

Original Article

Triptolide reduces podocytes injury through blocking ERK and JNK pathways in passive Heymann nephritis (PHN) model

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Abstract: Triptolide, a traditional Chinese medicine, obtained from *Tripterygium wilfordii* Hook F (TWHF), has been proved clinically effective in reducing proteinuria in chronic kidney disease in China. However, the effects and mechanisms of triptolide on membranous nephropathy (MN) have not been well understood. This study aimed to investigate the effects of triptolide on rat experimental membranous nephropathy and reveal its underlying molecular mechanism. To study the underlying mechanisms of podocytes injury, an in vivo rat model of passive Heymann nephritis and an in vitro model of C5b-9-induced podocytes injury were established. The rats were orally administered triptolide (100 and 200 µg/kg/d) started on d 5 and continued until d 32. Blood and urine samples were collected for analyses. Our results showed that treatment with triptolide markedly reduced the level of proteinuria and the serum albumin as well as the titer of circulating rat anti-rabbit IgG antibodies. And expression of the podocyte injury marker desmin decreased after triptolide treatment, whereas the expression of the podocyte marker synaptopodin, podocin and nephrin was increased. Furthermore, the TUNEL-positive cells and caspase-3 level of podocytes were reduced, which indicated that podocyte injuries in PHN could be reversed after treatment with triptolide. Using in vitro experiments, we also found that triptolide could reduce podocyte injury and inhibited the apoptosis. In addition, we found that triptolide treatment stimulated a strong decrease in phosphorylated JNK and ERK in podocytes, whereas triptolide treatment had no significant effect on phospho-p38 MAPK levels. Taken together, triptolide attenuates the progression of PHN and podocyte injuries in PHN rat model, and inhibits podocyte apoptosis partly by blocking the ERK and JNK pathways.

Keywords: Triptolide, passive Heymann nephritis, podocytes, apoptosis, ERK and JNK pathways

Introduction

Membranous nephropathy (MN) is one of the major causes of adult nephrotic syndrome [1]. MN is characterized by the podocytes injury, which leads to heavy proteinuria and hypoalbuminemia [2]. It was reported that podocytes apoptosis is a critical part in the process of MN development [3]. Although therapeutic strategies have been improved in recent years, at least one-third of MN patients will develop progressive renal disease, reflecting limited advances in our understanding of the pathogenesis of this disease [4, 5].

Extracts of *Tripterygium wilfordii* Hook F (TWHF) plays an important role in the treatment of kidney diseases in China. Triptolide, its major com-

ponent, has been increasingly studied and has been proved to be effective in experiments of kidney diseases [6, 7]. For example, it has been well documented that triptolide could effectively reduce proteinuria and remarkably improve C5b-9-induced podocyte lesion in experimental membranous nephropathy [6]. Recent observations showed that triptolide ameliorated puromycinaminonucleoside (PAN)-mediated podocyte injuries in vivo and in vitro [7]. But little is known about the therapeutic effects and underlying mechanisms of triptolide on MN because of lack of experimental data.

In previous study, mitogen activated protein kinases (MAPK) signaling pathway plays an indispensable role in regulating renal function [8]. Aberrant activation of MAPK signaling pathway

has been observed in many renal failure models, both in vivo and in vitro. Liu M et al. found that triptolide treatment significantly attenuated cardiac fibrosis and improved cardiac function through suppressing the MAPK signaling pathway in isoprenaline-induced cardiac remodeling rats [9]. A recent study has indicated that the activation of MAPK appears to be cytoprotective against complement-mediated podocytes injury [10]. However, whether MAPK signaling pathway participates in the therapeutic effects of triptolide on MN is unknown.

In this research, we found that triptolide treatment suppresses proteinuria and podocyte injury in experimental membranous nephropathy in rats. Meanwhile, we explored the mechanism of triptolide on podocyte injury in vitro, we found that triptolide reduced podocytes injury through blocking ERK and JNK pathways.

Materials and methods

Cell culture

Conditionally immortalized mouse podocyte cells (MPCs) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Life Technologies), 100 U/mL penicillin G, and 100 mg/mL streptomycin. The mouse podocytes proliferated at 37°C and treated with 10 U/ml of mouse recombinant γ -interferon as previously described. After growing to 50-60% confluency, cells were transferred to 37°C in 10-14 days caused podocytes to stop proliferating and undertake a differentiated phenotype.

