Angiotensin II stimulates MCP-1 production in rat glomerular endothelial cells via NAD(P)H oxidase-dependent nuclear factor-kappa B signaling

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Angiotensin II (Ang II) plays a crucial role in the pathogenesis of renal diseases. The objective of the present study was to investigate the possible inflammatory effect of Ang II on glomerular endothelial cells and the underlying mechanism. We isolated and characterized primary cultures of rat glomerular endothelial cells (GECs) and observed that Ang II induced the synthesis of monocyte chemoattractant protein-1 (MCP-1) in GECs as demonstrated by Western blot. Ang II stimulation, at concentrations ranging from 0.1 to 10 µm, of rat GECs induced a rapid increase in the generation of reactive oxygen species as indicated by laser fluoroscopy. The level of p47^{phox} protein, an NAD(P)H oxidase subunit, was also increased by Ang II treatment. These effects of Ang II on GECs were all reduced by diphenyleneiodonium (1.0 µm), an NAD(P)H oxidase inhibitor. Ang II stimulation also promoted the activation of nuclear factor-kappa B (NF-κB). Telmisartan (1.0 µm), an AT_1 receptor blocker, blocked all the effects of Ang II on rat GECs. These data suggest that the inhibition of NAD(P)H oxidase-dependent NF-κB signaling reduces the increase in MCP-1 production by GECs induced by Ang II. This may provide a mechanistic basis for the benefits of selective AT_1 blockade in dealing with chronic renal disease.

Key words: Ang II; Glomerular endothelial cells; MCP-1; Reactive oxygen species; AT_1 receptor blocker; NF-κB

Introduction

Activation of the intrarenal renin-angiotensin system is a characteristic feature of the development and progression of chronic kidney disease (1). Angiotensin II (Ang II), the main effector of the renin-angiotensin system, is implicated in the pathogenesis of renal diseases (2,3). Ang II initiates its effects by interacting with at least two pharmacologically distinct subtypes of cell surface receptors, AT_1 and AT_2. The major functions of Ang II in the cardiovascular system are mediated by the AT_1 receptor (4,5).

An intriguing concept has emerged proposing that Ang II not only mediates intraglomerular hypertension but also behaves as a pro-inflammatory and growth-stimulating factor, contributing to renal hypertension and sclerosis

(6,7). Ang II has growth-stimulating properties with respect to different renal cell types. However, possible inflammatory effects of this vasoactive peptide on endothelial cells isolated from the glomerular microvasculature have not been systematically investigated. Glomerular endothelial cells (GECs), being exposed to the bloodstream, are the target of many factors that may alter their functional state and induce them to release inflammatory cytokines.

Inflammatory processes involve both the synthesis of inflammatory cytokines, such as monocyte chemoattractant protein-1 (MCP-1), and the activation of their distinct signaling cascades. Recent findings have suggested that Ang II activates intracellular signaling processes, including the polyol pathway and generation of reactive oxygen species (ROS) (8,9). In cell systems, a major source of

ROS derives from the membrane-bound NAD(P)H oxidase system. Activation of the NAD(P)H oxidase system requires the participation of p47^{phox}, one of the NAD(P)H oxidase subunits, which plays a central role in the scenario of NAD(P)H oxidase activation (10). Activation of nuclear factor-kappa B (NF-κB) is involved in the expression of pro-inflammatory genes (11).

In the present study, we investigated whether Ang II activates the NF-κB cascade by ROS and whether the ROS-dependent mechanism may be involved in Ang II-induced MCP-1 formation. We also determined whether telmisartan, an AT$_1$ receptor blocker, and diphenyleneiodonium (DPI), an inhibitor of NAD(P)H oxidase, modulate the endothelial inflammation and oxidative cell damage induced by Ang II-dependent stimuli in cultured rat GECs.

Material and Methods

Isolation and culture of rat glomerular endothelial cells

GECs were obtained from isolated, collagenase-treated rat glomeruli. Briefly, glomeruli were harvested from Sprague-Dawley rats weighing 100 to 120 g by filtration with 4°C phosphate-buffered saline (PBS) through an 80-, 100-, and 200-μm nylon mesh (12). Those retained in the sieve were collected by centrifugation (4°C, 700 *g*), incubated with 0.1% collagenase (type IV, Sigma, USA) for 40 min at 37°C under constant gentle shaking and then washed twice in Dulbecco's modified essential medium (DMEM, pH 7.4; Gibco, USA) with high glucose (25 mmol/L) concentration. The culture medium was supplemented with 20% fetal bovine serum (Gibco), 100 U/mL penicillin, 100 μg/mL streptomycin, 10 Mm HEPES, and 10 U/mL heparin (Sigma). Three to 5 μL of cell suspension was then plated onto 25-cm^2 tissue culture flasks previously coated with 1% gelatin and grown to confluence at 37°C in a 5% CO$_2$ humidified incubator. The cell medium was not changed for 3 days and then changed every other day.

