Reactive Oxygen Species-Initiated Autophagy Opposes Aldosterone-Induced Podocyte Injury

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Evidence has demonstrated that Aldosterone (Aldo) is involved in the development and progression of chronic kidney diseases (CKDs). The purpose of this 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 (3-MA) and NAC. Cell apoptosis was investigated by detecting Annexin V conjugates, apoptotic bodies, caspase-3 activity, and the alteration of podocyte protein nephrin. Autophagy was evaluated by measuring the expressions of LC3, p62, beclin-1 and Atg5. Aldo (10^{-7} \text{ 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-MA and Atg5 siRNA pretreatment. Moreover, Aldo time-dependently increased ROS generation, and H_2O_2 (10^{-4} \text{ mol/L}) application remarkably elevated podocyte autophagy. After treatment with NAC, the autophagy induced by Aldo or H_2O_2 was markedly attenuated, suggesting a key role of ROS in mediating the autophagy formation in podocytes. Inhibition of ROS also could lessen Aldo-induced podocyte injury. Taken together, our findings suggested 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.

**Keywords**  Podocyte, Aldosterone, Autophagy, Apoptosis, ROS.
Introduction

Podocytes not only participate in forming the mechanical and electric charge barrier of the glomerular filtration membrane, but also play key roles in maintaining the normal opening of the glomerular capillary loop, alleviating the impact of hydrostatic pressure, producing glomerular basement membrane matrix, and maintaining the homeostasis of glomerular basement membrane metabolism(13). Podocytes, as an important component of the filtration barrier, are terminally differentiated cells and do not proliferate(23). Consequently, the loss of podocytes can cause mesangial expansion, proteinuria, deterioration of glomerulosclerosis, and ultimately, the progressive loss of kidney function (15).

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 sub-membrane structures and the 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 the increased activity of the renin-angiotensin-aldosterone system. The clinical application of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin 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 non-hemodynamic actions (10). Podocytes are one of the targets of Aldo. Our previous study has reported that Aldo could induce podocyte apoptosis (31, 38).

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

The source of cells

The conditional immortalized mouse podocyte cell line MPC5 was gifted by Professor P. Mundel from the Albert Einstein College of Medicine, Bronx, NY, USA, and Professor Jie Ding from Peking University, Beijing, China.

Chemical reagents

RPMI 1640 medium, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and fetal bovine serum (FBS) were purchased from Gibco (USA). Interferon-γ, type I collagen, trypsin, aldosterone, H₂O₂, 3-methyladenine (3-MA) and N-acetylcysteine (NAC) were purchased from Sigma (USA). An enhanced chemiluminescence kit was purchased from Pierce (USA). A rabbit antibody against LC-3B was purchased from Sigma, Beclin1 antibody was from Cell Signaling (USA), and nephrin antibody was purchased from Abcam (USA). Mouse antibodies against β-actin and p62 were bought from Cell Signaling and Abcam, respectively. HRP-labeled goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Beijing Zhongshan Company. mRFP-GFP-LC3 adenovirus was purchased from Hanheng Biotechnology. Caspase3 activity assay kit was from KeyGEN BioTECH (China). SYBR Green PCR Master Mix was purchased from Applied Biosystems (USA).

Podocyte culture

Mouse MPC5 cells were cultured as previously described (21) with slight modification. Following rapid recovery, the podocytes were cultured and expanded in RPMI 1640 medium
containing 10% FBS and 10U/ml interferon-γ at 33°C. Then, these cells were seeded into 25-cm² tissue culture flasks (3.0 × 10⁵/flask) or 6-well plates (1.0 × 10⁵/well). The cells were induced to differentiate at 37°C, and the differentiated podocytes were used for the experiments. The following experimental groups of podocytes were established: the normal control group (vehicle), Aldo group (10⁻⁷ mol/L), H₂O₂ group (10⁻⁴ mol/L), 3-MA treatment groups (Aldo 10⁻⁷ mol/L + 3-MA 2 mmol/L; H₂O₂ 10⁻⁴ mol/L + 3-MA 2 mmol/L) and NAC treatment groups (Aldo 10⁻⁷ mol/L + NAC 750 µmol/L; H₂O₂ 10⁻⁴ mol/L + NAC 750 µmol/L).

