Aldosterone/Mineralocorticoid Receptor Stimulation Induces Cellular Senescence in the Kidney

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Recent studies demonstrated a possible role of aldosterone in mediating cell senescence. Thus, the aim of this study was to investigate whether aldosterone induces cell senescence in the kidney and whether aldosterone-induced renal senescence affects the development of renal injury. Aldosterone infusion (0.75 μg/h) into rats for 5 weeks caused hypertension and increased urinary excretion rates of proteins and N-acetyl-D-glucosaminidase. Aldosterone induced senescence-like changes in the kidney, exhibited by increased expression of the senescence-associated β-galactosidase, overexpression of p53 and cyclin-dependent kinase inhibitor (p21), and decreased expression of SIRT1. These changes were abolished by eplerenone (100 mg/kg/d), a mineralocorticoid receptor (MR) antagonist, but unaffected by hydralazine (80 mg/liter in drinking water). Furthermore, aldosterone induced similar changes in senescence-associated β-galactosidase, p21, and SIRT1 expression in cultured human proximal tubular cells, which were normalized by an antioxidant, N-acetyl L-cysteine, or gene silencing of MR. Aldosterone significantly delayed wound healing and reduced the number of proliferating human proximal tubular cells, while gene silencing of p21 diminished the effects, suggesting impaired recovery from tubular damage. These findings indicate that aldosterone induces renal senescence in proximal tubular cells via the MR and p21-dependent pathway, which may be involved in aldosterone-induced renal injury. (Endocrinology 152: 680–688, 2011)

Recent animal and clinical studies have suggested a role for aldosterone in the pathogenesis of renal injury (1–4). Rats treated with aldosterone and high salt develop severe proteinuria, glomerular injury, and tubulointerstitial fibrosis (1, 4). In hypertensive patients, treatment with a selective mineralocorticoid receptor (MR) antagonist, eplerenone, reduced albuminuria to a greater extent than treatment with an angiotensin-converting enzyme inhibitor, enalapril, with a similar hypotensive effect (2). Importantly, a greater increase in albuminuria in patients with primary aldosteronism compared with patients with essential hypertension has also been demonstrated (3). These data suggest that aldosterone plays a detrimental role in the development of renal injury.

Aging is an important factor in the development of chronic kidney disease (5). Although it is unclear whether the aging-related changes result from senescence itself or from accumulated environmental stress throughout life, cell senescence could be a common mechanism and marker for the renal dysfunction observed in the aging

Abbreviations: BrdU, 5′-Bromo-2′-deoxyuridine; CDK, cyclin-dependent kinase; FBS, fetal bovine serum; HPTC, human proximal tubule cell; MR, mineralocorticoid receptor; NAC, N-acetyl l-cysteine; NAG, N-acetyl-β-D-glucosaminidase; SA-βGal, senescence-associated β-galactosidase; SBP, systolic blood pressure; siRNA, small interfering RNA; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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kidney. Cellular senescence is characterized by irreversible growth arrest, which is regulated by telomerase, cyclin, cyclin-dependent kinase (CDK), and CDK inhibitors such as p21 (6). In fact, an age-dependent increase in CDK inhibitor expression was observed in kidneys in animals (7, 8), indicating that cell senescence occurs in parallel with aging. Recently, Westhoff et al. (9) reported that the expression of p16\(^{INK4a}\), another cell cycle inhibitor, was increased in the kidney of 18-week-old rats with deoxycorticosterone acetate-salt–induced hypertension, and that antihypertensive treatment (hydrochlorothiazide, reserpine, and hydralazine) or treatment with spironolactone at a nonhypotensive dose partially ameliorated the increased expression of p16\(^{INK4a}\) and hypertensive renal damage. These findings indicate that environmental stress (e.g., hypertension) can induce cell senescence in the kidneys and the treatment could prevent it. Furthermore, recent studies have revealed that vascular senescence is strongly associated with the progression of atherosclerosis in apoE-knockout mice and that the deletion of p21 could attenuate senescence or atherosclerotic changes (10). This indicates that cellular senescence may decrease the innate ability of vessels to protect against pathological factors. However, the effects of cellular senescence on renal function, whether it causes or accelerates damage, and whether it results from the disease state, remain unclear.

