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ABSTRACT

Membranous nephropathy (MN) is the most common cause of nephrotic syndrome in adults without diabetes. Primary MN has been associated with circulating antibodies against native podocyte antigens, including phospholipase A2 receptor (PLA2R); however, precision therapy targeting the signaling cascade of PLA2R activation is lacking. Both PLA2R and the mammalian target of rapamycin (mTOR) exist in podocytes, but the interplay between these two proteins and their roles in MN warrants further exploration. This study aimed to investigate the crosstalk between PLA2R activation and mTOR signaling in a human podocyte cell line. We demonstrated that podocyte apoptosis was induced by Group IB secretory phospholipase A2 (sPLA2IB) in a concentration- and time-dependent manner via upregulation of phosphoinositide 3-kinase (PI3K), protein kinase B (AKT), and mTOR, and inhibited by rapamycin or LY294002. Furthermore, aberrant activation of the PI3K/AKT/mTOR pathway triggers both extrinsic (caspase-8 and caspase-3) and intrinsic (Bcl-2-associated X protein [BAX], B-cell lymphoma 2 [BCL-2], cytochrome c, caspase-9, and caspase-3) apoptotic cascades in podocytes. The therapeutic implications of our findings are that strategies to reduce PLA2R activation and PI3K/AKT/mTOR pathway inhibition in PLA2R-activated podocytes help protect podocytes from apoptosis. The therapeutic potential of rapamycin shown in this study provides cellular evidence supporting the repurposing of rapamycin for MN treatment.

1. Introduction

Membranous nephropathy (MN) is a major cause of nephrotic syndrome in adults without diabetes [1]. MN is characterized (using light microscopy) by glomerular basement membrane thickening and formation of subepithelial "spikes" of basement membrane on the outer surface of the capillary wall. MN can be classified as primary (previously called idiopathic) or secondary, representing 70% and 30% of cases, respectively [2,3]. The secondary form is caused by several infections such as viral hepatitis B, C, or E, certain autoimmune diseases (systemic lupus erythematosus, vasculitis, rheumatoid arthritis, ankylosing spondylitis, systemic sclerosis, Hashimoto's thyroiditis, and Sjogren's syndrome), hyper-IgG4 syndrome, malignancies (lung, stomach, colon, prostate, and kidney), and medications (penicillamine, non-steroidal

anti-inflammatory drugs, adalimumab, and captopril) [4–10]. Primary MN is now recognized as a kidney-specific autoimmune glomerular disease. It has been reported to be associated with circulating antibodies against native podocyte antigens, including the neutral endopeptidase (NEP), the M-type receptor for secretory phospholipase A2 (PLA2R), and thrombospondin type-1 domain-containing 7A (THSD7A) [11–13], but the pathways from antigen-antibody binding to podocyte injury remain largely unknown. In an in vitro model, Tomas et al. incubated murine glomerular epithelial cells with rabbit anti-THSD7A antibodies and found that antigen-antibody binding led to cytoskeleton rearrangement and activation of focal adhesion signaling [14,15]. PLA2R has been shown to co-localize with S100A10 at the cell surface and in the extracellular vesicles of cultured immortalized human podocytes, but the binding of PLA2R-S100A10 does not cause podocyte injury [16].

Current treatment strategies are mainly based on the 2012 Kidney Disease Improving Global Outcomes (KDIGO) guidelines that recommend offering optimal renal protection including using renin-angiotensin-aldosterone blockades, and control of hypertension, dyslipidemia, excess weight, and other cardiovascular risk factors, while limiting the use of immunosuppressive drugs to patients at the highest risk of kidney failure [17]. Analysis of the long-term outcomes of this restrictive strategy showed very low complete remission rates of 5%, 24%, and 38% at 1, 3, and 5 years, respectively; the ten-year cumulative incidence rates were 3% for renal replacement therapy and 10% for death [18]. A recent consensus document on treating MN suggests testing PLA2R antibody levels for possible eligibility for immunosuppressive therapy, monitoring treatment, and follow-up [19]. This progress highlights the role of the PLA2R antibody in MN, but it remains important to delineate the signal cascades from antigen-antibody binding to podocyte injuries. Developing novel treatment strategies based on more clearly dissected pathomechanisms may lead to better treatment outcomes.

The mammalian target of the rapamycin (mTOR) pathway is a central regulator of mammalian metabolism and physiology. It is dysregulated in human diseases such as diabetes, obesity, depression, and certain cancers [20,21]. Rapamycin inhibits mTOR by associating with its intracellular receptor FKBP12 [22] and has been used successfully to maintain graft function in renal transplantation. It has also been reported to ameliorate proteinuria-associated tubulointerstitial inflammation and fibrosis in experimental MN [23], but its role in podocytes remains unclear. mTOR is a downstream target of the phosphoinositol 3 kinase (PI3K)/protein kinase B (AKT) pathway. Activation of the PI3K/AKT/mTOR pathway has been reported in lupus nephritis [24], polycystic kidney disease [25] and diabetic nephropathy [26]. Analysis of homogenized kidney tissue from patients with lupus nephritis revealed upregulation of PI3K, AKT, and mTOR [24]. In polycystic kidney disease, over-activation of the PI3K/AKT/mTOR pathway in tubule epithelial cells is responsible for cyst formation [25]. Lu et al. demonstrated that high glucose levels induced the upregulation of AKT and mTOR in rat tubule epithelial cells [26]. To date, information on the roles of the PI3K/AKT/mTOR pathway in MN and podocytes has been very limited. In recent years, podocytes have emerged as therapeutic targets for MN. Interestingly and notably, PLA2R and mTOR both exist in podocytes [27] but the interplay between these two is yet to be investigated. An important paper highlighted that PLA2R activation stimulates podocyte apoptosis by activating extracellular signal-regulated kinase (ERK)1/2 signaling [28], reported to crosstalk with mTOR pathways [29]. Therefore, our study aimed to investigate the crosstalk between PLA2R activation and PI3K/AKT/mTOR signaling in podocytes.

