Aldosterone Stimulates Reactive Oxygen Species Production through Activation of NADPH Oxidase in Rat Mesangial Cells

Kayoko Miyata,* Matlubur Rahman,‡ Takatomi Shokoji,† Yukiko Nagai,§ Guo-Xing Zhang,† Guang-Ping Sun,‡ Shoji Kimura,‡ Tokihiro Yukimura,‖ Hideyasu Kiyomoto,† Masakazu Kohno,† Youichi Abe,‡ and Akira Nishiyama‡

*Radioisotope Research Center, †Second Department of Internal Medicine, ‡Department of Pharmacology and §Research Equipment Center, Kagawa Medical University, Kagawa, Japan; ‖Department of Pharmacology, Osaka City University Graduate School of Medicine, Osaka, Japan

It has recently been shown that glomerular mesangial injury is associated with increases in renal cortical reactive oxygen species (ROS) levels in rats treated chronically with aldosterone and salt. This study was conducted to determine the mechanisms responsible for aldosterone-induced ROS production in cultured rat mesangial cells (RMC). Oxidative fluorescent dihydroethidium was used to evaluate intracellular production of superoxide anion (O$_{2}^{-}$) in intact cells. The lucigenin-derived chemiluminescence assay was used to determine NADPH oxidase activity. The staining of dihydroethidium was increased in a dose-dependent manner by aldosterone (1 to 100 nmol/L) with a peak at 3 h in RMC. Aldosterone (100 nmol/L for 3 h) also significantly increased NADPH oxidase activity from 232 ± 18 to 346 ± 30 cpm/5 × 10⁴ cells. Immunoblotting data showed that aldosterone (100 nmol/L for 3 h) increased p47phox and p67phox protein levels in the membrane fraction by approximately 2.1- and 2.3-fold, respectively. On the other hand, mRNA expression of NADPH oxidase membrane components, p22phox, Nox-1, and Nox-4, were not altered by aldosterone (for 3 to 12 h) in RMC. Pre-incubation with the selective mineralocorticoid receptor (MR) antagonist, eplerenone (10 μmol/L), significantly attenuated aldosterone-induced O$_{2}^{-}$ production, NADPH oxidase activation and membranous translocation of p47phox and p67phox. These results suggest that aldosterone-induced ROS generation is associated with NAPDH oxidase activation through MR-mediated membranous translocation of p47phox and p67phox in RMC. These cellular actions of aldosterone may play a role in the pathogenesis of glomerular mesangial injury.

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Address correspondence to: Dr. Akira Nishiyama, Department of Pharmacology, Kagawa Medical University, 1750-1 Ikenobe, Miki-cho, Kita-gun, Kagawa 761-0793, Japan. Phone: +81-87-898-5111 ext. 2502; Fax: +81-87-891-2126; E-mail: akira@kms.ac.jp

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oxidase-dependent ROS generation after AngII stimulation. Further studies by Callera et al. (22) showed that aldosterone increased NADPH oxidase activity in vascular smooth muscle cells, which were prevented by treatment with eplerenone.

Recently we demonstrated that in aldosterone/salt-treated rats, glomerular injury, characterized by mesangial matrix expansion and cell over-growth, is associated with increases in renal tissue ROS levels (5). We also observed that treatment with an antioxidant, tempol, or eplerenone normalized ROS levels and markedly attenuated glomerular injury in these animals (5). These results suggest that the glomerular mesangium is a target for injuries induced by aldosterone and the MR, and prompt us to perform further in vitro experiments to investigate possible mechanisms responsible for the aldosterone and MR-induced ROS generation. In this study, we hypothesized that aldosterone has a direct effect on ROS generation through MR-dependent activation of NADPH oxidase in glomerular mesangial cells. To test this hypothesis, we examined the effects of aldosterone and MR blockade on O$_2^-$ generation and NADPH oxidase activity in cultured rat mesangial cells (RMC).

Materials and Methods

Reagents

Aldosterone was purchased from Across Organics (Geel, Belgium). Dihydroethidium was obtained from Molecular Probes Inc. (Eugene, OR). Diphenyleneiodonium, apocynin, lucigenin, NADPH and anti–β-actin antibody were obtained from Sigma Chemical Co. (St. Louis, MO). Anti-p22phox and p67phox antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Eplerenone was provided by Pfizer Inc. (New York, NY).