Aldosterone is reported to up-regulate p21 in several cell types, including cancer cells, (11) vascular smooth muscle cells (12), and renal epithelial cells (13). Therefore, we hypothesized that chronic exposure to excessive aldosterone levels accelerates senescence in the kidney through a p21-dependent pathway and that aldosterone-induced renal senescence contributes to the development of renal injury. To address this hypothesis, we assessed in vivo–“premature” senescence in the kidney of aldosterone-infused rats in this study. The target cells identified in vivo, proximal tubular cells, were then cultured in vitro to evaluate the direct effects of aldosterone on cellular senescence and the contribution to aldosterone-induced renal injury.

Materials and Methods

Animal preparation

All experimental procedures were performed under the guidelines for the care and use of animals established by the Kagawa University. The experiments were performed on male Sprague Dawley rats (CLEA, Tokyo, Japan). The 10-week-old rats underwent right uninephrectomy under anesthesia with sodium pentobarbital (50 mg/kg, ip). After a 10-d recovery period, rats weighing 322–363 g were randomly divided into the following four groups and were maintained throughout the 5-week experimental period: group 1, vehicle (2% ethanol as vehicle, sc, n = 10); group 2, aldosterone (0.75 μg/h, sc, n = 8), Across Organics, Geel, Belgium); group 3, aldosterone + eplerenone (100 mg/kg/d, po, n = 10); group 4, aldosterone + hydralazine (80 mg/liter in drinking water, n = 10). All groups received 1% NaCl in drinking water throughout the experimental period. Rats were anesthetized with sodium pentobarbital (50 mg/kg, ip), and osmotic minipumps were implanted subcutaneously at the dorsum of the neck to infuse vehicle or aldosterone. The doses of drugs were determined on the basis of results from previous rat studies (1, 14).

Systolic blood pressure (SBP) was measured in the conscious state by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan) at a day before uninephrectomy and at weeks 0, 1, 3, and 5 during the treatment period. Twenty-four-hour urine samples were collected starting after a 24-hr acclimatization period in metabolic cages. Rats were euthanized by an excessive dose of sodium pentobarbital. Kidneys were perfused by chilled sterile PBS solution, and a piece of cross-sectioned kidney tissue was used for senescence-associated β-galactosidase (SA-βGal) staining (15). The remaining kidney was snap-frozen in liquid nitrogen and stored at −80°C until processing for protein or mRNA extraction.

Urinary protein excretion was determined using a protein assay kit (microTP-test; Wako Co., Osaka, Japan). Urine and plasma creatinine values were analyzed using a Jaffe assay kit (Creatinine-test; Wako Co.). The measurement of N-acetyl-β-D-glucosaminidase (NAG) in urine was performed using a colorimetric assay kit (Shionogi, Osaka, Japan).

SA-βGal staining

Kidneys were fixed for 3–5 min at room temperature in 2% formaldehyde/0.2% glutaraldehyde, washed twice with PBS, and incubated for 24 h at 37°C in freshly prepared β-Gal staining solutions (pH 6.0) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, 5 mmol/liter potassium ferrocyanide, 150 mmol/liter NaCl, 2 mmol/liter MgCl\(_2\), 0.01% sodium deoxycholate, and 0.02% Nonidet-40 (8, 16, 17). After staining, the samples were immersed in OCT compounds (Sakura Finetek, Torrance, CA) and snap-frozen in chilled acetone to prepare cryostat sections. Three cross sections (6 μm thickness per section 100 μm apart) from one kidney were subjected to analyze the senescent cells. SA-βGal–positive cells were counted in five randomly chosen fields per section at ×100 magnification and assessed in terms of the development of blue color.

Western blotting

Protein expression of p53, p21, and MR was measured by Western blotting (10, 18, 19). Protein (50 μg) was separated by 10% or 15% SDS-PAGE, transferred to a nitrocellulose membrane, and immunoblotted with antibodies specific for p53 (1:1000; Cell Signaling, Beverly, MA), p21 (1:1000; BioSource International, Camarillo, CA), or MR (1:50000 Santa Cruz Biotechnology, Santa Cruz, CA). Equal loading was confirmed by reprobing the membranes with an antibody against β-actin (1:10,000 dilution; Sigma Chemical). Data are expressed as the relative differences between each group after normalization for β-actin expression. The protein bands were visualized with an ECL system (Amersham Biosciences). Densitometric analysis was performed using National Institutes of Health image software (20).