2. Materials and methods

2.1. Cell culture and reagents

The human podocyte cell line was a kind gift from Dr. Hsiang-Hao Hsu and was cultured as previously described [30,31]. Briefly, cells were cultured in RPMI-1640 growth medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), $1\times$ penicillin-streptomycin (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1 mM L-glutamine, and $1\times$ insulin, transferrin, and selenium (ITS; Invitrogen, Grand Island, NY, USA) at a permissive temperature (33 °C). After reaching approximately 80% confluence, the cells were transferred to a medium without ITS at 37 °C in a humidified atmosphere with 5% CO_2 for 14 days. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

2.2. Cell treatment

To activate PLA2R in podocytes, cells were starved with 0.5% FBS in a complete RPMI-1640 medium for 24 h. Cells were then treated with Group IB secretory phospholipase A2 (sPLA2IB) in a medium containing 3% FBS at various time points. For the time-course experiment, podocytes were treated with 10^{-6} M sPLA2IB at 0, 0.5, 1, 2, and 6 h. For the concentration-effect experiment, podocytes were incubated in media containing various concentrations of sPLA2IB (0, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M) for 2 h. In the inhibition experiments, cells were seeded at a density of 1×10^6 cells in a 10-cm dish. After overnight incubation, cells were pre-treated with or without PI3K/AKT inhibitor LY294002 and/or rapamycin for 30 min, and then sPLA2IB was added to a final concentration of 10^{-6} M and incubated for 2 h.

2.3. Cellular apoptotic assay

In the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, podocytes were seeded in 24-well plates with glass coverslip bottoms at a density of 3×10^4 cells/well in 0.1 mg/mL type I collagen and allowed to differentiate at 37 °C for 14 days. Before use in experiments, cells were starved with 0.5% FBS in a complete medium for 24 h. Cells were then treated with sPLA2IB at defined concentrations in a complete medium containing 3% FBS for 6 h. Deoxyribonuclease (DNase) treatment was used as the positive control. Cells were then fixed in 4% paraformaldehyde and treated with a blocking solution (10% FBS in phosphate-buffered saline [PBS]) for 30 min at 25 °C. Cells were permeabilized with 0.1% (vol/vol) Triton X-100 in PBS and then incubated with the TUNEL reaction mixture (Roche, Germany) for 1 h at 37 °C. TUNEL-positive apoptotic cells were detected using localized fluorescein isothiocyanate (FITC) fluorescence with 4,6-diamidino-2-phenylindole (DAPI) as a counterstain. After treatment, the cells were observed under a fluorescence microscope (Olympus BX51; Olympus America Inc., Melville, NY, USA). The fluorescence intensity of the cells was analyzed using ImageJ software and normalized to the cell numbers.

2.4. Western blot analysis

Western blot analysis was performed as described previously [32]. Briefly, cell pellets were homogenized in RIPA buffer and centrifuged at 13,000 rpm for 30 min at 4 °C. Proteins (40 μg) from the supernatant of each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene

difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% non-fat dried milk in Tris-buffered saline with Tween 20 (TBST) buffer for 1 h at room temperature and incubated overnight with the primary antibody at 4 °C. The blots were probed with primary antibodies, namely, anti-p-AKT (4060, Cell Signaling), anti-AKT (4691, Cell Signaling), anti-p-mTOR (5536, Cell Signaling), anti-mTOR (2983, Cell Signaling), anti-p-PI3K p85 (GTX132597, GeneTex), anti-PI3K p85 (4292, Cell Signaling), anti-PI3K p100 (sc-134986, Santa Cruz), anti-β-actin (MA5-15739, Invitrogen), anti-BH3-interacting domain death agonist (BID)/t-BID (2002, Cell Signaling), anti-BAX (sc-493, Santa Cruz), anti-BCL-2 (3498, Cell Signaling), anti-cytochrome C (sc-13156, Santa Cruz), anti-cleaved-caspase-3 (9661, Cell Signaling), anti-cleaved-caspase-8 (9496, Cell Signaling), and anti-cleaved-caspase-9 (7237, Cell Signaling), followed by incubation with secondary antibodies at an appropriate dilution. The blots were visualized with SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Inc., Foster City, CA, USA).

2.5. Statistical analysis

All experiments were independently repeated at least thrice. Quantitative data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using GraphPad Prism software (version 7.0; GraphPad Software Inc., San Diego, CA, USA). Pairwise comparisons were performed using t-tests. Statistical significance was set at $P < 0.05$. The level of significance is indicated as follows: *p < 0.05, **p < 0.01, and ***p < 0.001.

3. Results

3.1. sPLA2IB induces podocyte apoptosis in a concentration- and time-dependent manner

Because there are no commercially available PLA2R antibodies that mimic circulating autoantibodies identified in MN patients, Pan et al. used sPLA2IB to induce apoptosis of cultured human podocytes via the M-type phospholipase A2 receptor [28], known to be the target antigen in primary MN [11]. This model offers an excellent platform for investigating mechanisms at the cellular level. In this study, we used it and performed a series of time-course and concentration-response experiments, testing sPLA2IB at concentrations ranging from 10^{-8} to 10^{-5} M and treatment times of 0.5, 1, 2, and 6 h. We found that sPLA2IB induced podocyte apoptosis at a concentration of 10^{-7} M and the optimal concentration for inducing podocyte apoptosis was 10^{-6} M. Furthermore, we found that significant podocyte apoptosis occurred when incubated with sPLA2IB for 1 h. Two-hour incubation with sPLA2IB resulted in substantial podocyte apoptosis. Fig. 1 illustrates that sPLA2IB induced podocyte apoptosis in a concentration- and time-dependent manner.

