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The cytoprotective role of autophagy in puromycin aminonucleoside treated human podocytes



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ABSTRACT

Autophagy is a ubiquitous catabolic process involving degradation of damaged organelles and protein aggregates. It shows cytoprotective effects in many cell types and helps to maintain cell homeostasis. In many glomerular diseases, podocyte damage leads to the disruption of the renal filtration barrier and subsequent proteinuria. Puromycin aminonucleoside (PAN) which induces podocyte apoptosis *in vitro* and *in vivo* is widely used for studying the pathophysiology of glomerular diseases. It has been shown that PAN induces autophagy in podocytes. However, the relationship between autophagy and apoptosis in PAN treated human podocytes is not known and the role of PAN-induced autophagy in podocyte survival remains unclear. Here we demonstrate that PAN induced autophagy in human podocytes prior to apoptosis which was featured with the activation of mTOR complex 1 (mTORC1). When the PAN-induced autophagy was inhibited by 3-methyladenine (3-MA) or chloroquine (CQ), podocyte apoptosis increased significantly along with the elevation of active caspase-3. Under such circumstance, the podocyte cytoskeleton was also disrupted. Collectively, our results suggested that the induced autophagy may be an early adaptive cytoprotective mechanism for podocyte survival after PAN treatment.

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1. Introduction

Podocytes are highly differentiated cells which play an extremely important role in guarding the permeability of the tripartite renal filtration barrier. Many glomerular diseases, such as minimal change nephropathy and focal segmental glomerulosclerosis (FSGS), attribute to podocyte damage including apoptosis, cytoskeleton rearrangement and detachment [1,2]. Single gene mutations, such as those affecting nephrin and podocin, in podocytes have also been acknowledged in the pathogenesis of renal diseases [3,4]. Therefore, podocytes emerge as the therapeutic target of glomerular diseases. Since podocytes are terminally differentiated cells, they are able to cope with various adverse stimuli and repair themselves efficiently. The question of “How do podocytes survive longly?” initiated our investigation into the cellular protective mechanisms.

Macroautophagy (hereafter referred to as autophagy) is an evolutionary conserved catabolic process [5]. It is involved in processing unwanted cellular materials including misfold proteins, damaged organelles and invasive microorganisms to the lysosomes for degradation. The degraded products can be recycled for maintaining energy homeostasis [6]. Autophagy is being kept at basal levels in cells for safeguarding and promoting cell survival. This basal autophagy is one of the major processes that allow cells to respond rapidly to the metabolic stress. Under certain circumstances, the autophagy can be induced to a higher level to protect cells. For instance, the induction of autophagy under ischemia in neurons protects them from apoptosis [7]. Currently, the better known potential stimuli inducing autophagy include nutrient starvation, oxidative stress, mitochondrial dysfunction, ischemia–reperfusion and infection [8,9].

Mammalian target of rapamycin (mTOR) is an evolutionarily conserved serine/threonine kinase which is the core part of two complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Besides mTOR, mTORC1 also contains mLST8 and Raptor which recruits substrates such as p70 S6 Kinase (p70S6K) for the phosphorylation by the kinase domain of mTOR. mTORC1 plays multiple roles in regulating ribosome biogenesis, protein synthesis and tissue hypertrophy [10]. More importantly, the activation of

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mTORC1 inhibits autophagy. On the contrary, autophagy can be induced when mTORC1 is inhibited by the decreased growth factors and amino acids during starvation as well as some agents such as rapamycin [11,12].

Puromycin aminonucleoside (PAN) is widely used for studying glomerular diseases as it induces podocyte injury *in vitro* and *in vivo* [13,14]. Rats after a single dose of PAN injection will present with heavy proteinuria, effacement of foot processes and podocyte loss [15]. Thus, valuable knowledge for understanding the pathophysiology of renal diseases has been generated based on this reagent. Recently, it has been reported that PAN can induce autophagy in podocytes *in vitro* and *in vivo*. For instance, Asanuma et al. found that autophagy was induced in PAN treated conditionally immortalized mouse podocytes (MPCs) and in PAN nephrosis rat model [16]. Another study showed that mTOR-ULK1 pathway was involved in PAN-induced autophagy in MPCs [17]. In addition, Hartleben et al. demonstrated that autophagy deficient mice exhibited more severe albuminuria after PAN treatment than the wild type [18]. Taken together, these studies demonstrate that autophagy is essential for podocytes to maintain normal function. Even though PAN is known to induce podocyte apoptosis, cytoskeleton damage and autophagy, the relationship between these important processes remains unclear. In particular, the role of PAN-induced autophagy in podocyte survival has not been elucidated.

