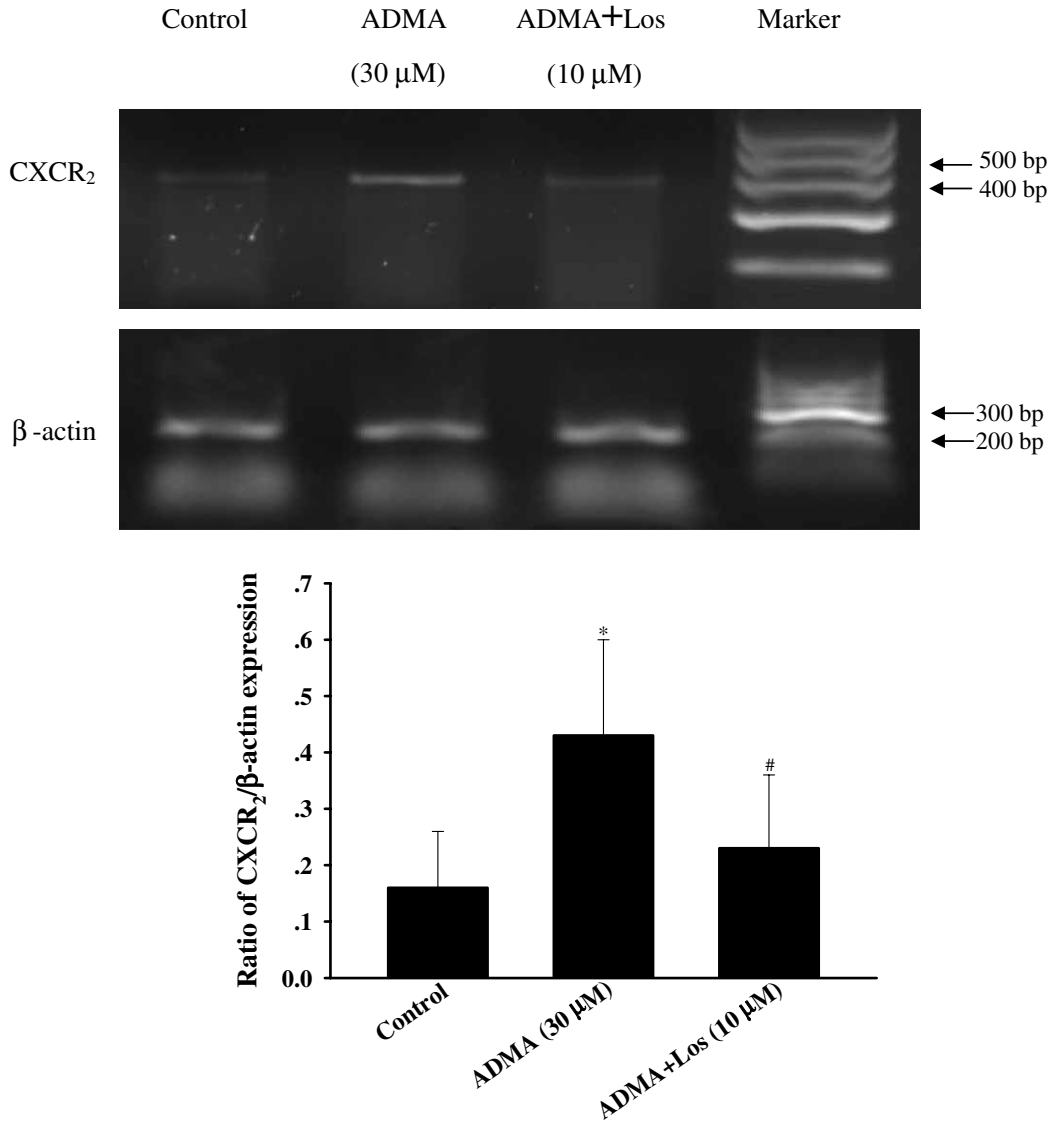

Fig. 6. Effect of ADMA on the expression of $CXCR_2$ mRNA in isolated PBMCs. PBMCs were incubated with ADMA (30 μM) for 4 h in the absence or presence of losartan (10 μM). $n = 6$. $^{*}P < 0.05$ vs. control; $^{\#}P < 0.05$ vs. ADMA (30 μM).

