Aldosterone induces mesangial cell apoptosis both in vivo and in vitro

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1Departments of Medicine, North Shore University Hospital and Long Island Jewish Medical Center, New Hyde Park; 2Renmin Hospital of Wuhan University, Hubei, China; and 3Department of Pathology, New York Medical College, Valhalla, New York

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Mathew JT, Patni H, Chaudhary AN, Liang W, Gupta A, Chander PN, Ding G, Singhal PC. Aldosterone induces mesangial cell apoptosis both in vivo and in vitro. Am J Physiol Renal Physiol 295: F73–F81, 2008. First published May 7, 2008; doi:10.1152/ajprenal.00435.2007.—Both clinical and experimental reports indicate that aldosterone contributes to the progression of renal failure independent of its hemodynamic effects. In the present study, we evaluated effect of aldosterone on human mesangial cell (MC) growth. Aldosterone induced apoptotic and mitogenic effects on MCs. Aldosterone promoted MC apoptosis in a dose- and time-dependent manner. Spironolactone, a mineralocorticoid receptor antagonist, inhibited aldosterone-induced MC apoptosis. Similarly, antioxidants and free radical scavengers partially attenuated proapoptotic effects of aldosterone. Aldosterone also enhanced dephosphorylation of phospho-Bad and accumulation of cytosolic cytochrome c in MCs. In vivo studies, rats were randomly assigned to receive normal saline, aldosterone, or eplerenone + aldosterone for 28 days. Systolic blood pressure, urinary albumin excretion rate, serum creatinine, and aldosterone were measured. Aldosterone-infused rats developed elevated systolic blood pressure and albuminuria when compared with control rats. Aldosterone-treated rats also showed greater numbers of apoptotic MCs. This proapoptotic effect of aldosterone was inhibited by eplerenone, a selective aldosterone antagonist. These findings suggest that aldosterone, beside its hemodynamic effects, may also directly contribute to the occurrence of MC apoptosis.

ROLE OF ALDOSTERONE IN THE PROGRESSION OF RENAL FAILURE

Aldosterone receptor blockade leads to slowing of the progression of renal lesions in patients with diabetic nephropathy (16, 37). Although hemodynamic effects of aldosterone remain a mainstay for progression of renal lesions, direct biological effects of aldosterone are also implicated in the development of glomerulosclerosis (16). Recently, spironolactone has been shown to attenuate glomerulosclerosis by modulating MC expression of connective tissue growth factor (cTGF) (16). However, these investigators did not evaluate the effect of aldosterone on glomerular cell apoptosis. In the present study, we evaluated the effect of aldosterone on MC apoptosis in vitro and in vivo. In addition, we studied the involved molecular mechanism of aldosterone-induced MC apoptosis.

MATERIALS AND METHODS

Mesangial Cell Culture

Primary cultures of human MCs were obtained from ScienCell (San Diego, CA). These cells were maintained in a special media

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(ScienCell) supplemented with 10% fetal calf serum, 14 mM HEPES (GIBCO), 50 U/ml penicillin, and 50 µg/ml streptomycin (GIBCO). Cells in third to seventh passages were used in all experiments.

**Experimental Agents**

Aldosterone and spironolactone were obtained from Sigma-Aldrich (St. Louis, MO). Ascorbic acid, diphenylethionamide chloride (DPI), and N-acetyl-L-cysteine (NAC) were also obtained from Sigma.

Eplerenone was a gift from Pfizer Pharmaceuticals.

**MTT assay.** Conversion of the formazan dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to assess the dose-response effect of aldosterone on the proliferation and/or viability of cells. Equal numbers of growth-arrested MCs (preincubated in serum-free medium containing 1% insulin, transferrin, and selenium for 48 h) were incubated in medium (with 1% serum) containing either vehicle or variable concentrations of aldosterone (10⁻¹² to 10⁻⁶ M) for 24 h. At the end of the incubation period, 10 µl of MTT reagent in PBS (final concentration of 0.5 mg/ml) were added to each well, and the plates were incubated in a humidified atmosphere for 5 h. At the end of each incubation period, the medium with the unconverted dye was removed, and the converted dye in the cells was measured with solubilization solution (1% SDS in 0.01 M HCl). The absorption of this solution was measured at 550 nm with a microplate reader with background subtraction for unconverted dye (620 nm reference). Three sets of experiments were carried out.

