Am J Physiol Renal Physiol 293: F1065–F1071, 2007.
First published August 1, 2007; doi:10.1152/ajprenal.00147.2007.

Aldosterone promotes proximal tubular cell apoptosis: role of oxidative stress

Hitesh Patni,[1] Jayant T. Mathew,[1] Liming Luan,[1] Nicholas Franki,[1] Praveen N. Chander,[2] and Pravin C. Singhal[1]

[1]*Department of Medicine, North Shore University Hospital and Long Island Jewish Medical Center, New Hyde Park; and* [2]*Department of Pathology, New York Medical College, Valhalla, New York*

Submitted 28 March 2007; accepted in final form 26 July 2007

Patni H, Mathew JA, Luan L, Franki N, Chander PN, Singhal PC. Aldosterone promotes proximal tubular cell apoptosis: role of oxidative stress. *Am J Physiol Renal Physiol* 293: F1065–F1071, 2007. First published August 1, 2007; doi:10.1152/ajprenal.00147.2007.—Aldosterone has attracted significant consideration for its role in the progression of renal injury. Since apoptotic cell loss contributes to the deterioration of renal function, we examined the effect of aldosterone on tubular cell apoptosis. To determine dose and time course effect, human renal proximal tubular (HK2) cells were treated with aldosterone at different doses and for variable time periods followed by evaluation for apoptosis. To determine the role of mineralocorticoid receptors (MR) and oxidative stress, HK2 cells were treated with either vehicle or aldosterone in the presence or absence of spironolactone/antioxidants/free radical scavengers (FRS) followed by evaluation for apoptosis. The presence of MR was evaluated using RT-PCR. Reactive oxygen species (ROS) generation was evaluated using redox-sensitive dyes. Effect of aldosterone was evaluated on dephosphorylation of phospho-Bad and accumulation of cytosolic cytochrome c. Human tubular cells express MR. Aldosterone promotes tubular cell apoptosis in a dose- and time-dependent manner. This effect of aldosterone is mediated through MR and associated with generation of ROS. Antioxidants and FRS partially attenuated the proapoaptotic effect of aldosterone. Aldosterone enhanced dephosphorylation of phospho-Bad and accumulation of cytosolic cytochrome c. We conclude that aldosterone-induced tubular cell apoptosis is mediated through the activation of the mitochondrial pathway and generation of ROS.

reactive oxygen species; mineralocorticoid receptor

THE RENIN-ANGIOTENSIN-ALDOSTERONE system (RAAS) plays an important role in the control of blood pressure and fluid electrolyte balance (13, 27, 34). Aldosterone promotes the retention of sodium and the loss of potassium and activates the sympathetic nervous system and myocardial and vascular fibrosis (13, 27, 28, 34). It has been thought to be produced solely by the adrenal cortex in response to ANG II, making it an important component of the circulating RAAS. Recently, aldosterone has also been reported to be produced in extra adrenal tissues, including the heart (27, 36), brain (7), blood vessels (29), and kidneys (35). Thus local production of aldosterone may be contributing directly to its cellular effects.

Several clinical and experimental data suggest a contribution of aldosterone to the progression of renal injury, many of which cannot be explained by its hemodynamic effects (2, 4, 8). Exogenous infusion of aldosterone can reverse the renoprotective effects of ANG II blockade in remnant kidney hypertensive rats (8). The addition of spironolactone to angiotensin-converting enzyme inhibitors has been shown to markedly attenuate the urinary protein excretion rate without significant hemodynamic effects in patients with chronic renal failure (4). Aldosterone can also stimulate cardiac ventricular myocyte apoptosis both in vivo and in primary culture (5). Campbell et al. (3) reported in aldosterone-treated rats that aldosterone caused myocyte necrosis by mitochondrial injury and sarcomeric contraction (3). Aldosterone has also been shown to cause apoptosis of rat thymocytes (1).

A role of oxidative stress in the initiation and propagation of apoptosis is indicated by studies where a rather low dose of reactive oxygen species (ROS) triggered apoptosis directly, and antioxidants such *N*-acetylcysteine (NAC) attenuated the proapoptotic effect of ROS (14, 17). The role of membrane NADPH oxidase in apoptosis has been increasingly recognized. During the immune process, assembly and activation of NADPH oxidase has been demonstrated to occur in response to various stimuli (6, 10). Activation of NADPH oxidase and production of superoxide contribute to apoptosis in various cell types (30). Aldosterone has been shown to increase renal cortical NADPH oxidase expression, and generation of ROS thus contribute to the progression of glomerular injury (18).

An altered balance between forces promoting survival and death usually determines the fate of a cell. Loss of tubular cells has been suggested to be an underlying mechanism for the development of tubular atrophy. Tubular cell apoptosis has also been shown to be an important factor in the progression of renal failure in patients with polycystic kidney disease (36). Similarly, occurrence of tubular cell apoptosis contributes to the development of tubular cell atrophy in the model of unilateral ureteral obstruction (UUO; see Ref. 11). Spironolactone has been demonstrated to slow down tubular injury in a mouse model of UUO (31), suggesting a role of aldosterone in the progression of tubulointerstitial fibrosis in a UUO model.

The present study was carried out to examine whether aldosterone has a potential to induce apoptosis in renal proximal tubular cells. In addition, we studied the involved molecular mechanism in aldosterone-induced tubular cell apoptosis.