Assembly of C5b-9 membrane attack complex on podocytes in vitro

Assembly of C5b-9 on the membranes of podocyte cells was done with using purified complement complex, including C5b6, C7, C8 and C9, according to the manufacturer's instructions (Calbiochem). The formation of C5b-9 on the membrane of podocytes were verified by confocal microscopy (LSM 510, Carl Zeiss, Jena, Germany) and flow cytometry (FACS Aria, Becton Dickinson, San Jose, CA, USA) using antibody to C5b-9 (DAKO). For the protective effect experiments, podocytes were pre-incubated for 30 min with triptolide (10 ng/ml) before C5b-9 exposure. There were three groups, including normal control, vehicle (C5b-9 injury) and triptolide pretreatment groups.

Induction of passive Heymann nephritis and treatment

Fx1A was prepared from renal cortices of Sprague-Dawley (SD) rat by sieving and ultracentrifugation, as described previously [6]. Rabbit anti-Fx1A was prepared by Beijing Biosynthesis Biotechnology. All experiments were performed in male SD rats (Beijing HFK Biotechnology) with an initial body weight of 150-180 g. Rats were then injected with anti-Fx1A serum (1 ml/100 g body weight; antibody titer: 1:250) into the caudal vein, followed by one further injection after 1 h. The control rats were injected with the same volume of normal saline.

Treatment with triptolide started 5 days after antiserum injection when proteinuria was already present. PHN rats were divided into 4 groups (n=8 per group): vehicle group (saline), triptolide treated groups (100 μ g/kg/day) and triptolide treated groups (200 μ g/kg/day). Eight of the healthy SD rats were chosen as normal controls. On 0, 7, 14, 21, and 28 d post PHN induction, blood and urine samples were collected. The blood samples were taken from the retrobulbar plexus of the pentobarbital sodium-anesthetized rats. The 24-h urine collections were done on individual rats in metabolic cages. At the endpoint of the animal experiment, 28 d after PHN induction, all of the rats were sacrificed and kidneys were collected.

Biochemical parameters and circulating rat anti-rabbit antibody

The protein content in the urine was measured by the Bradford method. The serum albumin (ALB) level was determined by the HITACHI-7080 automatic biochemical analyzer (Hitachi, Tokyo, Japan).

The amount of circulating antibodies to rabbit IgG was determined using ELISA. Briefly, plates were coated with 4 μ g/mL rabbit IgG (Sigma-Aldrich Co LLC, St Louis, MO, USA) in sodium carbonate buffer (pH 9.6) and incubated 20 h at 4°C. After blocking with 1% BSA and then washing the plates with PBST, rat serum of PHN rats with or without treatment with triptolide were added and incubated for 1 hour at room temperature. After washing, the HRP labeled sheep polyclonal antibody against rat (1:2000, Abcam, Cambridge, UK) was applied and incubated for 1 h. The absorbance was measured

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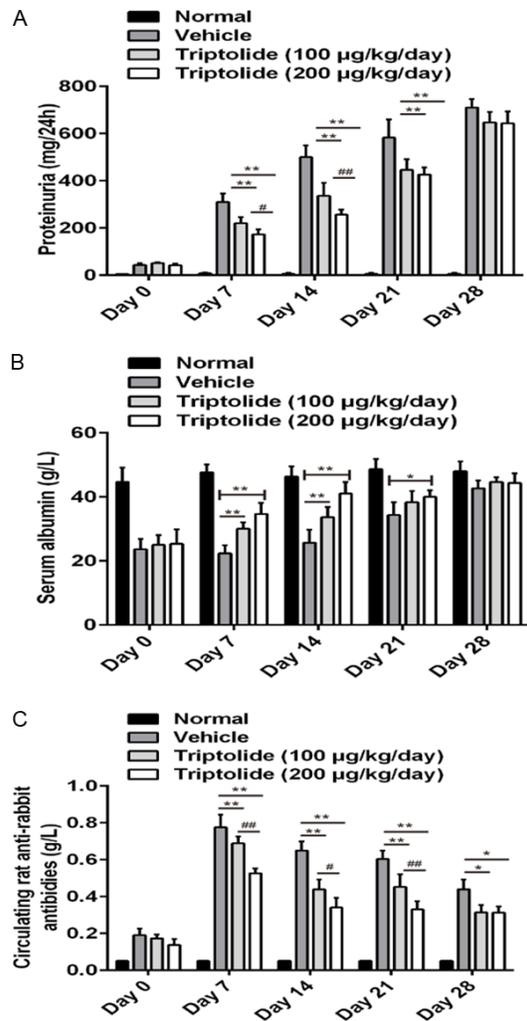


