Reactive oxygen species-initiated autophagy opposes aldosterone-induced podocyte injury

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1Department of Nephrology, Nanjing Children’s Hospital, Nanjing Medical University, Nanjing, China; 2Institute of Pediatrics, Nanjing Medical University, Nanjing, China; and 3Department of Nephrology, The First Affiliated Hospital of Nanjing Medical University, Nanjing, China

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Bai M, Che R, Zhang Y, Yuan Y, Zhu C, Ding G, Jia Z, Huang S, Zhang A. Reactive oxygen species-initiated autophagy opposes aldosterone-induced podocyte injury. Am J Physiol Renal Physiol 310: F669–F678, 2016. First published January 13, 2016; doi:10.1152/ajprenal.00409.2015.—Evidence has demonstrated that aldosterone (Aldo) is involved in the development and progression of chronic kidney diseases. The purpose of the present study was to investigate the role of autophagy in Aldo-induced podocyte damage and the underlying mechanism. Mouse podocytes were treated with Aldo in the presence or absence of 3-methyladenine and N-acetylcysteine. Cell apoptosis was investigated by detecting annexin V conjugates, apoptotic bodies, caspase-3 activity, and alterations of the podocyte protein nephrin. Autophagy was evaluated by measuring the expressions of light chain 3, p62, beclin-1, and autophagy-related gene 5. Aldo (10^{-7} mol/l) induced podocyte apoptosis, autophagy, and downregulation of nephrin protein in a time-dependent manner. Aldo-induced apoptosis was further promoted by the inhibition of autophagy via 3-methyladenine and autophagy-related gene 5 small interfering RNA pretreatment. Moreover, Aldo time dependently increased ROS generation, and H_{2}O_{2} (10^{-4} mol/l) application remarkably elevated podocyte autophagy. After treatment with N-acetylcysteine, the autophagy induced by Aldo or H_{2}O_{2} was markedly attenuated, suggesting a key role of ROS in mediating autophagy formation in podocytes. Inhibition of ROS could also lessen Aldo-induced podocyte injury. Taken together, our findings suggest that ROS-triggered autophagy played a protective role against Aldo-induced podocyte injury, and targeting autophagy in podocytes may represent a new therapeutic strategy for the treatment of podocytopathy.

Various pathogenic factors, such as infection, toxins, and drugs, can cause oxidative stress, DNA damage, and mitochondrial dysfunction in podocytes (23). These defects lead to the accumulation of a large number of abnormal organelles in the cell, which ultimately disrupt the cellular structure and function. Autophagy is a catabolic process that involves the dynamic changes of submembrane structures and lysosome-mediated degradation of intracellular proteins and organelles (14). Autophagy is performed by cells to promote survival by balancing anabolism and catabolism. Podocytes, as terminally differentiated cells, can effectively degrade damaged proteins and organelles by activating autophagy (1).

Both autophagy and apoptosis are self-destructive cellular processes that maintain the stability of the intracellular environment, and both processes are initiated by cellular exposure to certain extracellular or intracellular signals or stimulating factors. In autophagy, long-lived proteins and organelles are degraded, whereas in apoptosis, individual damaged cells are removed. In some cases, autophagy, as an adaptive response to external stimulation, inhibits the initiation of apoptosis (7). In contrast, excessive activation of autophagy can be toxic and may cause type II programmed cell death (18, 36). The type of death that a cell undergoes is usually determined by the degree or strength of the stimulus.

The main cause for the progression of chronic kidney disease is increased activity of the renin-angiotensin-aldosterone system. The clinical application of angiotensin-converting enzyme inhibitors (ACEIs) and ANG II receptor blockers (ARBs) significantly reduces proteinuria and slows the progression of various diseases (17). Some patients who receive long-term treatment with ACEIs and ARBs for hypertension develop the “Aldo escape” phenomenon at the plasma or tissue level, which attenuates the beneficial effects of ACEIs and ARBs on the targeted organs (16, 28). Treating patients with the Aldo escape phenomenon with a low-dose Aldo receptor antagonist produces a protective effect on the target organs independently of a reduction of blood pressure (6). Aldo is an important pathogenic factor for chronic renal injury through hemodynamic and/or nonhemodynamic actions (10). Podocytes are one of the targets of Aldo. Our previous studies have reported that Aldo could induce podocyte apoptosis (31, 38).

