Autophagy modulates endoplasmic reticulum stress-induced cell death in podocytes: A protective role

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Abstract

Endoplasmic reticulum stress occurs in a variety of patho-physiological mechanisms and there has been great interest in managing this pathway for the treatment of clinical diseases. Autophagy is closely interconnected with endoplasmic reticulum stress to counteract the possible injurious effects related with the impairment of protein folding. Studies have shown that glomerular podocytes exhibit high rate of autophagy to maintain as terminally differentiated cells. In this study, podocytes were exposed to tunicamycin and thapsigargin to induce endoplasmic reticulum stress. Thapsigargin/tunicamycin treatment induced a significant increase in endoplasmic reticulum stress and of cell death, represented by higher GADD153 and GRP78 expression and propidium iodide flow cytometry, respectively. However, thapsigargin/tunicamycin stimulation also enhanced autophagy development, demonstrated by monodansylcadaverine assay and LC3 conversion. To evaluate the regulatory effects of autophagy on endoplasmic reticulum stress-induced cell death, rapamycin (Rap) or 3-methyladenine (3-MA) was added to enhance or inhibit autophagosome formation. Endoplasmic reticulum stress-induced cell death was decreased at 6 h, but was not reduced at 24 h after Rap+TG or Rap+TM treatment. In contrast, endoplasmic reticulum stress-induced cell death increased at 6 and 24 h after 3-MA+TG or 3-MA+TM treatment. Our study demonstrated that thapsigargin/tunicamycin treatment induced endoplasmic reticulum stress which resulted in podocytes death. Autophagy, which counteracted the induced endoplasmic reticulum stress, was simultaneously enhanced. The salvational role of autophagy was supported by adding Rap/3-MA to mechanistically regulate the expression of autophagy and autophagosome formation. In summary, autophagy helps the podocytes from cell death and may contribute to sustain the longevity as a highly differentiated cell lineage.

Keywords: Endoplasmic reticulum stress, autophagy, autophagosome, podocyte

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Introduction

Endoplasmic reticulum (ER) is responsible for proper folding and processing the proteins to be secreted, and its capacity to keep the balance of proteins folding is vital to functional integrity of a cell.¹ Many metabolic and inflammatory situations may disrupt this balance and result in ER stress that is eventually implicated in a variety of clinical diseases, including metabolic diseases,² neurodegenerative diseases,³ etc. To maintain homeostasis of ER function, eukaryotic cells have evolved and generated a critical pathway of restoration called unfolded protein response (UPR).⁴ The UPR is composed of a series of adaptive mechanisms to cope with protein-folding challenges. However, when these mechanisms fail to recover normal ER function due to

excessive loads, other stress-response pathways may be initiated to selectively remove the damaged cells or cellular component.⁵

It is increasingly clear that ER stress leads to the induction of autophagy, a multistep catabolic process to degrade large protein aggregates and damaged organelles in autophagosomes.⁶ Besides its role to accommodate to the external challenges, autophagy is also vital to provide the basal turnover of long-lived proteins and organelles and maintain the normal cellular homeostasis.⁷ The seemingly conflicting pro-survival and pro-death roles of autophagy are potentially interchangeable, depending on the interactions of external stimuli and interior cellular ability to maintain survival.⁸

Typically, cells that turn over rapidly show high rates of autophagy, while terminally differentiated cells like neurons exhibit low rates of autophagy.⁹ Glomerular podocytes, recognized to be terminally differentiated, are apparently unusual as they demonstrate a high basal rate of autophagy.¹⁰ This has been supported by finding numerous vesicles resembling the double-membrane autophagosomes in the cytoplasm of healthy podocytes.¹¹ It is stated that the podocytes, as the major filtration barriers of plasma proteins, require autophagy to sustain their terminally differentiated state while they have to confront the ever-lasting stimuli coming from the glomerular filtration. This may be especially important because recent studies have shown significant role of ER stress in kidney diseases, especially proteinuric kidney diseases caused by podocytes injury.¹²

