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Angiotensin II induces NF-κB, JNK and p38 MAPK activation in monocytic cells and increases matrix metalloproteinase-9 expression in a PKC- and Rho kinase-dependent manner

H. Yaghooti[1], M. Firoozrai[2], S. Fallah[2] and M.R. Khorramizadeh[3]

[1]Department of Laboratory Medical Sciences, Faculty of Paramedicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran
[2]Department of Biochemistry, School of Medicine, Iran University of Medical Sciences, Tehran, Iran
[3]Department of Medical Biotechnology, School of Advanced Medical Technologies, Tehran University of Medical Sciences, Tehran, Iran

Abstract

Angiotensin II (ANG II), the main effector of the renin-angiotensin system, is implicated in endothelial permeability, recruitment and activation of immune cells, and also vascular remodeling through induction of inflammatory genes. Matrix metalloproteinases (MMPs) are considered to be important inflammatory factors. Elucidation of ANG II signaling pathways and of possible cross-talks between their components is essential for the development of efficient inhibitory medications. The current study investigates the inflammatory signaling pathways activated by ANG II in cultures of human monocytic U-937 cells, and the effects of specific pharmacological inhibitors of signaling intermediates on MMP-9 gene (*MMP-9*) expression and activity. *MMP-9* expression was determined by real-time PCR and supernatants were analyzed for MMP-9 activity by ELISA and zymography methods. A multi-target ELISA kit was employed to evaluate IκB, NF-κB, JNK, p38, and STAT3 activation following treatments. Stimulation with ANG II (100 nM) significantly increased *MMP-9* expression and activity, and also activated NF-κB, JNK, and p38 by 3.8-, 2.8- and 2.2-fold, respectively ($P < 0.01$). ANG II-induced *MMP-9* expression was significantly reduced by 75 and 67%, respectively, by co-incubation of the cells with a selective inhibitor of protein kinase C (GF109203X, 5 µM) or of Rho kinase (Y-27632, 15 µM), but not with inhibitors of phosphoinositide 3-kinase (wortmannin, 200 nM), tyrosine kinases (genistein, 100 µM) or of reactive oxygen species (α-tocopherol, 100 µM). Thus, protein kinase C and Rho kinase are important components of the inflammatory signaling pathways activated by ANG II to increase *MMP-9* expression in monocytic cells. Both signaling molecules may constitute potential targets for effective management of inflammation.

Key words: Angiotensin II; Signaling; MMP-9; Monocytic cell

Introduction

Angiotensin II (ANG II) is the major bioactive hormone of the renin-angiotensin system and has a central role in controlling cardiovascular homeostasis. Potentially, it can influence all vascular cells including endothelial cells, smooth muscle cells (SMCs), fibroblasts, monocyte/macrophages, and myocytes (1). In pathological states, ANG II mediates hypertension, endothelial dysfunction, and vascular inflammation (2).

Matrix metalloproteinases (MMPs) are specialized enzymes for the degradation of extracellular matrix. In the vessel wall, dysregulated functions of MMPs often lead to impaired endothelial barrier function, infiltration of inflammatory cells, migration and proliferation of SMCs, and the development of atherosclerosis (3). Major causes of vascular remodeling, such as hemodynamic stress, oxidative stress, and inflammatory and vasoactive agents regulate MMP expression and activation (4).

It has been demonstrated that ANG II induces expression of MMP genes (*MMPs*) via different signaling pathways in different cell types (5-7). Stimulation of ANG II type-1 re-

Correspondence: M.R. Khorramizadeh, Department of Medical Biotechnology, School of Advanced Medical Technologies, Tehran University of Medical Sciences, 100 Poursina Ave., Keshavarz Blvd., Tehran, Iran. Fax: +98-21-8899-1118-20.
E-mail: khoramza@sina.tums.ac.ir

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ceptor (AT_1R) activates both G- and non-G protein-related signaling pathways. ANG II acts through Ca^{2+} mobilization, mitogen-activated protein kinases (MAPK), receptor and non-receptor tyrosine kinases, Janus family kinases-signal transducers and activators of transcriptions (JAK-STATs), small G proteins (Ras, Rho, Rac, etc.), plus activation of NADPH oxidase (8,9).