Figure 1. Effect of triptolide on proteinuria, serum albumin and circulating rat anti-rabbit IgG antibodies in rats with passive Heymann nephritis. After treatment with triptolide for 7, 14, 21 and 28 days, the blood and urine samples from control rats and PHN model rats were collected. A. The protein content in the urine was measured by the Bradford method. B. Serum samples were analyzed for serum albumin content using Coomassie Brilliant Blue G-250. C. The titer of circulating rat anti-rabbit antibody was determined by ELISA. Data are presented as means \pm S.D. (n=12). *P<0.05, **P<0.01 vs vehicle group, #P<0.05, ##P<0.01 vs triptolide group.

chromatically at 492 nm with an ELISA-reader (ThermoMultiskan Spectrum, Waltham, MA, USA).

TUNEL staining

Apoptosis cells were detected with the In Situ Cell Death Detection kit, Fluorescein (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's protocol. Briefly, 4

mm cryostat kidney sections were fixed with 4% paraformaldehyde and incubated with permeabilization solution for 2 min on ice. The negative control sections were incubated only in label solution and the positive control sections were treated with 50 U/ml DNaseI (Sigma) prior to labeling procedures. Then sections were incubated with enzyme solution. Finally, sections were observed by immunofluorescence microscopy.

Apoptosis assay

The percentages of apoptosis in the different groups of cells were determined using an FITC-Annexin V/PI Apoptosis Detection Kit, according to the manufacturers' instruction (Nanjing KeyGen Biotech, Nanjing, China). Briefly, the different groups of cells were stained in duplicate with FITC-Annexin V/PI for 15 min in the dark. Apoptotic cells were measured by the flow cytometry (FC500, Beckman Coulter) and expressed as a percentage of total cells.

Western blot

Cells were harvested with ice-cold PBS containing 5 mM EDTA and lysed in a compound buffer. The protein concentration was measured using the Bradford assay as a standard. A total of 50 μ g total protein was separated by 6-15% SDS-PAGE and then transferred to a membrane, which was blocked with 5% skim milk, probed with a primary antibody overnight at 4°C, and incubated with a horseradish peroxidase-conjugated secondary antibody. The specific protein bands were scanned and normalized to β -actin (Beyotime Bio-Tech, Shanghai, China) to quantitate Western blot analysis.

Statistical analysis

Statistical analyses were performed with SPSS software. Results were expressed as mean \pm S.D. Student's t-test was used to compare differences between groups. P<0.05 was considered statistically significant, and P<0.01 was considered highly statistically significant.

Results

Triptolide improves the general state of PHN rats

Rat passive Heymann nephritis (PHN) is the most commonly used rodent model of human membranous nephropathy. Therefore, we used