**PCNA immunocytochemistry.** Mesangial cells grown on chamber slides were treated under control and experimental conditions. At the end of the incubation period, the cells were fixed in 3.7% formalin, washed in PBS, and treated with normal horse serum for 60 min followed by incubation with mouse monoclonal anti-PCNA antibody (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) for 60 min at room temperature. Sections were washed in PBS and treated with biotinylated horse anti-mouse antibody for 60 min, followed by incubation with avidin-biotin peroxidase complex (Vector, Burlingame, CA) for 60 min. Diaminobenzidine (DAB; Dako) was used as a chromogen, and sections were counterstained with methyl green. Negative control included omitting the primary antibody and replacing it with normal horse serum. The extent of PCNA staining was quantified by 200 magnification per section. The number of positively stained nuclei (brown) was then quantified.

**Apoptosis Studies**

Morphological evaluation of MC apoptosis was performed by staining cells with H-33342 (Molecular Probes, Eugene, OR) and propidium iodide (PI; Sigma). H-33342 is an ultraviolet light-excitable nuclear stain that brightly stains condensed chromosomes of apoptotic cells, whereas PI can only enter cells with compromised plasma membrane. Thus H-33342 stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence. Double staining by these two agents provides the percentage of live, apoptotic, and necrosed cells. Cells were prepared under control and experimental conditions followed by treatment with H-33342 (1.0 µg/ml) for 7 min at 37°C. Subsequently, PI (final concentration of 1.0 µg/ml) was added to each well. Cells were incubated with the dyes for 10 min on ice, protected from light, and then examined under ultraviolet light. Percentage of live, apoptotic, and necrosed cells was recorded in eight random fields in four wells for each variable by two observers unaware of the experimental conditions (40, 41). Three sets of experiments were carried out, each in quadruplicate.

**TUNEL Assay**

Detection of DNA fragmentation using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (40) was performed with TACS terminal deoxynucleotidyl transferase (TdT) kit (R&D Systems, Minneapolis, MN).

**DNA Fragmentation Assay: Gel Electrophoresis**

To confirm the effect of aldosterone, equal numbers (10⁶ cells/100-mm Petri dish) of MCs were prepared under control and experimental conditions. At the end of the incubation period, cells were centrifuged at 1,600 g for 10 min at room temperature, and the pellets were resuspended in DNA lysis buffer (1% Nonidet P-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5; 10 µl per 10⁶ cells). After centrifugation, the supernatant was collected, and the extraction was repeated. SDS in a final concentration of 1% was added to the supernatants before the samples were treated with RNase A (final concentration of 5 µg/µl) at 36°C. This was followed by digestion with proteinase K (Promega, Madison, WI) for 2 h at 37°C. After addition of 0.5 vol of 10 M ammonium acetate, the DNA was precipitated with 2.5 vol ethanol, dissolved in gel-loading buffer, and separated by electrophoresis in 1.6% agarose gels.

**Protein Extraction and Western Blot Analysis**

Mesangial cells grown in 100-mm Petri dishes were treated under control and experimental conditions for 16 h. At the end of the incubation period, cells were harvested and washed with PBS. The cell pellet was resuspended in mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 10 mM HEPES, 1 mM EDTA, pH 7.5; supplemented with 1% protease inhibitor (Sigma), 1 mM PMSF, phosphatase inhibitor cocktail (CT1 and CT2; Sigma)) and homogenized on ice. The homogenate was spun at 500 g for 5 min at 4°C, followed by resuspension of the pellet in mitochondrial buffer. The supernatants were pooled and spun at 1,500 g for 5 min, and the resulting supernatant was spun at 10,000 g for 5 min. At this juncture, the supernatant (cytosolic fraction) was separated from the pellet (mitochondrial fraction), and the protein concentrations were determined with the BCA (Pierce) kit. The supernatant (cytosolic) and pellet (mitochondrial) fractions were separately loaded onto a 10% polyacrylamide gel. After transfer to a cellulose membrane, the proteins were probed with rabbit anti-cytochrome c antibody (1:1,000; Santa Cruz Biotechnology) overnight at 4°C. In parallel series of experiments, cells were treated with either vehicle or aldosterone, followed by isolation of cytosolic fraction as mentioned above. Equal amounts of proteins were loaded onto 10% polyacrylamide gel followed by transfer to cellulose membrane. The proteins were probed with anti-phospho-Bad antibody (1:1,000; Cell Signaling, Beverly, MA) or anti-Bad (1:1,000; BD Biosciences, San Diego, CA) antibodies overnight at 4°C.