3.2. sPLA2IB activates PI3K/AKT/mTOR pathways

Based on the results shown on Fig. 1, the optimal concentration (10^{-6} M) and inducing time (2 h) of sPLA2IB treatment were determined for the subsequent pathway investigation experiments. Using western blot analysis (Fig. 2), we demonstrated that sPLA2IB upregulated PI3K, AKT, and mTOR in podocytes. Compared to the control group, sPLA2IB induced significant upregulation of phospho-PI3K (p-PI3K) and PI3K, irrespective of detection from regulatory (p85) subunits (Fig. 2b) or

catalytic (p100) subunits (Fig. 2c). Fig. 2d and e show the sPLA2IB-induced upregulation of phospho-AKT (p-AKT) and phospho-mTOR (p-mTOR), respectively.

3.3. Inhibition of PI3K/AKT/mTOR attenuates sPLA2IB-induced podocyte apoptosis

After demonstrating that sPLA2IB activates the PI3K/AKT/mTOR pathway (Fig. 2) and causes podocyte apoptosis, we tested whether inhibition of this pathway reduced sPLA2IB-induced apoptosis in podocytes. We employed LY294002 that inhibits the PI3K/AKT pathway and the phosphorylation of AKT, and also rapamycin, an inhibitor of the mammalian target of rapamycin complex 1 (mTORC1), at concentrations of either LY294002 or rapamycin of 5, 10, and 20 μM. Fig. 3 shows that pretreatment with LY294002 or rapamycin dose-dependently attenuated sPLA2IB-induced podocyte apoptosis. The best therapeutic effects were observed in a single blockade with 20 μM LY294002 or rapamycin.

As shown in Fig. 3c, a single blockade with either LY294002 or rapamycin at a concentration of 20 μM substantially rescued podocytes from PLA2R activation-mediated apoptosis. Interestingly, we found that dual blockade of the PI3K/AKT/mTOR pathway by pretreatment with both inhibitors appeared to reduce apoptosis back to the control baseline level (Fig. 4).

3.4. Aberrantly activated PI3K/AKT/mTOR pathway activates both extrinsic and intrinsic apoptotic pathways in podocytes

To further delineate the mechanisms by which inhibition of the PI3K/AKT/mTOR pathway attenuated apoptosis in PLA2R-activated podocytes, we examined the expression patterns of both extrinsic and intrinsic apoptotic pathways in these cells. The concentration of rapamycin and LY294002 were both 20 μM. The pretreatment time was 30 min. It is known that cellular apoptosis may occur via the extrinsic or intrinsic pathway. The extrinsic pathway is triggered by perturbations of the extracellular microenvironment detected by plasma membrane receptors. This process will then be propagated by caspase-8 and precipitated by executioner caspases, mainly caspase-3. The intrinsic pathway is initiated by internal cellular stress These internal stimuli induce the activation of BH3 (BCL-2 homology 3) proteins including BID. BH3 proteins interact and activate BH3 pro-survival or pro-apoptotic proteins, which may determine the interruption or continuation of apoptosis. When the pro-survival proteins, e.g., BCL-2 prevail, cell death mechanisms will be interrupted at some given point. However, activation of the pro-apoptotic protein BAX will result in mitochondrial outer membrane permeabilization, release of cytochrome-c and activation of caspase-9 and then caspase-3 [33,34]. We hypothesized that the binding of sPLA2IB to M-type PL2AR on the podocytes may activates the extrinsic apoptotic pathway and may cause intracellular stress to initiate the intrinsic apoptotic pathway. In our studies, when sPLA2IB (10^{-6} M) bound to the receptors on the podocytes, we identified the activated extrinsic apoptotic pathway evidenced by the upregulation of caspase-8 and caspase-3. Intriguingly, in our sPLA2IB-treated podocytes, we also discovered the downregulation of pro-survival protein BCL-2 and upregulation of pro-apoptotic protein BAX, cytochrome c, caspase-9, and caspase-3. This expression pattern suggests activation of the intrinsic apoptotic pathway in sPLA2IB-treated podocytes. Notably, the altered expression of extrinsic and intrinsic apoptotic pathway
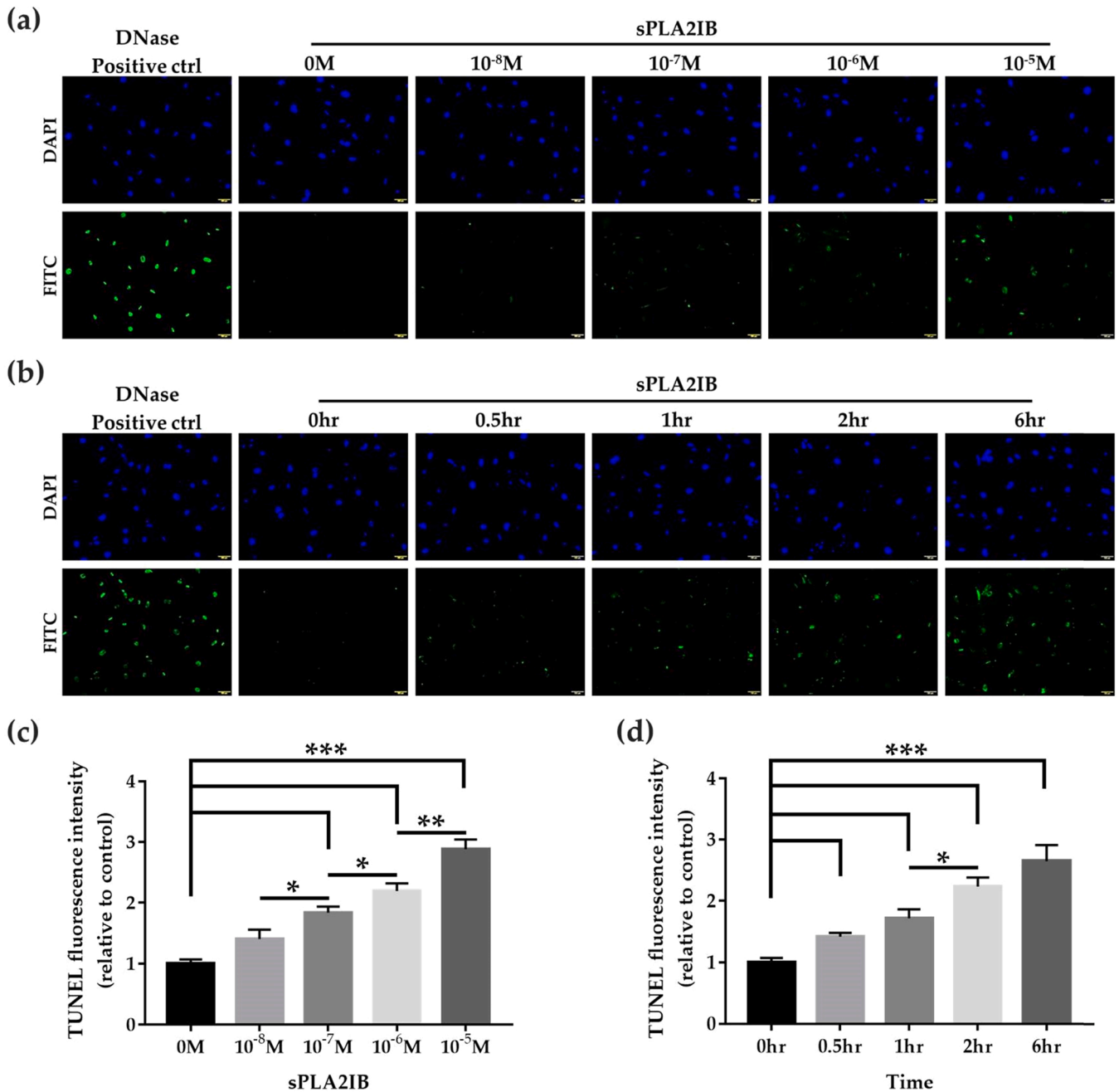
Fig. 1. sPLA2IB induces podocyte apoptosis in a concentration- and time-dependent manner. The ligand of PLA2R, sPLA2IB, was used to induce podocyte apoptosis; TUNEL-positive apoptotic cells were detected by localized FITC-fluorescence. DAPI was used as a counterstain. DNase was used as a positive control for the assay. (a) and (c) show that sPLA2IB induces podocyte apoptosis in a concentration-dependent manner. (b) and (d) illustrate that sPLA2IB (10^{-6} M) induces podocyte apoptosis in a time-dependent manner. Pair-wise comparisons were performed using the *t*-test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. The scale bar for all micrographs is 100 μm.