Seventy-five to 85% confluence cells were subcultured at 1:2 at 7-day intervals. GECs were identified by their typical cobblestone morphology and by immunofluorescence staining with monoclonal antibodies against Von Willebrand factor (Immunotech, France). Cells between passages 3 to 6 were used for all experiments. The cells were growth-arrested in serum-free DMEM for 24 h to synchronize cell growth prior to Ang II stimulation. GECs were pretreated with 1.0 μM telmisartan or 1.0 μM DPI for 30 min prior to Ang II treatment.

Western blot analysis

GECs were stimulated with Ang II at concentrations ranging from 0.1 to 10 μM for the times indicated in the legends to the Figures. The proteins of GECs were separated by SDS-PAGE, transferred to membranes and probed with primary antibodies (1:500) against MCP-1 (Biolegend, USA) and the NAD(P)H oxidase subunit P47^{phox} (Sigma). After washing, the membrane was incubated with horseradish peroxidase-conjugated second antibodies (1:2000). After successive washes, the protein bands were detected by an enhanced chemiluminescence method (Pierce, USA). The intensities of the immunoblot band were quantified using the Quantity One software (Bio-Rad, USA).

Reactive oxygen species detection

Generation of ROS was monitored by oxidation of carboxy-dichlorofluorescein diacetate (DCFH-DA) to DCF by laser fluoroscopy at 488/525 nm (Fluoroscan, Labsystems, Japan) and 4 x 104 GECs per well were plated under serum-free conditions and incubated with inhibitors. DCFH-DA (10 μM) was added before 10 μM Ang II stimulation, and developing fluorescence was determined. Each experiment was performed in triplicate.

Immunohistochemistry

GECs grown in 24-well plates (Invitrogen, USA) were washed three times, fixed in 2% paraformaldehyde for 15 min and incubated at 37°C for 1 h with PBS containing 3% BSA (Sigma) to block non-specific binding sites. GECs were then incubated with primary antibodies for von Willebrand factor and NF-κB P65 (1:200; Immunotech; Santa Cruz Biotechnology, USA). After washing, a second antibody conjugated with biotin or alex-586 was applied and mounted with DAPI (Vector, USA), followed by 0.3% H$_2$O$_2$, ABC (Vector). Images were obtained with a light and fluorescence microscope (Nikon, Japan).

Statistical analysis

Data are reported as means ± SEM. Results were analyzed by one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni test. A P value <0.05 was considered to be statistically significant.

Study design

GECs were randomly assigned to untreated control (normal), Ang II-treated, telmisartan-treated, and DPI-treated groups.

In the control group, GECs were incubated with serum-free DMEM. In the Ang II-treated group, GECs were treated with exogenous Ang II at concentrations ranging from 0.1 to 10 μM, with treatment times ranging from 6 to 12 and 24 h. In the other two groups, GECs were pretreated with the specific AT$_1$ receptor blocker telmisartan (1.0 μM) and with

the NAD(P)H oxidase inhibitor DPI (1.0 µM) for 30 min prior to exposure to Ang II for 24 h.

Results

Isolation and characterization of rat glomerular endothelial cells

Phase-contrast microscopy of vital cells in a culture flask demonstrated the typical cobblestone appearance of GECs grouped in growth islands (Figure 1A). Figure 1B shows the immunofluorescence expression of von Willebrand factor, a typical feature of endothelial cells.