In another set of experiments, proteins were extracted from MCs. Proteins were loaded onto 10% polyacrylamide gel and probed with anti-MR (H-300) antibody (1:200; Santa Cruz) overnight at 4°C. A horseradish peroxidase-conjugated appropriate secondary antibody was applied for 1 h at room temperature. The blots were then developed with an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT AR film. Quantitative densitometry was performed on the identified band using a computer-based measurement system. To determine the loading, blots were stripped and probed for β-actin.

**In Vivo Studies**

Twenty-four male Sprague-Dawley rats weighing between 180 and 220 g were housed in cages and maintained in a temperature-conditioned room with a 12:12-h light-dark cycle, with free access to tap water and standard rat chow for 2–4 wk. All experimental procedures were performed in accordance with the guidelines of the Long Island Jewish Medical Center and Renmin Hospital of Wuhan University, Animal Care and Use Committee. For celiac mini-pump implantation (Alzet model 2002 or 2004; Alza, Mountain View, CA), rats were selected at random to be subjected to aldosterone infusion at 100 ng·kg⁻¹·min⁻¹ for 28 days (n = 8), eplerenone infusion at 100 mg·kg⁻¹·day⁻¹ + aldosterone infusion at 100 ng·kg⁻¹·min⁻¹ for 28 days (n = 8), or normal saline infusion for 28 days (n = 8).
Systolic Blood Pressure Measurement

The systolic blood pressure was monitored by tail-cuff plethysmography in conscious, trained, and preheated rats on days 14 and 28.

Sample Collection and Preparation

Urine samples were collected throughout a 24-h period assay for albuminuria on days 14 and 28. Blood and kidney samples were harvested on day 28. Trunk blood was collected into prechilled tubes and then centrifuged at 4,000 rpm for 30 min at 4°C. Plasma fractions were removed and assayed for creatinine and aldosterone. After decapsulation, the kidneys were washed with ice-cooled saline and blotted dry. The kidneys were cross sectioned and fixed in 10% formalin in PBS (pH 7.2) for histochemical, TUNEL, and immunolabeling studies.

PCNA Immunolabeling

After paraffin dewaxing was completed, renal cortical sections (5 μm) were incubated with 3% H2O2 for 30 min, followed by 0.1% Triton X-100 in PBS for 15 min at room temperature. Subsequently, sections were washed in PBS and treated with normal horse serum for 60 min followed by incubation with rabbit IgG anti-PCNA antibody (1:50; Zymed) for 60 min at room temperature. Sections were washed in PBS, treated with biotinylated anti-goat antibody for 60 min, and in PBS, treated with avidin-biotin peroxidase complex (Vector, Burlingame, CA) for 60 min. DAB (Dako) was used as a chromogen, and sections were counterstained with hematoxylin. Negative control included omitting the primary antibody and replacing it with normal horse serum. PCNA-positive cells from single cross-sections through glomerulus were counted with the Weibel-Gomez method.

TUNEL Studies

DNA fragmentation in renal cortical apoptotic cells was detected with the TUNEL assay. After paraffin dewaxing was completed, sections (5 μm) were incubated with 3% H2O2 for 30 min followed by 0.1% Triton X-100 in PBS for 15 min at room temperature. Sections were washed and exposed to TdT buffer (Roche) for 1 h at room temperature. Sections were then washed in PBS for 15 min. The sections were incubated for 30 min with streptavidin-biotin-peroxidase-conjugated anti-digoxigenin-11-dUTP antibody, and antibody binding sites were visualized with DAB. The slides were counterstained with hematoxylin. Negative controls included the omission of TdT; positive controls included the pretreatment of sections with 0.1 U/μl deoxyribonuclease-I before TdT staining. Apoptotic MCs from single cross-sections through glomerulus were counted with the Weibel-Gomez method.