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Real-time RT-PCR
The mRNA expressions of β-actin, SIRT1, p53, p21, TNF-α, and collagen type I and III were analyzed by RT-PCR using a LightCycler FastStart DNA Master SYBR Green I kit (Applied Biosystems, Foster City, CA) (21). Briefly, cDNA was initially denatured at 95°C for 30 sec and then amplified by PCR for 40 cycles (95°C for 15 sec, 60°C for 40 sec) (21). The primer sequences were as follows: p21 forward, 5'-AGGCCCGTATGATGACATG-3', reverse, 5'-CCCGGATGCAAGAGAT-3'; p53 forward, 5'-GGCTCCGATTACCTACATCCA-3', reverse, 5'-TGTC- CCGTCCAGAAGATTC-3'; SIRT1 forward, 5'-TGACTCTCAAGGATGTT-3', reverse, 5'-GACAGAGGATGGCTGAATGT-3'; TNF-α forward, 5'-GGAGAGAGAACATCCACCTT-3', reverse, 5'-CCCTAAGCCCCAACATTCT-3'; collagen III forward, 5'-CTGTGACTCAGGATCCGTTCTCT-3', reverse, 5'-TTGAGGCCGTCTGGGCAAA-3'; and β-actin forward, 5'-GCCAGATAGGCACCAACATC-3', reverse, 5'-ACTGCCTGGTCCTTAGCA-3'. All data are expressed as relative differences to vehicle-infused rats after normalization for β-actin expression.

Cell culture and treatment
Human proximal tubule cells (HPTCs) immortalized by temperature-sensitive SV40 large T antigen containing adenovirus (TSlm-HPTC) and HPTCs cultured from non-hypertensive patients (N-HPTC, passage 3) were maintained in a growth medium of Click's Medium: RPMI-1640–free containing 3.5 ng/ml dexamethasone and 40 ng/ml dexamethasone for 24 h at 37°C for both HPTCs and at 37°C for N-HPTCs. After 80% confluence in growth medium, the cells were transferred to 0.2% FBS Click's Medium: RPMI-1640 containing with 1% insulin-transferrin-selenium and 40 ng/ml dexamethasone, 10 ng/ml epidermal growth factor, 2% fetal bovine serum (FBS), and 2% penicillin-streptomycin in humidified atmosphere of 5% CO2 at 33°C for TSIm-HPTCs and at 37°C for N-HPTCs. Scramble vector was used as a control. For RNA interference, interfering RNA (siRNA) for MR or p21. In another group of experiments, an antioxidant, acetyl L-cysteine (NAC, 10 nmol/liter) was pretreated for 1 h before aldosterone treatment.

Wound healing assay
Wound healing was performed as described previously (22). HPTCs were rendered quiescent by incubation for 24 h in medium containing 0.2% FBS. The medium was then removed and wounding was induced by a single pass with a sterile yellow pipette tip. Medium containing 0.2% FBS with or without 10 nmol/liter aldosterone was added to the wells, and wound closure was monitored every 12 h with a TS100 inverted microscope fitted with a digital camera (Nikon, ECLIPSE TS100, Tokyo, Japan). The area of the wound was measured in arbitrary units using Image J 1.32 software.

5′-Bromo-2′-deoxyuridine (BrdU) labeling
HPTCs were treated with BrdU (10 μmol/liter, Sigma, St. Louis, MO) from 36–48 h after making the wound, and immunoreactivity was analyzed at 48 h after wounding (23). After fixing the cells in paraformaldehyde (3.7% for 30 min), cells were incubated with methanol/acetic acid for 10 min. After blocking, the sections were incubated with primary antibodies for 2 h at 37°C. Antibodies were visualized by fluorescein isothiocyanate. The average BrdU-positive nuclei numbers around the wound in five different examinations was calculated using WinROOF image software.

Statistical analysis
Results are expressed as means ± st. Statistical significance was assessed using ANOVA, followed by Bonferroni’s test. A value of P < 0.05 was considered to be statistically significant.

