RESEARCH ARTICLE

Protosappanin-A and oleanolic acid protect injured podocytes from apoptosis through inhibition of AKT-mTOR signaling

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Abstract

Protosappanin-A (PrA) and oleanolic acid (OA), which are important effective ingredients isolated from Caesalpinia sappan L., exhibit therapeutic potential in multiple diseases. This study focused on exploring the mechanisms of PrA and OA function in podocyte injury. An in vitro model of podocyte injury was induced by the sC5b-9 complex and assays such as cell viability, apoptosis, immunofluorescence, quantitative real-time polymerase chain reaction, and western blot were performed to further investigate the effects and mechanisms of PrA and OA in podocyte injury. The models of podocyte injury were verified to be successful as seen through significantly decreased levels of nephrin, podocin, and CD2AP and increased level of desmin. The sC5b-9-induced podocyte apoptosis was inhibited in injured podocytes treated with PrA and OA, accompanied by increased protein levels of nephrin, podocin, and CD2AP and increased level of desmin. The p-AKT/p-mTOR levels were also reduced by treatment of PrA and OA while AKT/mTOR was unaltered. Further, the effects of PrA and OA on injured podocytes were similar to that of LY294002 (a PI3K-AKT inhibitor). PrA and OA were also seen to inhibit podocyte apoptosis and p-AKT/p-mTOR levels induced by IGF-1 (a PI3K-AKT activator). Our data demonstrate that PrA and OA can protect podocytes from injury or apoptosis, which may occur through inhibition of the abnormal activation of AKT-mTOR signaling.

Keywords: AKT-mTOR pathway; apoptosis/proliferation; LY294002/IGF-1; podocyte injury; protosappanin-A/oleanolic acid

Introduction

Podocytes, highly differentiated glomerular visceral epithelial cells, are important in glomerular filtration barrier (GFB) function. Generally, the major processes and foot processes (FPs) of podocytes are interlinked by slit diaphragm (SD) molecules (Shankland, 1999; Guan et al., 2004). Podocyte injury and loss are an initiating cause of numerous renal diseases. Loss or injury of podocytes leads to proteinuria, which is the major risk factor for the progression of end-stage renal disease (Hemmelgarn et al., 2010). Therefore, injury to podocytes is a central event in the development of multiple diseases including membranous nephropathy (MN)

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Abbreviations: CD2AP, CD2-associated protein; DN, diabetic nephropathy; FITC, fluorescein isothiocyanate; FPs, foot processes; GFB, glomerular filtration barrier; IF, intermediate filament; MN, membranous nephropathy; NHS, normal human serum; OA, oleanolic acid; PI, propidium iodide; PrA, protosappanin-A; SD, slit diaphragms

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and diabetic nephropathy (DN) (Durvasula and Shankland, 2006; Shankland, 2006). Currently, several drugs such as rapamycin and triptolide are reported to have therapeutic potential for podocyte injury. However, the limitations of currently available drugs highlight the need for further studies in podocyte injury.