Cell Culture

All experimental procedures were performed according to the guidelines for the care and use of animals established by Kagawa Medical University. RMC were isolated from male Sprague-Dawley rats and were maintained according to published methods (23,24). Control solutions always contained the appropriate amount of vehicle (ethanol for aldosterone and DMSO for eplerenone, apocynin, and diphenyleneiodonium). After stimulation, protein or mRNA was extracted as described previously (23,24). In some RMC, membrane fraction was isolated as described previously (20,25). The protein concentration was determined using the Bradford or Lowry protein assay (Pfizer Inc. New York, NY).

Dihydroethidium Staining

The oxidative fluorescence dihydroethidium was used to evaluate intracellular O$_2^-$ levels as described previously (25). Briefly, RMC were plated in a glass-bottom dish (Matsunami Glass Ind. Ltd., Kishiwada, Japan) and allowed to adhere for at least 18 h. At the appropriate time after stimulation, dihydroethidium (10 μmol/L) was added to the medium, and the incubation was continued for 15 min. Then, cells were washed with PBS and images were obtained with a laser scanning confocal microscope system (Bio-Rad Laboratories). The averages of fluorescence intensity values from 20 to 30 cells of 5 to 8 different examinations were calculated using image software from the National Institutes of Health (NIH).

NADPH Oxidase Activity

NADPH oxidase-dependent O$_2^-$ production by intact RMC was measured by lucigenin-enhanced chemiluminescence (22,25). Briefly, RMC were detached from the culture dishes using 0.25% trypsin/EDTA (1 mmol/L), washed with PBS, and resuspended at 1 × 10$^6$ cells/ml in Krebs-HEPES buffer. Fifty microliter of cell suspension (5 × 10$^4$ cells) was transferred into glass scintillation vials containing 5 μmol/L lucigenin in Krebs-HEPES buffer (950 μl). The chemiluminescence value was recorded at 30-s intervals over 10 min (BLR-301, Aloka Co., Tokyo, Japan), and readings in the last 5 min were averaged. After measurement of background lucigenin chemiluminescence, NADPH (100 μmol/L) was added to the incubation medium as a substrate for O$_2^-$ production.

Real-Time Reverse Transcriptase-PCR

mRNA expression levels of the NADPH membrane components, p22phox, Nox-1, and Nox-4, were analyzed by real-time PCR (5). All data were normalized by the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The primers for p22phox, Nox-1, Nox-4, and GAPDH were synthesized as described previously (5).

Western Blotting Analysis

The protein expression of p47phox and p67phox was measured by Western blotting analysis as described previously (20,25). All values were normalized by setting the densitometry of control samples to 1.0. In samples from total lysates, blotting membranes were re-probed with an antibody against β-actin to check for equal loading.

Statistical Analyses

The values are presented as means ± SEM. One-way ANOVA was used to determine significant differences among groups, after which the modified t test with the Bonferroni correction was used for comparison between individual groups. P < 0.05 was considered statistically significant.

Results

Effects of Aldosterone on O$_2^-$ Production

To determine whether aldosterone stimulates ROS production in RMC, intracellular O$_2^-$ levels were measured using dihydroethidium and fluorescence microscopy. Figure 1A shows the time course of aldosterone-induced increases in dihydroethidium staining (n = 5 to 6 for each). Aldosterone (100 μmol/L)-induced increases in dihydroethidium staining peaked at 3 h. Figure 1B shows the concentration-dependent effects of aldosterone (3 h) on the staining of dihydroethidium (n = 5 to 6 for each). Aldosterone-induced increases in dihydroethidium staining were maximal at 100 nmol/L (4.1 ± 0.9-fold). Representative results of dihydroethidium staining are shown in Figure 1C.

To investigate the role of MR in aldosterone-induced O$_2^-$ production, the effect of eplerenone on the staining of dihydroethidium was examined (n = 5 to 7 for each). RMC were pretreated with eplerenone (10 μmol/L) for 60 min before incubation with aldosterone (100 nmol/L). As shown in Figure 2A, eplerenone significantly attenuated aldosterone-induced increases in dihydroethidium staining. In addition, aldosterone-induced enhancement of dihydroethidium signal was abolished by preincubation with NADPH oxidase inhibitors, apocynin (300 μmol/L, n = 7) or diphenyleneiodonium (10 μmol/L, n = 5). Representative results of dihydroethidium staining are shown in Figure 2B. The preincubation time and doses of eplerenone, apocynin, and diphenyleneiodonium were determined on the basis of results from previous studies in vitro (22,24–27).
Effects of Aldosterone on NADPH Oxidase Activity