Prevention of plasma proteins from loss into urine depends largely on the functional and structural integrity of the glomerular podocytes. Maintenance of survival is vital to podocytes because they usually do not proliferate and are unable to replenish themselves.¹⁰ In this study, tunicamycin (TM) and thapsigargin (TG) were used to induce the expression of biomarkers of ER stress, cell death, and also autophagy.¹³ We further demonstrated that both up- and down-regulation of autophagy were linked to alterations of cell death of podocytes. Our results supported the statement that autophagy is important in maintaining cellular longevity and the consequent functional integrity, and therefore, could be a potential target of pharmaceutical management.

Materials and methods

Reagents, chemicals and antibodies

RPMI 1640 medium, 100 × penicillin/streptomycin solution, and fetal bovine serum (FBS) were purchased from GIBCO BRL (Invitrogen, Burlington, Ontario, Canada). Rat tail collagen type I was purchased from BD Biosciences (Bedford, MA, USA). Mouse recombinant γ -interferon was purchased from PeproTech (Rocky Hill, NJ, USA). The ER stress-inducing chemical compounds TM and TG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rapamycin was purchased from LC Laboratories (Woburn, MA, USA). 3-Methyladenine (3-MA), monodansylcadaverine (MDC), and propidium iodide (PI) were purchased from Sigma-Aldrich (St. Louis, MO, USA). WST-1 assay kit was obtained from BioVision (Mountain View, CA). M-PER Mammalian Protein Extraction Reagent and Halt Protease inhibitor cocktail were purchased from Thermo Fisher Scientific (Rockford, IL, USA). Immobilon western chemiluminescent HRP substrate kit (WBKLS0050) and immobilon polyvinylidene difluoride (PVDF) membranes were purchased from Millipore (Bedford, MA, USA). Antibodies against LC3B, phospho-mTOR (ser2448), mTOR (Cell Signaling Technology, Beverly, MA, USA), GADD153 (Santa Cruz Biotech, CA, USA), GRP78 (Abcam, Cambridge, UK), GAPDH, and β -actin (Chemicon, Temecula, CA) were used as primary antibodies. The following secondary antibodies were used: horseradish peroxidase-conjugated

anti-mouse or anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, PA, USA).

Podocyte cell culture and treatment

Conditionally immortalized mouse podocyte cell lines were kindly provided by Dr. Peter Mundel (Massachusetts General Hospital, Boston, MA). Briefly, podocytes were propagated at 33°C on collagen I-coated flasks in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin solution, and 10 unit/ml mouse recombinant γ -interferon. Differentiated podocytes were cultured at 37°C for 10–14 days without γ -interferon in RPMI culture medium.¹⁴ For inducing ER stress, cells were exposed to 2 μ M of TG or 5 μ g/ml of TM for the indicated time periods. To evaluate whether autophagy has any effect on ER stress-inducing cell death, we pretreated with 5 nM of rapamycin to enhance autophagy formation, and 2 mM of 3-MA used to inhibit autophagy formation for 2 h before ER stress inducers incubation.

Western blot analysis

Cells were washed with phosphate buffered saline (PBS), harvested and lysed in M-PER Mammalian Protein Extraction Reagent with halt protease inhibitor cocktail. The lysates were then incubated on ice for 30 min and centrifuged at 12,000 g for 10 min. Equal amounts of protein were subjected to 10–15% SDS-PAGE for electrophoresis, followed by transfer onto an Immobilon-PVDF membrane. The membrane was blocked with 0.25% NET-gelatin (150 mm NaCl, 5 mm ethylenediaminetetraacetic acid, 50 mm Tris-HCl (pH 8.0), and 0.05% Triton X-100) and sequentially immunoblotted with each primary antibody overnight at 4°C. The membranes were washed with TBS/Tween-20, and then incubated with a horseradish peroxidase-conjugated secondary antibody. The corresponding bands were detected using an Immobilon western chemiluminescent HRP substrate kit. The chemiluminescence signal was captured on X-ray film. β -actin was used as internal controls. The exposed film was scanned and analyzed using ImageJ software.