Research has shown that cytoskeletal structure and intercellular junctions are disrupted when podocytes are injured, which is accompanied by decreased levels of nephrin, podocin and CD2-associated protein (CD2AP), and increased level of desmin (Machado et al., 2012). Nephrin usually functions as an intracellular signaling scaffold in podocytes. Podocin, is a supporting protein that maintains SD integrity and podocyte function (Kang et al., 2010), and is capable of modulating nephrin-signaling activity by binding to the cytoplasmic tail of nephrin (Boute et al., 2000; Huber et al., 2001; Schwarz et al., 2001; Roselli et al., 2002). CD2AP participates in congenital nephritic syndrome through its C-terminus interaction with podocin and nephrin (Li et al., 2000; Shih et al., 2001). Finally, CD2AP is also involved with various signaling molecules and assembly of cytoskeleton through its SH3 region (Patrakka et al., 2000; Kim et al., 2003; Wolf and Stahl, 2003). Podocytes were also found to respond to injury by altering their intermediate filament (IF) proteins such as desmin, which is enhanced in podocytes of glomerulosclerosis and DN (Floge et al., 1997; Hoshi et al., 2002). Furthermore, signaling pathways including phosphoinositide 3-kinase (PI3K)-AKT, ROS-NF-xB, and Wnt/β-catenin are also implicated in podocyte injury (Wang et al., 2011; Wei et al., 2015). The PI3K-AKT pathway is well-known to be the major signaling cascade regulating glucose metabolism, and activation of AKT regulates various processes involved in cancer, such as cell-cycle progression and growth (Hong et al., 2016). It is suggested that nephrin and CD2AP, together with podocin, interacts with PI3K, and further stimulates PI3K-dependent, serine-threonine kinase AKT signaling (Huber et al., 2003). Activation of PI3K-AKT signaling is also reported to participate in podocyte injury and apoptosis (Chuang and He, 2009; Zhang et al., 2016). Mammalian target-of-rapamycin (mTOR) is a key protein kinase downstream of PI3K-AKT. Through inhibition of mTOR-ULK1 signaling, rapamycin decreases podocyte injury (Lingling, 2013; Wu et al., 2013). Thus, the PI3K-AKT-mTOR signaling pathway is essential for the regulation of podocyte injury. *Caesalpinia sappan* L., a Chinese herb, belongs to the leguminous plant family. Studies have reported that *C. sappan* L. exhibits various biological activities and therapeutic potential for multiple diseases including diabetic complications, burning sensations, and dysentery (Baek et al., 2000; Badami et al., 2003; Kim et al., 2005). Protosappanin-A (PrA) is an important active ingredient isolated from an ethanol extract of *C. sappan* L. (Wu et al., 2008), and oleanolic acid (OA) is a pentacyclic triterpenoid compound that exists widely in food, medicinal herbs, and other plants (Liu, 1995; Pullier and Goossens, 2012). PrA has an immunosuppressive activity and is essential in the success and survival of transplanted hearts (Wu et al., 2008). In addition, PrA has been proven to inhibit CD4+/CD8+ ratios of peripheral T cells as well as expression of perforin and granzyme B (Wu et al., 2008, 2010). A study has also reported that OA could prevent the progression of DN induced by streptozotocin (Dubey et al., 2013). However, the effects of PrA and OA on podocyte injury remain unclear.

Previous studies have stated the suitable concentrations of Cs5b-6 in vitro model. Assembly and regulation of the membrane attack complex (MAC) were based on structures of Cs5b-6 and sCs5b-9, and in vitro concentrations relevance for the human in vivo situation has been suggested (Aleshin et al., 2012; Hadders et al., 2012). Cs5b-6 complex can combine with C7, C8, and multiple C9 molecules to construct the sublytic Cs5b-9 (sCs5b-9) complex. Chen et al. (2010) used 0.8 µg/mL of Cs5b-6, maintaining fixed concentrations of C7, C8, and C9 (10 µg/mL) to assemble sCs5b-9, which established injured podocyte model. sCs5b-9, a macromolecule complex, also known as MAC, can be inserted into the phospholipids bilayer membrane, forming a channel or leaking patch, thus, making the non-nuclear cells lethal, but nucleated cells such as podocytes will cause no fatal damage (Ma et al., 2013). Here, Cs5b-6 complex and normal human serum (NHS) were used to generate sCs5b-9 complex to induce podocyte injury in vitro.

In this study, after podocytes were injured by the sCs5b-9 complex, podocyte apoptosis was increased accompanied by decreased levels of nephrin, podocin, and CD2AP and increased level of desmin. However, treatment of PrA and OA protected podocytes from sCs5b-9-induced apoptosis, and treatment with both drugs inhibited apoptosis to a significantly greater degree than treatment with any one drug alone. Furthermore, high levels of p-AKT and p-mTOR induced by Cs5b-9 were significantly reduced by PrA and OA treatment, and were accompanied by altered expression of several associated genes. These data demonstrated the protective effect of PrA and OA on sCs5b-9 induced-apoptosis, which may be via regulation of AKT-mTOR signaling.