In the present study, we found that PAN induced autophagy prior to apoptosis which was featured with the activation of mTORC1 in human podocytes. Moreover, when the PAN-induced autophagy was being blocked by autophagy inhibitors, podocyte apoptosis increased significantly. In addition, we revealed that the proportion of podocytes with disrupted cytoskeleton dramatically increased after autophagy inhibition. These results suggested that the induced autophagy may be an early adaptive cytoprotective mechanism for podocyte survival after PAN treatment.

2. Materials and methods

2.1. Cell culture

Conditionally immortalized human podocytes AB8/13 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Life Technologies). The human podocytes proliferated at 33 °C, with the addition of Insulin–Transferrin–Selenium (Life Technologies). After growing to 50–60% confluency, cells were transferred to 37 °C for stopping proliferation and achieving full differentiation in 10–14 days. Fully differentiated human podocytes were treated with different reagents, such as PAN, in RPMI 1640 medium plus 10% FBS at 37 °C in a humidified atmosphere containing 5% CO₂.

2.2. Western blotting

Total protein was extracted by RIPA buffer containing a cocktail of protein inhibitors (Roche Applied Science) and phosphatase inhibitors (Calbiochem). Protein concentration was determined by the Bradford assay (Bio-Rad Laboratories). Equal amounts of denatured proteins were separated by 4–12% SDS–PAGE and transferred to PVDF membrane (Bio-Rad) which was incubated with primary antibody and secondary antibody sequentially, visualized using chemiluminescence substrate (Perkin Elmer). The image was captured by the ChemiDoc MP system (Bio-Rad) and analyzed with the Image J software (NIH, USA). LC3, p62, p-mTOR (Ser2448), T-mTOR, p-p70S6K (Thr389), p70S6K antibodies, anti-rabbit IgG, HRP linked antibody (Cell Signaling Technology) and β -actin antibody (Santa Cruz Biotechnology) have been used.

2.3. Immunofluorescence analysis

Podocytes were fixed with 4% paraformaldehyde, blocked with PBS + 2% BSA and incubated with anti-LC3 antibody (4 °C, 12 h). Then, Alexa Fluor 488 goat anti-rabbit IgG antibody (Life Technologies) was added (room temperature, 2 h) and washed. After being mounted with the ProLong[®] Gold Antifade Reagent with DAPI (Life Technologies), cells were visualized by confocal microscopy (EZ-C1, Nikon Instrument, Japan). In addition, Alexa Fluor 594 Phalloidin (Life Technologies) was used to stain F-actin. The percentage of podocytes showing accumulation of LC3 puncta (that is with at least five puncta per podocyte) and podocyte with disrupted cytoskeleton was quantified as the previous study described [19]. At least 100 cells were scored in each of six independent experiments.

2.4. Flow cytometry

The treated podocytes were harvested and the cell density was adjusted to 1×10^6 /ml for YO-PRO-1/PI assay (Life Technologies) and active caspase-3 assay (BD Pharmingen) according to manufacturers' recommendation. Apoptotic cells were measured by the flow cytometry (FC500, Beckman Coulter) and expressed as a percentage of total cells. Examples of plots and analysis are included in the [Supplementary Material](#).

2.5. Statistical analysis

Data were expressed as mean \pm SEM. Multiple groups were analyzed by Kruskal–Wallis test with post hoc procedures using the Prism 5.0 Software (GraphPad, San Diego, CA, USA). A *p* value < 0.05 was considered to be statistically significant.

3. Results

3.1. PAN induces autophagy prior to apoptosis in human podocytes

To investigate the relationship between autophagy and apoptosis in podocyte, conditionally immortalized human podocytes were used in the present study. The level of autophagy was evaluated by the expression of microtubule-associated protein 1A/1B-light chain 3 (LC3). LC3 is a soluble protein in mammalian cells including two forms (LC3-I and LC3-II). The elongation of autophagosome requires LC3-II which is the conjugated product of LC3-I and phosphatidylethanolamine (PE). Thus, the increased LC3-II is regarded as the standard marker for autophagy activation [20]. As shown in Fig. 1A, when podocytes were treated with different doses of PAN for 24 h, the expression of LC3-II increased significantly at the dose of 30 and 50 μ g/ml. There was no significant difference between these two doses. Hence, the concentration of 30 μ g/ml was selected for the subsequent experiments.