**Apoptosis analyzed by flow cytometry**

Apoptosis was determined by using the Annexin V-FITC/PI apoptosis detection kit. Cells were collected at the end of the experiment, washed two times with pre-chilled phosphate-buffered saline (PBS), and re-suspended in 200 µL pre-chilled binding buffer. Then 3 µL of 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 the 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 24h, cells were digested and collected by centrifugation. Then cells were fixed in 5% glutaraldehyde, dehydrated, embedded, sectioned, and stained. Subsequently, the cells were observed under transmission electron microscopy.

**Autophagy evaluated by mRFP-GFP-LC3 adenovirus**
Podocytes cultured on glass bottom cell culture dish (NEST, China) were transduced with Ad-mRFP-GFP-LC3 at 10MOI for 24 hours, after which they were treated with Aldo or H2O2 for 12h. Then the cells were washed with PBS, fixed with 4% paraformaldehyde, and viewed under a laser scanning confocal microscope.

Real-time quantitative PCR

Total RNA was extracted from mature podocytes using TRIzol (Invitrogen), according to the manufacturer’s instructions. Two micrograms of total RNA from each sample was used. cDNA was reverse transcribed according to the instructions of the kit. The target gene and reference gene were amplified by real-time quantitative PCR. A standard curve and melting curve were generated. Cycle threshold (Ct) values were used to calculate the relative amount of sample template. The 20-μL reaction mix consisted of 1 μL positive and negative primers (10 μmol/L each), 10 μL PCR master mix, 1 μL template, and DEPC water to achieve the final volume. The following temperature cycling conditions were used: 95°C for 30 s; 40 cycles of 95°C for 5 s; and 60°C for 32 s. Each experiment was repeated three times. Primer 3.0 software was used to design the primers (http://Frodo.wi.mit.edu). The sequences of the primers were as follows: beclin-1, upstream: 5’-TGCAGGTGAGCTTCTGC-3’, downstream: 5’-CTGGGCTGTGGTAAGTAATGGAG-3’; and GAPDH, upstream: 5’-CAAGTTCAACGGCACAGTCAA -3’, downstream: 5’-TGGTGAAGACGCCAGCTGACTC-3’.

Protein extraction and Western blotting

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. Then cell lysates were centrifuged at 4°C and 15,000 rpm for 25 min. Supernatants were then collected and 1/4 volume of 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 the total protein concentration. The same amount of protein (30 μg) in each sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. 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 (PBST) and then incubated with horseradish peroxidase-labeled secondary antibodies at room temperature for 1 h with shaking. The membrane was then washed with 1× PBST 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 (5mol/L) was prepared by adding 50 μg MitoSOX powder to 13 μl of dimethylsulphoxide. 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 the cells were washed once with serum-free medium. Then, pre-warmed MitoSOX working solution was added, and the cells were incubated at 37°C for 10 min in the dark. The
MitoSOX working solution was then removed, and the cells were washed three times with PBS. Fluorescence intensity was measured using laser-scanning confocal microscopy. Stained podocytes were routinely digested, washed with pre-warmed 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.

**SiAtg5 Transfection**

Podocytes were cultivated to 30-50% confluence in culture medium containing no penicillin or streptomycin. Atg5 siRNA 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, the cells were transfected with 50nM Atg5 siRNA or control siRNA 24h 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 v13.0 was used for the statistical analysis. All data analyses are represented as the mean ± standard deviation (X ± s). Comparisons between multiple groups were analyzed by
analysis of variance (ANOVA), and the Student’s t-test was used for comparisons between two groups. P values less than 0.05 were considered statistically significant.

Results

Aldo induced podocyte damage

Aldo (10^{-7} \text{ mol/L}) induced podocyte apoptosis in a time-dependent manner. After 24 h Aldo treatment, the apoptosis rate was increased significantly (P < 0.05) as compared with control group. After 48 h stimulation, the apoptosis rate was further increased by 41.8% (Fig. 1A&B). 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. 1C&D). After cells were stimulated with Aldo for 24 h, nephrin protein expression was significantly decreased (P < 0.05) as compared with control group.