**Materials and methods**

**Cell culture**

MPC5, a mouse podocyte cell line derived from kidneys, was obtained from the Biotechnology Co., Ltd. Shanghai Enzyme Research (Shanghai, China) and cultured in a 5% CO2 incubator (Thermo Forma 3111; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 33°C with RPMI-1640 (SH30809.01B; Hyclone) medium containing 10% fetal bovine serum (16000-044; Gibco), 100 U/mL penicillin and...
100 mg/mL streptomycin (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and 10 μM IFN-γ (315-05; Peprotech). A period of time later, cells were passaged and cultured with RPMI-1640 medium containing 10% fetal bovine serum and 1% double-antibiotic without interferon γ in a 5% CO₂ incubator at 37°C for 10–14 days to induce cell differentiation. The medium was replaced daily or every other day according to the growth of the cells.

Preparation of rabbit anti-mouse podocyte polyclonal antibody

Nearly, 2 × 10⁷ mouse podocytes were collected, resuspended in 0.5 mL phosphate-buffered saline (PBS), and sonicated in an ice bath (200w, ultrasound 3 s, and interval 10 s, repeated 30 times). The cell lysate was mixed with Freund’s complete adjuvant (FCA) in a 1:1 ratio by volume and emulsified, followed by subcutaneous injections on the skin around the shoulders of the rabbit with 0.1 mL per point. Two weeks later, immunity was strengthened, and the same cell lysate was mixed with Freund’s incomplete adjuvant (FIA) in a 1:1 ratio by volume and emulsified, followed by injection subcutaneously with 0.1 mL per point. The immunity was strengthened again every 2 weeks for three more times. One week after the last immunization, blood was collected from the ear artery, and the isolated serum was rabbit anti-mouse podocyte polyclonal antibody.

In vitro model of podocyte injury induced by the C5b-9 complex

Models of podocyte injury were established using the C5b-6 complex and NHS as a complement source. The venous blood collected from the healthy volunteers were incubated at room temperature for 1 h and then centrifuged at 3,000 rpm, 4°C for 15 min to obtain the supernatant. After 24 h treatment of serum-free RPMI-1640 medium, differentiated podocytes were sensitized by incubation with rabbit anti-mouse podocyte polyclonal antibody for 1 h at 37°C. Subsequently, cells were washed twice with serum-free RPMI-1640 medium, and podocytes were incubated with NHS (NHS: serum-free RPMI-1640 medium = 1:80) and C5b-6 complex (204906-50UGCN; Merck) for 1 h at 37°C.

Experimental grouping

Different concentrations of C5b-6 (0.1, 0.2, 0.4, 0.8 μg/mL) and NHS were used to generate sc5b-9 to induce the model of podocyte injury. After generation of the models, groups generated with low sc5b-9 (0.1 μg/mL) and High C5b-9 (0.8 μg/mL) were continually cultured with a normal medium for 48 h, while podocytes cultured in only the presence of medium was regarded as blank. Cells were subjected to cell apoptosis assays, western blot analysis, and immunofluorescence. Models obtained with 0.8 μg/mL C5b-9 were chosen for treatment with PrA (102036-28-2; Biopurify) and OA (42515; Sigma) at different concentrations (0, 10, 20, 40, 80, 160 μM). Podocytes cultured in medium without PrA and OA were considered blank. Models obtained with 0.8 μg/mL C5b-9 were also randomly treated with either medium; 80 μM PrA; 80 μM OA; 80 μM PrA combined 80 μM OA; or 5 μM LY294002 (a PI3K-AKT inhibitor, S1105; Selleck). Podocytes cultured in only medium were considered blank. Furthermore, podocytes were randomly treated with medium; 100 ng/mL insulin-like growth factor 1 (IGF-1) (a PI3K-AKT activator, 100-11; Peprotech); or 100 ng/mL IGF-1 + 80 μM PrA + 80 μM OA. After that, cell apoptosis assays and western blot analysis were carried out.