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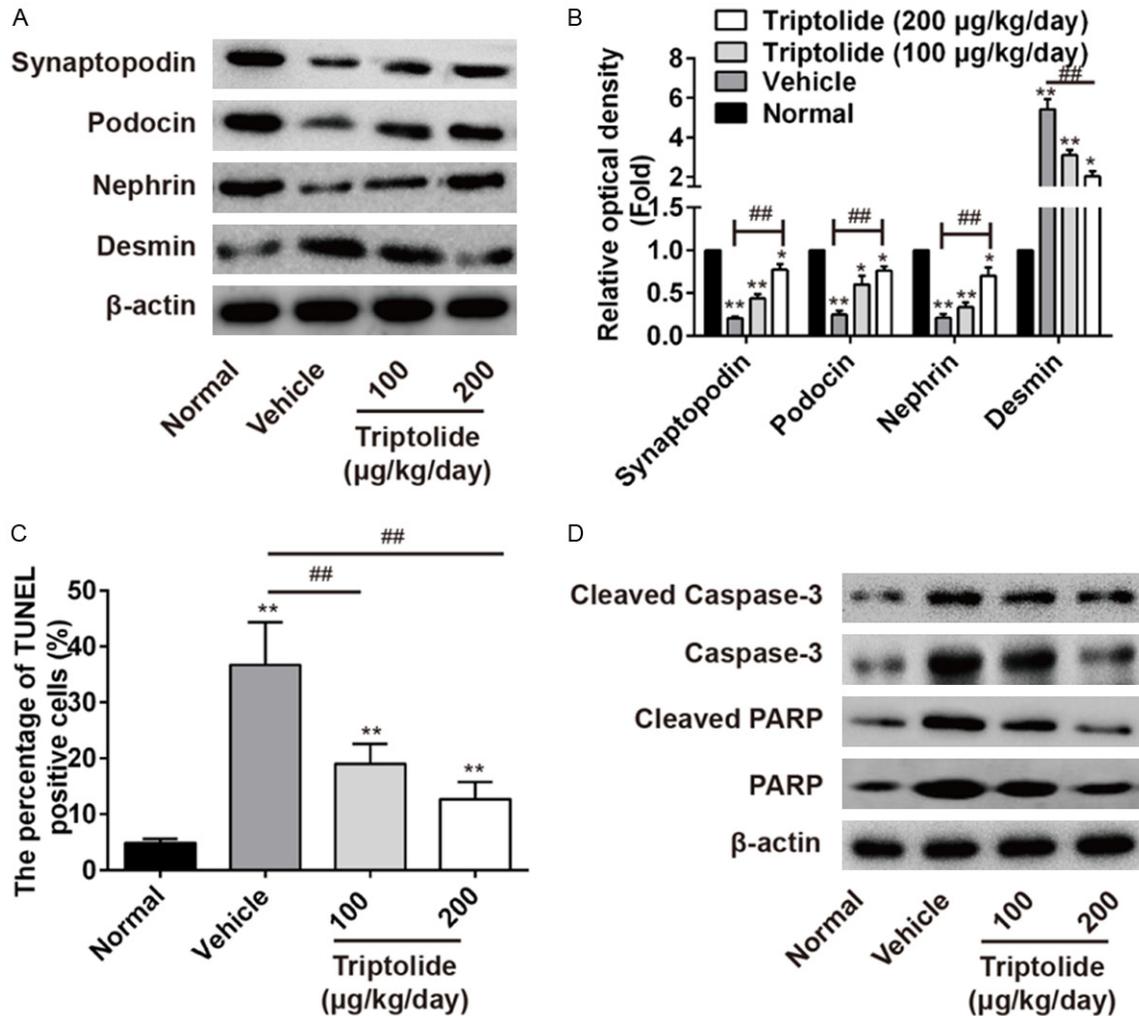


Figure 2. Triptolide treatment protected the podocyte in PHN rat kidneys. A, B. The Western blot results of synaptopodin, podocin, nephrin and desmin expression in podocytes of PHN rats after triptolide treatment. C. Deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay were used to detect apoptosis in podocytes of PHN rats. TUNEL positive cells were significantly decreased in podocytes of PHN rats after triptolide treatment. D. Western blotting assays were used to analyze the effects of triptolide on the expressions of apoptosis-associated proteins in podocytes of PHN rats. β -actin was used for loading control. Data are presented as means \pm S.D. ** $P < 0.01$ vs vehicle group, # $P < 0.05$, ## $P < 0.01$ vs triptolide group.

the PHN rat model to explore the effect of triptolide on MN. After treatment with triptolide for 7, 14, 21 and 28 days, the oral administration of triptolide at the doses of both 100 and 200 $\mu\text{g}/\text{kg}$, significantly reduced proteinuria (Figure 1A). The reducing effect of triptolide on proteinuria was persistent obviously at 14 days ($P < 0.01$) and maintained at 21 days ($P < 0.01$). At 28 days, proteinuria was restored to vehicle (Triptolide treatment groups versus vehicle group).

Consistent with the marked decrease of proteinuria, serum albumin (ALB) level was increased after treatment with triptolide. Triptolide

treatment restored the ALB obviously through the whole process (Figure 1B). In addition, triptolide treatment at the doses of both 100 and 200 $\mu\text{g}/\text{kg}$ showed a strong potential for reducing the circulating rat anti-rabbit antibodies (Figure 1C). Therefore, the results confirmed that triptolide treatment markedly ameliorated the symptoms of PHN rats.

Triptolide ameliorated podocyte injuries and inhibited podocyte apoptosis

Increasing evidence supports that podocyte injury leads to heavy proteinuria and hypoalbuminemia, and plays an important role in MN [5].