Effect of Ang II on MCP-1 formation in rat glomerular endothelial cells

The addition of 0.1 to 10 µM Ang II to the culture medium for rat GECs effectively stimulated the production of MCP-1. Western blot analysis revealed that exposure of GECs to Ang II stimulated MCP-1 formation over a 24-h period. A small increase in MCP-1 was detectable with 0.1 µM Ang II treatment at 12 h. A 10-fold higher concentration, 1.0 µM Ang II, stimulated MCP-1 production even more (Figure 2A). The dose-response effect of Ang II on MCP-1 formation reached a plateau at 10 µM. At a fixed dose of 10 µM Ang II, the stimulation of MCP-1 production was demonstrable at 6 h. Additional time of exposure to Ang II did not appear to increase the quantity of MCP-1 in the rat GECs any further, but the effect of Ang II persisted over the remainder of the 24-h incubation. The effect of Ang II on MCP-1 formation was reduced by pretreatment with 1.0 µM telmisartan and 1.0 µM DPI (Figure 2B).
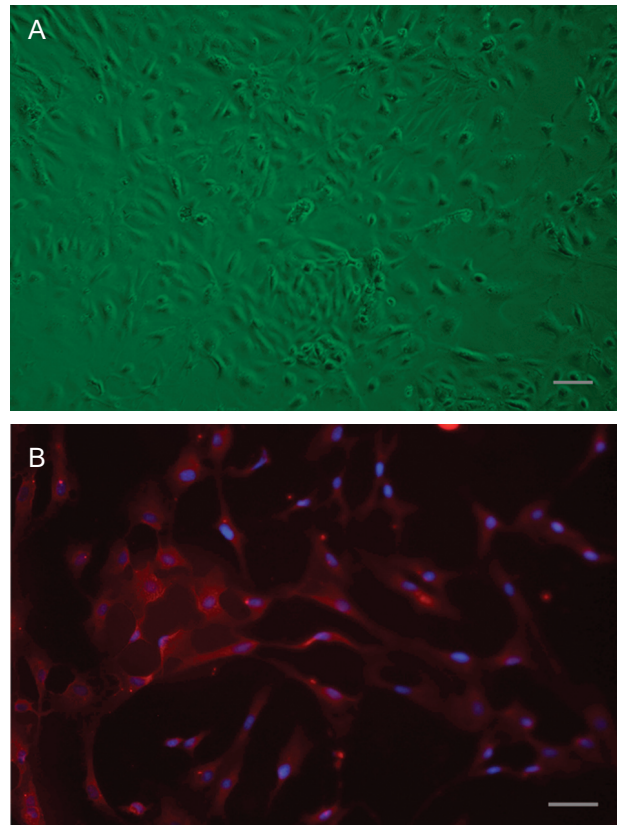
Figure 1. Light microscopy and immunohistochemical characterization of glomerular endothelial cells (GECs). *A*, Phase-contrast picture of GECs in culture shows typical cobblestone appearance with growth in the form of islands (original magnification 100X). *B*, Positive (cytoplasm and membrane) staining for von Willebrand factor (original magnification 100X; bars = 100 µm).

Figure 2. Angiotensin II (Ang II) stimulates the production of MCP-1 in rat GECs. The effect of Ang II on MCP-1 formation was reduced by pretreatment with TEL and DPI. *A*, Expression of MCP-1 protein evaluated by Western blot analysis. Cells were incubated with 10 µM Ang II$_1$, 1.0 µM Ang II$_2$, and 0.1 µM Ang II$_3$ for 12 h (left) and with 10 µM Ang II for 6, 12, and 24 h (right). The dose-response effect of Ang II on MCP-1 formation reached a plateau at 10 µM (0.53 ± 0.03, N = 3). *B*, Inhibition of NAD(P)H oxidase with DPI (1.0 µM) abolished the Ang II-induced MCP-1 synthesis, as well as the antagonist of the AT$_1$ receptor TEL (1.0 µM). MCP-1 = monocyte chemoattractant protein-1; GECs = glomerular endothelial cells; DPI = diphenyleneiodonium; TEL = telmisartan. *P < 0.01 *vs* control group; +P < 0.05 *vs* Ang II group (Bonferroni test).
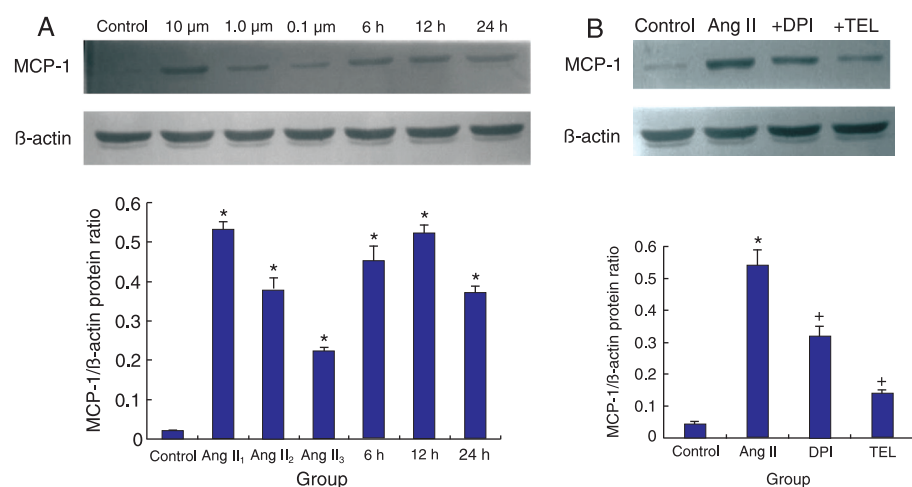
Effect of Ang II on p47^{phox} protein level