GADD153 and GRP78, ER stress biomarkers, were detected by Western blotting. Free form of microtubule-associated protein-1 light-chain 3 (LC3I) conversion to phosphatidylethanolamine-conjugated form of LC3I (LC3II) was also examined by Western blotting.¹¹

Analysis of autophagic vacuole by MDC assay

MDC is an autofluorescent compound that has been reported as a specific marker for autophagic vacuoles.¹⁵ After treatment, cells were incubated with freshly prepared 50 μ M MDC in medium at 37°C for 10 min in the dark. Cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. After washing with PBS, cells were mounted and examined by fluorescence microscopy (Zeiss Axio microscope) equipped with a filter system (excitation wavelength 380/420 nm; emission filter 450 nm). Contrast and brightness of the images were adjusted in Adobe Photoshop software.

Intracellular MDC was measured by fluorescence photometry. Mature podocytes were seeded (1000 cells/well) in black-wall 96-well plates at 37°C for 24 h. After treatment, cells were incubated with freshly prepared 50 μ M MDC in medium at 37°C for 10 min in the dark. The fluorescence was measured (excitation wavelength 380 nm; emission filter 525 nm) using FL $\times$ 800 fluorescence microplate reader (Biotek, Winooski, VT). To normalize the measurements to the number of cells present in each well, ethidium bromide (0.2 mM) was added and the DNA fluorescence was measured (excitation wavelength 530 nm, emission filter 590 nm). The fluorescence was then normalized to the number of cells. The MDC incorporated was expressed as specific activity (arbitrary units).¹⁶

Analysis of cell death by PI staining

Cell death in later stage of apoptosis following endonuclease activity was detected by PI staining. Endonucleases degrade DNA into small fragments of about 180 bp, which accumulate in the cell. Using PI staining, those cells may be detected by flow cytometry as a “sub-G1” population. After treatments, the collected cells were fixed with 0.5 ml of 70% ethanol at 4°C overnight. After washing twice with PBS, the cells were then permeabilized with 0.1% Triton-X-100, treated with 10 μ g/ml RNase A, and incubated at 37°C for 1 h before staining with 500 μ g/ml PI for 30 min at room temperature. At least 10,000 cells were measured by FACScan flow cytometer (Beckman Coulter, Brea, CA, USA) and analyzed with the Winmid2.9 software. The percentage of nuclei with hypodiploid content (sub-G1 DNA content) was evaluated as cell death.¹⁷

Cell viability assay

Cell viability was evaluated by the WST-1 assay. The WST-1 assay is based on the reductive cleavage of tetrazolium salt to the soluble formazan by a mitochondrial dehydrogenase that is active only in viable cells.¹⁸ Mature podocytes were seeded (1000 cells/well) in 96-well plates at 37°C for 24 h. After treatment, cell death was assayed by the WST-1 assay kit following the manufacturer's instruction. The amount of dye was measured at OD450 and referenced at OD620 using μ Quant microplate reader (BioTek, Winooski, VT).

Statistical analysis

All experiments were performed independently at least three times. All values are expressed as mean $\pm$ SD. Statistical significance was determined by one-way analysis of variance followed by Bonferroni posttest using GraphPad Prism 5.0 and differences were considered to be statistically significant when $p < 0.05$.

Results

Effects of ER stress on cell death

To validate whether cell death was caused by ER stress in podocyte, we treated podocyte with 2 μ M of TG and

5 μ g/ml of TM for the indicated time periods. As shown in Figure 1(a), GADD153 and GRP78 were analyzed to evaluate the effects of TG and TM to induce ER stress. GADD153 and GRP78 were increased at 6, 12, 24, and 48 h after TG or TM treatment. Then, cell death induced by ER stress was detected by PI staining and analyzed by FACS for sub-G1 peak (as a cell death indicator). Cell death was significantly increased at 6, 24, and 48 h after either TG or TM treatment (Figure 1(b)). The cell viability assay was undertaken to demonstrate the injuries by ER stress. WST-1 assay indicated that TG or TM treatment decreased cell viability at 6, 24, and 48 h (Figure 1(c)). These results showed that TG/TM could increase ER stress and might consequently induce cell death.