We found that LC3-II increased after PAN treatment and peaked at 24 h, but it decreased afterwards and returned nearly to the basal level at 48 h (Fig. 1B). To confirm the PAN-induced autophagy detected above, we performed LC3 immunofluorescence staining in PAN treated podocytes. It is known that LC3-II binds to the autophagosomal membrane and forms puncta in the formation of autophagosome. Therefore, immunofluorescence staining can be used to analyze LC3 distribution and monitor autophagy in cells. As shown in Fig. 1C, a large number of bright puncta were visualized in the cytoplasm of human podocytes at 24 h after PAN treatment. On the contrary, only very small scattered immunofluorescent dots were detected in the 0, 12 and 48 h post PAN treatment. The statistical results from LC3 staining are consistent with the data generated from Western blotting (Fig. 1D).

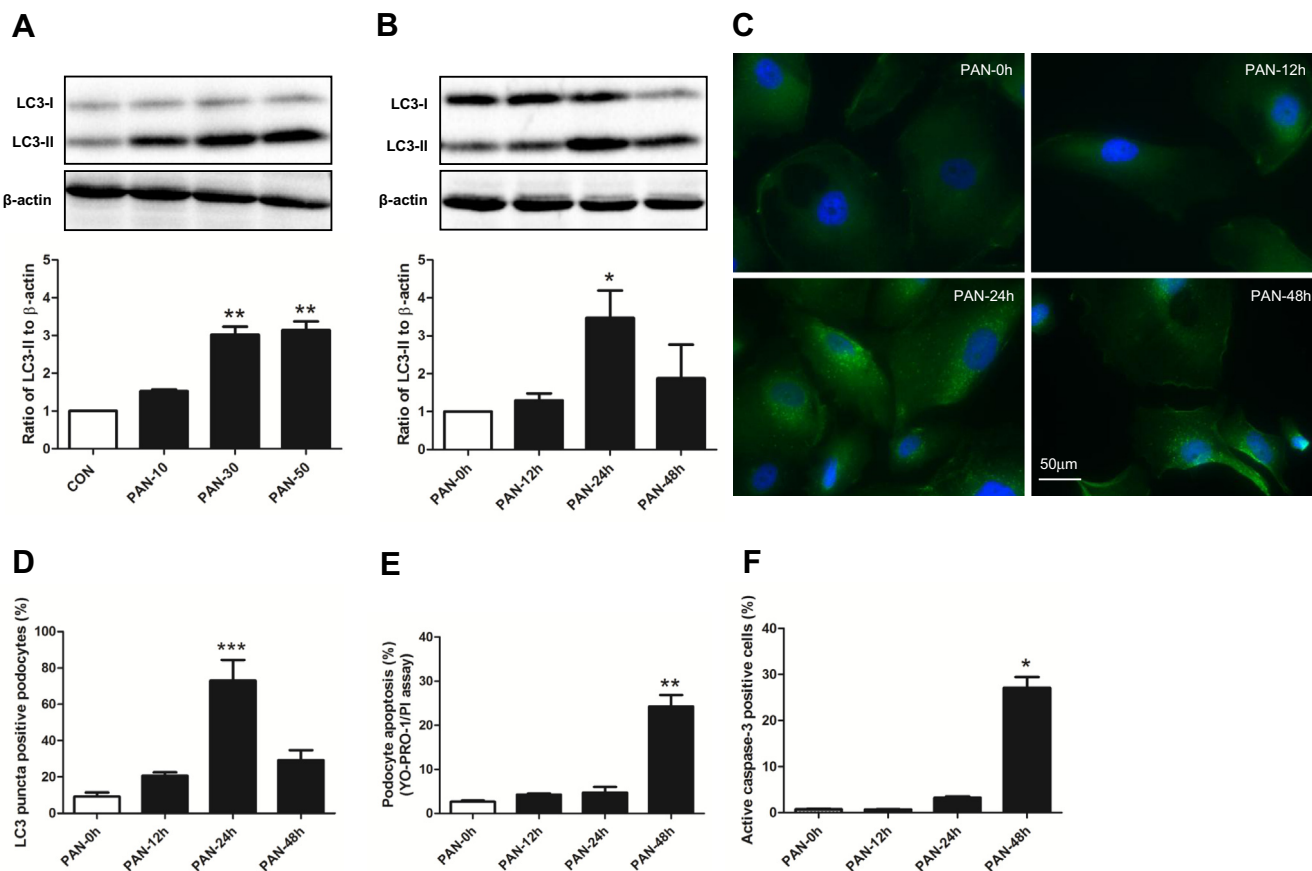


Fig. 1. PAN induces autophagy prior to apoptosis in human podocytes. (A) The LC3-II expression increased in a dose-dependent manner after PAN treatment. Conditionally immortalized human podocytes were treated with 10, 30 and 50 μ g/ml of PAN for 24 h. The LC3-II was measured by Western blotting. The data was expressed as the relative change compared with the control group (CON). A representative immunoblot is shown along with the quantitative data which represents the means \pm SEM ($n = 5$), ** $p < 0.01$ versus CON. (B) The LC3-II expression in podocytes peaked at 24 h after PAN treatment (30 μ g/ml). The results represent the means \pm SEM ($n = 4$), * $p < 0.05$ versus PAN-0 h. (C, D) Large number of bright LC3 puncta (green) at 24 h after PAN treatment (30 μ g/ml) were visualised by confocal microscopy. Representative LC3 immunostaining (green) and statistical results from 6 independent experiments were shown, *** $p < 0.001$ versus PAN-0 h. The nuclei of the podocytes were stained with DAPI (blue). (E) Active caspase-3 positive podocytes increased at 48 h after PAN treatment, flow cytometry was used for the measurement. $n = 4$, * $p < 0.05$ versus PAN-0 h. (F) Podocyte apoptosis peaked at 48 h after PAN treatment (30 μ g/ml). Apoptosis was measured by flow cytometry (YO-PRO-1/PI assay). The representative graphs are shown along with the statistical results. The results represent the means \pm SEM, $n = 8$, ** $p < 0.01$ versus PAN-0 h. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Apoptosis is typically characterized by altered membrane permeability as determined by YO-PRO-1/PI assay in this study. Our data showed that after PAN (30 μ g/ml) treatment, the percentage of apoptotic podocytes at 24 h was similar to the control (Fig. 1E), but it increased significantly at the 48 h time point. Moreover, active caspase-3 which is the cleaved product of caspase 3 indicates cell undergoing apoptosis. As shown in Fig. 1F, the percentage of podocytes with active caspase-3 increased significantly at 48 h after PAN treatment. Taken the data of autophagy and apoptosis together, it was clearly shown that PAN induced autophagy prior to apoptosis in human podocytes.

3.2. mTORC1 is activated in PAN treated human podocytes

To examine the activity of mTORC1 in PAN treated podocytes, we measured the levels of phospho-mTOR (p-mTOR) and its downstream target marker, phospho-p70S6K (p-p70S6K). It was shown that the expression of p-mTOR was maintained at the basal level in the first 12 h but increased gradually over time (Fig. 2A). Its expression was significantly higher than the control at 24, 36 and 48 h time points. Similarly, p-p70S6K was induced gradually over time and reached significantly high level after 24 h post PAN treatment (Fig. 2B).

3.3. Inhibition of PAN-induced autophagy increases apoptosis in human podocytes

Based on our observation of low percentage of apoptotic podocytes but high autophagy at 24 h after PAN treatment, we speculated that autophagy may play a cytoprotective role against podocyte apoptosis. To verify this hypothesis and investigate the potential mechanism, two inhibitors were used to suppress autophagy in PAN treated human podocytes. 3-Methyladenine (3-MA) blocks autophagy at the initial stage by suppressing class III Phosphatidylinositol 3-kinases (PI3K), whereas chloroquine (CQ) increases lysosomal pH, thereby inhibits autolysosomal degradation, which is featured with accumulation of the two autophagy substrates LC3-II and p62/SQSTM1 (p62). Additionally, polyubiquitinated proteins and aggregates can be oligomerized by p62, and binds to LC3 on the autophagosome membrane, eventually damaged organelles or unfolded proteins will be degraded in the autophagy pathway [21]. Hence, we also measured p62 to confirm the inhibition of autophagy by these inhibitors.