molecules was normalized when the aberrantly activated PI3K/AKT/mTOR pathway was inhibited by LY294002, rapamycin, and the combination of both (Fig. 5). However, we were unable to determine the altered expression of BID/truncated t-BID in sPLA2IB-treated podocytes.

4. Discussion

Up to 30% of MN patients with persistent nephrotic syndrome progress toward end-stage renal disease over 10 years [35,36]. Although primary MN is associated with circulating antibodies that bind to podocyte antigens, specific treatments targeting the cascades of
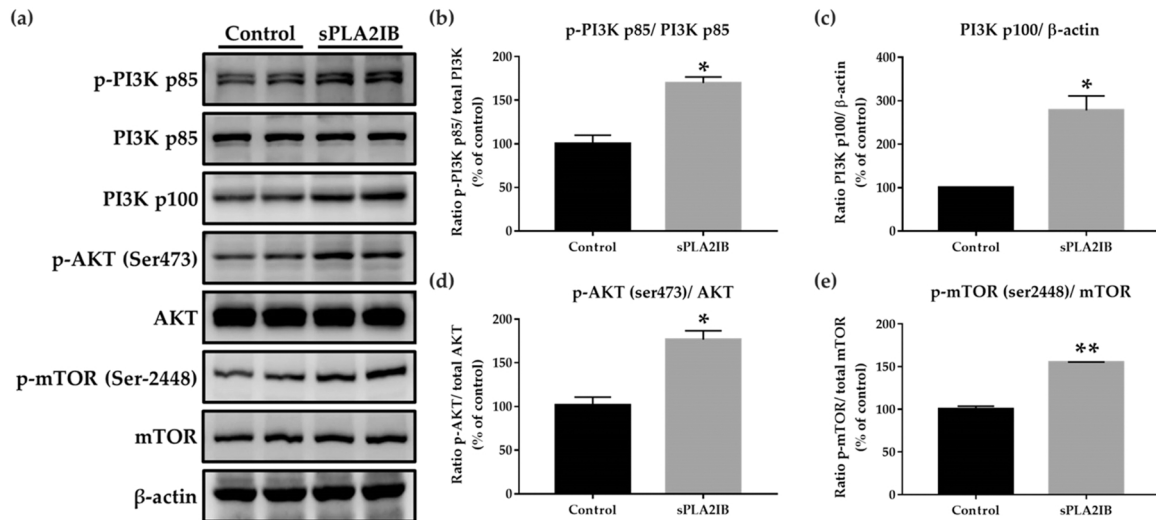
Fig. 2. sPLA2IB activates PI3K/AKT/mTOR pathways. (a) Representative western blot analysis comparing the expression of p-PI3K p85, PI3K p85, PI3K p100, p-AKT, AKT, p-mTOR, and mTOR in control and sPLA2IB-treated podocytes. β-actin was used for the normalization of protein loading. sPLA2IB was used at the concentration of 10^{-6} M and the inducing time was 2 h. The same cell treatments were repeated for several times. To demonstrate the consistent experimental results, protein lystaes obtained from replicates of these experiments were subjected to western blotting and are illustrated on the same blot. The left two lanes are for controls and the right two lanes are for sPLA2IB-treated cells. (b to e) Densitometric comparison of the average expression of p-PI3K, PI3K, p-AKT, and p-mTOR. Values are presented as mean ± SEM. *$p < 0.05$; **$p < 0.01$.