Cell Count Kit 8 (CCK-8) assay

The CCK8 Signalway Antibody LLC (SAB), CP002 assay was applied to assess podocyte proliferation. After digestion with 0.25% trypsin-ethylenediaminetetraacetic acid (T1300-100; Solarbio), fully differentiated podocytes were inoculated in a 96-well culture plate at 5 × 10³ cells/well. Four gradient concentrations of C5b-6 complex (0.1, 0.2, 0.4, 0.8 μg/mL) and NHS were put into different wells to induce podocyte injury, podocytes treated with medium were considered blank. After 0, 24, 48, and 72 h, a 100 μL mixture of CCK8 (CCK8: serum-free medium = 1:10) was added to each well, followed by incubation for 1 h in a 5% CO₂ incubator at 37°C. A microplate reader was used to detect the 450 nm absorption. An optimal concentration of sc5b-9 complex was chosen for all following experiments. Likewise, CCK-8 was also used to investigate the effects of PrA and OA at different concentrations on the cell viability of damaged podocytes, and an optimal concentration was chosen for further study.

Quantitative real-time polymerase chain reaction (qRT-PCR)

After 24 h treatment of PrA and OA, TRIZol Reagent (1596-026; Invitrogen) was utilized to extract the total RNA from treated podocytes. Following quantification, the integrity of isolated RNA was detected by electrophoresis with a 1% agarose gel. Subsequently, reverse transcriptase (#K1622; Fermentas) was utilized to synthesize complementary DNA, and qRT-PCR reactions were conducted on the ABI Prism 7300 (ABI-7300; ABI) with a SYBR Green PCR kit (#K0223; Thermo Fisher Scientific). After normalizing to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the messenger RNA levels of several associated genes were analyzed by the 2⁻ΔΔCT method. Primers were designed using the AlleleID software (PREMIER Biosoft) and the sequences are as follows: nephrin (5’-GGACCCACACTACTACTC-3’ and 5’-CTCTCCACCTCGTCATAC-3’), desmin (5’-GGTGA TGAGGCCACTCTAGC-3’ and 5’-TTAGCCCGATGGTCT TCATAC-3’), podocin (5’-TGTGTTCTCGCTCCTTC-3’).
and 5'-TGCCCTGGGACTACTTTC-3'), CD2AP (5'-AAGG AGAAGAATAGGGAGCA-3' and 5'-CCGTTTGA TGGGCAATTTGCTCA-3'), Bcl2 (5'-TGGGATAG GTGTCAGG-3' and 5'-CCATTATCATGCGTGAG-3'), Bax (5'-TTGTACAGGTTCATC-3' and 5'-ATT GTGTCAGGACTACTTTC-3'), GAPDH (5'-CTGCCCGAA CATCATCC-3' and 5'-CTCGAGAGTCGGTTCTC-3'). The PCR procedure was carried out as follows: 95°C for 10 min at 4°C, 12,000 rpm for 30 min at 4°C. After centrifugation for 30 min, the supernatants were collected and the intact cells were resuspended in 1% BSA (A8010; Solarbio) and labeled with fluorescein isothiocyanate (FITC) at 4°C in the dark,