As shown in Fig. 3A, PAN-induced podocyte autophagy was indicated by the increased LC3-II and decreased p62 levels at 24 h. The addition of 3-MA successfully suppressed the PAN-induced autophagy which was characterized by the low LC3-II and

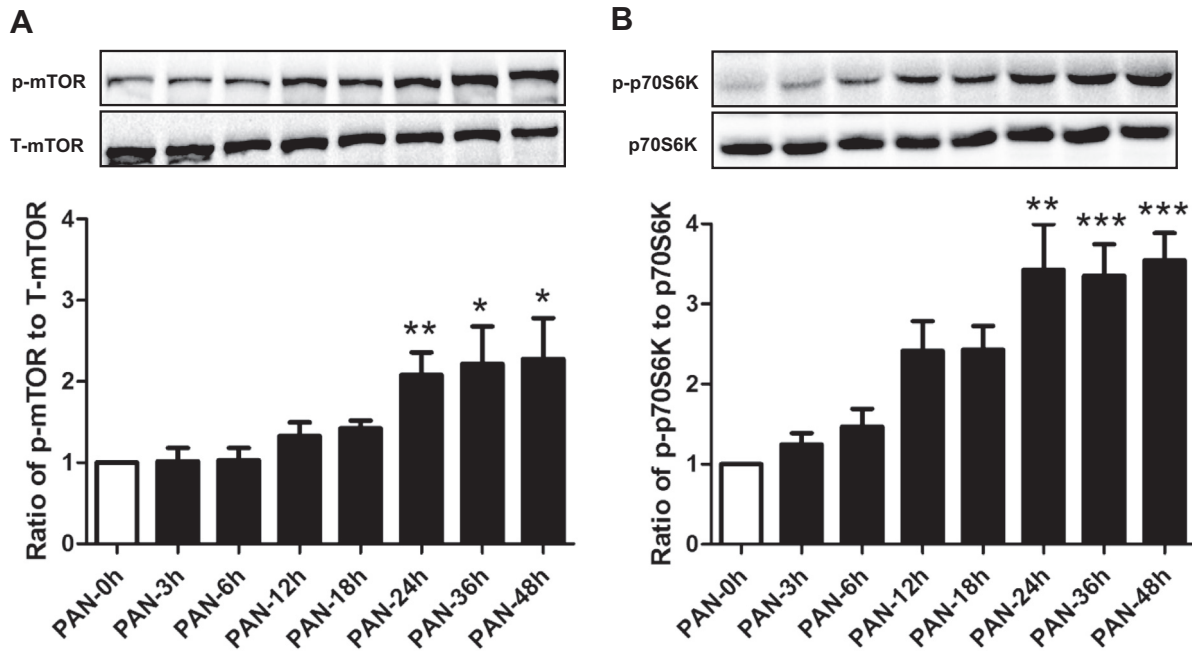


Fig. 2. mTORC1 is activated in PAN treated podocytes. (A) The expression level of p-mTOR (Ser2448) in PAN (30 µg/ml) treated podocytes increased significantly at 24, 36 and 48 h. The p-mTOR expression level was measured by Western blotting. The data was expressed as the relative change compared with PAN-0 h. A representative immunoblot is shown along with the quantitative data which represent the means \pm SEM ($n = 13$), * $p < 0.05$; ** $p < 0.01$ versus PAN-0 h. (B) Similarly, the expression level of p-p70S6K (Thr389) increased significantly at the same time points. A representative immunoblot is shown along with the quantitative data which represent the means \pm SEM ($n = 9$), ** $p < 0.01$; *** $p < 0.001$ versus PAN-0 h.