ER stress induces autophagy

To examine whether autophagosomes were formed after ER stress induction, MDC was used to evaluate the abundance of autophagic vacuoles in the cytoplasm, mainly perinuclear as previously reported. We found by fluorescence microscope that MDC-labeled vacuoles were increased at 6 and 24 h after ER stress induced by TG and TM (Figure 2(a)). MDC can also be used to quantify autophagy formation. Analyzed by flow cytometry, MDC-positive podocytes were significantly increased after treatment with TG and TM for 6 and 24 h (Figure 2(b)). Further, LC3I to LC3II conversion is commonly used as a marker of autophagosome formation. TG/TM induced significant expression of LC3II at 6, 12, 24, and 48 h (Figure 2(c)). From the above findings, we showed that ER stress could promote autophagosome formation.

Rapamycin enhances autophagosome formation in podocyte

Mammalian target of rapamycin (mTOR) is a protein kinase that controls cell growth and is a negative regulator of autophagy. Rapamycin is able to induce autophagy via by inhibiting mTOR activity. To test whether rapamycin could enhance autophagy induction in podocytes, podocytes were incubated in medium containing vehicle (Ctl), 0.5, 1, 5, and 10 nM of rapamycin (Rap). Compared with Ctl, the mTOR activation was significantly inhibited, while the amount of LC3II conversion was increased by various concentrations of Rap after 2 h incubation (Figure 3(a)). Then, cells were stained with MDC and analyzed by microscopy (Figure 3(b)) and flow cytometry (Figure 3(c)). MDC activity was significantly increased by 0.5, 1, 5, and 10 nM Rap. These findings indicated that Rap promoted autophagosome formation by inhibition of mTOR pathway; 5 nM Rap was used in the subsequent experiments.

Effect of rapamycin on ER stress-induced cell death

As shown in Figure 4(a), LC3II levels increased in TG/TM treatment groups. However, LC3II levels did not change at 6 and 24 h in Rap + TG/TM treatment groups. Cell death was detected by PI staining and analyzed by FACS for sub-G1 peak. As shown in Figure 4(b), cell death was

Figure 1 TG and TM caused ER stress and cell death. Podocytes were incubated with TG (2 μ M) or TM (5 μ g/ml) for the indicated time periods. (a) GADD153 and GRP78 were up-regulated at 6, 12, 24, and 48 h after TG or TM treatment. Quantification of GADD153 and GRP78 was expressed as the ratio of target protein over β -actin. Data are presented as mean $\pm$ SD, $N=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with loading control. (b) Cell death was detected by PI staining and analyzed by FACS for sub-G1 peak. Cell death was significantly increased at 6, 24, and 48 h after TG or TM treatment. Values were mean $\pm$ SD, $N=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with controls. (c) Cell viability was evaluated by WST-1 assay. TG and TM significantly decrease cell viability at 6, 24, and 48 h. Values were mean $\pm$ SD, $N=3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with controls

significantly increased by TG and TM treatment for either 6 or 24 h. However, the enhanced cell death was ameliorated in the Rap-pretreated podocytes while the cells were incubated with TG and TM for 6 h (upper panel). Such an effect to lessen the enhanced cell death from Rap pretreatment was absent if the cells were incubated with TG and TM for 24 h (lower panel). The cell viability assay by WST-1 follows a similar pattern of findings in cell death experiment: Rap pretreatment reduced ER stress-induced cell viability at 6 h, but not at 24 h of TG/TM treatment (Figure 4(c)). GADD153 is recognized as one of the

representatives of UPR. As shown in Figure 4(a), the expression of GADD153 was significantly increased by TG and TM treatment for either 6 or 24 h. Rap decreased the levels of GADD153 in TG/TM treated podocyte at 6 h, but not at 24 h. These findings indicated that Rap might protect podocytes from the ER stress-induced cell death through the enhanced autophagy at early stage, but not in later stage. The statement was partially supported by the findings in Figure 4(b) that Rap treatment alone did not influence cell death in 6 h, but increased cell death in 24 h.