Results
Systolic blood pressure, renal function, and tubular injury in aldosterone-infused rats
The general characteristics of the rats are presented in Table 1. Aldosterone infusion induced a significant elevation of SBP at week 5 compared with vehicle infusion, whereas treatment with eplerenone or hydralazine suppressed the aldosterone-induced SBP elevation. Aldosterone infusion significantly increased the kidney/body weight ratio, urinary protein/creatinine ratio, and urinary NAG activities and plasma creatinine levels (Table 1) compared with vehicle infusion. Eplerenone treatment markedly suppressed the effects of aldosterone. Hydralazine partially prevented the increase in kidney/body weight and urinary protein/creatinine ratio but failed to suppress the increase in urinary NAG and plasma creatinine levels.

Aldosterone affected senescence-associated β galactosidase activity and expression of p53, p21, and SIRT1 in the kidney
An increase in SA-βGal staining, which is typically observed in senescent cells, was detected in the kidney, particularly the proximal tubules, of aldosterone-infused rats, but not in vehicle-infused rats (Fig. 1). However, SA-βGal staining was hardly detected in the glomeruli in any group. Eplerenone prevented the aldosterone-stimulated increase in SA-βGal staining in the kidney. Hydralazine tended to reduce the staining area, but the staining area was still significantly greater than those in the vehicle-treated control group and eplerenone-treated group, suggesting that...
the staining was augmented by a blood pressure–independent pathway in this experimental animal model.

Because p53 is an important molecule that regulates the cell cycle, we analyzed the expression of p53 in renal cortical tissues. Aldosterone infusion markedly increased the expression of p53 mRNA and protein (Fig. 2, A and B). Next, we analyzed the expression of p21, a transcriptional target of p53 and an important cell cycle regulator, in renal cortical tissues (Fig. 2, C and D). Aldosterone strongly increased the expression of p21 mRNA and protein. Eplerenone abolished the increase in p53 and p21, suggesting that aldosterone regulates cell cycle mediators via the MR in the renal cortex. Hydralazine significantly suppressed the mRNA level of p21 but failed to affect the expression of p53 mRNA and protein and p21 protein. Either mRNA or protein level of p21 in hydralazine-treated group was greater than that in eplerenone-treated group. On the other hand, the gene expression of SIRT1, an enzyme that inactivates p53 by deacetylation, was reduced in aldosterone-infused rats compared with that in vehicle-infused rats (0.51 ± 0.06-fold vs. 1.00 ± 0.09-fold, P < 0.01), and was significantly normalized by eplerenone (0.87 ± 0.15-fold, P < 0.05), but not by hydralazine (0.63 ± 0.07-fold, P < 0.05 compared with eplerenone).

**Aldosterone-induced senescence in human proximal tubular cells**

To further determine the effect of aldosterone on cellular senescence, we investigated the effect of aldosterone in cultured HPTCs because the proximal tubules showed

**FIG. 1.** Staining for senescence-associated β-galactosidase (SA-βGal) in rat renal cortex (n = 8–10). SA-βGal is labeled by bright-blue staining in the tubular epithelial cells. Aldosterone-infused rats showed greater staining for SA-βGal than vehicle-infused rats. Eplerenone suppressed the increased staining in aldosterone-infused rats. *, P < 0.05 vs. vehicle; #, P < 0.05 vs. aldosterone; †, P < 0.05 vs. aldosterone + eplerenone.

**FIG. 2.** mRNA and protein expression levels of p53 and p21 (n = 8–10). Aldosterone infusion increased the mRNA (A and C) and protein (B and D) expression of p53 (A and B) and p21 (C and D) in renal cortical tissues. These changes were suppressed by eplerenone. Values were normalized for the value of vehicle-infused rats. *, P < 0.05 vs. vehicle; †, P < 0.05 vs. aldosterone; †, P < 0.05 vs. aldosterone + eplerenone.