MATERIALS AND METHODS

Proximal Tubular Cell Culture

Primary cultures of human renal proximal tubular cells (HRPTEC) were obtained from Science Cell (San Diego, CA). These cells were maintained in a special media provided by Science Cell. Immortalized human proximal tubular (HK2) cells were obtained from American

Address for reprint requests and other correspondence: P. C. Singhal, Division of Kidney Diseases and Hypertension, Long Island Jewish Medical Center, 410 Lakeville Rd., Ste. 105, New Hyde Park, NY 11042 (e-mail: singhal@lij.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

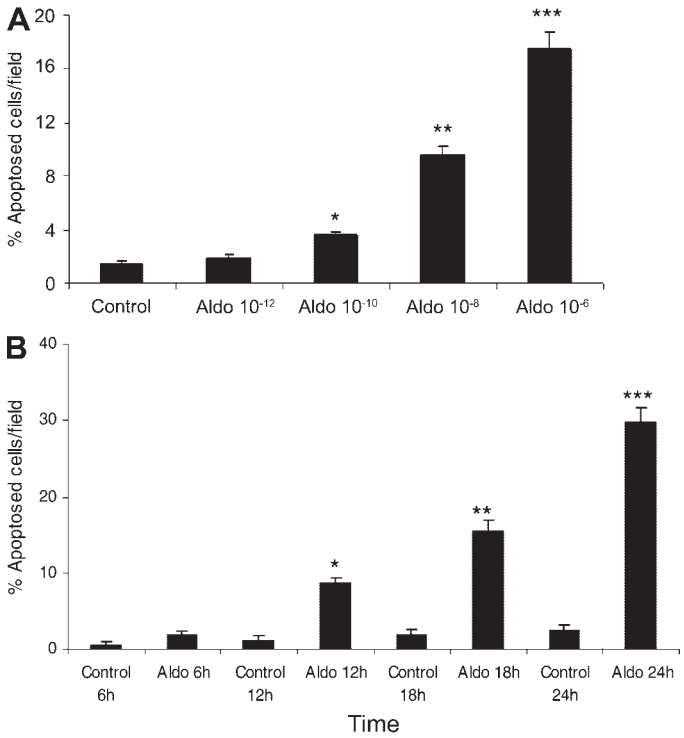

Fig. 1. *A*: dose-response effect of aldosterone on tubular cell apoptosis. An equal number of tubular (HK2) cells were incubated in media containing either buffer (control) or variable concentrations of aldosterone (Aldo, 10^{-12} to 10^{-6} M) for 18 h. At the end of the incubation period, cells were evaluated for apoptosis. Results (means ± SE) are from 4 series of experiments, each carried out in triplicate. *$P < 0.05$ compared with control. **$P < 0.01$ compared with control and Aldo, (10^{-12} to 10^{-10} M). ***$P < 0.001$ compared with control and Aldo (10^{-12} to 10^{-8} M). *B*: time course effect of aldosterone on tubular cell apoptosis. Equal numbers of tubular (HK2) cells were incubated in media containing either buffer (control) or aldosterone (10^{-6} M) for variable time periods (6, 12, 18, and 24 h). At the end of the 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. *$P < 0.001$ compared with respective control and aldosterone (6 h). **$P < 0.001$ compared with respective control and aldosterone (6 and 12 h). ****$P < 0.001$ compared with respective control and aldosterone (6, 12, and 18 h).

Type Culture Collection (Manassas, VA) and maintained in culture in keratinocyte media supplemented with growth factors (Invitrogen, Grand Island, NY) and 1% penicillin-streptomycin at 37°C in 5% CO_2. All studies were performed in HK2 cells except for determining expression of mineralocorticoid receptors (MR) in HRPTECs as well.

Apoptosis Studies

In the present study we have used three types of assays

Assay 1: Detection of apoptosis by cell nuclear staining. Morphological evaluation of tubular cell apoptosis was carried out by staining the cell nucleus with H-33342 (Molecular Probes, Portland, OR) and propidium iodide (Sigma, St. Louis, MO). H-33342 stains the nuclei of live cells and identifies apoptotic cells by increased fluorescence, whereas propidium iodide costains the necrotic cells (pink). Double staining by these two dyes provides the percentage of live, apoptotic, and necrotic cells under control and experimental conditions (15).

Assay 2: Detection of DNA fragmentation. To determine DNA fragmentation, we have used terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay (15). It was performed by using the TACS TdT kit (R & D Systems, Minneapolis, MN).

Assay 3: Flow cytometry for annexin V-stained cells. Normally phosphatidylserine resides on the inner surface of cell membrane. However, with the occurrence of apoptosis, it relocates on the outer surface. Annexin V preferentially binds negatively charged phospholipids like phosphatidylserine.

Detection of Apoptosis by Cell Nuclear Staining

Briefly, at the end of the incubation period, the cells were stained with H-33342 (1 μg/ml) for 7 min at 37°C. Next, the cells were placed on ice with the addition of propidium iodide (1 μg/ml). The cells were incubated with both dyes for 10 min, and the incubation was protected from light. The stained cells were then examined under ultraviolet light with a Hoechst filter (Nikon, Melville, NY). The percentage of live, apoptotic, and necrotic cells was recorded in eight random fields by two observers who were unaware of the experimental conditions.

FACS Analysis

HK2 cells were incubated in media containing either buffer, aldosterone (10^{-6} M), or aldosterone + spironolactone (10^{-5} M) for 16 h. At the end of incubation, cells were stained with annexin V using a kit (Annexin V-PE apoptosis detection kit; BD Pharmingen, San Diego, CA) and analyzed by flow cytometry.

Protein Extraction and Western Blot Analysis

Tubular cells were plated in 100-mm petri dishes in triplicates and grown to subconfluence. Cells were treated with aldosterone, aldosterone + spironolactone or vehicle for 16 h. At the end of the incubation period, cells were harvested and washed with ice-cold PBS. The cell pellet was resuspended in ice-cold mitochondrial buffer [MB: 210 mM mannitol, 70 mM sucrose, 10 mM HEPES, and 1 mM EDTA, pH 7.5; supplemented with 1× protease inhibitor cocktail (Sigma), 1 mM phenylmethylsulfonyl fluoride, phosphatase inhibitor cocktail (CT1