Statistical Analysis

Statistical analysis was performed with GraphPad Instat software. A Newman-Keuls multiple comparison test was used, and P values were calculated. Testing between two groups was performed by Student’s t-test. Results are expressed as means ± SE except in Tables 2, 3, and 4, where results are expressed as means ± SD. P < 0.05 was considered statistically significant.

Table 1. Effect of aldosterone on mesangial cell growth

<table>
<thead>
<tr>
<th>Control</th>
<th>10^{-16} M</th>
<th>10^{-14} M</th>
<th>10^{-12} M</th>
<th>10^{-10} M</th>
<th>10^{-8} M</th>
<th>10^{-6} M</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of cells, ×10,000 cells/well</td>
<td>96.9 ± 12.3</td>
<td>81.2 ± 8.7</td>
<td>97.2 ± 10.2</td>
<td>108.7 ± 13.9</td>
<td>104.3 ± 10.2</td>
<td>99.3 ± 10.1</td>
</tr>
</tbody>
</table>

Values are means ± SE from 3 sets of experiments. Equal numbers of growth arrested mesangial cells (preincubated in serum-free media containing 1% insulin, selenium, and transferrin solution for 48 h) were incubated in 1% serum containing media with either buffer (control) or variable concentrations of aldosterone (10^{-16} to 10^{-6} M) for 48 h. At the end of the incubation periods, cells were trypsinized and counted in a hemocytometer.

RESULTS

PCNA-Positive Glomerular Cells Detected by Immunolabeling

As shown in Table 1, aldosterone did not alter the total number of cells. These findings were also confirmed by MTT assay (data not shown). However, it increased the percentage of PCNA-positive cells in a dose-dependent manner (Fig. 1). These findings are consistent with previously reported observations (45).

MC Apoptosis Detected by TUNEL Assay

Because the total number of cells did not increase in response to aldosterone (despite increased number of PCNA-positive cells), we suspected loss of cells in response to aldosterone. As shown in Fig. 1, aldosterone increased percentage of TUNEL-positive MCs in a dose-dependent manner.

Apoptosis Studies

To confirm the effect of aldosterone on MC apoptosis, equal numbers of MCs were incubated either with buffer (control) or variable concentrations of aldosterone (10^{-12} to 10^{-6} M) for 18 h. At the end of the incubation period, cells were evaluated for apoptosis. Aldosterone promoted MC apoptosis in a dose-dependent manner (Fig. 2A).
Presence of MR in MCs has been reported previously by various investigators (15). In the present study, we confirmed the presence of MR by Western blot (data not shown).

Role of Oxidative Stress in Aldosterone-Induced MC Apoptosis

To determine the role of oxidative stress, equal numbers of MCs were incubated in medium containing either buffer (control), DPI (10 μM), ascorbic acid (100 μM), or NAC (50 μM) in the presence or absence of aldosterone (10⁻⁶ M) for 18 h, followed by apoptosis assay. As shown in Fig. 4A, ascorbic acid, NAC, and DPI partially inhibited proapoptotic effects of aldosterone on MCs.

To further confirm the role of oxidative stress, equal numbers of MCs were incubated in medium containing either buffer, superoxide dismutase (50 μg/ml), or catalase (2,000 U) in the presence or absence of aldosterone (10⁻⁶ M) for 18 h. At the end of the incubation period, cells were evaluated for apoptosis. Aldosterone promoted MC apoptosis; however, this effect of aldosterone was inhibited by both superoxide dismutase and catalase (Fig. 4B).