**Western blot analysis**

Treated podocytes were lysed in RIPA buffer (R0010; Solarbio), supplemented with protease and phosphatase inhibitors, for 30 min at 4°C. After centrifugation for 10 min at 4°C, 12,000 rpm, proteins were obtained and quantified by a BCA kit (PICPI23223; Thermo), followed by separation using 10% and 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (Jrdun Biotechnology Co., Ltd., Shanghai, China) and semi-dry transferred onto polyvinylidene fluoride (PVDF) membranes (HATF00010; Millipore). The PVDF membranes containing target bands were blocked with 5% bovine serum albumin (BSA) in phosphate-buffered saline with Tween 20 (PBST) for 1 h at room temperature, and then incubated with specific primary antibodies against nephrin (1:500, Sc-377246; Santa Cruz Biotechnology), podocin (1:1,500, Ab50339; Abcam), CD2AP (1:1,000, #2135; Cell Signaling Technology [CST]), desmin (1:1,000, #4024; CST), Bcl2 (1:500, Sc-493; Santa Cruz Biotechnology), Bax (1:500, Sc-493; Santa Cruz Biotechnology), p-AKT (1:1,000, #9271; CST), AKT (1:1,000, #9272; CST), p-mTOR (1:1,000, #2974; CST), mTOR (1:1,000, #2972; CST), or GAPDH (1:2,000, #5174; CST) at 4°C overnight with gentle shaking. After 5–6 washes, the membranes were then incubated in secondary antibodies (1:5,000; Beyotime) for 2 h in the dark at room temperature. Membranes were again washed 5–6 times and incubated with chemiluminescence detection reagent in the dark for 5 min. The bands of target proteins were visualized through an ECL imaging system (Tanon, Shanghai, China).

**Flow cytometry (FCM) detection**

Podocyte apoptosis was detected by FCM analysis (BD) with a double staining of annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). After centrifugation, about 5–10 million cells were resuspended in 195 µL annexin V-FITC binding buffer. Following a 15 min incubation with 5 µL annexin V-FITC at 4°C in the dark, cells were incubated with 5 µL PI staining solution for 5 min at 4°C in the dark. A tube without annexin V-FITC/PI was used as a negative control. Through FCM analysis, the rates of apoptotic cells were determined. Annexin V-FITC was green in fluorescence and marked early apoptotic cells. PI was red in fluorescence. Annexin V/PI indicated late apoptotic or necrotic cells, Annexin V/PI indicated viable cells.

**Immunofluorescence**

Injured podocytes cultured on glass coverslips were washed with 0.02 M PBS and then fixed in 4% paraformaldehyde (10010018; National Medicines Corporation Ltd.) for 30 min. Following 10 min permeabilization with 0.5% Triton X-100 (T8200; Solarbio), cells were blocked in 1% BSA (A8010; Solarbio) for 1 h at room temperature. Subsequently, the cells were incubated in specific primary antibodies against sC5b-9 (1:5,000 dilution; ab55811; Abcam), nephrin (1:200 dilution; sc-377246; Santa Cruz Biotechnology), or podocin (1:1,000 dilution; ab50339; Abcam) at 4°C overnight in a humidifying box. Residual antibodies on the cells were removed by washing with PBST for three times. Next, the cells were incubated in secondary antibodies (1:1,000 dilution; Beyotime) and labeled with fluorescein for 2 h at room temperature. Finally, cells were sealed with mounting medium (P0126; Beyotime). A laser-scanning microscope was used to take all of the images.

**Lactate dehydrogenase (LDH) activity detection**

After modeling for inducing podocyte injury, the supernatants were collected. According to the instruction, the activity of LDH in supernatants after MAC deposition was detected by LDH Test Kit (Nanjing Institute of Bioengineering, Nanjing, China).

**Statistical analysis**

All statistical analyses in this paper were carried by GraphPad prism 7.0. Student’s t test was used to compare the two groups, while multiple comparisons were analyzed using one-way analysis of variance followed by Tukey’s multiple comparisons. All data values were obtained from at least three independent experiments and shown as mean ± standard deviation. The P < 0.05 is considered statistically significant.