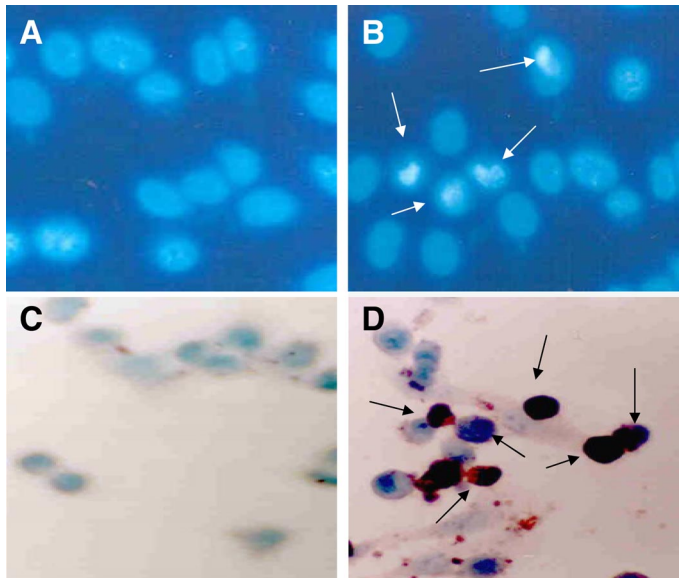

Fig. 2. Representative micrographs showing the effect of aldosterone on tubular cell apoptosis. Equal numbers of tubular (HK2) cells were incubated in media containing either vehicle (control) or aldosterone (10^{-8} M) for 18 h. Subsequently, cells were stained with either H-33342 or prepared for terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. *A*: control HK2 cells stained with H-33342 showing fluorescent labeling of nuclei. *B*: HK2 cells treated with aldosterone and stained with H-33342. Apoptotic cells show bright fluorescence and condensed nuclei as indicated by arrows. *C*: control tubular cells stained for TUNEL assay. *D*: aldosterone-treated tubular cells stained for TUNEL assay. Apoptotic cells show dark nuclei, indicated by arrows.

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 MB. 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. The supernatant (cytosolic fraction) was separated from the pellet (mitochondrial fraction), and the protein concentrations were determined using the BCA (Pierce) kit. The cytosolic and mitochondrial fractions were loaded on a 10% polyacrylamide gel and probed with rabbit anti-cytochrome c (1:1,000; Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C.

In a parallel series of experiments, cells were treated with either vehicle or aldosterone, followed by isolation of the cytosolic fraction. Equal amount of proteins were loaded on 10% polyacrylamide gel and probed with anti-phospho-Bad (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 HK2 cells and HRPTEC. Proteins were loaded on 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 using a chemiluminiscence detection kit (ECL; 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 actin.

RT-PCR for MR

Total RNA was extracted from primary human proximal tubular cells and HK2 cells using TRI reagent (Sigma). cDNA was synthesized using a Qiagen omni script RT kit. The PCR reaction for the MR was performed using the following primers: sense, 5′-CAACAGTACTCAAGGAAGCA-3′ and antisense, 5′-TTAACAATGGCGCGCATGAC-3′. The RT-PCR reaction product was then separated on a 1.5% agarose gel in Tris-borate-EDTA buffer and visualized using ethidium bromide staining.

Immunofluorescent Detection of Oxidative Stress

Aldosterone-induced oxidative stress in tubular cells (HK2 cells) was studied by trafficking of 2,3,4,5,6-pentafluorodihydrotetramethylrosamine or Redox Sensor red CC-1 (Molecular Probes) using fluorescence microscopy. Tubular cells were incubated in media containing either vehicle or aldosterone (10^{-6} M). At the end of the incubation period, cells were stained with Redox Sensor red CC-1 (1 μM) and a mitochondria-specific fluorescent dye, MitoTracker green FM (50 nM; Molecular Probes), in PBS at 37°C for 20 min. Redox Sensor red CC-1 is oxidized in the presence of $O_2^{\bullet -}$ and H_2O_2. Culture slides were washed and mounted with aqueous mount and visualized under a fluorescence microscope (Nikon Eclipse E800) equipped with a triple filter cube and charge-coupled device camera (Nikon DXM 1200). Images were captured by using Nikon ACT-1 (version 1.12) software.

RESULTS

Effect of Aldosterone on Tubular Cell Apoptosis

Equal numbers of tubular (HK2) cells grown to subconfluence in 24-well plates 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. As shown in Fig. 1*A*, aldosterone promoted tubular cell apoptosis in a dose-dependent manner. A representative photograph showing the morphologic effect of aldosterone (10^{-6} M) on tubular cells is shown in Fig. 2*B*.

To determine the time course effect, equal numbers of tubular cells were incubated in media containing either buffer or aldosterone (10^{-6} M) for variable periods (6, 12, 18, and 24 h) followed by morphologic assay for apoptosis. As shown in Fig. 1*B*, aldosterone promoted tubular cell apoptosis in a time-dependent manner. A representative morphological view of aldosterone-induced tubular cell apoptosis (at 24 h) is shown in Fig. 2*D*.