Using lucigenin chemiluminescence, a very low level of $O_2^-$ was detected in intact RMC in the absence of NADPH ($n = 4$, Figure 3). Addition of NADPH (100 $\mu$mol/L) resulted in substantially increased levels of $O_2^-$ in RMC ($232 \pm 18$ cpm/$5 \times 10^4$ cells, $n = 6$). Treatment with aldosterone (100 nmol/L) for 3 h significantly enhanced NADPH-dependent $O_2^-$ production ($346 \pm 30$ cpm/$5 \times 10^4$ cells, $n = 6$). The aldosterone-induced enhancement of NADPH-dependent $O_2^-$ production was markedly attenuated by preincubation with eplerenone (10 nmol/L).
Treatment with aldosterone (100 nmol/L) for 3 to 12 h did not alter p22phox, Nox-1, and Nox-4 expression in RMC (114 ± 13% and 112 ± 12% of control at 12 h, respectively, n = 6 for each). Similarly, no significant changes in p22phox, Nox-1, and Nox-4 mRNA expression were observed in RMC treated with aldosterone for 24 and 48 h (data not shown, n = 6 for each).

Effects of Aldosterone on Membranous Translocation of p47phox and p67phox

Activation of NADPH oxidase requires the translocation of the cytosolic components of p47phox and p67phox to the cell membrane (28,29). Therefore, we examined the effects of aldosterone on membranous translocation of p47phox and p67phox proteins in RMC by Western blotting analysis. As shown in Figure 5A, aldosterone (100 nmol/L)-induced increases in p47phox and p67phox protein levels in the membrane fraction peaked at 3 h (n = 4 for each). On the other hand, aldosterone treatment did not change the protein levels of p47phox and p67phox (Figure 5A) and -actin (data not shown) in total lysates (n = 4 for each), indicating aldosterone-induced membranous translocation of p47phox and p67phox. As shown in Figure 5B, aldosterone treatment for 3 h increased p47phox and p67phox protein levels in the membrane fraction by 2.1 ± 0.5-fold and 2.3 ± 0.2-fold, respectively. Aldosterone-induced membranous translocation of p47phox and p67phox was markedly attenuated by treatment with eplerenone (10 μmol/L) or apocynin (300 μmol/L) (n = 6 to 7 for each, Figures 5B).

Discussion

In a recent study, we demonstrated that in rats treated with aldosterone and salt, glomerular injury was associated with exaggerated ROS production in damaged renal tissue, suggesting that ROS are involved in the progression of aldosterone-induced glomerular injury (5). Further in vitro studies demonstrated a significant expression of MR in cultured RMC (24). Our study provides, for the first time, evidence that aldosterone

Figure 3. Effects of aldosterone, eplerenone, apocynin, and diphenyleneiodonium on NADPH oxidase activity in RMC. NADPH oxidase-dependent O$_2^-$ production by intact RMC was measured by lucigenin-enhanced chemiluminescence. Incubation with aldosterone (100 nmol/L) for 3 h significantly enhanced NADPH-dependent O$_2^-$ production. The aldosterone-induced enhancement of NADPH-dependent O$_2^-$ production was markedly attenuated by eplerenone (10 μmol/L), apocynin (300 μmol/L), or diphenyleneiodonium (10 μmol/L) (n = 4 to 7 for each).

Figure 4. Effects of aldosterone on mRNA Expression of p22phox, Nox-1, and Nox-4

Figure 4 shows mRNA expression of the NADPH oxidase membrane components, p22phox, Nox-1, and Nox-4, in RMC.
directly induces O$_2^-$ generation in RMC. In addition, we found that aldosterone-induced O$_2^-$ production was accompanied by increases in NADPH oxidase activity and the translocation of p47phox and p67phox to the RMC membrane. These findings are consistent with the hypothesis that NADPH oxidase contributes to the aldosterone-induced ROS generation in RMC. Because these effects of aldosterone were markedly attenuated by treatment with eplerenone, the MR may play a role in mediating these effects of aldosterone.