**Results**

**Successful modeling of podocyte injury induced by the sC5b-9 complex**

After treatment of different concentrations of C5b-6 complex and NHS, the cell viability of podocytes was evaluated using a CCK8 assay at 0, 24, 48, and 72 h. As shown in Figure 1A, when sC5b-9 < 0.8 μg/mL,
immunoﬂuorescence showed that the deposition of sC5b-9 on the surface of podocytes smaller increased, and the activity of LDH in the culture supernatant was low, indicating that the cell lysis was very small at this time. When sC5b-9 = 0.8 µg/mL, sC5b-9 deposition on podocytes signiﬁcantly increased, and LDH activity in the culture supernatant was low, indicating that the cell lysis was very small at this time. When sC5b-9 = 0.8 µg/mL, sC5b-9 deposition on podocytes signiﬁcantly increased, and LDH activity in cells also increased signiﬁcantly, indicating the higher activity of MAC in the system. Furthermore, the sC5b-9 complex had a signiﬁcant inhibitory effect on podocyte proliferation in a dose- and time-dependent manner. The cell viability of podocytes treated with 0.8 µg/mL sC5b-9 was remarkably lower than that of cells treated with other concentrations of sC5b-9 (Figure 1B). Therefore, it preliminarily suggested that an in vitro model of podocyte injury was successfully induced by C5b-6 and NHS, and an optimal concentration of 0.8 µg/mL sC5b-9 was chosen for further study (Chen et al., 2010).

The effects of sC5b-9 complex on podocyte apoptosis and several associated genes

Cells in the blank, low sC5b-9 (0.1 µg/mL), and high sC5b-9 (0.8 µg/mL) groups were used to explore the effect of the sC5b-9 complex on podocyte apoptosis and several associated genes. FCM analysis revealed that the sC5b-9 complex induced early apoptosis of podocyte, and its induction of apoptosis was enhanced with increasing concentrations (Figure 2A). The protein levels of nephrin, podocin, and CD2AP were signiﬁcantly reduced by the sC5b-9 complex, while desmin was elevated (Figure 2B). Moreover, immunoﬂuorescence of nephrin and podocin further showed the inhibitory effects of the sC5b-9 complex on nephrin (Figure 2C) and podocin (Figure 2D) expression. All data suggested that the sC5b-9 complex injured podocytes by promoting apoptosis and regulating the expression of associated genes.

PrA and OA have a protective effect on podocyte apoptosis induced by podocyte injury

After 0, 24, 48, and 72 h treatment of PrA and OA, the cell proliferation of injured podocytes was detected by a CCK8 assay. As shown in Figure 3, both PrA (Figure 3A) and OA (Figure 3B) signiﬁcantly promoted the cell proliferation of injured podocytes in a dose- and time-dependent manner and reached their peak effects at 80 µM. The same dose of OA showed a stronger protective effect than that of PrA. These results indicated that there was a protective effect of PrA and OA on podocyte injury through inhibition of podocyte apoptosis. An optimal concentration of 80 µM was chosen for further study.

PrA and OA protect injured podocytes from apoptosis through the regulation of several associated genes and changes in signaling

Injured podocytes were treated, either alone or in combination, with PrA and OA. Early apoptotic cells of injured podocytes were obviously reduced by PrA and OA (Figure 4A), accompanied by markedly increased levels of nephrin, podocin, CD2AP, and B-cell lymphoma 2 (Bcl2) along with decreased levels of desmin and Bcl-2-associated genes.