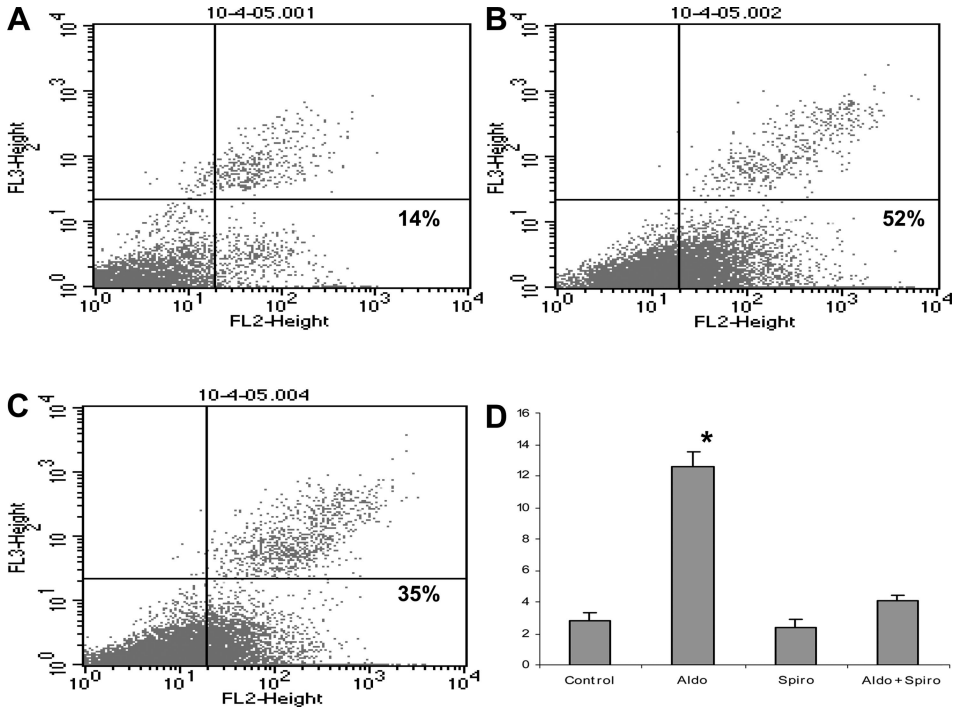

Fig. 3. Effect of spironolactone on aldosterone-induced tubular cell apoptosis. Equal numbers of cells were incubated in media containing either buffer, aldosterone (10^{-6} M), or aldosterone + spironolactone (10^{-5} M) for 16 h. At the end of incubation, cells were stained with annexin V and 7AAD and analyzed by flow cytometry. *A*: control cells (apoptotic cells, 14%). *B*: aldosterone-treated treated cells (apoptotic cells, 52% cells). *C*: spironolactone + aldosterone-treated cells (apoptotic cells, 35%). *D*: equal numbers of cells were incubated in media containing buffer, aldosterone (10^{-6} M), spironolactone (Spiro, 10^{-5} M), or aldosterone + spironolactone (10^{-5} M) for 18 h. Subsequently, cells were prepared for TUNEL assay. Results (means ± SE) are from 3 series of experiments. *$P < 0.001$ compared with all other variables.

Evaluation of the Role of MR in Aldosteone-Induced Tubular Cell Apoptosis

To determine the role of MR in aldosterone-induced tubular cell apoptosis, equal numbers of cells were incubated in media containing either buffer, aldosterone (10^{-6} M), or aldosterone + spironolactone (10^{-5} M) for 24 h. At the end of incubation, cells were stained with annexin V and 7-amino-actinomycin D and analyzed by flow cytometry. As shown in Fig. 3, aldosterone-treated cells showed 3.5-fo1d greater apoptosis (apoptotic cells 52%; Fig. 3*B*) when compared with control cells (apoptotic cells 14%; Fig. 3*A*). However, spironolactone partially attenuated this effect of aldosterone (apoptotic cells, 35%; Fig. 3*C*).

In a parallel series of experiments, equal numbers of cells were incubated in media containing buffer, aldosterone (10^{-6} M), spironolactone (10^{-5} M), or aldosterone + spirolactone (10^{-5} M) for 18 h. Subsequently, the cells were prepared for TUNEL assay. As shown in Fig. 3*D*, aldosterone promoted apoptosis, but spironolactone alone did not modulate tubular cell apoptosis. However, spironolactone attenuated aldosterone-induced tubular cell apoptosis.

Expression of MR by Tubular Cells

To determine the presence of MR in HRPTEC and HK2 cells, total RNA was extracted, and RT-PCR was performed. As shown in Fig. 4*A*, MR was expressed in both HRPTEC (*lanes 2* and *6*) and HK2 cells (*lanes 3* and *5*). Similarly in Western blotting studies, both HK2 cells and HRPTECs showed expression of MR (Fig. 4*B*).

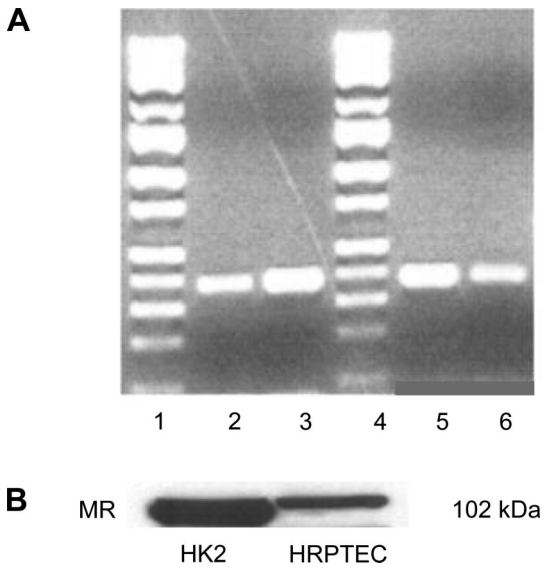

Fig. 4. Determination of expression of mineralocorticoid receptor (MR) by tubular cells. *A*: total RNA was extracted from equal numbers of human renal proximal tubular cells (HRPTECs) and HK2 cells. Subsequently, RT-PCR studies were carried out. *Lanes 1* and *4* show the marker. *Lanes 2* and *6* show a single band of 385 bp in RNAs extracted from HRPTECs. Similarly, *lanes 3* and *5* show a single band of 385 bp in RNAs extracted from HK2 cells. *B*: proteins were extracted from subconfluent HK2 cells and HRPTECs and probed for MR. Both HK2 cells and HRPTECs showed expression of MR.