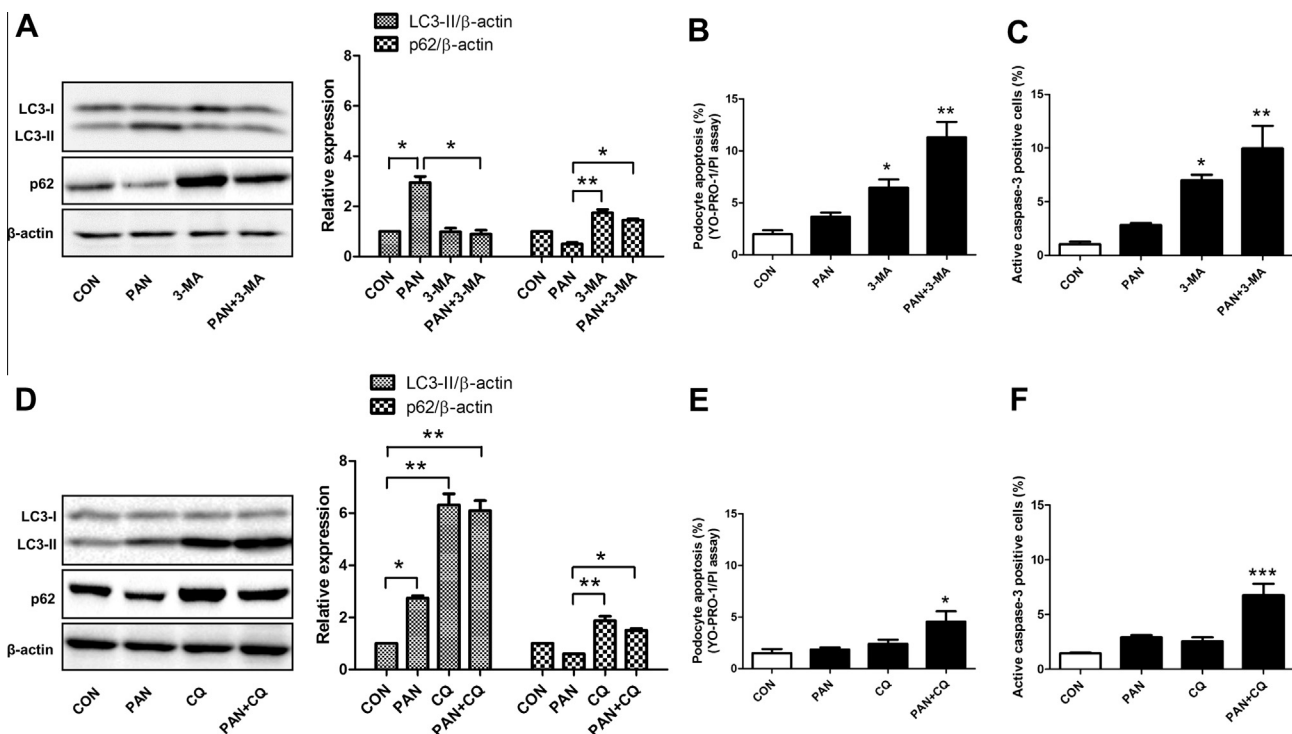


Fig. 3. The inhibition of PAN-induced autophagy increases podocyte apoptosis. (A) PAN-induced autophagy was inhibited by 3-MA. The expression of LC3-II increased in podocytes which were treated with PAN (30 µg/ml) for 24 h, it was decreased by 3-MA (5 mM). The expression of p62 decreased in PAN treated podocytes, but it increased significantly after the addition of 3-MA. The representative immunoblot is shown along with the quantitative data which represents the means \pm SEM ($n = 5$), * $p < 0.05$, ** $p < 0.01$. (B) Podocyte apoptosis increased significantly at 24 h after autophagy inhibition. The results represent the means \pm SEM ($n = 4$), * $p < 0.05$, ** $p < 0.01$ versus CON. (C) Active caspase-3 positive podocytes increased after autophagy inhibition. Flow cytometry was used for the measurement. The data represent the means \pm SEM ($n = 4$), * $p < 0.05$, ** $p < 0.01$ versus CON. (D) PAN-induced autophagy was inhibited by CQ. The PAN-induced autophagy was blocked by CQ (25 µM) which resulted in the increased LC3-II expression. The expression of p62 decreased in PAN treated podocytes, but it increased significantly after the addition of CQ. The representative immunoblot is shown along with the quantitative data which represents the means \pm SEM ($n = 5$), * $p < 0.05$, ** $p < 0.01$. (E, F) The changes in podocyte apoptosis and the percentage of active caspase-3 positive podocytes after autophagy inhibition by CQ were similar to 3-MA treatment. $n = 5, 4, 4$ respectively, * $p < 0.05$, *** $p < 0.001$ versus CON.