Aldo induced early activation of podocyte autophagy

Typical autophagosomes appeared in podocytes 24 h after aldosterone treatment, manifesting as vesicular structures formed by the cytosolic free monolayer or bilayer membrane containing 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 fluorescence. So 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 12h, both autophagosomes and autolysosomes were significantly increased. (Fig. 2B&C). Microtubule-associated protein 1 light chain 3 (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 an ubiquitin-like system including Atg7 and Atg3, which results in the production of LC3-II (16 kDa) that translocates into the autophagosome. The induction of autophagy can be detected by monitoring the change of LC3-II/LC3-I ratio. The p62 protein serves as a link between LC3 and ubiquitinated substrates and it incorporates into the completed autophagosome and is degraded in autolysosome. Results from Fig. 3A-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, prior to the occurrence of apoptosis which began after 24 h Aldo treatment. Beclin-1 regulates autophagy by forming a complex with type III phosphatidylinositol 3-kinase (PI3K). Beclin-1 mRNA expression was significantly upregulated 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 prior to Aldo
administration for 24 h, the Aldo-induced enhancement of LC3-II/LC3-I ratio and beclin-1
mRNA expression were strikingly inhibited (Fig. 4A-C). Meanwhile, the decrease of p62 was
markedly blocked (Fig. 4A&B). These results suggest that 3-MA inhibits Aldo-induced
autophagy in podocytes. Aldo-induced podocyte damage following autophagy inhibition was
further characterized. After inhibiting autophagy with 3-MA, nephrin expression in
Aldo-induced podocytes was further reduced by 19.5% (Fig. 4A&B, P < 0.05), and the
apoptosis rate was further increased by 39% in comparison to podocytes treated with Aldo
alone (Fig. 4D, P < 0.05). 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 inhibiting autophagy
with 3-MA, caspase-3 activation was further significantly increased in comparison to
podocytes treated with Aldo alone (P < 0.05). Autophagy-related gene (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% in comparison to podocytes
treated with Aldo alone (Fig. 5B & C, P < 0.05). Nephrin expression in Aldo-induced
podocytes was further reduced by 39.5% (Fig. 5D, P < 0.05), and Moreover, the application of
siAtg5 could cause podocyte apoptosis and nephrin reduction (Fig. 5C&D, P<0.05).

Inhibition of autophagy led to more rapid occurrence of podocyte damage following

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 as compared to the cells treated with Aldo alone (at 24 h) (Fig. 6A&B). Meanwhile, the downregulation of nephrin expression appeared at 12 h after 3-MA and Aldo treatment (Fig. 6C&D).

