Aldosterone promotes proximal tubular cell apoptosis: role of oxidative stress

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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 apoptotic effect of ROS. 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.

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**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 and propidium iodide (Sigma, St. Louis, MO). H-33342 stains the nuclei, indicated by arrows.

**Assay 2: Detection of DNA fragmentation.** To determine DNA fragmentation, we have used terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay. A: control HK2 cells stained with H-33342 showing fluorescent labeling with propidium iodide (Sigma, St. Louis, MO) and 1% penicillin-streptomycin at 37°C in 5% CO2. All studies were performed in HK2 cells except for determining expression of mineralocorticoid receptors (MR) in HRPTECs as well.

**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 1X protease inhibitor cocktail (Sigma), 1 mM phenylmethylsulfonyl fluoride, phosphatase inhibitor cocktail (CT1

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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; 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-Bad (1:1,000; BD Biosciences, San Diego, CA) overnight at 4°C.

A horseradish peroxidase-conjugated appropriate secondary antibody was applied for 1 hr at room temperature. The blots were then developed using a chemiluminescence detection kit (ECL; Amersham, Arlington Heights, IL) and exposed to Kodak X-OMAT AR film. Blots were stripped and probed with anti-MR (H-300) antibody (1:200; Santa Cruz) overnight at 4°C.

Immunofluorescent Detection of Oxidative Stress

Aldosterone-induced oxidative stress in tubular cells (HK2 cells) was studied by trafficking of 2,3,4,5,6-pentafluorodihydrotetramethyl-rosamine 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$_2^•$ and H$_2$O$_2$. 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 DFX 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 subconfluency 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. 1A, 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. 2B.

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. 1B, 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. 2D.

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%). C: spironolactone + aldosterone-treated cells (apoptotic cells, 35%). D: equal numbers of cells were incubated in media containing buffer, aldosterone (10^{-6} M), spironolactone (Spiron. 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 Aldosterone-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-fold greater apoptosis (apoptotic cells 52%; Fig. 3B) when compared with control cells (apoptotic cells 14%; Fig. 3A). However, spironolactone partially attenuated this effect of aldosterone (apoptotic cells, 35%; Fig. 3C).

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 + spironolactone (10^-5 M) for 18 h. Subsequently, the cells were prepared for TUNEL assay. As shown in Fig. 3D, 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. 4A, 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. 4B).

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 (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 < 0.01 compared with control. **P < 0.01 compared with aldosterone.

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

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 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 < 0.01 compared with control. **P < 0.01 compared with aldosterone and catalase alone. ***P < 0.01 compared with control and SOD alone.
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 fluorescence 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 cytosolic accumulation of cytochrome c, equal numbers of tubular cells were treated either with buffer, aldosterone (10^{-6} M), or spironolactone (10^{-5} M) or 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 cytosolic cytochrome c.

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 fluorescence was seen in the aldosterone-treated cells (E), which colocalized with mitochondrial green fluorescence showing yellow-orange fluorescence (F).

Fig. 7. Effect of aldosterone 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 aldosterone 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) or 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.

UO model is often used to evaluate the mechanism of progressive tubulointerstitial fibrosis (12). Trachtman et 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 tem- pol, a cell membrane-permeable radical scavenger, prevented the elevation of ROS levels and MAPK activities and amelio- rate 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 main- tained in phosphorylated 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 tubu- lar 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 cyto- chrome c.

Aldosterone has been demonstrated to cause myocyte apop- tosis through a calcineurin-dependent pathway (12). Cal- cineurin affects the function of the proapoptotic protein Bad and thus accelerates the mitochondrial death signaling pathway (33). Recent studies suggested that MR blockade with spirono- lactone 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 aldosterone in myocardium was found to be 17 times higher than plasma (24, 27). Because tubular cells carry aldosterone synthase (unpublished observations), it is likely that the renal concentration of aldo- steroid may also be higher than plasma. Therefore, the aldo- steroid 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.

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GRANTS

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REFERENCES

ALDOSTERONE PROMOTES TUBULAR CELL APOPTOSIS