antigen-antibody activation are currently unavailable. A better understanding of the molecular mechanisms underlying the pathway from antigen-antibody binding to podocyte injury could be a solution to this bottleneck. Podocytes have recently been recognized as novel therapeutic targets for various known glomerulopathies [37–40]. Our study confirmed that PLA2R activation induces podocyte apoptosis. Using different apoptotic assays on the same PLA2R activation podocyte model, we confirmed Pan's important finding that sPLA2IB activated PLA2R and caused podocyte apoptosis in a concentration- and time-dependent manner [28]. The presence of more PLA2R ligands and longer duration of PLA2R activation lead to increased podocyte apoptosis. The therapeutic implications of these laboratory findings in the clinical scenario are that adequate suppression of the production of PLA2R ligands and early removal of the produced ligands help protect podocytes from apoptosis. In line with the therapeutic implications we proposed, immunoadsorption has been successfully used to remove THSD7A antibodies in two patients with primary MN [41]. The removal of PLA2R autoantibodies by immunoadsorption is currently being examined in clinical trials [42].

A more important finding of our study is the molecular pathways by which PLA2R activation-mediated podocyte apoptosis occurred. We observed that once PLA2R was activated by sPLA2IB, the PI3K/AKT/mTOR pathway, seldom investigated in MN, was activated. Hall et al. reported that hyperactivation of the PI3K/AKT/mTOR signaling axis and activation of mTOR-driven endoplasmic reticulum stress in mutated anillin (ANLN)$_{R431C}$-expressing podocytes accounted for the pathogenesis of the familial form of focal segmental glomerulosclerosis [43]. Zheng et al. showed that p66Shc, a protein adapter that regulates the production of reactive oxygen species, inhibits podocyte autophagy and induces apoptosis through the Notch-PTEN-PI3K/AKT/mTOR signaling pathway in a high-glucose environment [44]. Using the passive Heymann nephritis rat model, Tu et al. demonstrated that curcumin could attenuate the development of MN by reducing renal oxidative stress and inducing autophagy through the PI3K/AKT/mTOR pathway [45]. Notably, however, this experimental MN rat model did not involve PLA2R activation that is the key mechanism of primary MN. Using human podocytes expressing M-type PLA2R, we offer cellular evidence that highlights the crucial roles of the aberrantly activated PI3K/AKT/mTOR pathway in PLA2R activation-mediated podocyte apoptosis.

We also examined the therapeutic potential of LY294002 and rapamycin on PLA2R activation-mediated podocyte apoptosis. In our study, LY294002 and rapamycin showed similar therapeutic effects (Fig. 3), and dual blockade of this pathway seemed to offer podocytes better protection from apoptosis (Fig. 4). LY294002 is the first synthetic molecule known to inhibit PI3K [46] and has been widely used in various experimental models. However, there are many downstream targets of the PI3K/AKT pathway that govern a wide variety of biological events. The use of PI3K inhibitors in MNs may cause unexpected adverse effects. Many clinical trials using PI3K inhibitors to treat a variety of cancers are ongoing [47]. The results from these clinical trials suggest that the side effects of most studied PI3K inhibitors outweigh their efficacy. In addition, the PI3K/AKT pathway plays a complex role in podocyte physiology. LY294002 may induce podocyte apoptosis in the presence of high glucose levels [48]. In contrast to LY294002, rapamycin has been commonly and safely used for solid organ transplantation [49–52]. Our study has demonstrated its efficacy in protecting PLA2R-activated podocytes against apoptosis by attenuating the aberrantly activated PI3K/AKT/mTOR pathway. This finding warrants further research into the therapeutic efficacy of rapamycin in patients with primary MN.