Studies Pertaining to Apoptotic Pathway

To determine the effect of aldosterone on cytosolic dephosphorylation of phospho-Bad, equal numbers of MCs were treated with either vehicle or aldosterone (10⁻⁶ M) for 16 h. Subsequently, cytosolic fractions were isolated and probed for

Fig. 2: A: dose-response effect of aldosterone (Aldo) on MC apoptosis. Equal numbers of MCs were incubated with either buffer (control) or variable concentrations of aldosterone (10⁻¹² to 10⁻⁶ M) for 18 h. At the end of the incubation period, cells were evaluated for apoptosis. Results (means ± SE) are from 4 series of experiments, each carried out in triplicate. *P < 0.05 compared with control; **P < 0.001 compared with control and aldosterone (10⁻¹² and 10⁻¹⁰ M); ***P < 0.001 compared with control and aldosterone (10⁻¹² to 10⁻⁸ M). B: time course effect of aldosterone on MC apoptosis. Equal numbers of MCs were incubated in media containing either vehicle (control) or aldosterone (10⁻⁶ M) for variable time periods (12, 24, and 48 h). At the end of incubation period, cells were stained with H-33342 and propidium iodide. Results (means ± SE) are from 4 series of experiments, each carried out in triplicate. *P < 0.05 compared with respective control; **P < 0.001 compared with respective control and aldosterone 6 h; ***P < 0.001 compared with respective control and aldosterone 6 and 12 h; ****P < 0.05 compared with aldosterone 18 h.

To determine time course effect, equal numbers of MCs were incubated in media containing either buffer or aldosterone (10⁻⁶ M) for variable periods (12, 18, and 24 h) followed by morphological assay for apoptosis. As shown in Fig. 2B, aldosterone promoted MC apoptosis in a time-dependent manner.

Role of MRs in Aldosterone-Induced MC Apoptosis

To determine the role of MR in aldosterone-induced MC apoptosis, equal numbers of cells were incubated in medium containing either buffer, aldosterone (10⁻⁶ M), spironolactone (10⁻⁶ M), or aldosterone + spironolactone (10⁻⁵ M) for 24 h. Subsequently, cells were prepared for TUNEL assay. Percentage of TUNEL-positive cells were counted per field. As shown in Fig. 3A, Aldosterone promoted MC apoptosis. However, this effect of aldosterone was inhibited by spironolactone.

In a parallel series of experiments, cells were treated under similar conditions and then DNA was extracted and electrophoresed. As shown in Fig. 3B, aldosterone-treated cells showed multiple integers of 180 base pairs in the form of a ladder pattern. However, spironolactone attenuated this effect of aldosterone.

Fig. 3: A: effect of spironolactone (Spiro) on aldosterone-induced MC apoptosis. Equal numbers of MCs grown on chamber slides were incubated in media containing vehicle (control), aldosterone (10⁻⁶ M), spironolactone (10⁻⁶ M), or spironolactone + aldosterone for 18 h followed by preparation of cells for TUNEL assay. Percentage of TUNEL-positive cells was counted in 8 random fields. Results (means ± SE) are from 3 sets of experiments. *P < 0.001 compared with all other variables. B: effect of spironolactone on aldosterone-induced MC DNA fragmentation. Equal numbers of MCs were incubated in media containing vehicle (control), aldosterone (10⁻⁶ M), spironolactone (10⁻⁶ M), or spironolactone + aldosterone for 18 h followed by DNA extraction and gel electrophoresis.
control and eplerenone + aldosterone-treated rats. Similarly, aldosterone-receiving rats showed increased ($P < 0.05$) urinary albumin excretion rates compared with control and eplerenone + aldosterone-treated rats.

**Serum creatinine and aldosterone levels.** Aldosterone-infused rats showed sixfold increased levels of aldosterone compared with levels shown in control rats (Table 3). However, there was no difference in mean serum creatinine levels between control and aldosterone-treated rats (Table 3).

**PCNA-positive and TUNEL-positive glomerular cells in aldosterone-treated rats.** Renal cortical sections of aldosterone-treated rats showed greater numbers of PCNA-positive cells than shown in sections from control rats (Table 4). However, treatment with eplerenone inhibited this mitogenic effect of aldosterone. Representative photomicrographs are shown in Fig. 6, A–C.

Renal cortical sections of aldosterone-infused rats showed increased numbers of TUNEL-positive MCs compared with that shown in control rats (Table 4). However, eplerenone attenuated this effect of aldosterone (Table 4). Representative photomicrographs are shown in Fig. 6, D–F.