Oxidative stress induced activation of podocyte autophagy

In order to evaluate the effect of oxidative stress on autophagy, we treated podocytes with H2O2 (10^{-4} mol/L) for different time points. We found H2O2 treatment also significantly increased Beclin1, LC3-II/LC3-I ratio, and decreased p62 (Fig. 7A-D). In podocytes transfected with Ad-mRFP-GFP-LC3, H2O2 increased both green and red dots, and the merged images showed that H2O2 increased yellow dots representing autophagosomes and red dots representing autolysosomes (Fig. 8). These results suggest that H2O2 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 induced mitochondrial ROS generation in podocytes (Fig. 9A), as well as total ROS (H2O2) (Fig. 9B). N-Acetylcysteine (NAC) is a widely used anti-oxidant that can inhibit the generation of ROS in podocytes. When cells were pretreated with 5mmol/L NAC for 1 h prior to 24 h Aldo or H2O2 administration, the conversion of LC3-I to LC3-II, induction of beclin-1, and reduction of p62 were markedly attenuation (Fig. 9C-E and Fig. 10A-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 the activity of caspase3 were decreased significantly (P < 0.05) as compared with Aldo group (Fig. 11B & C) and nephrin expression was significantly increased (Fig. 11D, P < 0.05). These suggest that inhibition of ROS can protect 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 over-produced and serves as an independent risk factor for kidney damage (2, 8, 25). Aldo generates biological effects through classical genetic functions as well as non-genetic functions. Aldo elevates glomerular blood pressure, activates glucocorticoid-induced protein kinase 1 (SGK1), and induces the oxidative stress response, which causes podocyte damage leading to proteinuria and glomerulosclerosis (22, 32, 40). In present study, administration of Aldo resulted in the 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 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 autophagosome formation in podocytes in several glomerular diseases (26, 27). In 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 48h 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. Autophagy related gene-5 (Atg5) is an E3 ubiquitin ligase which 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 present study, Aldo induced obvious autophagy and apoptosis in podocytes. Although
Mao et al had reported that ginsenoside Rg1 could relieve Aldo-induced oxidative stress and indirectly inhibit podocyte autophagy (19). They did not clarify the relation between autophagy and apoptosis. In our study, we found that podocyte autophagy occurred after 12h Aldo administration, whereas the 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 hours 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, alteration 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 expression was downregulated in a time-dependent manner following Aldo treatment and that inhibition of autophagy further increased its reduction. 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 characteristic of H2O2 in quick degradation, a relatively large dose of 100μM was applied in the experiments. Following treatment, we found that it induced autophagy as evidenced by increased LC3-II and decreased p62. Similar as Aldo experiment, H2O2 induced
autophagy at 12 h. NAC, a widely used anti-oxidant, directly captures unpaired electrons via reducing thiol to suppress superoxide anion formation, and NAC can also bind and inactivate the active forms of H$_2$O$_2$, O$_2^-$, 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 H$_2$O$_2$ and also protected 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 anti-apoptotic protein family that includes Bcl-2. Beclin-1 competes with the pro-apoptotic protein Bad for binding with the anti-apoptotic protein Bcl-XL to form beclin-1-Bcl-XL complexes, thereby performing an anti-apoptotic role by improving mitochondrial permeability (20, 33). In this 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. discovered that treating tumor cells with H$_2$O$_2$ and 2-methoxyestradiol upregulated beclin-1 gene expression and mediated cell autophagy (4). In the present study, we also found that H$_2$O$_2$ could upregulate beclin1 gene expression. These suggest that ROS are involved in Aldo-induced beclin-1 upregulation which could promote the autophagic response. Under the physiological condition, promoted autophagy could result in autophagic cell death (7, 29). However, accumulating evidence demonstrated a protective role of autophagy under the challenges of pathological insults via
getting rid of the damaged organelles and the maintenance of 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 the Aldo-mediated podocyte injury, but also provide some potential targets for the treatment of podocytopathy.

Conflict of Interests

There is no conflict of interests to disclose. No conflicts of interest, financial or otherwise, are declared by the authors.

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Figure legends

**Fig. 1.** Aldo induced podocyte damage. (A) Representative images of flow cytometry in podocyte after aldo treatment. (B) Apoptosis was evaluated by flow cytometry at 0 h, 6 h, 12 h, 24 h, and 48 h after Aldo administration. (C) Western blots of nephrin and β-actin. (D) Densitometric analysis of nephrin expression. N=6 per group. *, P < 0.05 vs. 0h.

**Fig. 2.** Aldosterone induces podocyte autophagy. (A) Images of autophagosome bodies detected using transmission electron microscopy. Cont: Image of normal cells with a uniform cytoplasm and mitochondria with clear structural boundaries. Aldo: Mitochondria in podocytes swelled after 24-h treatment with aldosterone. An autophagosome, containing cellular components that were ready to be degraded, formed in the cytoplasm. Magnification, ×50000. (B) Autophagosome determined by Ad-mRFP-GFP-LC3. Podocytes were transduced with Ad-mRFP-GFP-LC3 for 24h and were subjected to Aldo for 12h. Autophagosome bodies were detected using laser scanning confocal microscope. (C) Quantification the mean density of fluorescence.

**Fig. 3.** Aldo induced podocyte autophagy. (A) P62 and conversion of autophagy marker protein LC3-I to LC3-II were detected by Western blotting. (B) Densitometric analysis of p62 expression. (C) Densitometric analysis of the conversion of LC3-I to LC3-II. (D) The expression of beclin-1 was determined by qRT-PCR. N=6 per group. *, P < 0.05 vs. 0h.