As published research on the association between Aldo-induced podocyte autophagy and apoptosis is sparse, the present study sought to investigate the relationship between autophagy and Aldo-induced podocyte damage as well as the role of ROS in this process.

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MATERIALS AND METHODS

Source of cells. The conditional immortalized mouse podocyte cell line MPC5 was a gift from Dr. P. Mundel (Albert Einstein College of Medicine, Bronx, NY) and Prof. Jie Ding (Peking University, Beijing, China).

Chemical reagents. RPMI 1640, HEPES, and FBS were purchased from Gibco. Interferon-γ, collagen type I, trypsin, aldosterone, H2O2, 3-methyladenine (3-MA), and N-acetylcysteine (NAC) were purchased from Sigma. An enhanced chemiluminescence kit was purchased from Pierce. A rabbit antibody against light chain (LC)-3B was purchased from Sigma, beclin-1 antibody was from Cell Signal Technology, and goat anti-mouse secondary antibodies were purchased from Beyotime.

Podocyte culture. Mouse MPC5 cells were cultured as previously described (21) with slight modifications. After a rapid recovery, podocytes were cultured and expanded in RPMI 1640 containing 10% FBS and 10 U/ml interferon-γ at 33°C. Cells were then seeded into 25-cm² tissue culture flasks (3.0 × 10⁶ cells/flask) or six-well plates (1.0 × 10⁵ cells/well). Cells were induced to differentiate at 37°C, and the differentiated podocytes were used for experiments. The following experimental groups of podocytes were established: normal control group (vehicle), Aldo (10⁻⁷ mol/l)-treated group, H2O2 (10⁻⁴ mol/l)-treated group, 3-MA-treated groups [Aldo (10⁻⁷ mol/l) + 3-MA (2 mmol/l)] and H2O2 (10⁻⁴ mol/l) + 3-MA (2 mmol/l)] and NAC-treated groups [Aldo (10⁻⁷ mol/l) + NAC (750 μmol/l)] and H2O2 (10⁻⁴ mol/l) + NAC (750 μmol/l)].

Apoptosis analysis by flow cytometry. Apoptosis was determined using the annexin V-FITC/propidium iodide (PI) apoptosis detection kit. Cells were collected at the end of the experiment, washed two times with prechilled PBS, and resuspended in 200 μl prechilled binding buffer. Then, 3 μl annexin V-FITC and 3 μl PI were added to the cell suspension and mixed. This reaction was kept in the dark for 15 min, and cells were then analyzed in a flow cytometer to detect the rate of apoptosis.

Autophagy evaluation by transmission electron microscopy. After Aldo (10⁻⁷ mol/l) treatment for 24 h, cells were digested and collected by centrifugation. Cells were then fixed in 5% glutaraldehyde, dehydrated, embedded, sectioned, and stained. Subsequently, cells were observed with transmission electron microscopy.

Autophagy evaluated by mRFP-GFP-LC3 adenovirus. Podocytes cultured on glass-bottom cell culture dishes (NEST) were transduced with mRFP-GFP-LC3 adenovirus (where mRFP is monomeric red fluorescent protein and GFP is green fluorescent protein) and the kit. The target gene and reference gene were amplified by software was used to design the primers (http://frodo.wi.mit.edu).

The sequences of the primers were as follows: beclin-1, upstream 5'-TGCAAGTGACGTTGCGT-3' and downstream 5'-CTGGG-TGGGTAATGTGGAG-3' and GAPDH, upstream 5'-CAA-GTCCAACGCCACAGTCAA-3' and downstream 5'-TGGTGGAAGAGCCGAGTAGAC-3'.