Western blot analysis revealed that 10 μM Ang II induced p47^{phox} synthesis. The level of p47^{phox} protein was increased in a time-dependent way (Figure 3A), with a peak being reached at 8 h. This increase was reduced by 1.0 μM DPI and 1.0 μM telmisartan treatment (Figure 3B).

Effect of Ang II on reactive oxygen species generation

Laser fluoroscopy experiments revealed an Ang II-dependent increase in ROS formation peaking at 15 min. The inhibitor of NAD(P)H oxidase DPI and the AT$_1$ receptor antagonist telmisartan both reduced Ang II-induced ROS

generation in GECs. Serum-free controls showed no increase in ROS generation (Figure 4).

Ang II activates NF-κB signaling

Ang II stimulation increased the translocation of the NF-κB P65 subunit from the cytoplasm to the nucleus and promoted the activation of NF-κB in rat GECs. Immunofluoroscopy experiments revealed the expression of the NF-κB P65 immunofluorescence in the cytoplasm and nucleus of the control group (Figure 5A) and in the 10 μM Ang II-treated group. The Ang II-dependent activation of NF-κB was most evident at 30 min (Figure 5B). Blockade of the AT$_1$ receptor by telmisartan reduced Ang II-induced
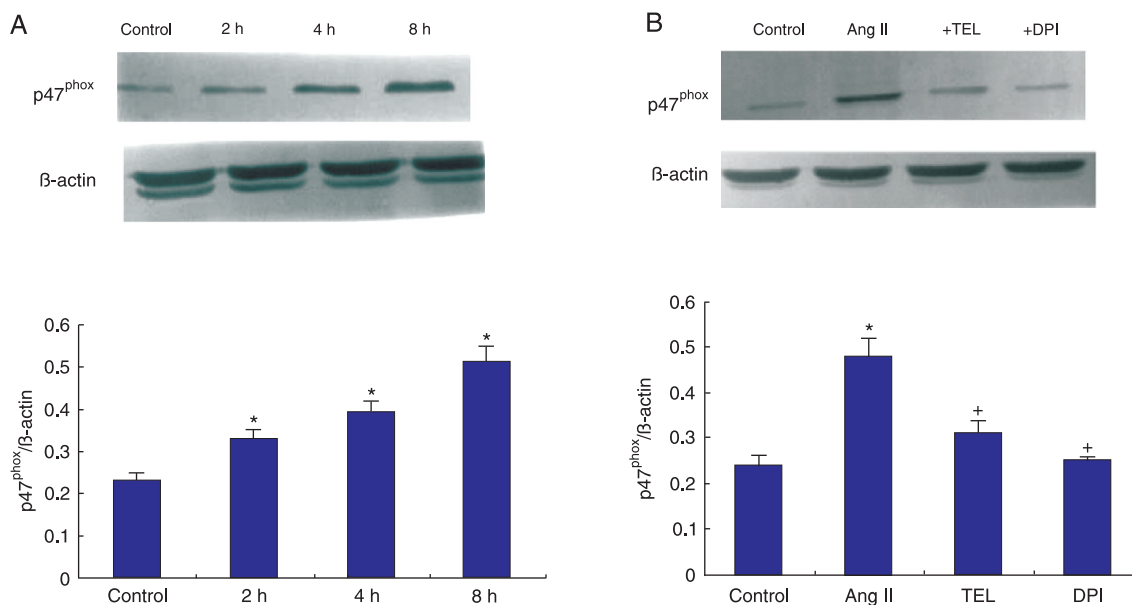