NADPH oxidase is one of the major sources of O$_2^-$ in a variety of cells (28,29). Our study showed that aldosterone-induced O$_2^-$ production was accompanied by increases in NADPH oxidase activity in RMC. Furthermore, O$_2^-$ production as well as NADPH oxidase activation induced by aldosterone were virtually abolished by pretreatment with the NADPH oxidase inhibitors, apocynin or diphenyleneiodonium. Keidar et al. (20) showed that increased NADPH oxidase activity was observed in macrophages isolated from aldosterone-treated apolipoprotein E-deficient mice, and this increase was prevented by concurrent administration of apocynin (17). These in vivo observations as well as those in the in vitro experiments support the hypothesis that aldosterone stimulates ROS generation via the NADPH oxidase-dependent pathway. Our study also showed that eplerenone markedly attenuated aldosterone-induced increases in NADPH oxidase activity and ROS generation in RMC. These results are consistent with those observed in previous animal studies (15–18).

NADPH oxidase is composed of membrane-associated components (gp91phox, Nox-1, Nox-4, and p22phox) and cytosolic regulatory subunits (p40phox, p47phox, p67phox, and Rac) (28). Activation of NADPH oxidase requires the translocation of the cytosolic components to the cell membrane (28,29). Recent studies have shown that macrophages from aldosterone-treated apolipoprotein E-deficient mice exhibited higher membranous translocation of the cytosolic p47phox compared with those derived from placebo-treated mice (20). Our study showed that aldosterone directly induces the translocation of p47phox and p67phox to the RMC membrane. Of note, the effects of aldosterone on O$_2^-$ production, NADPH oxidase activation and membranous translocation of p47phox and p67phox were shown within a similar time span. Thus, these data are consistent with the concept that membranous translocation of p47phox and p67phox is involved, at least in part, in the overall increased NADPH oxidase activity resulting in O$_2^-$ production in RMC. The finding that aldosterone-induced translocation of p47phox and p67phox were markedly attenuated by treatment with eplerenone indicates the potential contribution of the MR to these effects of aldosterone.

Weber and co-workers (30,31) showed that immunohistochemical staining for gp91phox and 3-nitrotyrosine (a marker of nitro-
sative stress) were significantly increased in the heart of aldosterone/salt-treated uninephrectomized rats. In these animals, increased mRNA expression of p22phox was also observed in the aortic tissues (17). Similarly, we previously showed that in kidneys of aldosterone/salt-treated rats, elevated renal tissue ROS levels were associated with increased mRNA expression of p22phox, Nox-4, and gp91phox (5). Therefore, we anticipated that these components would be increased by aldosterone in RMC. However, we observed that aldosterone did not affect mRNA expression of p22phox, Nox-1, and Nox-4 in RMC. In this study, apparent mRNA expression of gp91phox was not detected in RMC (data not shown). At present, we can not explain the discrepancy between previous in vivo and current in vitro data, but it may be due to differences in the experimental conditions or cell types. Alternatively, the exposure time of aldosterone alone would not be enough for the overexpression of NADPH oxidase membrane components in RMC. Further in vitro studies in different cells and time courses will be needed to determine the effects of aldosterone on the expression of NADPH oxidase membrane components.

AngII also induces ROS generation through the NADPH oxidase-dependent pathway (28,29). Recent studies have indicated that MR interacts with AngII-induced NADPH oxidase activation and ROS production. Schiffrin et al. (15) showed that increased vascular NADPH oxidase activity and ROS production in AngII-induced hypertensive rats were markedly attenuated by treatment with spironolactone. Schiffrin et al. also showed that aldosterone-induced increases in vascular NADPH oxidase activity and ROS production were attenuated by AT1 receptor antagonist (16). Further in vitro studies demonstrated that AngII-induced ROS generation is attenuated by spironolactone in vascular smooth muscle cells (21). Although the mechanisms by which MR interacts with AngII-dependent NADPH oxidase activation are not clear, it is possible that the beneficial effects of aldosterone/MR blockade on AngII-induced glomerular injury reported in previous studies (21,22,24,26) are mediated, at least partially, through inhibition of the MR to these effects of aldosterone. These findings might provide novel insights into the mechanisms responsible for aldosterone-induced ROS generation during the progression of renal injury. In addition, it can be speculated that some BP-independent renoprotective effects of MR antagonists reported in recent clinical studies (13,14,36 to 38) are mediated through their direct antioxidant actions on renal cells. Further in vitro studies will be performed to determine the specific roles of ROS in mediating aldosterone-dependent renal cell damage.

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