Protein extraction and Western blot analysis. Cells were washed twice with PBS. Protease inhibitors were added to the cell lysis buffer at a ratio of 1:100. Cells were scraped from the tissue culture plate and lysed on ice for 20 min. Cell lysates were then centrifuged at 4°C and 15,000 rpm for 25 min. Supernatants were then collected, and 1/4 volume of the sample buffer was added to each supernatant. Proteins were then denatured in a 100°C water bath for 10 min. The Coomassie brilliant blue method was used to measure total protein concentration. The same amount of protein (30 μg) in each sample was separated by SDS-PAGE. After electrophoresis, proteins were transferred to a nitrocellulose membrane, and the membrane was blocked in 5% nonfat dry milk at room temperature for 1 h. Primary antibody was added, and the membrane was slowly shaken at 4°C overnight. The membrane was washed with 1× PBS-Tween 20 and then incubated with horseradish peroxidase-labeled secondary antibodies at room temperature for 1 h with shaking. The membrane was then washed with 1× PBS-Tween 20 and finally scanned using a gel imaging system.

ROS measurement. Because the fluorescent probe MitoSOX can be oxidized by superoxide to generate red fluorescence after entering mitochondria, MitoSOX can selectively detect the amount of superoxide in the mitochondria of living cells. A MitoSOX stock solution (5 mol/l) was prepared by adding 50 μg MitoSOX powder to 13 μl dimethylsulfoxide. The MitoSOX working solution was prepared by adding an appropriate amount of stock solution to serum-free medium for a final concentration of 5 μmol/l. The culture medium was removed, and cells were washed once with serum-free medium. Prewarmed MitoSOX working solution was then added, and cells were incubated at 37°C for 10 min in the dark. The MitoSOX working solution was then removed, and cells were washed three times with PBS. Fluorescence intensity was measured using laser scanning confocal microscopy. Stained podocytes were routinely digested, washed with prewarmed PBS three times, and repetitively pipetted into single cell suspensions. Their fluorescence value was determined with a fluorescence microplate reader, and the excitation and emission wavelengths were 510 and 580 nm, respectively.

Autophagy-related gene 5 small interfering transfection. Podocytes were cultivated to 30~50% confluence in culture medium containing no penicillin or streptomycin. Autophagy-related gene (Atg)5 Small interfering (si)RNA and vehicle siRNA were synthesized by GenePharma. Cells were then transfected with siRNA using siRNA-Mate (GenePharma) according to the manufacturer’s instructions. In particular, cells were transfected with 50 nM Atg5 siRNA or control siRNA 24 h before Aldo treatment.

Measurement of caspase-3 activity. A caspase-3 activity assay kit was used to measure caspase-3 activity in podocytes through cleavage of a colorless substrate specific for caspase-3 [Ac-DEVD-p-nitroaniline (pNA)] and release of the chromophore pNA. The absorbance of pNA was examined at 405 nm.

Statistical analysis. SPSS (version 13.0) was used for the statistical analysis. All data analyses are represented as means ± SD. Comparisons between multiple groups were analyzed by ANOVA, and Student’s t-test was used for comparisons between two groups. P values of <0.05 were considered statistically significant.

RESULTS

Aldo induced podocyte damage. Aldo (10⁻⁷ mol/l) induced podocyte apoptosis in a time-dependent manner. After 24 h of Aldo treatment, the apoptosis rate was significantly increased (P < 0.05) compared with the control group. After 48 h of stimulation, the apoptosis rate was further increased by 41.8%
Nephrin, which is a transmembrane protein in the slit diaphragm of podocytes, participates in signal transduction in podocytes, and the reduced expression level of nephrin can be used as a marker of podocyte damage. We found that Aldo reduced nephrin expression in podocytes in a time-dependent manner (Fig. 1, C and D). After cells were stimulated with Aldo for 24 h, nephrin protein expression was significantly decreased \( (P < 0.05) \) compared with the control group.