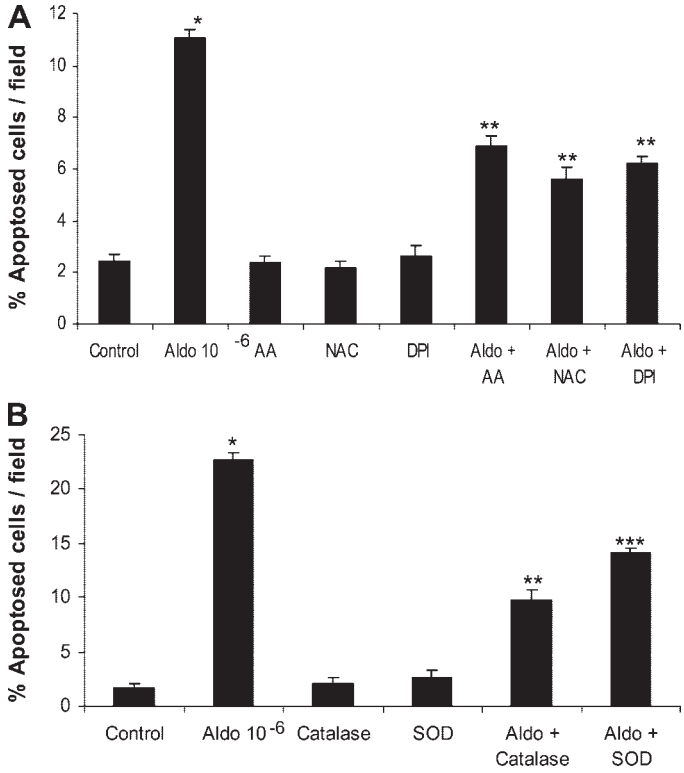

Fig. 5. *A*: effect of antioxidants on aldosterone-induced tubular cell apoptosis. Equal numbers of HK2 cells were incubated in media containing either buffer (control), DPI (10 μM), ascorbic acid (AA, 100 μM), or *N*-acetylcysteine (NAC, 50 μM) with or without aldosterone (10^{-6} M) for 18 h followed by apoptosis assay. Results (means ± SE) are from 4 series of experiments. *$P <$ 0.001 compared with control. **$P < 0.01$ compared with aldosterone. *B*: effect of free radical scavengers on aldosterone-induced tubular cell apoptosis. Equal numbers of HK2 cells were incubated in media containing either buffer, superoxide dismutase (SOD, 50 μM), or catalase (CAT, 2,000 units) 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. Results (means ± SE) are from 4 series of experiments. *$P <$ 0.001 compared with control. **$P <$ 0.01 compared with aldosterone and catalase alone. ***$P < 0.01$ compared with control and SOD alone.

Evaluation of the Effect of Antioxidants on Aldosterone-induced Tubular Cell Apoptosis

To determine the role of oxidative stress, equal numbers of tubular (HK2) cells were incubated in media 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 24 h followed by apoptosis assay. As shown in Fig. 5*A*, ascorbic acid, NAC, and diphenyleneiodonium chloride partially inhibited the proapoptotic effect of aldosterone on tubular cells.

Evaluation of the Effect of Free Radial Scavengers on Aldosterone-induced Tubular Cell Apotposis

To evaluate the role of free radical scavengers, equal numbers of tubular (HK2) cells were incubated in media containing either buffer, superoxide dismutase (SOD, 50 μg/ml), or catalase (2,000 units) 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 tubular cell apoptosis; however, this effect of aldsoterone was inhibited by both SOD and catalase (Fig. 5*B*).

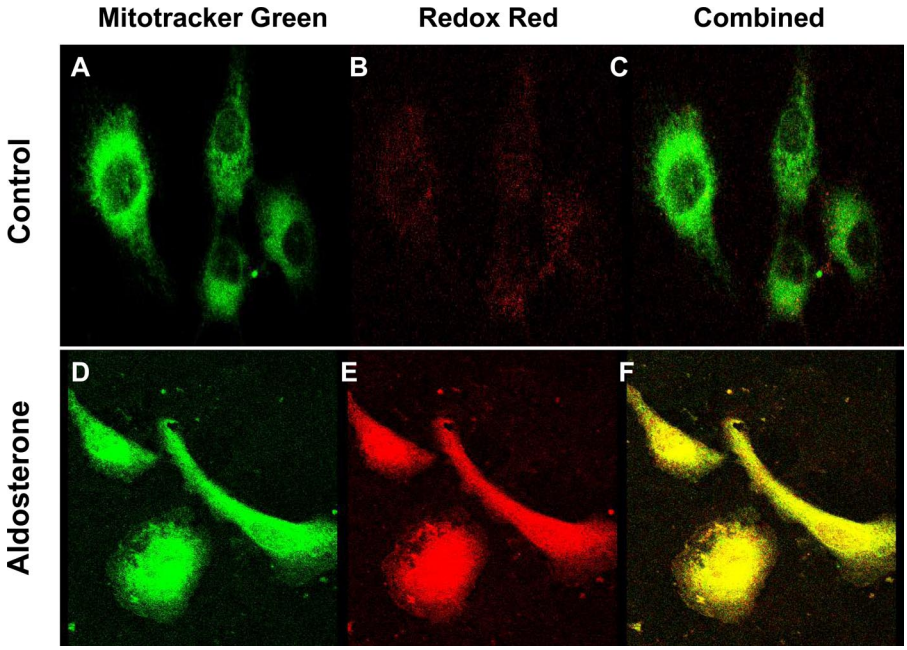

Fig. 6. Aldosterone-induced tubular cell generation of reactive oxygen species (ROS). Equal numbers of HK2 cells were incubated in media containing either vehicle (*A–C*) or aldosterone (10^{-6} M, *D-F*) for 16 h. At the end of the incubation period, cells were loaded with the oxidant-sensitive dye Redox Sensor Red and Mitotracker Green FM. Bright red florescence was seen in the aldosterone-treated cells (*E*), which colocalized with mitochondrial green fluorescence showing yellow-orange fluorescence (*F*).

Evaluation of Oxidative Stress Induced by Aldosterone

To determine the effect of aldosterone on tubular cell ROS generation, equal numbers of HK2 cells were incubated in media containing either vehicle or aldosterone (10^{-6} M) for 2, 6, 12, and 16 h. At the end of the incubation period, cells were stained with the oxidant sensitive dye Redox Sensor Red and Mitotracker Green FM. Aldosterone-treated cells showed bright red fluorescence from 6 h onward (data not shown). As shown in Fig. 6 (at 16 h), bright red florescence was seen in the aldosterone-treated cells, which colocalized with mitochondrial green fluorescence showing yellow-orange fluorescence. These findings indicate that aldosterone stimulates production of tubular cell ROS generation.

Evaluation of the Effect of Aldosterone on Tubular Cell Cytosolic Dephosphorylation of Phospho-Bad

To determine the effect of aldosterone on cytosolic dephosphorylation of phospho-Bad, equal numbers of tubular cells were treated either with vehicle or aldosterone (10^{-6} M) for 16 h. Subsequently, cytosolic fractions were isolated and probed for phospho-Bad and Bad. As shown in Fig. 7, aldosterone promoted dephosphorylation of cytosolic phospho-Bad when compared with vehicle-treated cells.