In addition to identifying the aberrantly activated pathway, we have demonstrated that sPLA2IB activated both the extrinsic and intrinsic apoptotic pathways (Fig. 5). The extrinsic apoptotic pathway refers to the activation of caspases that are initiated at the plasma membrane upon ligand-receptor binding, whereas the intrinsic apoptotic pathway refers to the activation of caspases that are initiated in the mitochondria [53,54]. It was not surprising that our study showed that sPLA2IB activated caspases 8 and 3 via the extrinsic pathway. In most cases, when caspase-8 is activated, the cytosolic BID is cleaved into truncated t-BID that translocates into the mitochondria [55]. However, we were unable to find increased expression of t-BID in sPLA2IB-treated podocytes. An increasing number of BID-independent mitochondrial activation mechanisms for caspase-8 and BAX have been identified. These reported mechanisms include cardiolipin, sequestration of caspase-8 on the outer mitochondrial surface, nucleo-cytoplasmic translocation of Fas-associated death domain-like IL-1β–converting enzyme (FLI-CE)-associated proteins, and caspase activation and recruitment domain (CARD)-containing proteins [56–60]. Further research is required to determine the BID-independent mitochondrial activation mechanism
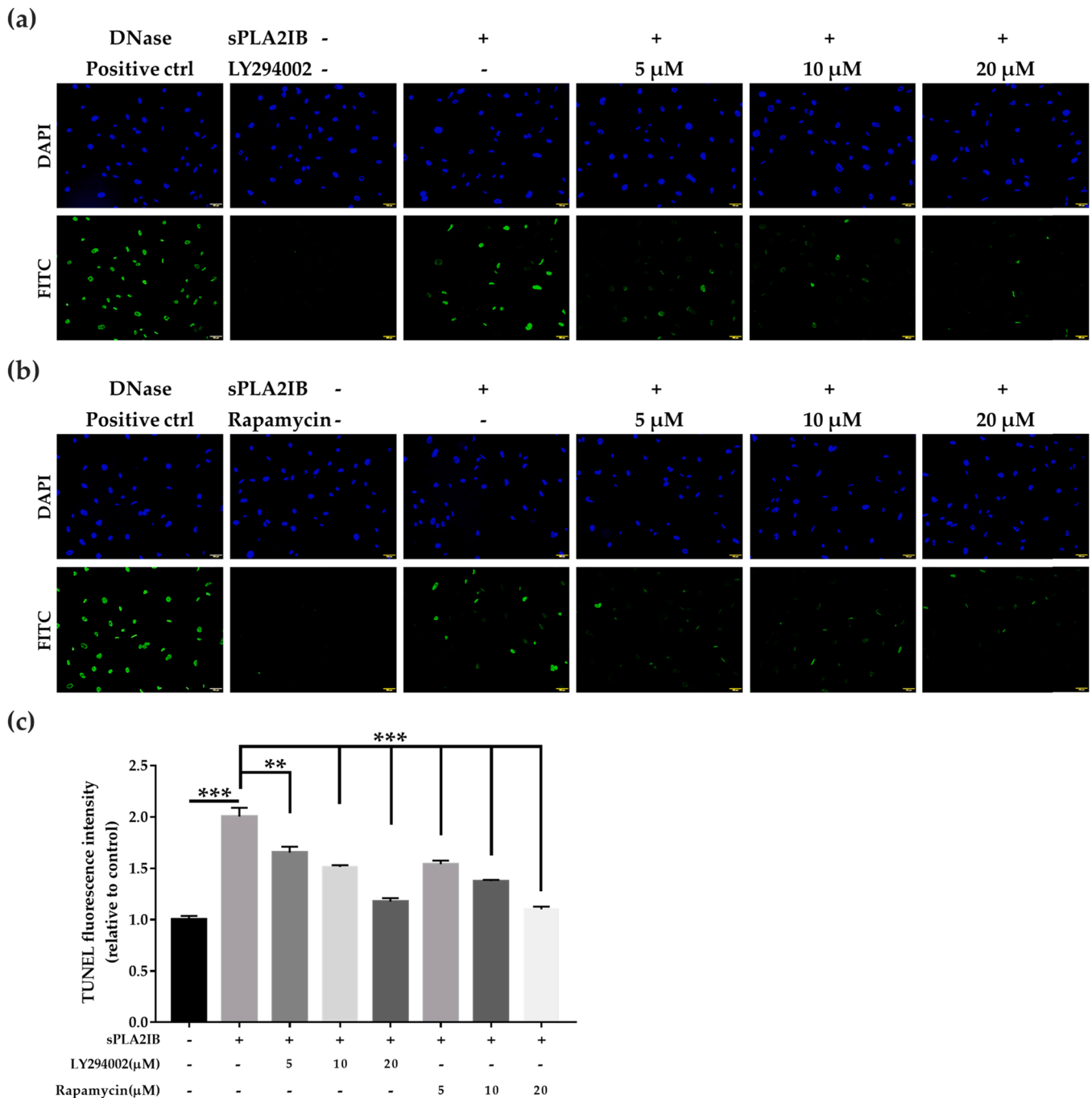
Fig. 3. Inhibition of PI3K/AKT/mTOR attenuates sPLA2IB-induced podocyte apoptosis. The micrographs of TUNEL assays show that the sPLA2IB-induced podocyte apoptosis was ameliorated by the PI3K/AKT inhibitor, LY294002 (a), and by the mTOR inhibitor, rapamycin (b). Panel (c) illustrates the concentration-dependent effects of these two inhibitors. **p < 0.01; ***p < 0.001. The scale bar for all micrographs is 100 μm.

responsible for the observed activation of the intrinsic apoptotic pathway in sPLA2IB-treated podocytes. Nevertheless, a notable finding of our study is that LY294002, rapamycin, or a combination of both normalizes the altered expression of both extrinsic and intrinsic apoptotic pathways in PLA2R-activated podocytes (Fig. 5). Taking together the main findings of our study, we propose a mechanism (Fig. 6) for the therapeutic potential of rapamycin in PLA2R activation-mediated podocyte apoptosis.