**Fig. 4.** Inhibition of autophagy exacerbated Aldo-induced podocyte damage. (A) Western blot
analysis of p62, LC3, and nephrin expressions in podocytes pretreated with 3-MA for 1 h followed by Aldo. (B) Densitometric analysis of p62, LC3-II/LC3-I and nephrin. (C) qRT-PCR analysis of beclin-1. (D) Flow cytometry analysis of apoptosis in podocytes pretreated with 3-MA for 1 h followed by Aldo treatment for 24 h. (E) Analysis of caspase-3 activity. Con: Control; Aldo: Aldo-treated group; 3MA+Aldo: group pretreated with 3-MA for 1 h followed by Aldo. N=6 per group. *, P < 0.05 vs. Con group; #, P < 0.05 vs. Aldo group.

**Fig. 5.** SiAtg5 aggravated Aldo-induced podocyte injury. The podocytes were transfected with Atg5 siRNA 24h before Aldo treatment. (A) Western blot analysis of Atg5 after Aldo treatment for different time. 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. N=6 per group. *, P < 0.05 vs. con or nc; #, P<0.01 vs con; *#, P<0.05 vs Aldo.

**Fig. 6.** Inhibition of autophagy caused more rapid occurrence of Aldo-induced podocyte damage. The podocytes were pretreated with 3MA. (A) Flow cytometry analysis of podocyte apoptosis at different time points. (B) Apoptosis analysis of Aldo-treated cells with or without 3-MA pretreatment. (C) Western blotting analysis of nephrin expression at different time points. (D) Densitometric analysis of nephrin. N=6 per group. *, P < 0.05 vs. 0h.
**Fig. 7.** H2O2 induced podocyte autophagy. (A) P62 and LC3 were detected by Western blotting. (B) Densitometric analysis of p62 expression. (C) Quantification of the ratio of LC3-II to LC3-I. (D) The expression of beclin-1 was determined by qRT-PCR. N=6 per group. *, P < 0.05 vs. 0h.

**Fig. 8.** Ad-mRFP-GFP-LC3 was used to evaluate the autophagy induced by H2O2 in podocytes. After transfected with Ad-mRFP-GFP-LC3 for 24h, the podocytes were subjected to H2O2 for 12h. Autophagosome bodies were detected using laser scanning confocal microscope and quantificated the mean density using ImagJ.

**Fig. 9.** Inhibition of ROS production reduced Aldo-induced autophagy in podocytes. (A) Representative images of MitoSOX in podocyte after aldosterone treatment. (B) Total ROS generation in Aldo-treated podocytes was measured using a fluorescence microplate reader. (C) qRT-PCR analysis of beclin-1 mRNA expression. (D) Western blot analysis of p62, LC3, and Beclin1 expressions in Aldo-treated podocytes with or without NAC pretreatment. (E) Densitometric analysis of p62, LC3-I to LC3-II conversion, and beclin-1. N=6 per group. *, P < 0.05 vs. Con group; #, P < 0.05 vs. Aldo group.

**Fig. 10.** H2O2-induced autophagy was attenuated by inhibition of oxidative stress. (A) Western blot analysis of p62, LC3, and Beclin1 expressions in H2O2-treated podocytes with or without NAC pretreatment. (B-D) Densitometric analysis of p62 (B), LC3-I to LC3-II conversion (C), and beclin-1. (D). (E) qRT-PCR analysis of beclin-1 mRNA expression. N=6
per group. *, P < 0.05 vs. Con group; #, P < 0.05 vs. Aldo group.

Fig. 11. Inhibition of ROS production protected Aldo-induced podocyte injury. The podocytes were pretreated with NAC. (A) ROS production measured by fluorescence microplate reader. (B) Apoptosis analysis of Aldo-treated cells with or without NAC pretreatment. (C) Analysis of caspase-3 activity. (D) Western blot analysis of nephrin. (E) Densitometric analysis of nephrin. N=6 per group. *, P < 0.05 vs. Con group; #, P < 0.05 vs. Aldo group.
Fig. 1.

**A**

Con  |  Aldo
--- | ---

**B**

Apoptosis (fold over control)

0  |  6  |  12  |  24  |  48h
--- | --- | --- | --- | ---
1.0 | 1.0 | 1.5 | 2.0 | 2.0

**C**

Nephrin  |  β-actin
--- | ---

0  |  6h  |  12h  |  24h  |  48h
--- | --- | --- | --- | ---
85KD  |  85KD  |  85KD  |  85KD  |  85KD

42KD  |  42KD  |  42KD  |  42KD  |  42KD

**D**

Relative nephrin expression

0  |  6  |  12  |  24  |  48h
--- | --- | --- | --- | ---
1.0 | 1.0 | 1.0 | 1.0 | 1.0

* Indicates significant difference compared to control.
Fig. 2.