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clearance of ADMA to L-citrulline and dimethylamine. It has been reported that the increase in the level of ADMA by LDL, ox-LDL, TNF-α or shear stress was due to increase in PRMT expression and/or decrease in DDAH activity [13–14]. Elevated plasma ADMA level occurred in rats with chronic kidney disease (CKD) and patients with coronary heart disease (CHD) was associated with reduction of DDAH activity/expression and upregulation of PRMT expression [15]. Over-expressing DDAH gene and improving DDAH activity could attenuate elevated levels of ADMA and ADMA-induced biological effects [16–17]. It was reported that Ang II could increase ADMA production via upregulation of PRMT expression and reduction of DDAH activity in cultured endothelial cells [18] and treatment with ACEI (angiotensin-converting enzyme inhibitor) or ARB (angiotensin receptor blocker) reduced the elevated ADMA level in patients with hypertension, X syndrome and CKD [19–21]. Monocytes are important inflammatory cells contributing to the development of AS, however, the effect of Ang II on the levels of ADMA in monocytes is unknown. Thus, we hypothesized that there is the enzyme system of metabolizing ADMA in monocytes. Our study has shown that the expression of PRMT protein, the levels of ADMA in the medium and the activity of DDAH in untreated monocytic THP-1 could be detected. Thus, the present results first revealed that there exists the PRMT/DDAH system of metabolizing ADMA in THP-1 cells. In addition, incubation of THP-1 with Ang II significantly elevated ADMA levels, accompanied with the increased expression of PRMT protein and the decreased activity of DDAH, which was attenuated by pretreatment with ARB losartan.

Chemokines are classified into four distinct subfamilies as CXC, CC, C and CX_3C. MCP-1, the subfamily of CC chemokines, is most important in the recruitment of monocytes to atherosclerotic lesion. Recently, it was reported that MCP-1 and CCR_2 expression was increased with age in rats [22] and the expression of CCR_2 mRNA in circulating monocytes was increased in hypercholesterolemic patients and hypertensive animals [1,23]. A 4-fold increase in leukocyte CCR_2 expression was detected in aortic lesion area [24] and blockade of the MCP-1/CCR_2 pathway could abolish MCP-1-induced vascular smooth muscle cells (VSMC) invasiveness [22] and prevent vascular inflammation as well as destabilization of established lesions in hyperlipidemic mice [2,25]. IL-8 and its receptor $CXCR_2$, the subfamily of CXC chemokines, profoundly increase stable adhesion of monocytes to endothelium [26]. Previous studies have demonstrated that $CXCR_2$ was strongly expressed on monocytes and macrophages in atherosclerotic lesion [2] and ox-LDL increased the adhesion of monocytes via upregulation of the $CXCR_2$ expression [27]. Blockade or deficiency of $CXCR_2$ could inhibit the recruitment of inflammatory cells to endothelium induced by Ang II [4] and delay the progression of AS in mice [3]. These studies suggest that MCP-1/CCR_2 and IL-8/$CXCR_2$ pathways are important activators of monocytes. In the present study, Ang II increased the release of MCP-1 and IL-8 and the expression of CCR_2 and $CXCR_2$ and induced monocytic adhesion, concomitantly with an increase in the levels of ADMA. Pretreatment with ARB losartan could abolish the effect mediated by Ang II. In order to further confirm the contribution of endogenous ADMA in Ang II-induced monocytic adhesiveness, exogenous ADMA was used in the present study. Nowadays, common opinions consider ADMA as a novel inflammatory factor which contributes to the development of AS. In cultured endothelial cells, ADMA increased the levels of MCP-1