Evaluation of the Effect of Aldosterone on Tubular Cell Cytosolic Accumulation of Cytochrome C

To evaluate the effect of aldosterone on cyosolic accumulation of cytochrome c, equal numbers of tubular cells were treated either with buffer, aldosterone (10^{-6} M), or spironolactone (10^{-5} M) + aldosterone (10^{-6} M) for 16 h. Subsequently, mitochondrial and cytosolic fractions were isolated; proteins were extracted and probed for cytochrome c and actin. As shown in Fig. 8, aldosterone enhanced accumulation of

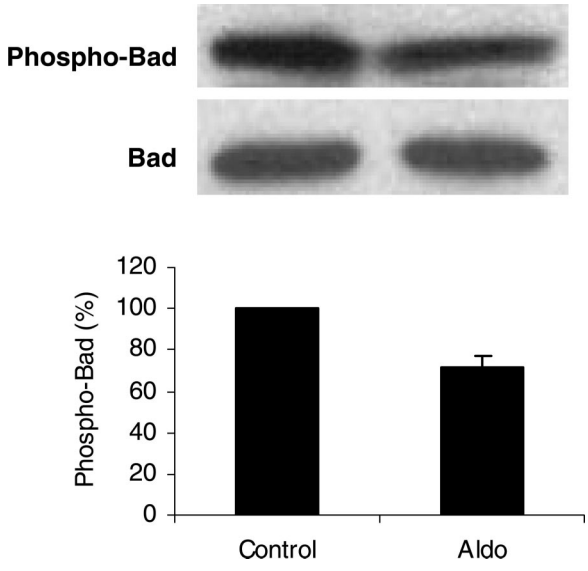

Fig. 7. Effect of aldosteone on tubular cell cytosolic content of phospho-Bad. Equal numbers of tubular cells were incubated in media containing either buffer or aldosterone (10^{-6} M) for 16 h. Subsequently, cytosolic fractions were isolated and probed for phospho-Bad and Bad. *Top*: tubular cell expression of phospho-Bad. *Middle*: tubular cell expression of Bad. *Bottom*: cumulative data of 3 sets of experiments showing %phospho-Bad of control cells.

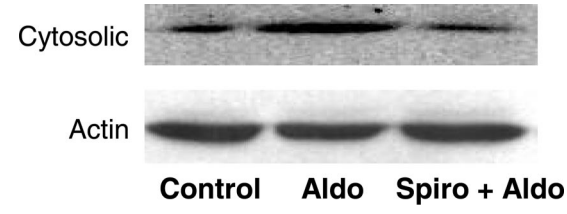

Fig. 8. Effect of aldosteone on tubular cell cytosolic cytochrome c. Equal numbers of tubular cells were incubated in media containing either buffer (control), aldosterone (10^{-6} M), or spironolactone (10^{-5} M) + aldosterone (10^{-6} M) for 16 h. Subsequently, mitochondrial and cytosolic fractions were isolated, and proteins were extracted and probed for cytochrome c and actin. *Top*: cytochrome c content in cytosolic fraction. *Bottom*: actin content in cytosolic fraction.

cytochrome c in the cytosolic fraction when compared with control and spironolactone + aldosterone-treated cells.

DISCUSSION

The present study demonstrates that human renal tubular cells express MR. Aldosterone induces oxidative stress in tubular cells. Aldosterone also induces tubular cell apoptosis in a dose- and time-dependent manner. However, this effect of aldosterone was attenuated by both oxidative stress antagonists and free radical scavengers. Because inhibition of MR was associated with reduction of aldosterone-induced apoptosis, it appears that the proapoptotic effect of aldosterone is mediated through MR. In addition, aldosterone stimulates dephosphorylation of cytosolic phospho-Bad and associated accumulation of cytosolic cytochrome c.

MR antagonists have been shown to have renoprotective effects independent of their hemodynamic effects (2, 4, 8, 21, 22, 38). Greene et al. (8) reported that aldosterone plays a role in the development of renal injury in the remnant kidney model of chronic renal failure. They demonstrated that the exogenous administration of aldosterone completely reversed the ability of combined treatment with enalapril and losartan to attenuate proteinuria, hypertension, and glomerular sclerosis. Chronic administration of spironolactone significantly delayed the development of proteinuria in the remnant kidney model of renal failure although the ultimate development of glomerular sclerosis was not prevented. Rocha et al. (23) showed that aldosterone infusion completely reverses the renal protective action of captopril in saline-drinking stroke-prone hypertensive rats. Similarly, spironolactone has been shown to slow down renal fibrosis in an animal experimental model of diabetic nephropathy (9, 25). Interestingly, Han et al. (9) demonstrated that spironolactone ameliorates renal injury and connective tissue growth factor expression in type II diabetic rats. These investigators demonstrated that this effect of spironolactone is independent of TGF-β production.

UUO model is often used to evaluate the mechanism of progressive tubuloinerstitial fibrosis (12). Trachtman el al. (31) demonstrated that spironolactone slows down progression of renal injury in this model. We presume that spironolactone might have modulated occurrence of apoptosis in this model. However, investigators did not evaluate this aspect in their studies. On the other hand, in an in vitro model of aldosterone-induced rat cardiac myocyte apoptosis, spironolactone completely blocked the occurrence of apoptosis (5). The present study further supports the role of aldosterone in the induction of cellular apoptosis.

Nishiyama et al. (19) demonstrated that renal injury is associated with increases in renal cortical ROS levels and the activation of mitogen-activated protein kinase (MAPK) in aldosterone/salt hypertensive rats and pretreatment with tempol, a cell membrane-permeable radical scavenger, prevented the elevation of ROS levels and MAPK activities and ameliorated renal injury (19). We found that DPI, an inhibitor of NADPH oxidase, partially attenuated apoptosis. Similarly, the free radical scavengers SOD and catalase also partially inhibited aldosterone-induced tubular cell apoptosis. These finding confirm that oxidative stress played a role in aldosterone-induced tubular cell injury. However, antioxidants could provide only partial protection against aldosterone-induced tubular cell apoptosis. These findings suggest that aldosterone might be contributing to tubular cell injury by some other mechanisms also.