Figure 1 Successful modeling of podocyte injury induced by the C5b-9 complex. After 0, 24, 48, and 72 h treatment of C5b-6 complex and normal human serum (NHS) at four gradient concentrations (0.1, 0.2, 0.4, 0.8 µg/mL), cells were stained for immunofluorescence and lactate dehydrogenase (LDH) activity in supernatants was detected (A; magniﬁcation: ×400), and cell viability of podocytes at 450 nm was measured by a cell counting kit-8 (CCK-8) assay (B). Blank: podocytes treated with only medium. Data shown are representative of at least three independent experiments, data are expressed as mean ± standard deviation (*P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 compared with blank).
Figure 2 The effects of the C5b-9 complex on podocyte apoptosis and several associated genes. Successful modeling with 0.1 and 0.8 µg/mL C5b-6 were chosen to continually treat with a normal medium for 48 h. Apoptotic cells were analyzed by flow cytometry (FCM) (A) while western blot analysis was performed to quantify the protein levels of associated genes (B). Lower right quadrant shows early apoptotic cells stained with annexin-V; upper right quadrant represents late apoptotic or necrotic cells that were stained double positive with two dyes annexin-V and propidium iodide (PI); lower left quadrant represents viable cells. Immuno-fluorescence staining of nephrin (C) and podocin (D) was performed (magnification: ×400). Blank: podocytes treated with the only medium. Data shown are representative of at least three independent experiments. All data are shown as mean ± standard deviation (**P and ###P < 0.01; ####P < 0.0001 compared with blank).

Figure 3 Protosappanin-A (PrA) and oleanolic acid (OA) has a protective effect on podocyte apoptosis induced by podocyte injury. After treated with different concentrations of PrA (0, 10, 20, 40, 80, 160 µM) and OA (0, 10, 20, 40, 80, 160 µM) for 0, 24, 48, and 72 h, the cell proliferation of injured podocytes was detected by a cell counting kit-8 (CCK-8) assay; podocytes treated with only medium are considered as blank. Data are representative of at least three independent experiments and results are shown as mean ± standard deviation (SD) (**P < 0.01, ****P < 0.0001 compared with blank; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 compared with sC5b-9 [0.8 µg/mL]).
X proteins (Bax). The effects of the combination of the two drugs were significantly better than treatment with any single drug (Figures 4B and 4C). Studies have reported that Bcl2 is an anti-apoptotic protein in cancers, whereas Bax has a pro-apoptotic effect, and the ratio of Bax to Bcl-2 is used to determine whether the cells will commit to apoptosis (Green and Reed, 1998; Gao and Dou, 2000). Further, C5b-9-induced increase of p-AKT (Ser473)/p-mTOR (Ser2481) levels in damaged podocytes was also remarkably reversed by PrA and OA, while the levels of AKT and mTOR remained unchanged (Figure 4D). These results were also consistent with the effects of the PI3K/AKT inhibitor LY294002 on injured podocytes in our study. Therefore, we conjectured that podocyte injury was likely to cause abnormal activation of AKT/mTOR signaling, and PrA and OA protected injured podocytes from apoptosis mainly through inhibiting phosphorylation of AKT/mTOR and the regulation of expression in associated genes.

PrA and OA suppress podocyte apoptosis by inhibiting the AKT/mTOR signaling pathway

Podocytes were treated with IGF-1 (a PI3K-AKT activator) and PrA combined with OA. As shown in Figure 5, a significant increase of early apoptosis in podocytes was detected after IGF-1 treatment, and IGF-1-induced apoptosis was remarkably inhibited by PrA and OA (Figure 5A). Furthermore, p-AKT/p-mTOR levels in podocytes were significantly increased by IGF-1 while levels of AKT and mTOR were unaltered. Increases in p-AKT/p-mTOR levels were also markedly reversed by PrA and OA (Figure 5B). These results further demonstrate that inhibition of podocyte apoptosis by PrA and OA may be via inhibition of AKT/mTOR signaling.

Discussion

Podocytes, a size-selective filtration barrier in the glomerulus, are injured in a great number of glomerular diseases.
Several effective monomers isolated from *C. sappan* L. have been reported to exhibit therapeutic potential on these diseases. Ursolic acid is reported to improve podocyte injury (Xu et al., 2017). In addition, OA and N-acetylcysteine can ameliorate the progression of DN (Lee et al., 2016). Here, we aimed to investigate the effects as well as the mechanism of PrA and OA on podocyte injury.