**Aldo induced early activation of podocyte autophagy.** Typical autophagosomes appeared in podocytes 24 h after Aldo treatment, manifesting as vesicular structures formed by the cytosolic free monolayer or bilayer membrane containing the cytoplasm or damaged organelles (Fig. 2A). Autophagy is a dynamic process, including autophagosomes and autolysosomes. Ad-mRFP-GFP-LC3 staining is a very useful tool for evaluating the extent of autophagic flux (12). GFP fluorescence is quenched in the acidic pH of the lysosomal compartment, but mRFP continues to fluoresce. Thus, mRFP-LC3 can be used to identify both autophagosomes and autolysosomes. The overlap of red dots and green dots appearing yellow in merged images indicates autophagosomes, and the red dots that do not overlay green dots and appear red in merged images indicate autolysosome formation. After Aldo treatment for 12 h, both autophagosomes and autolysosomes were significantly increased (Fig. 2, B and C). Microtubule-associated protein-1

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**Fig. 1.** Aldosterone (Aldo) induced podocyte damage. A: representative images of flow cytometry in podocyte after Aldo treatment. B: apoptosis was evaluated by flow cytometry at 0, 6, 12, 24, and 48 h after Aldo administration. C: Western blots of nephrin and \( \beta \)-actin. D: densitometric analysis of nephrin expression. Con, control. \( n = 6 \) per group. \( *P < 0.05 \) vs. 0 h.

**Fig. 2.** Aldo induced podocyte autophagy. A: images of autophagosome bodies detected using transmission electron microscopy. The control (Con) image shows normal cells with a uniform cytoplasm and mitochondria with clear structural boundaries. The Aldo image shows mitochondria in podocytes swelled after 24 h of treatment with Aldo. An autophagosome, containing cellular components that were ready to be degraded, formed in the cytoplasm. Magnification: \( \times 50,000 \). B: autophagosomes determined by Ad-mRFP-GFP-LC3 (where Ad is adenovirus, mRFP is monomeric red fluorescent protein, GFP is green fluorescent protein, and LC is light chain). Podocytes were transduced with Ad-mRFP-GFP-LC3 for 24 h and subjected to Aldo for 12 h. Autophagosome bodies were detected using laser scanning confocal microscope. C: quantification of the mean density of fluorescence.
LC3 is considered to be a molecular marker of autophagy. In the process of autophagy, LC3-I protein, with a molecular weight of 18 kDa, is processed and modified by a ubiquitin-like system including Atg7 and Atg3, which results in the production of LC3-II (16 kDa), which translocates into the autophagosome. The induction of autophagy can be detected by monitoring changes of the LC3-II-to-LC3-I ratio. p62 protein serves as a link between LC3 and ubiquitinated substrates, and it incorporates into the completed autophagosome and is degraded in autolysosome. The results shown in Fig. 3, A–C, demonstrated a significant conversion from LC3-I to LC3-II and a remarkable reduction of p62 after cells were treated with Aldo for 12 h before the occurrence of apoptosis, which began after 24 h of Aldo treatment. Beclin-1 regulates autophagy by forming a complex with type III phosphatidylinositol 3-kinase (PI3K). mRNA expression of beclin-1 was significantly up-regulated by Aldo. Inhibition of autophagy exacerbated Aldo-induced podocyte damage. A: Western blot analysis of p62, LC3, and nephrin expression in podocytes pretreated with 3-methyladenine (3-MA) for 1 h followed by Aldo. B: densitometric analysis of p62 expression. C: densitometric analysis of the conversion of LC3-I to LC3-II. D: expression of beclin-1 was determined by quantitative RT-PCR. *P < 0.05 vs. 0 h.

Fig. 3. Aldo induced podocyte autophagy. A: p62 and conversion of the autophagy marker protein LC3-I to LC3-II were detected by Western blot analysis. B: densitometric analysis of p62 expression. C: densitometric analysis of the conversion of LC3-I to LC3-II. D: expression of beclin-1 was determined by quantitative RT-PCR. *P < 0.05 vs. 0 h.
regulated after Aldo treatment for 6 h ($P < 0.05$) and peaked at 24 h (Fig. 3D). This result further suggests that Aldo induced early activation of autophagy in podocytes.