Apoptosis is a regulated pathway that is the net outcome of balance between death-promoting and cell survival forces. Bad is a death-promoting protein; however, Bad is usually maintained in phoshphorylated and sequestered form in the cytosol by 14-3-3 proteins and cannot exert its death-promotive action (37). Nevertheless, when Bad is dephosphorylated by upstream apoptotic signals, it heterodimerizes with Bcl-2 and Bcl-xL and reduces their survival signals (33, 37). In the present study, aldosterone enhanced cytosolic dephosphorylation of cytosolic phospho-Bad. We propose that aldosterone-induced dephosphorylation of tubular cell cytosolic phospho-Bad tilted the balance toward apoptosis.

Mitochondria contain the porin channel (voltage-dependent anion channel) on its outer membrane (26, 33). Usually, binding of Bcl-xL protein to this channel stabilizes the porin pore. Nevertheless, when Bad migrate into mitochondria and heterodimerizes with Bcl-xL, anion channel would open and release cytochrome c in the cytosol. In the present study, aldosterone promoted cytochrome c accumulation in the tubular cell cytosol, suggesting heterodimerization of Bcl-xL and opening of the mitochondrial anion channel. Because this effect of aldosterone was attenuated by spironolactone, there seems to be a causal relationship between aldosterone-induced downstream signaling and cytosolic accumulation of cytochrome c.

Aldosterone has been demonstrated to cause myocyte apoptosis through a calcineurin-dependent pathway (12). Calcineurin affects the function of the proapoptotic protein Bad and thus accelerates the mitochondrial death signaling pathway (33). Recent studies suggested that MR blockade with spironolactone prevented renal dysfunction and reduced renal injury in both acute and chronic cyclosporine nephrotoxicity in rats (20). In these studies, spironolactone reduced both tubulointerstitial fibrosis and renal cell apoptosis.

The level of aldosterone in plasma can be as high as 10^{-7} mol/l under stimulatory conditions such as in patients with heart failure; moreover, the level of alodosterone in myocardium was found to be 17 times higher than plasma (24, 27). Because tubular cells carry alodsterone synthase (unpublished observations), it is likely that the renal concentration of aldosterone may also be higher than plasma. Therefore, the aldosterone concentrations used in the present study are considered clinically relevant.

We conclude that aldosterone causes apoptosis of human proximal tubular cells through oxidative stress, and this effect of aldosterone is mediated via MR.

ACKNOWLEDGMENTS

Part of this work was presented as an oral presentation at the Annual Meeting of the American Society of Nephrology held in San Diego in 2006.

GRANTS

This work was supported by grant RO1 DA-12111 from National Institutes of Health, Bethesda, MD.

REFERENCES

1. **Ben Rhouma K, Schimchowitsch S, Stoeckel ME, Felix JM, Sakly M.** Implication of type II glucocorticoid receptors in aldosterone induced apoptosis of rat thymocytes. *Arch Physiol Biochem* 105: 216–224, 1997.