Monocytes are important target cells of ANG II and express both AT_1R and AT_2R. These cells have substantial roles in promoting vascular inflammation, foam cell formation and MMP secretion (10,11). Inhibition of harmful activity of vascular monocytes especially in terms of MMP-9 secretion may be a useful strategy to counteract ANG II-mediated inflammation and remodeling. As a consequence, multiple approaches such as immunosuppression and inhibition of AT_1R signaling mediators have emerged to regulate receptors of ANG II (12).

Elucidation of ANG II signaling pathways and their major intermediates is necessary for the development of efficient therapies against its harmful effects. Here, we measured the effect of ANG II on MMP-9 production in a monocytic cell line and analyzed the activation of inflammatory signaling pathways following ANG II treatment. We further studied the possible association of key signaling kinases (protein kinase C, tyrosine kinases, phosphoinositide 3-kinase, and Rho kinase) and reactive oxygen species (ROS) in ANG II-induced *MMP-9* expression by application of pharmacological kinase inhibitors and α-tocopherol, which was used as an antioxidant to inhibit oxidant-mediated signaling.

Material and Methods

Reagents

Angiotensin II, lipopolysaccharide (LPS) from *Escherichia coli* 055:B5, and α-tocopherol were all purchased from Sigma (USA). Genistein (a specific inhibitor of tyrosine-specific protein kinases), wortmannin (a phosphoinositide 3-kinase inhibitor), GF109203X (a selective inhibitor of protein kinase C), and Y-27632 dihydrochloride (a selective inhibitor of the Rho-associated protein kinase) were purchased from Tocris Bioscience (UK). Cell culture reagents were purchased from Gibco (Invitrogen, USA).

Cell culture

The human monocytic U-937 cell line was obtained from the cell bank of the Pasteur Institute of Iran (NCBI). Cells were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2. Cultures were provided with fresh media every 2-3 days. Experiments were performed following 24 h of starving the cells in a low serum medium (1% FBS). Following centrifugation and resuspension in fresh RPMI containing 1% FBS and antibiotics, the cells were then seeded in 12-well plates at a density of 1×10^6 cells/mL and treated either with ANG II (100 nM) or LPS (100 ng/mL), in combination with inhibitors of signaling kinases or α-tocopherol.

ELISA

To determine the effects of LPS and ANG II stimulation on MMP-9 secretion in the culture media, we measured MMP-9 levels in the supernatants using a commercial sandwich ELISA kit from R&D Systems (USA) following an overnight incubation. Procedures were performed according to the manufacturer protocol.

Zymography

Assessment of MMP-9 activity in the culture media was performed by a zymography method as previously described (13). Briefly, equal amounts of different conditioned media were loaded onto sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) containing 0.1% gelatin type B. Electrophoresis was performed under non-reducing conditions. Gels were washed in 2.5% Triton X-100 for 30 min twice and incubated in substrate buffer (50 mM Tris-HCl, 5 mM $CaCl_2$, 0.01% NaN_3, pH 7.6) for 24 h at 37°C. Gels were then stained with 1% Coomassie blue R250 for 1 h and destained with 45% methanol and 10% acetic acid. Areas of enzymatic activity appeared as clear bands over a dark blue field. Gels were photographed and images were analyzed with the NIH ImageJ software. Data are reported as fold changes compared to control.