**Inhibition of autophagy exacerbated Aldo-induced podocyte damage.** Next, we investigated the effects of autophagy inhibition on podocyte damage via 3-MA pretreatment. When podocytes were pretreated with 2 mmol/l 3-MA before Aldo administration for 24 h, the Aldo-induced enhancement of the LC3-II-to-LC3-I ratio and mRNA expression of beclin-1 were strikingly inhibited (Fig. 4, A–C). Meanwhile, the decrease of p62 was markedly blocked (Fig. 4, A and B). These results suggest that 3-MA inhibits Aldo-induced autophagy in podocytes. Aldo-induced podocyte damage after autophagy inhibition was further characterized. After inhibition of autophagy with 3-MA, nephrin expression in Aldo-induced podocytes was further reduced by 19.5% ($P < 0.05$; Fig. 4, A and B), and the apoptosis rate was further increased by 39% compared with podocytes treated with Aldo alone ($P < 0.05$; Fig. 4D). Moreover, the application of 3-MA alone did not cause podocyte apoptosis (data not shown). Caspase-3 is the implementation protease of the apoptosis cascade whose activation induces apoptosis. As shown in Fig. 4E, after inhibition of autophagy with 3-MA, caspase-3 activation was further significantly increased compared with podocytes treated with Aldo alone ($P < 0.05$). Atg5 is a gene required for the formation of autophagosomes. Atg5 expression was significantly upregulated after Aldo treatment for 6 h ($P < 0.05$) and peaked at 24 h (Fig. 5A). After transfection with Atg5 siRNA, the apoptosis rate was further increased by 35% compared with podocytes treated with Aldo alone ($P < 0.05$; Fig. 5, B and C). Nephrin expression in Aldo-induced podocytes was further reduced by 39.5% ($P < 0.05$; Fig. 5D). Moreover, application of Atg5 siRNA could cause podocyte apoptosis and a reduction in nephrin ($P < 0.05$; Fig. 5, C and D).

**Inhibition of autophagy led to a more rapid occurrence of podocyte damage after Aldo treatment.** Podocyte injury in 3-MA-pretreated cells was determined at different time points after Aldo induction. The results demonstrate that Aldo-induced podocyte damage occurred earlier (at 12 h) in the context of 3-MA pretreatment compared with cells treated with Aldo alone (at 24 h; Fig. 6, A and B). Meanwhile, downregulation of nephrin expression appeared at 12 h after 3-MA and Aldo treatment (Fig. 6, C and D).

![Fig. 5. Autophagy-related gene (Atg5) small interfering (si)RNA (siAtg5) aggravated Aldo-induced podocyte injury. Podocytes were transfected with Atg5 siRNA 24 h before Aldo treatment. A: Western blot analysis of Atg5 after Aldo treatment for different times. Top, representative immunoblots; bottom, densitometric analysis. B: Western blot analysis of Atg5 after transfection with Atg5 siRNA. Top, representative immunoblots; bottom, densitometric analysis. C: flow cytometry analysis of podocyte apoptosis. D: Western blot analysis of nephrin. Left, representative immunoblots; right, densitometric analysis. *P < 0.05 vs. control or nc; #P < 0.01 vs. control; *#P < 0.05 vs. Aldo.](http://ajprenal.physiology.org/ by 10.220.33.6 on August 28, 2017)
Oxidative stress induced activation of podocyte autophagy.

To evaluate the effect of oxidative stress on autophagy, we treated podocytes with H$_2$O$_2$ (10$^{-4}$ mol/l) for different time points. We found that H$_2$O$_2$ treatment also significantly increased beclin-1 and the LC3-II-to-LC3-I ratio and decreased p62 (Fig. 7, A–D). In podocytes transfected with Ad-mRFP-GFP-LC3, H$_2$O$_2$ increased both green and red dots, and the merged images showed that H$_2$O$_2$ increased yellow dots, representing autophagosomes, and red dots, representing autolysosomes (Fig. 8). These results suggest that H$_2$O$_2$ induced activation of autophagy in podocytes.

ROS participated in Aldo-induced podocyte autophagy.

Staining with the dye MitoSOX, which can detect mitochondrial ROS, indicated that Aldo could induce mitochondrial ROS generation in podocytes (Fig. 9A) as well as total ROS (H$_2$O$_2$; Fig. 9B). NAC is a widely used antioxidant that can inhibit the generation of ROS in podocytes. When cells were pretreated with 5 mmol/l NAC for 1 h before 24 h of Aldo or H$_2$O$_2$ administration, the conversion of LC3-I to LC3-II, induction of beclin-1, and reduction of p62 were markedly attenuated (Figs. 9, C–E, and 10, A–E). These data suggest that ROS participate in Aldo-induced autophagy formation in podocytes.