Figure 2 ER stress-induced autophagosome formation. (a) MDC assay. MDC labeling (green) at 6 and 24 h after TG or TM treatment was shown. Images are taken at 630 $\times$ magnification. Scale bar represents 50 μ m. (b) Intracellular MDC uptake was significantly increased after incubation with TG or TM for 6 and 24 h. Values are presented as mean $\pm$ SD, $N = 3$. * $p < 0.05$, ** $p < 0.01$ compared with controls. (c) Cell lysates were analyzed for LC3-I/LC3-II conversion. LC3II was significantly increased at 6, 12, 24, and 48 h by TG or TM. Data show mean $\pm$ SD, $N = 3$. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with controls. (A color version of this figure is available in the online journal.)

Inhibition of autophagy formation exacerbates ER stress-induced cell death

3-MA is a known inhibitor of autophagosome formation. Figure 5(a) shows that LC3II increased after TG/TM treatment. Then, LC3II formation decreased at 6 and 24 h in the groups of co-treatment of 3-MA and TG/TM. As shown in Figure 5(b), intracellular MDC-labeled vacuoles were

significantly decreased at 24 h after 3-MA treatment (Figure 5(b)). To test whether inhibition of autophagosome formation altered ER stress-induced cell death, podocytes were pretreated with vehicle (Ctl), or 2 mM of 3-MA for 2 h and then TG or TM was added for 6 h or 24 h. Sub-G1 peaks were increased after TG or TM treatment at 6 and 24 h. With 3-MA pretreatment, cell death increased further compared

Figure 3 Rapamycin enhanced autophagosome formation in podocytes. (a) Effect of rapamycin on mTOR activation (p-mTOR/mTOR) and LC3 conversion. The mTOR activation was significantly inhibited and the amount of LC3II was increased. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with controls. (b) Rapamycin increased MDC expression in a dose-dependent manner. Images are taken at 630 $\times$ magnification. Scale bar is 50 μ m. (c) MDC activity was significantly increased in 0.5, 1, 5, and 10 nM rapamycin shown by fluorescence photometry. Values are presented as mean $\pm$ SD, $N = 3$. *** $p < 0.001$ compared with controls. (A color version of this figure is available in the online journal.)

with non-pretreated cells at 6 h or 24 h (Figure 5 (c)). Furthermore, 3-MA alone was noted to significantly increase cell death either at 6 or 24 h. The result in Figure 5(d) shows that cell viability experiment generally followed the pattern in cell death experiment. As shown in Figure 5(a), the expression of GADD153 was significantly increased by TG and TM treatment for either 6 or 24 h. Pretreatment with 3MA could also increase the levels of GADD153 in TG/TM treated podocyte at 6 h, especially in 24 h. The above findings indicated that the inhibition of autophagy contributed to increased cell death.

Discussion

ER stress is involved in a wide range of physiological and pathological conditions, and accumulating evidence demonstrates that ER stress contributes to glomerular and tubular injuries in kidney disease.¹⁹ Cells react to ER stress by activating a series of integrative pathways, including autophagy.²⁰ The present experiments have applied TG and TM to treat glomerular podocytes to induce ER stress, which was represented by significant expression of GADD153 and GRP78. The induced ER stress was associated with the increased podocyte cell death. However, TG/TM stimulation also enhanced the autophagosome formation, shown by intracellular MDC staining and LC3 conversion. Treatment with Rap increased the autophagosome formation and inhibited the TG/TM-induced podocyte cell death. Oppositely, 3-MA inhibited the formation of autophagosomes and increased the TG/TM-induced cell death. We conclude that autophagy is enhanced along with increased ER stress and cell death, and it may be purposefully protective for maintenance of podocytes survival.