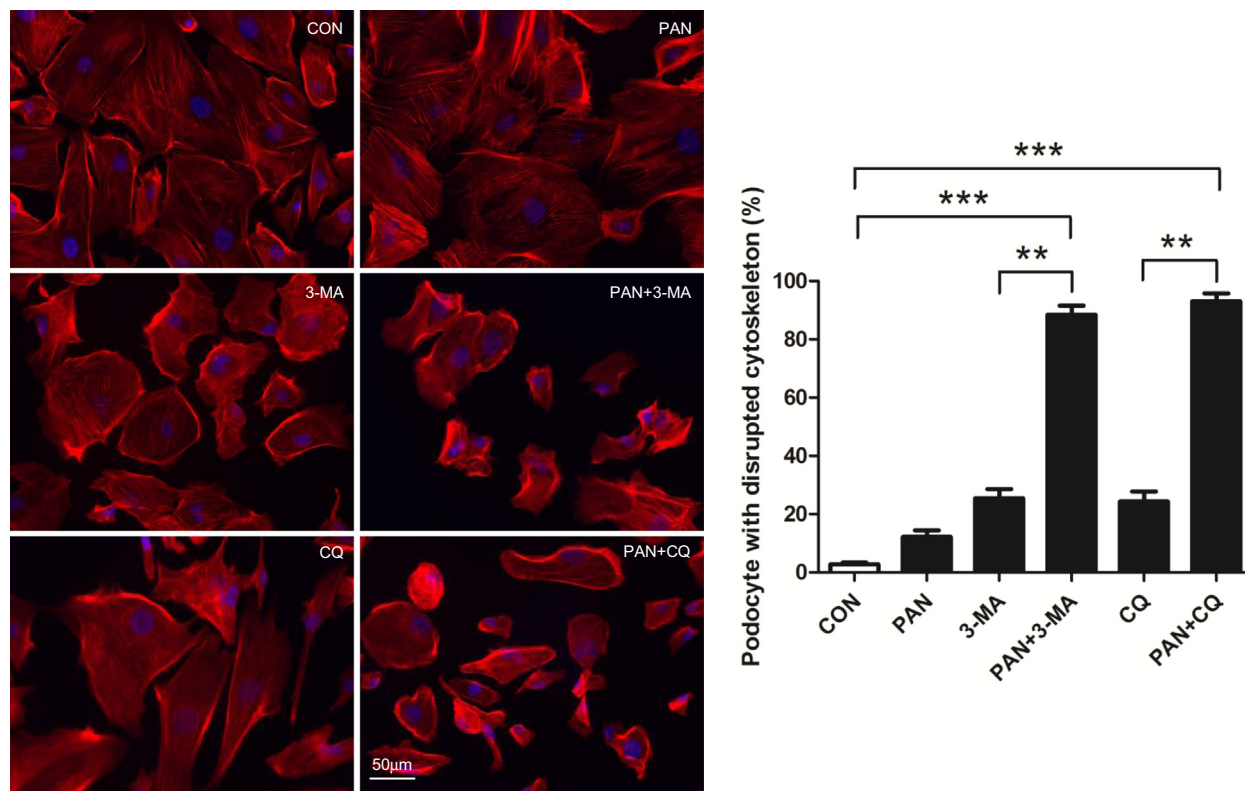


Fig. 4. The inhibition of PAN-induced autophagy disrupts podocyte cytoskeleton. The podocytes with disrupted F-actin increased when PAN-induced autophagy was inhibited by 3-MA (5 mM) or CQ (25 μ M) for 24 h. Regular bundles of intracellular actin filaments (red) were observed in untreated cells (CON). The nuclei of the podocytes were stained with DAPI (blue). PAN, 3-MA and CQ alone slightly disrupted the actin filaments in podocytes at 24 h. However, cell retraction, the loss of actin-stress fibre organization and the formation of cortical cytoskeleton were observed in the PAN + 3-MA and PAN + CQ groups. The representative staining pictures are shown along with the quantitative data which represent means \pm SEM from 6 independent experiments, ** p < 0.01, *** p < 0.001 versus CON. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

accumulation of p62. Concurrently, there was a significant increase in podocyte apoptosis (Fig. 3B), along with the percentage of active caspase-3 positive podocytes (Fig. 3C).

Similarly, CQ arrested autophagy which was presented with LC3-II and p62 accumulation (Fig. 3D). The blockage of autophagy by CQ also led to an increase in podocyte apoptosis and the percentage of active caspase-3 positive cells (Fig. 3E and F).

3.4. Inhibition of PAN-induced autophagy disrupts podocyte cytoskeleton

Disruption of cytoskeleton is the hallmark of many glomerular diseases and lead to the podocyte effacement resulting in proteinuria [22]. To test whether the inhibition of induced autophagy affects the stability of podocyte cytoskeleton, we checked the status of F-actin in human podocytes when the PAN-induced autophagy was inhibited by 3-MA or CQ at 24 h. As shown in Fig. 4, podocyte cytoskeleton damage was featured with cell retraction, the loss of actin-stress fibre organization and the formation of cortical cytoskeleton. The percentage of podocytes with disrupted cytoskeleton increased significantly after the inhibition of PAN-induced autophagy.

4. Discussion

Autophagy is a conserved catabolic process for keeping cell homeostasis and serves as the quality control mechanism which is particularly important for the long lived cells such as neurons. Similarly, podocytes are terminally differentiated cells characterized by

the high basal level of autophagy [18], suggesting the significance of autophagy in keeping podocyte homeostasis. Previously PAN has been shown to induce autophagy in murine podocytes [16,17]. Here we reveal the role of the induced autophagy in PAN treated human podocytes.