<table>
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<tr>
<th>No.</th>
<th>Vehicle</th>
<th>Aldosterone</th>
<th>Aldosterone + eplerenone</th>
<th>Aldosterone + hydralazine</th>
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<td>8</td>
<td>10</td>
<td>10</td>
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<td>Systolic blood pressure, mm Hg</td>
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<td>191 ± 7*</td>
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<tr>
<td>Body weight, g</td>
<td>556 ± 13</td>
<td>509 ± 6*</td>
<td>517 ± 10*</td>
<td>508 ± 11*</td>
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<td>Left kidney weight, g</td>
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<td>4.7 ± 0.3*</td>
<td>3.0 ± 0.1*</td>
<td>3.5 ± 0.2**</td>
</tr>
<tr>
<td>Left kidney weight/body weight, mg/g</td>
<td>5.4 ± 0.2</td>
<td>9.2 ± 0.6*</td>
<td>5.8 ± 0.2*</td>
<td>7.0 ± 0.3**</td>
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<td>Urinary protein excretion, mg/d</td>
<td>14 ± 6</td>
<td>334 ± 64*</td>
<td>57 ± 6*</td>
<td>191 ± 19*</td>
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<td>Urinary protein/creatinine ratio, mg/mg</td>
<td>0.5 ± 0.1</td>
<td>6.1 ± 2.6*</td>
<td>1.2 ± 0.3*</td>
<td>2.0 ± 0.2**</td>
</tr>
<tr>
<td>Urinary NAG, U/mmol creatinine</td>
<td>0.6 ± 0.2</td>
<td>2.4 ± 0.5*</td>
<td>0.9 ± 0.1*</td>
<td>1.7 ± 0.1*</td>
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<tr>
<td>Plasma creatinine, µmol/liter</td>
<td>44 ± 3</td>
<td>65 ± 4*</td>
<td>51 ± 5*</td>
<td>61 ± 3*</td>
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</table>

Values are means ± SE.

* P < 0.05 vs. vehicle; †, P < 0.05 vs. aldosterone.
extensive staining for SA-βGal. We used HPTCs immortalized by temperature-sensitive SV40 large T antigen containing adenovirus (TSIm-HPTC), or HPTCs cultured from non-hypertensive patients (N-HPTC, passage 3). The TSIm-HPTCs were able to approach the Hayflick limit, a limitation of cell mitosis, at 37°C (Supplemental Fig. 1, A–C, published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org/), indicating that cells were not immortalized at this temperature. Both TSIm- and N-HPTCs used in this study showed higher proximal tubular markers such as sodium glucose transporter 2 (SGLT2) expression and alkaline phosphatase activity, than renal mesangial cells (Supplemental Fig. 1D). Aldosterone (1 and 10 nmol/liter for 3 d) dose-dependently increased the percentage of SA-βGal–expressing HPTCs compared with vehicle treatment in both TSIm-HPTCs and N-HPTCs (Supplemental Fig. 2, A and B). In addition, aldosterone time-dependently increased SA-βGal staining, which reached statistical significance when the cells were treated for more than 2 d (Supplemental Fig. 2, C and D). Furthermore, aldosterone dose-dependently decreased the SIRT1 expression and increased the acetylated p53 (Supplemental Fig. 3). The effect of aldosterone on SA-βGal expression was not affected by transfection with the scramble vector, but was markedly reduced by siRNAs for MR and p21 in both TSIm-HPTCs (Fig. 3A) and N-HPTCs (Supplemental Fig. 4). Aldosterone decreased the expression of SIRT1 (Fig. 3B) and increased the expression of p21 (Fig. 3C) in TSIm-HPTCs, and these effects were strongly suppressed in cells treated with siRNA for the MR (Fig. 3). These data support the in vivo results that aldosterone/MR may induce cell senescence in the kidney via an MR/SIRT1/p53/p21-dependent pathway.

Aldosterone is known to induce MR-mediated superoxide production in mesangial cells (24) and fibroblasts (25). Aldosterone also significantly increased the fluorescence intensity of dihydroethidium (DHE) staining after 3 d of treatment, which was prevented by siRNA for the MR (Supplemental Fig. 5) or the addition of an antioxidant, NAC (10 mmol/liter) (Supplemental Fig. 6). Because oxidative stress has been implicated in cellular senescence, (26) we next evaluated the effect of exposure to an antioxidant on the effects of aldosterone. Aldosterone-induced changes in SA-βGal staining and the expression of p21 and SIRT1 were significantly inhibited by exposure to NAC, indicating that oxidative stress is involved in aldosterone/MR-induced senescence in TSIm-HPTCs (Fig. 4).