Currently, the immunosuppressant protocols for treating patients with primary MN only include steroids, cyclophosphamide, calcineurin inhibitors, and rituximab [61]. The first choice recommended by KDIGO is a combination of steroids and cyclophosphamide based on the

Ponticelli regimen. The relapse rate of this regimen is 20–30% [17,62]. The second choice recommended by KDIGO is the combination of steroids and calcineurin inhibitors, but the relapse rate of this regimen is 40–50% [17,63]. As research evidence on PLA2R and its autoantibody in MN has accumulated rapidly in recent years, an antibody-guided diagnosis and treatment algorithm for primary MN has been proposed [61]. Rituximab, capable of reducing the production of PLA2R autoantibodies, is superior to cyclosporine in maintaining proteinuria remission for up to 24 months in primary MN [64]. This promising immunosuppressant is likely to be suggested as the first-line treatment option for primary MN [65]. However, the ideal dosing of rituximab and its combination with other immunosuppressants needs to be carefully assessed.
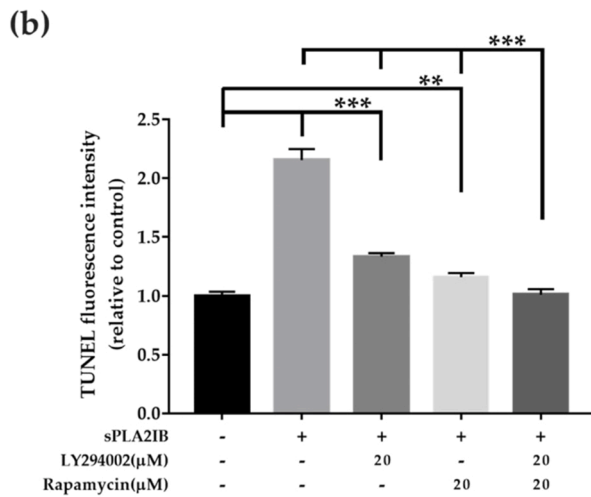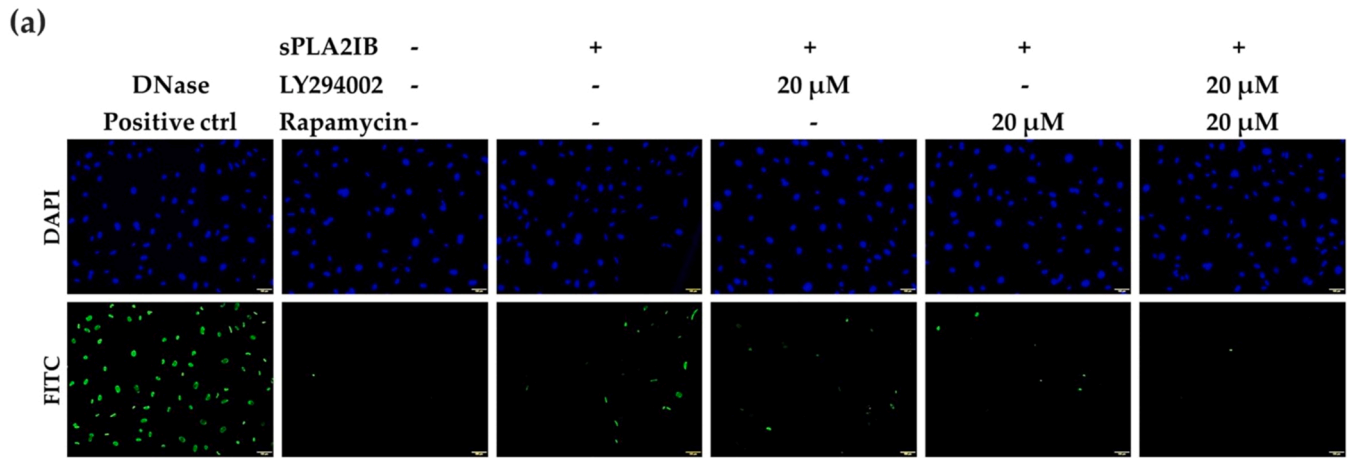
Fig. 4. Dual blockade on the PI3K/AKT/mTOR pathways rescues podocytes from PLA2R activation-mediated apoptosis. The micrographs of TUNEL assay (a) show that the sPLA2IB-induced podocyte apoptosis was ameliorated by LY294002, rapamycin, or a combination of these two inhibitors. Panel (b) depicts that the comparable TUNEL fluorescence intensity between control and the simultaneous use of LY294002 and rapamycin in podocytes. **p < 0.01; ***p < 0.001. The scale bar for all micrographs is 100 μm.
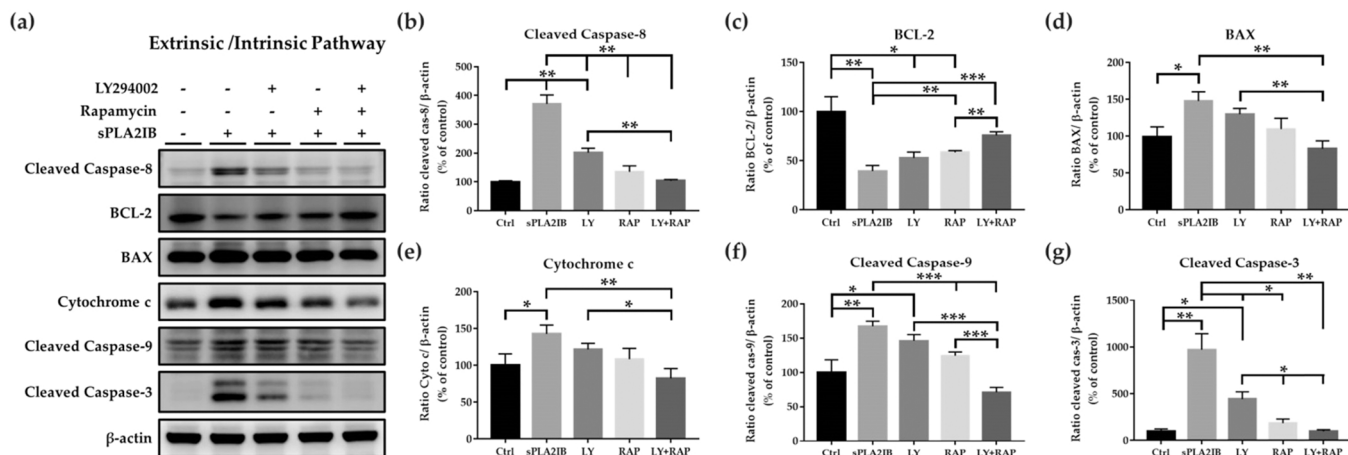
Fig. 5. Inhibition of the aberrantly activated PI3K/AKT/mTOR pathways normalizes both the altered extrinsic and intrinsic apoptotic pathways in PLA2R-activated podocytes. The concentration of rapamycin and LY294002 were both 20 μM. The pretreatment time was 30 min. Representative western blot analysis (a) shows that sPLA2IB upregulated caspase-8 and caspase-3. It also led to upregulation of BAX, downregulation of BCL-2, and upregulation of cytochrome c, caspase-9, and caspase-3. These altered expression patterns were normalized by LY294002, rapamycin, or both. β-actin was used for the normalization of protein loading. Densitometric comparisons (b to g) illustrate the therapeutic effects of inhibitors on individual molecules on the extrinsic and intrinsic apoptotic pathways. Values are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001. LY, LY294002; RAP, rapamycin.