2. **Blasi ER, Rocha R, Rudolph AE, Blomme EA, Polly ML, McMahon EG.** Aldosterone/salt induces renal inflammation and fibrosis in hypertensive rats. *Kidney Int* 63: 1791–1800, 2003.
3. **Campbell SE, Janicki JS, Matsubara BB, Weber KT.** Myocardial fibrosis in the rat with mineralocorticoid excess. *Am J Hypertens* 6: 487–495, 1993.
4. **Chrysostomou A, Becker G.** Spironolactone in addition to ACE inhibition to reduce proteinuria in patients with chronic renal disease. *N Engl J Med* 345: 925–926, 2001.
5. **De Angelis N, Fiordaliso F, Latini R, Calvillo L, Funicello M, Gobbi M, Mennini T, Masson S.** Appraisal of the Role of Angiotensin II and Aldosterone in Ventricular Myocyte Apoptosis in Adult Normotensive Rat. *J Mol Cell Cardiol* 34: 1655–1665, 2002.
6. **Finkel T.** Redox-dependent signal transduction. *FEBS Lett* 476: 52–54, 2000.
7. **Gomez-Sanchez CE, Zhou MY, Cozza EN, Morita H, Foecking MF, Gomez-Sanchez EP.** Aldosterone Biosynthesis in the Rat Brain. *Endocrinology* 138: 3369–3373, 1997.
8. **Greene EL, Kren S, Hostetter TH.** Role of aldosterone in the remnant kidney model in the rat. *J Clin Invest* 98: 1063–1068, 1996.
9. **Han KH, Kang YS, Han SY, Jee YH, Lee MH, Han JY, Kim HK, Kim YS, Cha DR.** Spironolactone ameliorates renal injury and connective tissue growth factor expression in type II diabetic rats. *Kidney Int* 70: 111–120, 2006.
10. **Kaul JF, Choi J, Forman HJ.** Transmembrane redox signaling activates NF-kappaB in macrophages. *Free Radic Biol Med* 24: 202–207, 1998.
11. **Kim JH, Yang JI, Jung MH, Hwa JS, Kang KR, Park DJ, Roh GS, Cho GJ, Choi WS, Chang SH.** Heme oxygenase-1 protects rat kidney from ureteral obstruction via an antiapoptotic pathway. *J Am Soc Nephrol* 17: 1373–1381, 2006.
12. **Klahr S, Morrissey J.** Obstructive nephropathy and renal fibrosis. *Am J Physiol Renal Physiol* 283: F861–F875, 2002.
13. **Laragh JH.** Hormones in the pathogenesis of congestive heart failure: vasopressin, aldosterone, angiotensin II. *Circulation* 25: 1015–1023, 1962.
14. **Lennon SV, Martin SJ, Cotter TG.** Dose-dependent induction of apoptosis in human tumor cell lines by widely diverging stimuli. *Cell Prolif* 24: 203–214, 1991.
15. **Lodha S, Dani D, Mehta R, Bhaskaran M, Reddy K, Ding G, Singhal PC.** Angiotensin II-induced Mesangial Cell Apoptosis: Role of Oxidative Stress. *Mol Med* 8: 830–840, 2002.
16. **Mano A, Tatsumi T, Shiraishi J, Keira N, Nomura T, Takeda M, Nishikawa S, Yamanaka S, Matoba S, Kobara M, Tanaka H, Shirayama T, Takamatsu T, Nozawa Y, Matsubara H.** Aldosterone directly induces myocyte apoptosis through calcineurin-dependent pathways. *Circulation* 110: 317–323, 2004.
17. **McGowan AJ, Fernandes RS, Samali A, Cotter TG.** Anti-oxidants and apoptosis. *Biochem Soc Trans* 24: 229–233, 1996.
18. **Nishiyama A, Abe Y.** Aldosterone and renal injury. *Nippon Yakurigaku Zasshi* 124: 101–109, 2004.
19. **Nishiyama A, Yao L, Nagai Y, Miyata K, Yoshizumi M, Kagami S, Kondo S, Kiyomoto H, Shokoji T, Kimura S, Kohno M, Abe Y.** Possible contributions of reactive oxygen species and mitogen-activated protein kinase to renal injury in aldosterone/salt-induced hypertensive rats. *Hypertension* 24: 841–848, 2004.
20. **Pérez-Rojas J, Blanco JA, Cruz C, Trujillo J, Vaidya VS, Uribe N, Bonventre JV, Gamba G, Bobadilla NA.** Mineralocorticoid receptor blockade confers renoprotection in preexisting chronic cyclosporine nephrotoxicity. *Am J Physiol Renal Physiol* 292: F131–F139, 2007.
21. **Rachmani R, Slavachevsky I, Amit M, Levi Z, Kedar Y, Berla M, Ravid M.** The effect of spironolactone, cilazapril and their combination on albuminuria in patients with hypertension and diabetic nephropathy is independent of blood pressure reduction: a randomized controlled study (Abstract). *Diabetic Medicine* 21: 471, 2004.
22. **Rocha R, Chander PN, Khanna K, Zuckerman A, Stier CT Jr.** Mineralocorticoid blockade reduces vascular injury in stroke-prone hypertensive rats. *Hypertension* 31: 451–458, 1998.
23. **Rocha R, Khanna K, Burstein J, Cho J, Zuckerman A, Chander PN, Stier CT Jr.** Aldosterone reverses the renal protective effect of ACE inhibition in stroke-prone hypertensive rats (Abstract). *J Am Soc Nephrol* 8: A1407, 1997.
24. **Rousseau MF, Gurné O, Duprez D, Van Mieghem W, Robert A, Ahn S, Galanti L, Ketelslegers JM.** Beneficial neurohormonal profile of spironolactone in severe congestive heart failure: results from the RALES neurohormonal substudy. *J Am Coll Cardiol* 40: 1596–601, 2002.
25. **Sato A, Hayashi K, Naruse M, Saruta T.** Effectiveness of aldosterone blockade in patients with diabetic nephropathy. *Hypertension* 31: 451–458, 2003.
26. **Shimizu S, Narita M, Tsujimoto Y.** Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 399: 483–487, 1999.
27. **Silvestre JS, Robert V, Heymes C, Aupetit-Faisant B, Mouas C, Moalic JM, Swynghedauw B, Delcayre C.** Myocardial production of aldosterone and corticosterone in the rat. Physiological regulation. *J Biol Chem* 273: 4883–4891, 1998.
28. **Swedberg K, Eneroth P, Kjekshus J, Wilhelmsen.** Hormones regulating cardiovascular function in patients with severe congestive heart failure and their relation to mortality: CONSENSUS Trial Study Group. *Circulation* 82: 1730–1736, 1990.
29. **Takeda Y, Miyamori I, Yoneda T, Iki K, Hatakeyama H, Blair IA, Hsieh FY, Takeda R.** Production of Aldosterone in Isolated Rat Blood Vessels. *Hypertension* 25: 170–173, 1995.
30. **Tammariello SP, Quinn MT, Estus S.** NADPH oxidase contributes directly to oxidative stress and apoptosis in nerve growth factor-deprived sympathetic neurons (Abstract). *J Neurosci* 20: RC53, 2000.
31. **Trachtman H, Weiser AC, Valderrama E, Morgado M, Palmar LS.** Prevention of renal fibrosis by spironolactone in mice with completer unilateral ureteral obstruction. *J Urol* 172: 1590–1594, 2004.
32. **Trudel M, Lanoix J, Barisoni L, Blouin MJ, Desforges M, L'Italien C, D'Agati V.** C-myc-induced apoptosis in polycystic kidney disease is Bcl-2 and p53 independent. *J Exp Med* 186: 1873–1884, 1997.
33. **Wang HG, Pathan N, Ethell IM, Krajewski S, Yamaguchi Y, Shibasaki F, McKeon F, Bobo T, Franke TF, Reed JC.** Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. *Science* 284: 339–343, 1999.
34. **Weber KT, Brilla CG.** Pathological hypertrophy and cardiac interstitial: fibrosis and renin-angiotensin-aldosterone system. *Circulation* 83: 1849–1865, 1991.
35. **Wu P, Liang X, Dai Y, Liu H, Zang Y, Guo Z, Zhang R, Lai W, Zhang Y, Liu Y.** Aldosterone biosynthesis in extra-adrenal tissues *Chin Med J Engl* 112: 414–418, 1999.
36. **Young MJ, Clyne CD, Cole TJ, Funder JW.** Cardiac steroidogenesis in the normal and failing heart. *J Clin Endocrinol Metab* 86: 5121–5126, 2001.
37. **Zha J, Harada H, Yang E, Jockel J, Koresmeyer MJ.** Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14–3-3 not Bcl-X(L). *Cell* 87: 619–628, 1996.
38. **Zhou X, Ono H, Ono Y, Frohlich ED.** Aldosterone antagonism ameliorates proteinuria and nephrosclerosis independent of glomerular dynamics in L-NAME/SHR model. *Am J Nephrol* 24: 242–249, 2004.