Signaling analysis

A Path-Scan ELISA kit (Cell Signaling Technology, USA) was employed to measure phospho-IκB-α, total nuclear factor-kappa B (NF-κB), phospho-NF-κB, phospho-Jun N-terminal kinase (JNK), phospho-p38, and phospho-STAT3 levels following ANG II treatments. After appropriate incubation times (5 min for phospho-IκB-α, 10 min for NF-κB, phospho-NF-κB, and phospho-STAT3, and 15 min for phospho-JNK and phospho-p38), cells were washed with ice-cold phosphate-buffered saline (PBS). Lysis buffer was applied and cells were maintained on ice for 5 min followed by sonication and centrifugation. Supernatants were diluted and loaded to the corresponding ELISA wells. The assay was performed according to the manufacturer protocol. In order to dilute the lysates properly and to assure that equal amounts of protein were loaded in the wells, lysates were assayed for their protein contents using Lowry's reagents (Pierce, USA) (14).

Gene expression analysis

In order to evaluate the efficacy of signaling inhibition during *MMP-9* expression, we used the pharmacological inhibitors of protein kinase C (PKC), phosphoinositide 3-kinase (PI3K), Rho kinase (ROCK), tyrosine kinases, and α-tocopherol as an antioxidant to identify oxidant-dependent signaling. Prior to ANG II treatment, cells were pre-incubated with genistein (100 µM), wortmannin (200 nM), GF109203X

(5 µM), or Y-27632 (15 µM) for 1 h. For α-tocopherol (100 µM), pre-incubation was performed for 12 h. *MMP-9* was analyzed for expression as a target gene.

RNA was isolated from 1 x 10^6 cells using the FAST Pure RNA extraction kit (Takara Bio Inc., Japan). Synthesis of cDNA was carried out at 37°C for 30 min using the Primescript RT enzyme from Takara. Real-time PCR amplifications were performed using specific primer pairs and Taqman probes (Alpha DNA, Canada) that are reported in the RTPrimer data base with the following sequences: *MMP-9* forward 5′-ACC TCG AAC TTT GAC AGC GAC-3′, reverse 5′-GAG GAA TGA TCT AAG CCC AGC-3′, probe FAM5′-TGC CCG GAC CAA GGA TAC AGT TTG TT-3′TAMRA, *GAPDH* forward 5′-GTG AAC CAT GAG AAG TAT GAC AAC-3′, reverse 5′-CAT GAG TCC TTC CAC GAT ACC-3′, and probe FAM5′-CCT CAA GAT CAT CAG CAA TGC CTC CTG-3′TAMRA. Reactions were carried out using a Rotor-gene 6000 thermocycler (Corbett Research, Australia) in a total volume of 20 µL, containing 2 µL cDNA, 10 µL Premix Ex-Taq (Takara Bio Inc.) and 0.2 µM of each primer and probe. Amplification efficiencies were validated and normalized against *GAPDH* expression. Relative quantification analysis was performed using the Rotor-gene 6000, version 1.7, software based on ΔΔCt calculations.

Cytotoxicity assay

To determine potential cytotoxic effects of the drugs used, supernatants were assayed for lactate dehydrogenase (LDH) activity using an LDH-based cytotoxicity detection kit (Roche, Germany). Procedures were performed according to the manufacturer protocol. Cytotoxicity, as percent, was determined by comparing LDH levels in treated and control cells following 18 h of incubation.

Statistical analysis

Data are reported as means ± SEM of three or four independent measurements. Data for the various groups were analyzed by ANOVA followed by the Tukey multiple comparison test. Differences with P values of <0.05 were considered to be significant.

Results

Angiotensin II increased MMP-9 levels in U-937 cell conditioned media

To determine the effects of ANG II on MMP-9 secretion from monocytic cells we employed ELISA and zymography techniques. As shown in Figure 1A, stimulation of the cells with ANG II for 24 h caused a 1.4-fold increase from 328 ± 25 to 787 ± 29 pg/mL in MMP-9 levels (P < 0.01).