Inhibition of ROS protected Aldo-induced podocyte injury.

After inhibition of ROS induced by Aldo with NAC (Fig. 11A), the apoptosis rate and activity of caspase-3 were significantly decreased (P < 0.05) compared with the Aldo-treated group (Fig. 11B and C), and nephrin expression was significantly increased (P < 0.05; Fig. 11D). These suggest that inhibition of ROS can protect against Aldo-induced podocyte damage.

DISCUSSION

The renin-angiotensin-aldosterone axis plays a critical role in the occurrence and progression of kidney disease (10, 34). In
recent years, the role of Aldo in kidney damage has attracted increasing attention. Under pathological conditions, Aldo is overproduced and serves as an independent risk factor for kidney damage (2, 8, 25). Aldo generates biological effects through classical genetic functions as well as nongenetic functions. Aldo elevates glomerular blood pressure, activates serum and glucocorticoid-induced protein kinase-1, and induces the oxidative stress response, which causes podocyte damage leading to proteinuria and glomerulosclerosis (22, 32, 40). In present study, administration of Aldo

Fig. 8. Ad-mRFP-GFP-LC3 was used to evaluate the autophagy induced by H$_2$O$_2$ in podocytes. After being transfected with Ad-mRFP-GFP-LC3 for 24 h, podocytes were subjected to H$_2$O$_2$ for 12 h. Autophagosome bodies were detected using laser scanning confocal microscopy, and the mean density was quantified using ImagJ.

Fig. 9. Inhibition of ROS production reduced Aldo-induced autophagy in podocytes. A: representative images of MitoSOX in podocyte after Aldo treatment. B: total ROS generation in Aldo-treated podocytes was measured using a fluorescence microplate reader. C: quantitative RT-PCR analysis of beclin-1 mRNA expression. D: Western blot analysis of p62, LC3, and beclin-1 expression in Aldo-treated podocytes with or without N-acetylcysteine (NAC) pretreatment. E: densitometric analysis of p62, the LC3-I to LC3-II conversion, and beclin-1. n = 6 per group. *P < 0.05 vs. the control group; #P < 0.05 vs. the Aldo-treated group.
resulted in severe apoptosis and downregulation of nephrin in mouse podocytes.

Autophagy is a multiple-step degradation process that functions to maintain the homeostasis of cell structure, function, and metabolism. Autophagic degradation occurs in membrane-bound vesicles containing the cytoplasm, cytoplasmic organelles, and proteins/peptides that need to be degraded (37). Glomerular visceral epithelial cells are terminally differentiated podocytes with a branch-like structure and exhibit a high basal level of autophagic activity (9, 26). Analysis of human biopsies showed evidence of increased autophagosomal formation in podocytes in several glomerular diseases (26, 27). In the present study, transmission electron microscopy and Ad-mRFP-GFP-LC3 showed that Aldo induced the formation of autophagosome bodies. Moreover, Western blot analysis demonstrated that the autophagy marker protein LC3-I (18 kDa) was converted to LC3-II (16 kDa), p62 was decreased, and Atg5 was increased in a time-dependent manner after cells were treated with Aldo. These results indicate that Aldo can induce podocyte autophagy. Beclin-1 is an important protein in autophagy due to its role in regulating the localization of other autophagy-related proteins in autophagosome precursor structures. In the present study, beclin-1 expression was significantly increased by Aldo, which further supported our hypothesis that Aldo induced autophagy in podocytes. Interestingly, beclin-1 expression decreased after 48 h of Aldo treatment, which could be due to the prolonged Aldo stimulation, which caused extensive apoptosis and excessive caspase activation. Activated caspases cleave beclin-1 (11), resulting in the reduction of beclin-1 protein expression. Atg5 is a kind of E3 ubiquitin ligase that is necessary for autophagy due to loss of Atg5 completely blocks the autophagy process (39). In addition, 3-MA suppresses autophagy by inhibiting the type III PI3K-beclin complex (30, 35). We used 3-MA and Atg5 siRNA to inhibit Aldo-induced podocyte autophagy, which also indirectly corroborates the induction of autophagy by Aldo.