Induction of ER stress has been demonstrated in various experimental animal models of glomerulonephritis with

proteinuria,²⁰ and markers of ER stress have been identified in human kidney biopsies of proteinuric glomerulonephritis.²¹ Glomerular podocytes, the terminally differentiated cell type among the cellular members in kidney, are relatively vulnerable to various damages. It is generally accepted that podocytes damage stand in the center of almost all the proteinuric glomerular diseases and contribute to the disease initiation and progression.²² The most particular structural features of podocytes are the interdigitating foot processes attached firmly to glomerular capillaries, and also the slit diaphragms bridging the neighboring foot processes. Structural and functional integrity of the foot processes along with slit diaphragms are by far the most important mechanism to prevent the loss of valuable plasma proteins into urine.²³ However, it does have a downside, that is, podocytes have to constantly contact with substances (toxic, pro- and inflammatory, etc.) that filter through the glomerular basement membrane, and consequently carry high risks of ER stress and survival threats. Minor injuries to podocytes can result in detachment and more severe damage may lead to cell apoptosis, or even cell death.²⁴ However, being terminally differentiated, podocytes usually do not proliferate to renew themselves. Therefore, it is mandatory for podocytes to persevere with their survival and resist the continuous plasma-derived complex milieu filtering through the glomerular filtration barrier. To execute these two seemingly contrasting tasks in the same time, podocytes have to equip themselves with efficient mechanisms. Study has shown that un-stimulated healthy podocytes displayed numerous intra-cytoplasmic vacuoles resembling autophagosomes, suggesting high basal rate of autophagy.²⁵ Typically, highly differentiated cells like neurons exhibit low basal rate of autophagy.²⁰ Unlike neurons in the brain exhibiting blood-brain barrier, podocytes have to confront all the

Figure 4 Effect of rapamycin on ER stress-induced cell death and viability in podocytes. (a) LC3II was increased after TG/TM treatment with or without Rap pretreatment at 6 and 24 h. Quantification of LC3II expressed as the ratio of target protein over β -actin. GADD153 significantly increased in TG and TM treatment groups and decreased at 6 h in Rap + TG and Rap+TM treatment groups. But at 24 h, the levels of GADD153 had no significant change between TG/TM treatment only and co-treat with Rap group. Quantification of GADD153 expressed as the ratio of target protein over β -actin. (b) Cell death analyzed by FACS. Upper panel: Rapamycin significantly reduced ER stress-induced cell death by TG or TM at 6 h. Lower panel: Rapamycin did not prevent cell death at 24 h. (c) Cell viability analyzed by WST-1 assay. Left: Rapamycin helped maintain cell viability after TG or TM at 6 h. Right: Rapamycin was not able to rescue cell viability by TG or TM at 24 h. All data above are presented as mean $\pm$ SD from three independent experiments. N.S. (not significant), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with indicated groups

plasma-derived filtered substances that may disturb the normal homeostasis, while maintaining cell survival without proliferation. The specific situations explain the rationale of the present study to clarify the crucial roles of autophagy in podocytes.

Autophagy can promote cell survival in response to nutrient deprivation and metabolic stress through recycling intracellular components in the short term, and it is regulated by mTOR in the PI3-kinase/AKT pathways.²⁶ Rate of new protein synthesis is tuned down to cope with the enhanced catabolic processes. In long term, however,

autophagy may potentially lead to cell death (type II programmed cell death) through the accumulated self-digestive damages.²⁷ It is not clear whether autophagy involves directly in the initiation of the autophagic cell death or if the cell death simply results from an exhausted attempt of autophagy to preserve cell viability. The seemingly contrasting functions of autophagy in survival and death may partially explain the difference of cell death induced by the enhanced autophagy at different time periods (as shown in Figure 4). The exact role of autophagy on cell survival depends on the balance between the external