For further confirmation of the increase of MMP-9, the gelatinolytic activities of conditioned media were also measured (Figure 1B). Cultured U-937 cells constitutively secreted MMP-2 and MMP-9 into the incubation medium as demonstrated by gelatin zymography. Similar to the

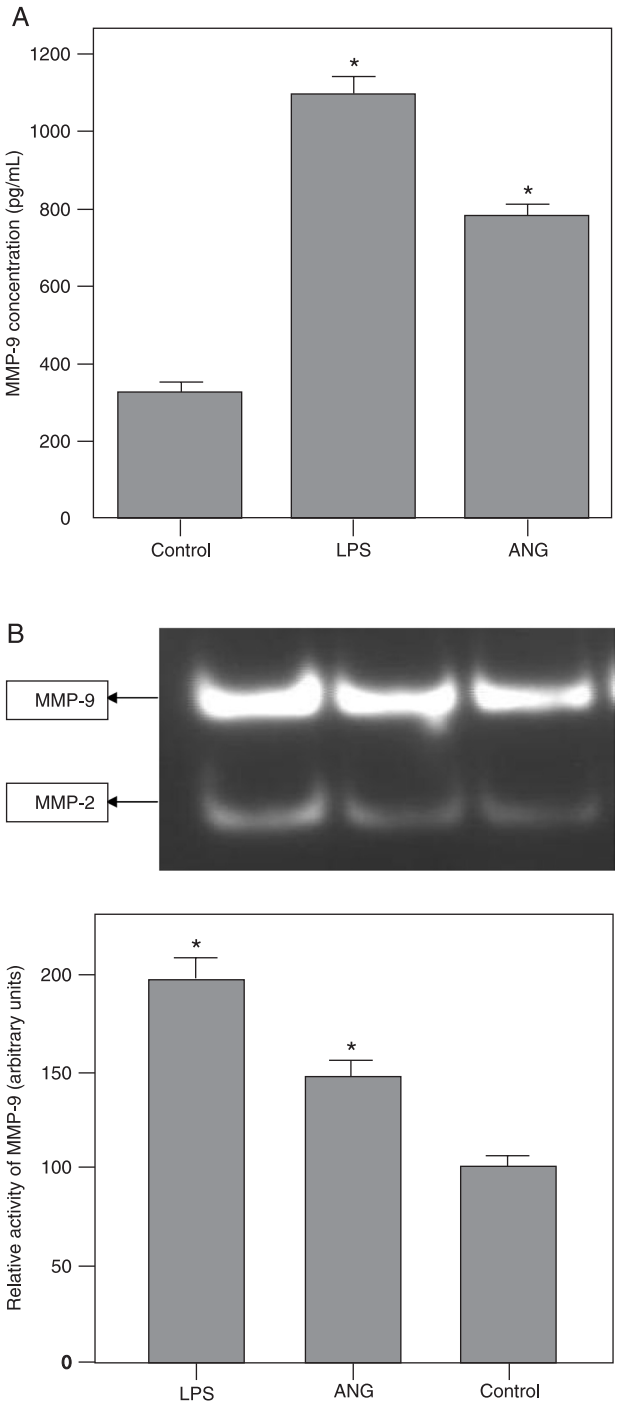

Figure 1. Measurement of matrix metalloproteinase-9 (MMP-9) levels in the cell-free conditioned media by ELISA (A) and zymography (B). Serum-starved cells (1 x 10^6) were seeded in 12-well plates and treated either with lipopolysaccharide (LPS, 100 ng/mL) or angiotensin II (ANG II, 100 nM) for 24 h. A representative zymogram is shown in the upper panel of Figure 1B. MMP-9 concentrations and relative gelatinolytic activities are reported as means ± SEM (N = 4). *P < 0.05 *vs* control (ANOVA followed by the Tukey test).

ELISA results, ANG II significantly increased the activity of MMP-9 in the supernatant up to 48% ($P < 0.05$). As expected, LPS, which was used as a positive control, substantially increased MMP-9 secretion by 2.4-fold from 328 ± 25 to 1116 ± 48 pg/mL ($P < 0.001$).