**DISCUSSION**

The present study demonstrates that aldosterone induces MC apoptosis in a dose- and time-dependent manner. The proapoptotic effect of aldosterone was attenuated by both antioxidants and free radical scavengers. Aldosterone stimulates dephosphorylation of cytosolic phospho-Bad and cytosolic accumulation of cytochrome $c$. Aldosterone-infused rats showed elevated levels of blood pressure and enhanced urinary albumin excretion rate. Renal cortical sections of aldosterone-receiving rats also showed greater numbers of TUNEL-positive MCs. Because inhibition of MRs in vitro and in vivo studies was associated with reduction in number of TUNEL-positive cells, it appears that the proapoptotic effect of aldosterone is mediated through MRs.

Reactive oxygen species (ROS) play a key role in the progression of renal injury (23, 28). Spontaneously hypertensive and cyclosporine A-induced hypertensive rats were found to have exaggerated ROS production in damaged renal tissue (8, 28). Aldosterone has been shown to increase ROS production in both renal and cardiovascular tissues (11, 37, 47). On the other hand, treatment with eplerenone has been shown to improve endothelial dysfunction and reduce vascular superoxide anion generation in diet-induced atherosclerosis (34). Recently, Miyata et al. (25) reported that aldosterone induced MC ROS production through the activation of NADPH oxidase. In vivo studies, aldosterone increased renal cortical NADPH oxidase expression and generation of ROS (26). Nishiyama et al. (29) demonstrated that renal injury was associated with increased renal cortical ROS levels in aldosterone/salt hypertensive rats. However, pretreatment with tempol, a cell membrane-permeable radical scavenger, prevented the elevation of ROS levels and ameliorated renal injury (29). Thus it appears that generation of free radicals plays an important role in aldosterone-induced renal cell injury. In the present study, DPI, an inhibitor of NADPH oxidase, partially attenuated apoptosis, suggesting a role of NADPH oxidase in aldosterone-induced MC apoptosis.
ANG II has been demonstrated to play a significant role in the development of glomerulosclerosis (19). Because ANG II stimulates aldosterone production by adrenal cells, it is logical to suggest that aldosterone may also contribute to the development of ANG II-mediated downstream effects. In the present study, we demonstrate direct effects of aldosterone on MCs in vitro. These studies suggest that aldosterone directly promotes MC apoptosis.

In in vivo conditions, serum aldosterone is often elevated in combination with elevated ANG II levels. However, serum aldosterone may also be elevated (without elevated levels of ANG II) in primary hyperaldosteronism and in the presence of aldosterone-producing tumors. Patients with these conditions have not been reported to develop glomerulosclerosis. Unfortunately, these conditions have not been critically evaluated for occurrence of MC injury. We propose that, in models of glomerulosclerosis with elevated levels of ANG II, aldosterone might have contributed to the development and progression of MC injury.

The Bcl-2 family plays an important role in maintaining a balance between cell survival and cell death (31). It comprises both cell survival proteins such as Bcl-2 and Bcl-xL and cell death proteins such as Bax and Bad. Bad is maintained in the cytosol by 14-3-3 proteins and cannot exert its death-promotive action (51). However, when Bad is dephosphorylated by upstream apoptotic signals, it heterodimerizes with Bcl-2 and Bcl-xL and reduces their survival-promoting action (31, 51). In the present study, aldosterone enhanced dephosphorylation of cytosolic phospho-Bad in MCs. It appears that dephosphorylation of cytosolic phospho-Bad tilted the balance toward apoptosis in aldosterone-treated MCs.

Mitochondria contain the voltage-dependent anion channel on its outer membrane (39, 48). Usually, binding of Bcl-xL protein to this channel stabilizes the pore. Nevertheless, when

**Table 2. Systolic blood pressure and urinary albumin excretion rate**

<table>
<thead>
<tr>
<th>Duration</th>
<th>Systolic Blood Pressure, mmHg</th>
<th>Urinary Albumin Excretion Rate, μg/min</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>103±7</td>
<td>0.78±0.31</td>
</tr>
<tr>
<td>28</td>
<td>109±12</td>
<td>0.75±0.37</td>
</tr>
<tr>
<td></td>
<td>Aldosterone</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>150±10*</td>
<td>3.64±0.44*</td>
</tr>
<tr>
<td>28</td>
<td>178±12*</td>
<td>10.31±0.31*</td>
</tr>
<tr>
<td></td>
<td>Eplerenone + aldosterone</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>131±9†</td>
<td>1.90±0.42†</td>
</tr>
<tr>
<td>28</td>
<td>156±10†</td>
<td>5.84±0.87†</td>
</tr>
</tbody>
</table>