Figure 5 Inhibition of autophagosome formation by 3-MA on ER stress-induced cell death. (a) LC3II increased after TG/TM treatment only and decreased at 6 and 24 h in co-treated with 3-MA group. Quantification of LC3II expressed as the ratio of target protein over β -actin. GADD153 increased at 6 and 24 h in TG/TM treatment groups. In co-treated with 3-MA and TG/TM groups, GADD153 significantly increased at 24 h. Quantification of GADD153 expressed as the ratio of target protein over β -actin. (b) Podocyte treated with TG or TM in the absence or presence of 3-MA for 24 h. Images are taken at 630 $\times$ magnification. Scale bar is 50 μ m. (c) Cell death was analyzed by FACS for sub-G1 peak. 3-MA promoted significantly more cell death than TG and TM. (d) Cell viability analyzed by WST-1 assay. Cell viability was decreased at TG+3-MA or TM+3-MA than TG or TM alone at 6 and 24 h. All values above represent mean $\pm$ SD of three independent experiments. N.S. (not significant), * p < 0.05, ** p < 0.01, *** p < 0.001 compared with indicated groups. (A color version of this figure is available in the online journal.)

challenges and the internal supportive mechanisms, and may not be straightforward.

Wang et al.'s²⁸ study in membranous nephropathy also found the up-regulated GRP78 and GRP94, while tubulin- β

was down-regulated by proteomics analysis. They indicated that up-regulated autophagy can repair ER stress-induced podocyte damage.²⁸ Our data also revealed that TG/TM activate LC3 up-regulation. Increased

autophagosome formation was induced by Rap. GADD153 levels were decreased at 6 h but not at 24 h (as shown in Figure 4(a)). Moreover, our data showed the enhanced autophagy by Rap decreased podocyte cell death at 6 h but not at 24 h (as shown in Figure 4(b) and (c)). The findings might show a complex role of autophagy in survival and death. Autophagy was often considered a mechanism of cell protection and homeostasis maintenance. However, recent studies have shown that autophagy may also promote cell death. We used 3-MA to inhibit autophagosome formation, and found GADD153 levels were significantly increased at 6 and 24 h (as shown in Figure 5(a)) and podocyte cell death was markedly increased at 6 and 24 h (as shown in Figure 5(b) and (c)). A recent study had demonstrated autophagy as a crucial regulatory mechanism of apoptotic cell death by modulating the balance between the pro-survival pathway and the pro-apoptotic pathway of ER stress.²⁹ The relevance between autophagy and ER stress in podocyte is worth future studies for confirmation.

Our study has its own limitations. First, the methods we used to assess cell death and cell viability were not specifically limited to the effects of autophagy and therefore, the presented data could potentially be exaggerated. For supplement, MDC and LC3 conversion were used to stain autophagosome, and 3-MA was applied to inhibit autophagosome formation. Further, we have not examined the morphological and functional integrity of podocytes and hence, are not able to show the functional significance of autophagy. Future works would have to include the functional aspects in either cellular or animal experiments to manifest the importance of autophagy in podocyte biology and in proteinuric kidney disease.

In conclusion, we showed that the induced ER stress might be related with podocyte cell death. However, autophagy would be simultaneously enhanced, and it mediated to salvage the injuries caused by ER stress in short term. Rapamycin increased the autophagosome formation and exhibited a similar influence on podocyte as the ER stress-related autophagy. We proposed that adequate, but not excessive, autophagy is crucial to help maintain the cell survival and viability of podocyte as a terminally differentiated cell lineage in glomerulus.

Authors' contributions: All authors contributed extensively to the work presented in this paper. CY-C and CC-A made contributions to conception and design this study. CY-C performed experiments, collected and analyzed data. CJ-M, CC-A and CY-C wrote the manuscript, and CH-C gave final approval of the version to be submitted and any revised version. All authors discussed the results and implications and commented on the manuscript at all stages.

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