NF-κB, JNK and p38 were differentially activated following angiotensin II stimulation

In order to investigate the activation of key pro-inflammatory signaling mediators after ANG II challenging, a commercial Path-scan ELISA kit was employed. As shown in Figure 2, levels of phospho-STAT3 and total NF-κB remained unchanged following exposure to ANG II. This indicates that activation of STAT3 was not implicated in MMP-9 up-regulation. ANG II significantly increased NF-κB, JNK, and p38 phosphorylation within 15 min (3.8-fold from 1.0 ± 0.18 to 4.8 ± 0.33, 2.8-fold from 1.0 ± 0.15 to 3.8 ± 0.28, and 2.2-fold from 1.0 ± 0.13 to 3.2 ± 0.24 in arbitrary units, respectively, relative to control, $P < 0.01$). Under non-stimulated conditions, the phosphorylated levels of these intermediates were not significant. IκB-α is an intracellular inhibitor of NF-κB that dissociates from it following phosphorylation, leading to NF-κB activation through a classical pathway. ANG II treatment partially increased IκB phosphorylation up to 0.9-fold from 1.0 ± 0.09 to 1.9 ± 0.35 in arbitrary units, a value that was not statistically significant.

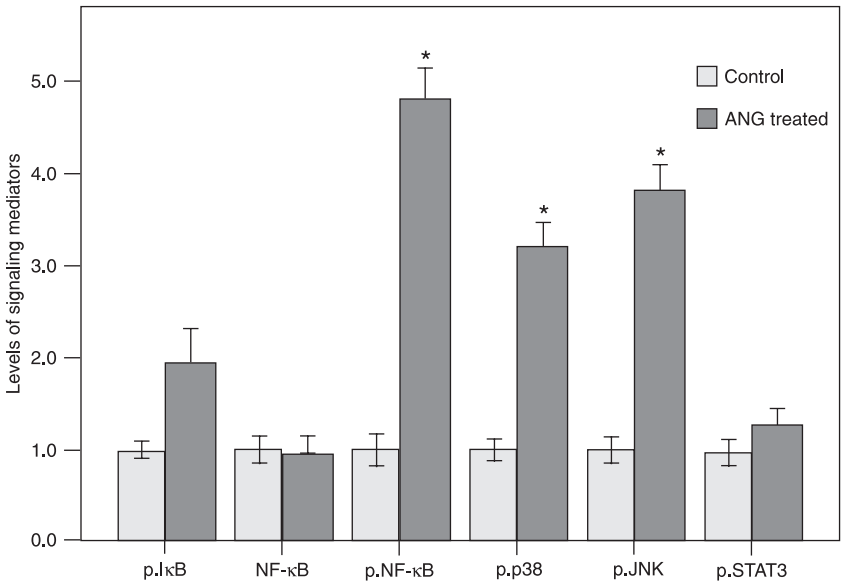

Figure 2. Activation levels of inflammatory signaling mediators in response to angiotensin II (ANG II). U-937 cells were cultured in a low-serum medium at a density of 1 x 10^6 cells/mL and treated with 100 nM ANG II. Following appropriate incubation times (5 min for p.IκB, 10 min for NF-κB, p.NF-κB, and p.STAT3 or 15 min for p.JNK and p.p38), cells were lysed and lysates were assayed for protein content. Signaling mediators in cell lysates were measured using a multi-target ELISA kit. Lysates were measured at a protein concentration of 0.5 mg/mL. p.IκB = phospho-intracellular inhibitor of total nuclear factor-kappa B (NF-κB); p.NF-κB = phospho-NF-κB; p.p38 = phospho-p38; p.JNK = phospho-Jun N-terminal kinase; p.STAT3 = phospho-STAT3. Data are reported relative to the non-stimulated levels of each analysis as means ± SEM (N = 3). *$P < 0.01$ *vs* control (ANOVA followed by the Tukey test).