In the present study, a relationship between autophagy and apoptosis was suggested in PAN treated podocytes. We found that the expression of LC3-II peaked at 24 h, the increased LC3 puncta were also observed in the cytoplasm at the same time point. Additionally, our study showed that the induction of autophagy was transient and the expression of LC3-II nearly dropped to the basal level at 48 h. Combining with our observation of low percentage of apoptotic cells at 24 h and peak apoptosis with the increased active caspase-3 positive podocytes at 48 h, we speculate that PAN induced cell stress in podocytes and triggered both autophagy and apoptosis. At the early time point, autophagy played a cytoprotective role to keep apoptosis at the low level. However, as cell stress persisted over time, autophagy was overridden by apoptosis at 48 h.

The changes of autophagy and apoptosis in PAN treated podocytes described above may be associated with mTORC1. We observed a significant increase in the level of p-mTOR and p-p70S6K at 24, 36 and 48 h, suggesting the activation of mTORC1 after PAN treatment. Recently it has been demonstrated that the mTORC1 activation in diabetic nephropathy leads to the mislocation of slit diaphragm proteins and an epithelial mesenchymal transition-like phenotypic switch [23]. Thus, we postulate that the activation of mTORC1 was responsible for the podocyte apoptosis. In addition, mTORC1 is also known to negatively regulate the autophagy. As expected, the increased activity of mTORC1

suppressed autophagy in the last 24 h of PAN treatment (from the time point of 24–48 h). However, it seems to be puzzling at the time point of 24 h that the peaked autophagy was coupled with the activated mTORC1. Actually, similar phenomenon has also been described recently by Huber et al. that high basal autophagy level was observed in podocytes despite the activation of mTOR [24]. This interesting finding could also be explained by the newly identified TOR-autophagy spatial coupling compartment (TASCC) which is located at the site of the Golgi apparatus [25]. TASCC sequesters cellular mTORC1 with autolysosomes and leads to a low concentration of mTORC1 in the environment outside the TASCC in favor for autophagy induction. Therefore, TASCC enables the simultaneous existing of the activation of mTORC1 and autophagy. In addition, it also can be not excluded that an mTOR-independent pathway exists in PAN treated podocytes.

Rapamycin, a mTOR inhibitor, has been shown to alleviate podocyte injury by inducing autophagy [17]. To verify the potential cytoprotective role of PAN-induced autophagy, we used 3-MA and CQ to inhibit the autophagy at the early and late stage respectively. Our results showed that 3-MA decreased the expression of LC3-II, while CQ led to the accumulation of LC3-II, suggesting that both of them can efficiently suppress PAN-induced autophagy. Consistently, p62 decreased at 24 h after PAN treatment but upregulated with the addition of 3-MA and CQ, further revealing that PAN can induce podocyte autophagy and 3-MA or CQ blocked the PAN-induced autophagy flux. Thus, it suggested that the damaged cellular components caused by PAN treatment accumulated in podocytes and could not proceed for degradation. Furthermore, it has been reported that the accumulation of p62 promotes cellular stress that leads to diseases [26]. As a consequence, podocyte apoptosis increased after 24 h, along with the upregulation of active caspase-3 positive podocytes, further showing the death pathway was activated. Therefore, the PAN-induced autophagy was essential for protecting podocyte against apoptosis in the first 24 h after treatment.

The disruption of podocyte cytoskeleton is commonly recognized as the key pathological changes in glomerular diseases [27]. It is also known that F-actin depolymerization occurs before the formation of apoptotic bodies and is an early step of apoptosis. In the present study, we showed that the percentage of podocytes with disrupted F-actin increased significantly when the PAN-induced autophagy was being inhibited. Therefore, it suggested that PAN-induced autophagy may play a critical role in maintaining the stability of podocyte cytoskeleton and prevented podocytes from undergoing apoptosis.

In this study, we have provided direct evidence that inhibition of PAN-induced autophagy increases podocyte apoptosis. The induced autophagy may be an early adaptive protective mechanism for PAN-treated podocytes. Since both apoptosis and autophagy were triggered by PAN, there may be common signals that involve similar downstream signaling molecules. The crosstalk between the cell death pathways may contribute to the functional link between autophagy and apoptosis. We reason that by modulating the molecules involved in podocyte autophagy, the effect of apoptosis will be ameliorated and this cytoprotective role of autophagy may be harnessed for the treatment of glomerular diseases in the future.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.bbrc.2013.12.015>.

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