A

[Image of Con and Aldo conditions]

B

GFP
mRFP
Merged

10 μm
10 μm
10 μm

C

Mean density

con
Aldo
Fig. 4.

A

<table>
<thead>
<tr>
<th>Protein</th>
<th>Con</th>
<th>Aldo</th>
<th>3-MA+Aldo</th>
</tr>
</thead>
<tbody>
<tr>
<td>p62</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC3I</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC3II</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nephrin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

B

Relative levels

<table>
<thead>
<tr>
<th>Protein</th>
<th>con</th>
<th>Aldo</th>
<th>3MA+Aldo</th>
</tr>
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<tbody>
<tr>
<td>p62</td>
<td>*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LC3II/LC3I</td>
<td>#</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nephrin</td>
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</tr>
</tbody>
</table>

C

Relative Beclin1 mRNA expression

<table>
<thead>
<tr>
<th>Condition</th>
<th>con</th>
<th>Aldo</th>
<th>3MA+Aldo</th>
</tr>
</thead>
<tbody>
<tr>
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</tbody>
</table>

D

Apoptosis (fold over control)

<table>
<thead>
<tr>
<th>Condition</th>
<th>con</th>
<th>Aldo</th>
<th>3MA+Aldo</th>
</tr>
</thead>
<tbody>
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</tbody>
</table>

E

caspase3

<table>
<thead>
<tr>
<th>Condition</th>
<th>con</th>
<th>Aldo</th>
<th>3MA+Aldo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Fig. 5.

A

Atg5

β-actin

0 6h 12h 24h

B

con SiAtg5

Atg5

β-actin

56KD 42KD

C

Apoptosis (fold over control)

con SiAtg5 Aldo SiAtg5+Aldo

D

Nephrin

β-actin

85KD 42KD

Con SiAtg5 Aldo SiAtg5+Aldo

Relative nephrin expression
Fig. 6.

A

Apoptosis (fold over control)

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>6h</th>
<th>12h</th>
<th>24h</th>
</tr>
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<tbody>
<tr>
<td>0</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12h</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td></td>
<td></td>
<td>*</td>
<td>#</td>
</tr>
</tbody>
</table>

B

Apoptosis (fold over control)

- Aldo
- 3MA+Aldo

C

Nephrin

<table>
<thead>
<tr>
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<th>12h</th>
<th>24h</th>
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β-actin

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<th>24h</th>
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</tr>
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</tr>
<tr>
<td>12h</td>
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</tr>
<tr>
<td>24h</td>
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</tbody>
</table>

D

Relative nephrin expression

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>6</th>
<th>12</th>
<th>24h</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>*</td>
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<tr>
<td>24h</td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Fig. 7.

A

B

C

D
Fig. 8.

Con

H$_2$O$_2$

GFP

mRFP

Merged

Mean density

Con Aldo
Fig. 9.

A. Con and Aldo images showing fluorescent staining.

B. Graph showing 
\[
\text{H}_2\text{O}_2 \text{ concentration (μmol/L)}
\]

- 0
- 2
- 4
- 8
- 12
- 24h

C. Graph showing relative Beclin1 mRNA expression:
- Con
- Aldo
- NAC+Aldo

D. Western blot analysis of p62, LC3I, LC3II, Beclin-1, and β-actin in Con, Aldo, and NAC+Aldo groups.

E. Graph showing relative levels of:
- p62
- LC3II/LC3I
- Beclin1

Legend:
- Black: con
- Gray: Aldo
- Dark gray: NAC+Aldo

* indicates statistical significance compared to control.
# indicates statistical significance compared to Aldo.
Fig. 10.
Fig. 11.

(A) ROS production

(B) Apoptosis (fold over control)

(C) caspase3

(D) Western blot analysis of nephlin and β-actin

(E) Relative nephrin expression

- con, Aldo, NAC+Aldo
- * indicates significance over control
- # indicates significance over Aldo
- 85KD and 42KD

- Con, Aldo, NAC+Aldo
- * indicates significance over control
- # indicates significance over Aldo