To explore the role of senescence of proximal tubular cells on the development of renal injury, we investigated the effect of aldosterone on wound healing. Aldosterone significantly delayed wound healing compared with the control, and the effect of aldosterone was significantly reduced by transfection with siRNA for p21 (Fig. 5, A and B), suggesting that aldosterone impaired tubular repair via p21, a cyclin-dependent kinase inhibitor. To support the hypothesis that aldosterone-induced senescence impairs the repair ability of proximal tubules, we assessed the proliferating cells around the wound using BrdU labeling (Fig. 5C). Aldosterone-treated cells showed less immunofluorescence than vehicle-treated cells. The decreased immunofluorescence...
was prevented by siRNA for p21, indicating that aldosterone caused cell senescence and decreased the number of cells undergoing proliferation.

Furthermore, we evaluated the role of cell senescence on the expression of apoptotic and fibrotic molecules because it has been reported that aldosterone induces apoptosis (27) and epithelial–mesenchymal transition (28) in proximal tubular cells. Aldosterone (10 nmol/liter for 3 d) significantly increased the gene expression of TNF-α and collagen types I and III (Fig. 6) and the TNF-α secretion (Supplemental Fig. 7) in TSIm-HPTCs. Gene silencing for p21 abolished these changes, suggesting the involvement of cell senescence in this process. Aldosterone (10 nmol/liter for 3 d) did not induce significant changes in terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive TSIm-HPTCs (Supplemental Fig. 8).

Discussion

Aldosterone is an important hormone that regulates electrolyte balance. The role of aldosterone in tissues has become an important issue because epidemiological studies [Randomized Aldosterone Evaluation Study (RALES) and Eplerenone Post-AMI Heart Failure Efficacy and Survival Study (EPHESUS)] revealed beneficial effects of MR inhibitors on patient outcomes compared with placebo control (29, 30). We previously demonstrated that the infusion of aldosterone induced glomerulosclerosis and tubulointerstitial fibrosis (1, 31). However, the precise mechanisms involved in aldosterone-induced renal injury are still under investigation. The current study showed that aldosterone-induced renal injury was associated with senescence in renal tissue, including in proximal tubular cells, and that the effect was regulated through an MR/reactive oxygen species/SIRT1/p53/p21–dependent/blood pressure–independent pathway. Furthermore, senescence delayed the repair of tubular cells in a wound. Our study did not explore whether this is a trigger of aldosterone-dependent injury because we observed senescence after the onset of renal injury in rats; however, we propose that aldosterone-induced proximal tubular senescence may accelerate aldosterone-induced renal injury by impairing the recovery of tubules and inducing the expression of fibrotic and apoptotic/inflammatory cytokines. This hypothesis may be supported by an earlier study showing that mice lacking the p21 gene developed less interstitial fibrosis with increased cell proliferation in a renal ablation model compared with normal mice (32).

It is known that cells have several pathways to protect themselves against differentiation into tumors when they are exposed to stress (33–35). Cells exposed to stress are temporally maintained in cell cycle arrest to allow repair of DNA damage (6). When cells are exposed to excessive stress that cannot be managed by the endogenous repair system, they are driven into senescence, dedifferentiate into other phenotypes, and ultimately undergo apoptosis (6, 36). In fact, Patni et al. (27) revealed that aldosterone induced tubular cell apoptosis by activating the mitochondrial pathway and generating reactive oxygen species in human proximal tu-
bular cells (HK2). On the other hand, aldosterone induced epithelial–mesenchymal transition via MR-mediated mitochondria-originating reactive oxygen species in HK2 cells (28). These studies, in addition to the current results, indicate that proximal tubules are a target of the deleterious effects of aldosterone. However, the mechanism by which aldosterone, as an exogenous stress, changes the cell fate to apoptotic death, epithelial–mesenchymal transition or senescence was unclear (37). In the present study, aldosterone induced cell senescence and increased the TNF-α and collagens, which is consistent with previous reports (38, 39), suggesting that aldosterone enhances autocrine/paracrine apoptotic/ inflammatory and fibrotic signals. Importantly, the increases in these factors were reduced by the knockdown of p21, an important molecule involved in cell senescence, indicating that the aldosterone-induced changes in the expression of TNF-α and collagens might result from p21-dependent cellular senescence. We failed to detect significant increase in TUNEL-positive cells in response to aldosterone. This result may be due to the sensitivity of TUNEL assay to detect the small changes in the number of apoptotic cells. The p21-dependent increase in TNF-α by the 3-d aldosterone treatment may not have caused severe apoptosis that could be detected by the TUNEL assay. Further study is needed to explore the detailed connection among aldosterone, cell senescence, and apoptosis.