Angiotensin II-induced *MMP-9* expression is PKC and Rho kinase dependent

Real-time PCR was used to analyze *MMP-9* expression in response to ANG II and inhibitor drug treatments. As shown in Figure 3, incubation of U-937 cells with ANG II for 12 h induced an increase (from 1.0 ± 0.1 to 2.6 ± 0.23 in arbitrary units) in *MMP-9* expression ($P < 0.05$). Pre-treatment of the cells

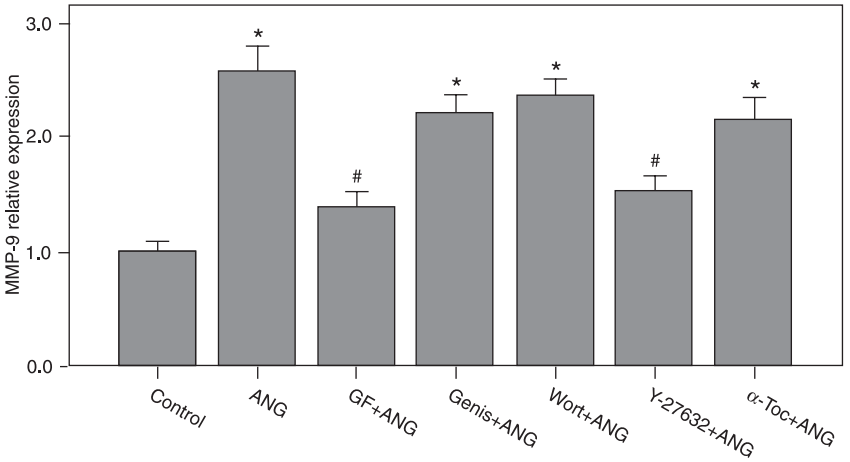

Figure 3. Inhibitory effects of α-tocopherol and kinase inhibitors on matrix metalloproteinase-9 (*MMP-9*) expression in response to angiotensin II (ANG II). Serum-starved cells (1.0 x 10^6) were pre-incubated with α-tocopherol (100 µM, 12 h), GF109203X (5 µM, 1 h), genistein (100 µM, 1 h), wortmannin (200 nM, 1 h), or Y-27632 (15 µM, 1 h), followed by ANG II treatment (100 nM) and incubated for 12 h. GF = GF109203X; Genis = genistein; Wort = wortmannin; α-Toc = α-tocopherol. Relative *MMP-9* expression data are reported as means ± SEM (N = 3). *$P < 0.05$ *vs* control; #$P < 0.05$ *vs* Ang II-treated cells (ANOVA followed by the Tukey test).

with GF109203X, genistein, wortmannin, Y-27632, or α-tocopherol before ANG II stimulation caused 75, 23, 13, 67, and 26% decreases in *MMP-9* expression, respectively, compared to the cells treated with ANG II alone. Among the inhibitors, GF109203X and Y-27632 significantly inhibited the stimulatory effect of ANG II ($P < 0.05$). These data demonstrate that the stimulatory effect of ANG II on *MMP-9* expression is mediated in part by PKC and Rho kinase activation.

To rule out any specific effects of the inhibitor drugs, α-tocopherol and dimethyl sulfoxide, on *MMP-9* expression, supplementary experiments were performed. U-937 cells that were treated with the inhibitor drugs did not show any significant differences in MMP-9 mRNA level compared to untreated controls (data not shown).

Treatments established no cytotoxicity

There were no significant differences in LDH levels between the controls and cultures treated with inhibitors in combination with ANG II. Cytotoxicity percentages for ANG II, GF109203X, genistein, wortmannin, Y-27632, and α-tocopherol were 2.2, 5.6, 4.2, 2.4, 4.0, and 1.0%, respectively.

Discussion

Vascular inflammation is evident in all conditions of ANG II-induced vascular damage. Recruitment of monocytes and neutrophils and secretion of cytokines and MMPs are hallmarks of ANG II-mediated inflammation (15). Here, we employed U-937 cells, a human monocytic cell line. These cells have been used to investigate modulation of cytokines, expression of inflammatory genes, and the corresponding signaling in response to various agents (16). Yuan et al. (17) used U-937 cells to evaluate AT_1R blockade to inhibit IL-1ß production. U-937 cells secrete considerable amounts of MMP-9 that could serve as a positive control for MMP-9 secretion.