We found that sC5b-9-induced apoptosis was significantly inhibited by the treatment of OA and PrA, and the combination of two drugs was significantly more efficacious than treatment with single drug. Treatment with drug was accompanied by a decrease in desmin level as well as the restoration of nephrin, podocin, and CD2AP. Nephrin, podocin, CD2AP, and desmin are important components of SD functioning in podocyte processes (Ruotsalainen et al., 1999; Huber et al., 2001; Schwarz et al., 2001; Shih et al., 2001; Roselli et al., 2002). Thus, we concluded that OA and PrA could protect podocytes from C5b-9 induced-injury by regulating nephrin, podocin, and CD2AP, and desmin expression.

AKT, a major downstream mediator of PI3K activity, was found to be required for the survival of various cells and blocked toxic stimuli-induced apoptosis. PI3K-AKT could control the regulation of gene expression, cell viability, growth, and other complex cellular programs.

**Figure 5** Protosappanin-A (PrA) and oleanolic acid (OA) suppress podocyte apoptosis by inhibiting AKT/mTOR signaling. After 24 h treatment with 100 ng/mL insulin-like growth factor 1 (IGF-1) (a PI3K-AKT activator) and IGF + 80 µM PrA + 80 µM OA, FCM analysis was carried out to assess podocyte apoptosis (A). Lower right quadrant shows early apoptotic cells stained with annexin-V; upper right quadrant represents late apoptotic or necrotic cells that were stained double-positive for two dyes annexin-V and propidium iodide (PI); lower left quadrant represents viable cells. AKT/mTOR signaling levels were quantified using western blot analysis (B). Blank: podocytes treated with only medium. Data shown are representative of at least three independent experiments, all data are shown as mean ± standard deviation (****P<0.0001 compared with blank; #P<0.01, ##P<0.001, ###P<0.0001 compared with IGF-1).
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(Cantley, 2002; Edinger and Thompson, 2002; Shioi et al., 2002). It was reported that Akt is activated in OA-treated vascular smooth muscle cells (VSMCs), which may be involved in OA-induced heme oxygenase-1 (HO-1) expression in VSMCs (Feng et al., 2011). Moreover, notoginsenoside R1 (NR1), the major component of a Chinese herbal medicine, was reported to ameliorate podocyte injury through activation of PI3K/AKT signaling (Huang et al., 2016). In our study, increased podocyte injury through activation of PI3K/AKT signaling from apoptosis or injury by inhibiting the abnormal activation of PI3K-AKT-mTOR signaling. Our conclusion is further supported by the observation that the effects of induced PI3K/AKT by IGF-1 on podocyte apoptosis and p-AKT/p-mTOR levels was reversed by OA and PrA. One limitation is that the model for inducing podocyte injury was not very good with low apoptosis levels. However, compared with normal podocytes, the injury model still showed similar protective effects of OA and PrA on OA and PrA-mediated inhibition of apoptosis or injury by inhibiting the abnormal activation of PI3K-AKT-mTOR signaling. We will further improve our model for podocyte injury to make our conclusions more convincing.

Conclusion
This paper found protective effects of OA and PrA on damaged podocytes in terms of cell apoptosis. In addition, a combination of the two drugs resulted in an additive effect, and may be through inhibition of the abnormal activation of the PI3K-AKT-mTOR signaling pathway. These results reveal a novel promising therapy for improvement and treatment of podocyte injury in glomerular diseases.

Acknowledgments and funding
This work was funded by the Heilongjiang Nature Science Foundation (key project) (Grant No. ZD2017018).

Conflict of interest
All authors declare that they have no conflict of interest.

Ethical approval
All procedures performed in studies involving humans were in accordance with the ethical standards of the institution or practice at which the study was conducted.

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Received 2 January 2019; accepted 18 August 2019.
Final version published online 8 September 2019.