Fig. 6. The proposed mechanism for the therapeutic potential of rapamycin on PLA2R activation-mediated podocyte apoptosis. When sPLA2IB activates the PLA2 receptor, the downstream PI3K/AKT/mTOR will be aberrantly activated. This will also cause activation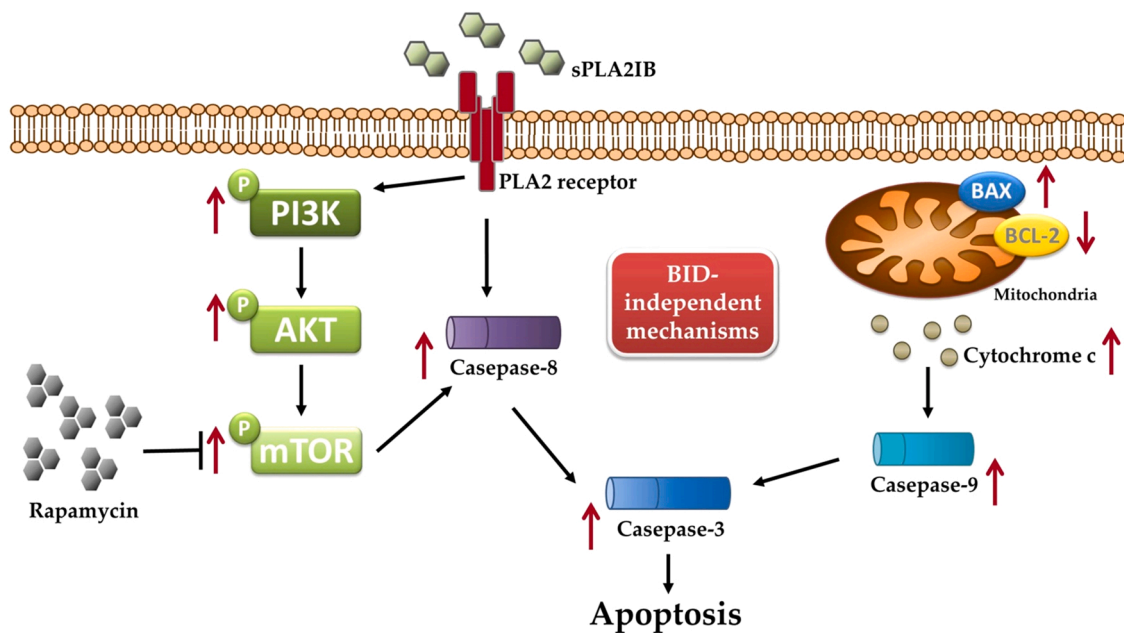 of both extrinsic and intrinsic apoptotic pathways via a BID-independent mechanism. By inhibiting mTOR, rapamycin was able to halt this pathogenic process and eventually attenuated the detrimental PLA2R activation, the key mechanism of primary MN.

The newly released clinical trial, the STARMEN trial, indicates that alternating treatment with corticosteroids and cyclophosphamide is superior to sequential treatment with tacrolimus and rituximab in primary MN [66]. Complementary to these updated findings, our study uncovered the signaling cascades from PLA2R activation to podocyte injury and characterized the fundamental role of rapamycin in PLA2R activation-mediated podocyte injury. Our findings open a new window for a well-known immunosuppressant, rapamycin, for primary MN treatment. Our work is performed in the cultured human podocytes. Although this culture system is currently an ideal cellular model for studying primary MN, result obtained based on using an in vitro model inevitably has its limitation. Testing the therapeutic effects of rapamycin in animals is required in the future when the appropriate animal model is developed. Solid preclinical data will then facilitate the clinical trial of rapamycin on the treatment of primary MN.

5. Conclusions

Our study has demonstrated that the aberrantly activated PI3K/AKT/mTOR pathway is responsible for apoptosis in PLA2R-activated podocytes. The therapeutic potential of rapamycin shown in this study provides cellular evidence supporting the repurposing of rapamycin for primary MN treatment.

CRediT authorship contribution statement

Terry Ting-Yu Chiou: Methodology, Validation, Investigation, Data curation, Writing – original draft, Visualization, Project administration, Funding acquisition. **You-Ying Chau**: Validation, Writing – original draft. **Jin-Bor Chen**: Conceptualization, Writing – review & editing, Supervision. **Hsiang-Hao Hsu**: Resources. **Shao-Pei Hung**: Software, Formal analysis, Data curation. **Wen-Chin Lee**: Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Conflict of interest statement

The authors declare no conflict of interest. The funders had no role in the study design, collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2021.112349.

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