Values are means ± SD. Sprague-Dawley rats in groups of 8 were randomly assigned to receive either vehicle (control), aldosterone (100 ng·kg⁻¹·min⁻¹), or eplerenone (100 mg·kg⁻¹·day⁻¹) + aldosterone (100 ng·kg⁻¹·min⁻¹) via mini-osmotic pumps for 4 wk. Systolic blood pressure and urinary albumin excretion rate were measured on days 14 and 28. *P < 0.05 compared with control of respective duration; †P < 0.05 compared with aldosterone alone of respective duration.

**Table 3. Serum creatinine and aldosterone in rats of different groups**

<table>
<thead>
<tr>
<th>Serum Creatinine, μmol/l</th>
<th>Aldosterone, ng/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>47±18</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>69±24</td>
</tr>
<tr>
<td>Eplerenone + aldosterone</td>
<td>56±23</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Serum Creatinine, μmol/l</th>
<th>Aldosterone, ng/l</th>
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<tbody>
<tr>
<td>Control</td>
<td>101±47</td>
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<tr>
<td>Aldosterone</td>
<td>651±99*</td>
</tr>
<tr>
<td>Eplerenone + aldosterone</td>
<td>643±75*</td>
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</table>

Values are means ± SD. Sprague-Dawley rats in groups of 8 were randomly assigned to receive either vehicle (control), aldosterone (100 ng·kg⁻¹·min⁻¹), or eplerenone (100 mg·kg⁻¹·day⁻¹) + aldosterone (100 ng·kg⁻¹·min⁻¹) via mini-osmotic pumps for 4 wk. Serum creatinine and aldosterone were measured on day 28. *P < 0.05 compared with control.
Bad migrates into mitochondria and heterodimerizes with Bcl-xL, anion channel would open and release cytochrome c into the cytosol. In the present study, aldosterone promoted cytochrome c accumulation into the cytosolic compartment of MCs, suggesting heterodimerization of Bcl-xL and opening of mitochondrial anion channel.

Aldosterone has been reported to promote cardiac myocyte apoptosis in both in vivo and in vitro studies (10). This effect of aldosterone has been attributed to the acceleration of the mitochondrial apoptotic pathway: activation of calcineurin and dephosphorylation of Bad (21). Campbell et al. (7) demonstrated that aldosterone caused myocyte injury by affecting integrity of mitochondria and sarcoceric contraction. Aldosterone has also been shown to induce thymocyte apoptosis (4). These findings are consistent with our studies.

The level of aldosterone in plasma is ~10⁻⁷ mol/l in patients with heart failure, whereas level of aldosterone in myocardium is ~17 times higher than the level in plasma (36, 42). Because atherosclerosis and glomerulosclerosis are often associated, we speculate that, in atherosclerotic-related cardiovascular diseases, including heart failure, MCs may be exposed to proapoptotic aldosterone levels. Therefore, aldosterone concentrations used in the present study are clinically relevant.

Recently, MR blockade has been shown to confer renoprotection in preexisting chronic cyclosporine toxicity (32). In this study, administration of spironolactone decreased tubulointerstitial fibrosis and renal cell apoptosis in cyclosporine A-treated rats. Cyclosporine A-induced nephrotoxicity was partly contributed through MR activation (46).

Several experimental reports suggest that aldosterone modulates MC function (26, 29, 46). Aldosterone promoted the proliferation of MCs by stimulating mitogen-activated protein kinase pathway (46). In addition, long-term administration of

| Table 4. Ratio of PCNA-positive to TUNEL-positive cells to number of nuclei in glomerulus cross section |
|-------------------------------|-----------------------------------------------|
| PCNA-Positive Cells, % | TUNEL-Positive Mesangial Cells, % |
| Control | 13.7±5.77 | 14.28±2.20 |
| Aldosterone | 21.03±8.20* | 21.29±2.41† |
| Eplerenone + aldosterone | 10.24±4.06† | 5.68±1.27§ |