In the present study, Aldo induced obvious autophagy and apoptosis in podocytes. Mao et al. (19) have previously reported that ginsenoside Rg1 could relieve Aldo-induced oxidative stress and indirectly inhibit podocyte autophagy (19). However, they did not clarify the relation between autophagy and apoptosis. In our study, we found that podocyte autophagy occurred after 12 h of Aldo administration, whereas podocyte apoptosis was initiated at 24 h, indicating that Aldo-induced podocyte autophagy occurs earlier than apoptosis. Moreover, inhibition of autophagy by 3-MA caused apoptosis to begin 12 h earlier and also enhanced the severity of apoptosis as well as Atg5 siRNA. Caspase-3 is at the center of the apoptosis signal transduction pathway, and activated caspase-3 digests various structural and functional proteins. Inhibition of autophagy promotes Aldo-induced caspase-3 activation in podocytes, which further suggests an inverse relationship between autophagy and apoptosis in podocytes.

As an important structural component in the slit diaphragm of podocytes, nephrin plays roles in adhesion and signal transduction in podocytes (24). Moreover, alterations in nephrin expression and distribution can cause proteinuria (24). A reduction in nephrin expression can be used as a marker for podocyte damage. Our study demonstrated that nephrin expres-
sion was downregulated in a time-dependent manner after Aldo treatment and that inhibition of autophagy further increased its reduction. Taken together, these results further confirm that the suppression of autophagy aggravates Aldo-induced podocyte damage.

ROS participate in the regulation of cell proliferation, differentiation, apoptosis, and other important biological activities. In our study, we treated podocyte with H2O2. In consideration of the characteristics of H2O2 in quick degradation, a relatively large dose of 100 μM was applied in the experiments. After treatment, we found that H2O2 induced autophagy, as evidenced by increased LC3-II and decreased p62. Similar to Aldo experiments, H2O2 induced autophagy at 12 h.

NAC, a widely used antioxidant, directly captures unpaired electrons by reducing thiol to suppress superoxide anion formation, and NAC can also bind and inactivate the active forms of H2O2, O2−, and OH− that are released into the medium. As expected, NAC remarkably inactivated the excessive ROS, which subsequently inhibited the occurrence of autophagy induced by Aldo or H2O2 and also protected against Aldo-induced podocyte injury. These results highly suggest that ROS directly induce podocyte damage and at the same time activate podocyte autophagy to protect podocytes against oxidative damage. In other words, autophagy may serve as a compensatory action of cells in response to excessive ROS production induced by Aldo.

Beclin-1 antagonizes apoptosis by regulating the antiapoptotic protein family that includes Bcl-2. Beclin-1 competes with the proapoptotic protein Bad for binding with the antiapoptotic protein Bcl-XL to form beclin-1–Bcl-XL complexes, thereby performing an antiapoptotic role by improving mitochondrial permeability (20, 33). In the present study, Aldo-induced podocyte autophagy was associated with an upregulation of beclin-1 expression. Whether the inhibition of Aldo-induced podocyte apoptosis occurs through the above mechanism remains to be determined. Chen et al. (4) discovered that treatment of tumor cells with H2O2 and 2-methoxyestradiol upregulated beclin-1 gene expression and mediated cell autophagy. In the present study, we also found that H2O2 could upregulate beclin-1 gene expression. These results highly suggest that ROS are involved in Aldo-induced beclin-1 up-regulation, which could promote the autophagic response. Under physiological conditions, promoted autophagy could result in autophagic cell death (7, 29). However, accumulating evidence has demonstrated a protective role of autophagy under the challenges of pathological insults by getting rid of the damaged organelles and the maintenance of the normal intracellular environment (3, 5).

In summary, Aldo induces significant autophagy before the occurrence of apoptosis in podocytes, and such an induction of autophagy is mediated by ROS overproduction. More importantly, ROS-mediated autophagy induction significantly counteracts Aldo-induced podocyte damage. These findings not only demonstrate a novel axis of ROS/autophagy in opposing Aldo-mediated podocyte injury but also provide some potential targets for the treatment of podocytopathy.

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DISCLOSURES
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Aldosterone in the pathogenesis of chronic kidney disease and proteinuria.

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