The mechanism by which aldosterone/MR/reactive oxygen species decreased SIRT1 expression was not clarified in the present study. Zhang et al. (37) reported that aldosterone decreased the SIRT1 expression through an MR-independent pathway in murine inner medullary collecting duct cells. However, we found that gene silencing of the MR and antioxidant treatment suppressed the aldosterone-induced reduction of SIRT1 mRNA in HPTCs, suggesting that aldosterone regulates SIRT1 expression via different mechanisms. One possible mechanism is that reactive oxygen species destabilize SIRT1 mRNA, as reported by Abdelmohsen et al. (40). They showed that reactive oxygen species induced the dissociation of SIRT1 mRNA from an RNA-binding protein, HuR, which stabilizes SIRT1 mRNA, and thus reduced the stability of SIRT1 mRNA (40). Further studies are needed to clarify the precise mechanism involved in aldosterone-induced decrease in SIRT1 expression and activity in HPTCs.

The present study also suggested that senescence, as well as these deleterious changes caused by aldosterone, in proximal tubular cells might be involved in the reduction in organ function in vivo. In our aldosterone infusion model, we observed a remarkable increase in urinary NAG excretion and in protein excretion, indicating that the proximal tubular cells were injured by aldosterone infusion. Proximal tubules play an important role in regulating the salt balance and protein uptake; therefore, these effects of aldosterone, such as senescence, may mediate the development and/or the maintenance of hypertension and proteinuria in the aldosterone-induced hypertensive model.

Clinical studies have shown poor graft survival of transplanted kidneys from elder donors (41, 42) and the increase in p21 expression in the kidney of allograft nephropathy patients (43). Such evidence may reflect the hypothesis that the senescent kidney is more susceptible to various diseases, particularly diseases affecting tubular function, such as acute kidney injury and proteinuria during the development of chronic kidney disease because of reduced tubular repair activity. Thus, patients with excessive aldosterone/MR levels in their kidney may show unfavorable outcomes, although this has not yet been evaluated in clinical studies.

Aldosterone is reported to stimulate cyclin-dependent kinase 2 and 4 and accelerate the cell cycle transition to the S and M phases, thus stimulating cell proliferation in cultured renal mesangial cells (44). This observation differs from our present study showing aldosterone-dependent cell cycle arrest. However, no study has shown that distal tubular cells, the main physiological target of aldosterone, proliferate excessively in response to aldosterone. We speculate that the difference may be due to differences in cell characteristics between tubular and mesangial cells, namely epithelial or non-epithelial cells. However, the precise mechanism by which aldosterone regulates the cell cycle warrants further studies to investigate differences in receptor-induced intracellular signaling and differences in transcriptional gene expression in response to aldosterone, for example.

An MR inhibitor, spironolactone, is reported to work as an inverse agonist in Langendorff heart (45). It is possible that eplerenone also induces beneficial effect via an inverse agonistic action. However, it is hard to distinguish antagonistic and inverse agonistic action in vivo because of the existence of ligands (aldosterone and corticosterone). In fact, an earlier study showed that eplerenone on normal rats did not exacerbate or ameliorate the renal function (46).

In summary, aldosterone-infused rats developed marked proteinuria with tubular injury, in addition to NAG excretion, which were accompanied by changes in SA-βGal expression in proximal tubules and the expression of SIRT1, p53, and p21 in the renal cortical tissues of rats. Aldosterone induced similar changes in cultured proximal tubular cells, indicating that the proximal tubule is one of the targets for aldosterone-induced renal senescence. The changes may contribute to the development
and progression of renal dysfunction as a consequence of chronic aldosterone exposure. An important issue that has not been addressed in the present study is whether patients with hyperaldosteronism exhibit senescent changes in the kidney and whether those changes can be treated or prevented by pharmacotherapy with MR blockers. On the other hand, aldosterone-independent MR stimulation may participate in the senescence of proximal tubules because a recent study showed that the activity of 11β-hydroxysteroid dehydrogenase 2, an enzyme that inactivates cortisol into cortisone, was reduced in an age-dependent manner, (47) suggesting that cortisol may stimulate the MR with aging, accelerate the MR-dependent senescent responses, and create a vicious cycle.

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