Figure 3. *A*, Angiotensin II (Ang II) stimulates the protein synthesis of p47^{phox} in rat GECs at a fixed dose of 10 μM. The p47^{phox} protein level increased in a time-dependent way, reaching the peak at 8 h. *B*, DPI and TEL reduced Ang II-induced p47^{phox} synthesis. GECs = glomerular endothelial cells; DPI = diphenyleneiodonium; TEL = telmisartan. *P < 0.01 *vs* control group; +P < 0.05 *vs* Ang II group (N = 3; Bonferroni test).
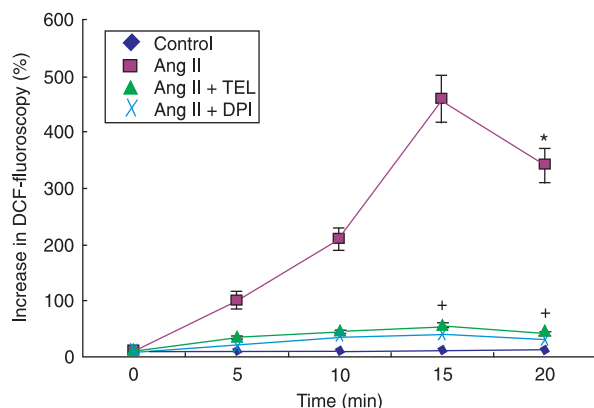

Figure 4. *A*, Angiotensin II (Ang II)-induced ROS generation was determined by DCFH-DA to DCF conversion, and results are reported as percent increase. Ang II-induced ROS generation reached the highest level (475 ± 42%, N = 5) at 15 min, which was greatly reduced by DPI. Serum-free controls showed no increase in fluoroscopic signal. Blockade of the AT$_1$ receptor by TEL reduced Ang II-induced ROS formation. ROS = reactive oxygen species; DCFH-DA = dichlorofluorescein diacetate; DPI = diphenyleneiodonium; TEL = telmisartan. *P < 0.01 *vs* control group; +P < 0.05 *vs* Ang II group (Bonferroni test).
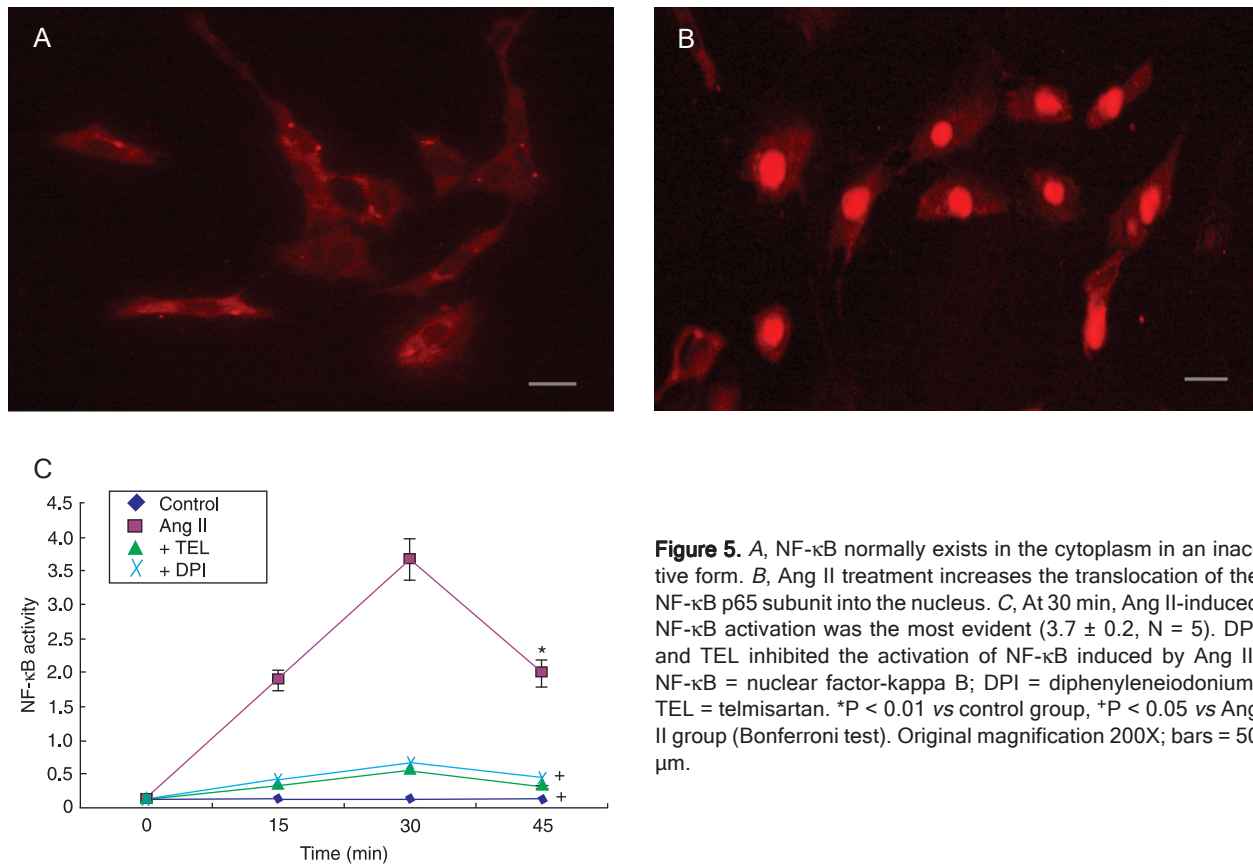
Figure 5. *A*, NF-κB normally exists in the cytoplasm in an inactive form. *B*, Ang II treatment increases the translocation of the NF-κB p65 subunit into the nucleus. *C*, At 30 min, Ang II-induced NF-κB activation was the most evident (3.7 ± 0.2, N = 5). DPI and TEL inhibited the activation of NF-κB induced by Ang II. NF-κB = nuclear factor-kappa B; DPI = diphenyleneiodonium; TEL = telmisartan. *P < 0.01 *vs* control group, +P < 0.05 *vs* Ang II group (Bonferroni test). Original magnification 200X; bars = 50 μm.