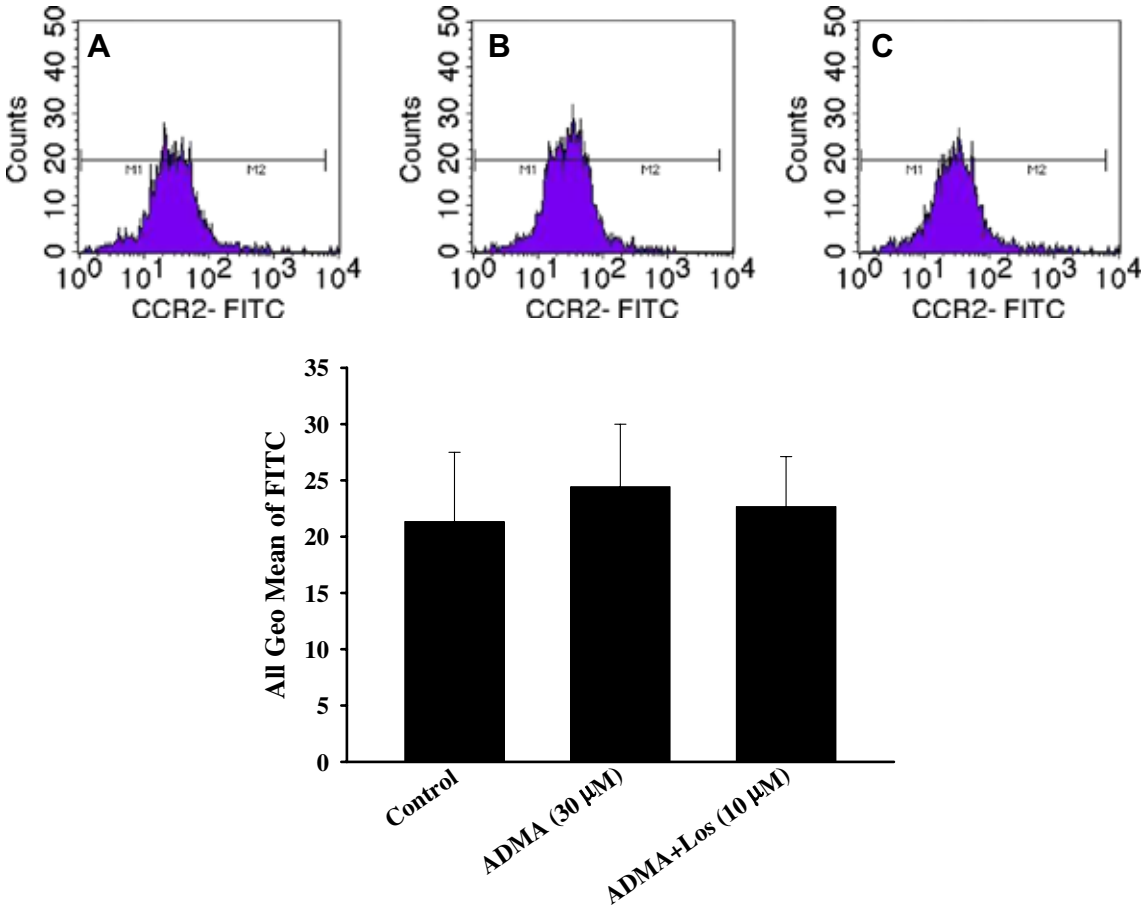

Fig. 7. Effect of ADMA on the protein expression of CCR_2 in isolated PBMCs by flow cytometry. PBMCs were incubated with ADMA (30 μM) for 4 h in the absence or presence of losartan (10 μM). (A) Control; (B) ADMA (30 μM); (C) ADMA + Los (10 μM). $n = 6$.

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and the adhesion of endothelial cells to monocytes, and in cultured macrophages, ADMA increased ox-LDL uptake to facilitate foam cell formation via increasing the expression of scavenger receptor [10,28]. The present results revealed that exogenous ADMA also directly induced a similar effect with Ang II. Incubation of with exogenous ADMA markedly increased the levels of MCP-1and IL-8 and upregulated the expression of CCR_2 and $CXCR_2$ in cultured monocytic THP-1, suggesting that ADMA, endogenous or exogenous, may induce monocytic adhesiveness via activation of chemokines and receptors. In addition, the study further investigated the effect of ADMA on the isolated human PBMCs from healthy controls. The results showed that exogenous ADMA induced a similar effect on the expression of $CXCR_2$. However, the expression of CCR_2 mRNA can not be tested in isolated PBMCs, thus flow cytometry was used to determine the protein expression of surface CCR_2. The data showed us that ADMA had no significant effect on the expression of CCR_2 protein. Too small amount of isolated cells and low expression of surface CCR_2 in monocytes may interpret these differences between two types of monocytes from different sources at least in part.

ROS has recently been as an intracellular signal molecule and plays an important role in the pathogenesis of AS. Under some inflammatory conditions, excessive amount of NO is produced by iNOS in monocytes/macrophages and has a deleterious effect on activity and function of cells. In turn, excessive NO may inactivate DDAH by nitrosylation of the -SH group and lead to accumulation of ADMA [29]. In addition, superoxide may react with excessive NO to generate peroxynitrite, and peroxynitrite has been shown to uncouple NOS and generate more oxygen free radicals. There is abundant evidence that oxidant stress sustained activation of NF-κB. It is known that NF-κB, a classical redox-sensitive proinflammatory transcription factor, plays a pivotal role in atherogenesis by regulating the expression of downstream redox-sensitive genes encoding adhesion molecules and chemokines. It is known that NF-κB is an important link between Ang II and inflammation and Ang II may activate NF-κB in monocytes and endothelial cells by a redox-sensitive mechanism involving NADPH oxidase pathway [30]. Previous study demonstrated that Ang II can elevate the levels of ADMA [18] and the elevated ADMA level is related to elevation of oxidative stress [31]. More direct evidence is that ADMA can induce the senescence and cell apoptosis via increasing the ROS generation in various cells [8–9,32]. ADMA, as a novel proinflammatory factor, could upregulate the expression of redox-sensitive genes and MCP-1 via activating the NF-κB pathway [28]. In the present study, incubation of monocytic THP-1 cells with Ang II elevated the levels of ADMA and endogenous or exogenous ADMA markedly increased intracellular ROS generation and activated NF-κB to switch the release or expression of inflammatory genes such as TNF-α, MCP-1, IL-8, CCR_2 and $CXCR_2$. As MCP-1, IL-8 or TNF-α contains NF-κB binding site on promoter region [33], pretreatment with antioxidant PDTC significantly attenuated Ang II or ADMA-induced oxidant stress and activation of NF-κB, in support of the hypothesis that the ROS/NF-κB pathway is involved in ADMA-induced monocytic activation.

In conclusion, the present study suggests that in monocytic THP-1 cells, ADMA may induce monocytic adhesion via upregulation of chemokine receptor expression, which ROS/NF-κB pathway is involved in. Thus, downregulation of chemokine receptors expression and inhibition oxidant stress might be a promising strategy to prevent atherosclerosis.

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