Values are means ± SD. Ratio of PCNA- to TUNEL-positive cells to number of nuclei was calculated in 10 randomly selected glomeruli from each cortical section. Sprague-Dawley rats in groups of 8 were randomly assigned to receive either vehicle (control), aldosterone (100 ng·kg⁻¹·min⁻¹), or eplerenone (100 mg·kg⁻¹·day⁻¹) + aldosterone (100 ng·kg⁻¹·min⁻¹) via mini-osmotic pumps for 4 wk. Renal cortical section sections were immunostained for PCNA- and TUNEL-positive cells. *P < 0.05 compared with control and eplerenone + aldosterone. †P < 0.05 compared with control. §P < 0.01 compared with control and eplerenone + aldosterone. ¶P < 0.01 compared with control and aldosterone.

Fig. 6. Effect of aldosterone on MC apoptosis in in vivo studies. Sprague-Dawley rats in groups of 8 were randomly assigned to receive either vehicle (control), aldosterone (100 ng·kg⁻¹·min⁻¹), or eplerenone (100 mg·kg⁻¹·day⁻¹) + aldosterone (100 ng·kg⁻¹·min⁻¹) via subcutaneous mini-osmotic pumps for 4 wk. Renal cortical sections were evaluated for PCNA-positive and TUNEL-positive MCs. A and D: representative photomicrographs of glomeruli from control rats. B and E: representative photomicrographs of glomeruli from aldosterone-treated rats. PCNA-positive cells are stained brown (×400). C and F: representative photomicrographs of glomeruli from eplerenone + aldosterone-treated rats. Apoptosed cells showed brown staining (×400).
aldosterone to rats induced MC injury in the form of MC proliferation and expansion of the mesangium (29). This effect of aldosterone was attributed to the generation of ROS (29). Because MC proliferation and expansion of the mesangium are considered to be the precursor of focal segmental glomerulosclerosis (FSGS), one may speculate that aldosterone has the potential to initiate the development of glomerulosclerosis independent of other injuries. However, in the latter part of the time course of FSGS, there is accumulated matrix and paucity of MCs. Thus it appears that stimulus, which was mitogenic to begin with, has succumbed to overwhelming proapoptotic forces. Because aldosterone also has proapoptotic properties, it may contribute to the latter part of FSGS, paucity of MCs in the mesangium.

In the present study, we have not ruled out the contributory role of high blood pressure on occurrence of renal cell apoptosis in aldosterone-infused rats. However, in a previously reported study, only ANG II-infused rats developed renal cell apoptosis, whereas norepinephrine-infused rats did not develop renal cell apoptosis, despite having comparable elevated levels of blood pressure (1). In the present study, we cannot rule out the role of aldosterone-induced elevated blood pressure on occurrence of MC apoptosis. Nevertheless, we want to emphasize that aldosterone, besides its hemodynamic effects, may also directly contribute to the occurrence of MC apoptosis.

In human and animal kidney disease models of glomerulosclerosis, hypertension and hyperlipidemia contribute to the progression of renal injury (14, 20). ANG II has been considered to be the mediator of hypertension, whereas lipoproteins are effector molecules in hyperlipidemic states. Both ANG II and lipoproteins stimulate aldosterone production by MCs (26, 29). It is likely that local production of aldosterone may contribute to MC injury in these instances. Thus MCs not only have the capability of producing aldosterone but also serve as potential targets for injury induced by aldosterone.

In in vivo studies, occurrence of MC apoptosis in association with proliferation has been demonstrated in a variety of human renal diseases, including postinfectious glomerulonephritis, IgA nephropathy, lupus nephritis, and systemic vasculitis (2, 43, 45). Similarly, in in vitro studies, occurrence of apoptosis has been noted in association with mitogenic effects of platelet-derived growth factor and gentamycin on MCs (19, 22). It has been suggested that generation of ROS may act as a second messenger for downstream signaling and may also be exert proapoptotic effects on vulnerable cells (22).

We conclude that aldosterone besides its hemodynamic effects can induce MC apoptosis. The present study provides a basis for the hypothesis that aldosterone antagonists may help in the modulation of the progression of renal injury.

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REFERENCES