NF-κB activation. Also, DPI, the inhibitor of NAD(P)H oxidase, inhibited the activation of NF-κB.

Discussion

The present study demonstrated that Ang II-induced ROS generation in rat GECs depends on the p47phox subunit of NAD(P)H oxidase. ROS are also required for Ang II-induced activation of NF-κB. Blockade of NAD(P)H oxidase by its inhibitor DPI significantly abolished Ang II-induced MCP-1 formation, indicating that NF-κB, when activated by ROS, participates in the Ang II-induced MCP-1 production.

It is now well accepted that Ang II may function as a potent pro-inflammatory mediator and be implicated in the pathogenesis of chronic renal disease (13,14). GECs are at the interface between blood and the adjacent cell population and play a crucial role in preserving kidney function. The monocyte-endothelium interaction induced by Ang II may contribute to the initiation of vascular inflammation (15). MCP-1, when expressed on the plasma membrane of endothelial cells, can mediate the initial capture of mono-

cytes (16). Our results suggest that Ang II can induce MCP-1 formation in rat GECs and promote monocyte adhesion to endothelial cells.

Ang II acts via AT_1 and AT_2 receptors. Both receptor subtypes have been demonstrated in rat GECs (17). Ang II exerts most of its already well-defined physiologic and pathophysiologic actions through AT_1 receptors (18). Our results showed that the effect of Ang II on MCP-1 formation was inhibited by the selective AT_1 receptor antagonist telmisartan.

In mammalian cells, a major source of ROS derives from the membrane-bound NAD(P)H oxidase system, which exists in nonphagocytic cells of the vascular wall such as fibroblasts, vascular smooth muscle cells and endothelial cells (19). Increased ROS generation can induce cell inflammation (20,21). The present study conducted on isolated rat glomerular endothelial cells demonstrated that the expression of p47phox protein, one of the NAD(P)H oxidase subunits, was increased by Ang II treatment according to the increased ROS generation. Moreover, blockade of NAD(P)H oxidase by DPI reduced p47phox protein synthesis and ROS generation.

Nuclear factor-κB normally exists in the cytoplasm in an inactive form bound to the inhibitory protein IκB. Upon cellular activation, IκB is rapidly degraded prior to the translocation of NF-κB into the nucleus and its subsequent activation, resulting in the transcriptional regulation of target genes encoding pro-inflammatory cytokines. NF-κB consists of two subunits, p50 and p65, with p65 containing a transcription domain (11,22). We examined the possible involvement of NF-κB activation in Ang II-induced MCP-1

production in rat GECs and observed that Ang II stimulation increased the translocation of the NF-κB P65 subunit to the nucleus, which was reduced by treatment with DPI. This observation suggests that Ang II-induced NF-κB activation is partially mediated by ROS.

The data reported here may provide a mechanistic basis for the benefits of selective AT$_1$ blockade in dealing with chronic renal disease.

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