In the present study, the data obtained from the ELISA, zymography, and gene expression experiments support the conclusion that ANG II stimulation significantly increased MMP-9 secretion. *MMPs* are considered to be inflammatory genes that are induced via different signaling cascades in various cell types. Based on the results of Path-scan ELISA, ANG II stimulation triggers the activation of NF-κB, JNK, and p38. Activation of these mediators may account for the up-regulation of MMP-9 in response to ANG II. Phosphorylated JNK activates *c-jun* expression, which, together with *c-fos*, forms active transcription factor AP-1. The *MMP-9* promoter region contains the binding elements for NF-κB, AP-1, Ets-1, and STAT transcription factors (18). It has been demonstrated that ANG II stimulates the secretion of MMP-1, MMP-3, and MMP-9 in SMCs via NF-κB and AP-1 activation in a redox-sensitive manner (19). In cardiac myocytes, ANG II signals for a mechanical stress to induce *MMP-2* and *MMP-14* expression via the JNK-STAT pathway (6). Luchtefeld and colleagues (7) have reported that ANG II-induced MMP-2 secretion is NADPH oxidase-dependent in endothelial cells. These findings suggest that ANG II induces multiple MMPs within the vascular wall, which, when combined, can enhance vascular inflammation, SMC migration and neointima formation.

The Path-scan ELISA detects the phosphorylated-p65-subunit (RelA) of the NF-κB complex. Phosphorylation of p65 suggests that NF-κB is activated through the classical pathway via IκB phosphorylation and degradation. However, in our experiments ANG II treatment did not increase IκB phosphorylation significantly. The lack of correlation may partly be explained by fast proteosomal degradation of phosphorylated IκB. Furthermore, alternative mechanisms could be proposed to account for NF-κB activation such as MAPK, Rho kinase and calcium-dependent pathways (20,21).

Pre-treatment with inhibitors of main up-stream kinases and ROS was performed to investigate their possible association in *MMP-9* expression in response to ANG II treatment. *MMP-9* expression was significantly increased in response to the ANG II challenge and this effect was significantly inhibited by GF109203X and Y-27632. Therefore, it could be postulated that PKC and Rho kinase play pivotal roles in mediating the effects of ANG II. It has been demonstrated that ANG II activates NADPH oxidase, a major source of ROS, via activation of PKC (22). PKC participates as an effector in the Ras/Raf/MEK/ERK pathway (23). Implication of PKC in Rho and JNK activation has also been demonstrated previously (24). ANG II-induced activation of JNK and p38 MAPK depends on $G\alpha_{12/13}$-mediated activation of Rho/Rho kinase (25). ANG II induces membrane NADPH oxidase in SMCs to produce ROS. These reactive species have been shown to be potent intracellular second messengers. Activation of p38 MAPK, Akt/PKB, Src, and transcription factors such as NF-κB, AP-1, and Nrf-2 are redox sensitive (26,27). Zalba et al. (28) have reported that activation of NADPH oxidase in human monocytes promotes MMP-9 secretion. In our study, α-tocopherol, as an antioxidant, showed no significant inhibitory effect on the ANG II-stimulated *MMP-9* expression. The activity level of NADPH oxidase varies in different cell types of the cardiovascular system. The functionality of NADPH oxidase and the nature of the target gene should be taken into account when interpreting the outcome of antioxidant pre-treatment. Further studies with the application of other antioxidant drugs, and with extended incubation times, are required to reach a firm conclusion about the benefits of antioxidant therapy.

The observations reported here provide further evidence that ANG II can activate multiple signaling pathways in monocytes-macrophages, as well as increased *MMP-9* expression and secretion. Importantly, the data show that not all signaling molecules activated by ANG II are implicated

in its ability to enhance *MMP-9* expression in these cells. Indeed, the evidence points to relevant roles of PKC and Rho kinase in the signaling pathways leading to ANG II-induced *MMP-9* expression. We, thus, propose that both of these signaling molecules might be implicated in the monocytic-dependent deleterious effects of ANG II, and that inhibitors, which selectively target PKC and Rho kinase, may provide an effective management of inflammation.

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