Triptolide reduces podocytes injury in PHN model

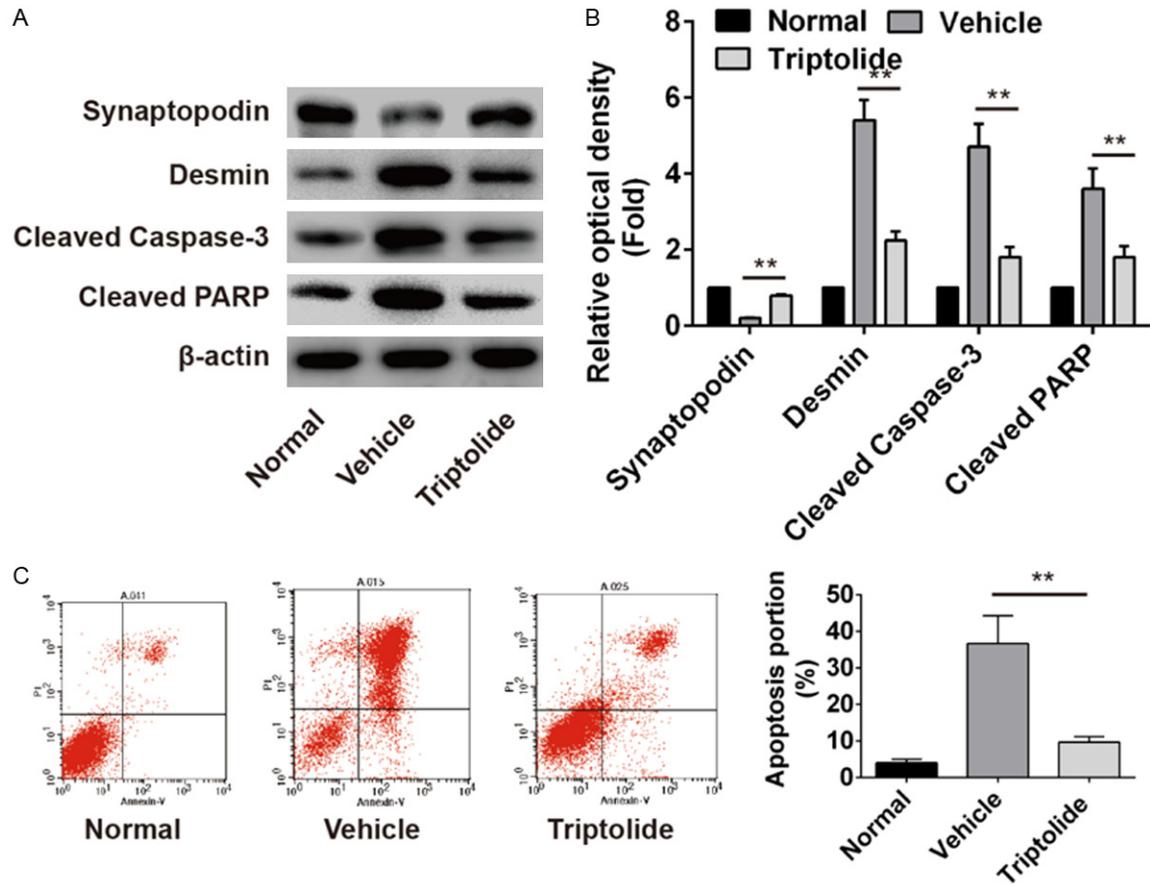


Figure 3. Triptolide attenuates C5b-9-induced MPCs injury. MPC podocytes were pre-treated with triptolide and subjected to sublytic C5b-9 attack for 48 h. Subsequently, the relative levels of synaptopodin, desmin, cleaved caspase-3 and cleaved PARP expression were determined by Western blot and the apoptosis of MPC5 cells were determined apoptosis assays. Data are representative images or expressed as the means \pm S.D. of each group from three separate experiments. A, B. The expression levels of synaptopodin, desmin, cleaved Caspase-3 and cleaved PARP in podocytes. C. The percentage of apoptotic cells was analyzed by fluorescence-activated flow cytometry analysis. $**P < 0.01$ vs vehicle group.

Therefore, we test whether triptolide improves podocyte injuries in PHN. Synaptopodin protein, podocin and nephrin play central role in keeping the normal morphology and function of podocyte [7, 11-13]. In addition, desmin is an important biomarker of podocyte injuries [14]. In this study, western blot analysis showed that the expression of synaptopodin protein, podocin and nephrin were significantly increased after triptolide treatment, whereas triptolide treatment resulted in a decrease in desmin expression (Figure 2A, 2B).

Apoptosis is one of the key factors in determining the number of podocytes in the glomeruli. And it was found in this study that podocyte apoptosis, assessed by TUNEL staining, was significantly decreased with triptolide treat-

ment (Figure 2C). Both caspase-dependent and -independent pathways are known to be involved in the process of apoptosis. In this experiments, we found that cleaved-caspase-3, caspase-3, cleaved PARP and PARP were markedly decreased after triptolide treatment (Figure 2D). These results showed that triptolide treatment efficiently protected the podocyte from injuries in PHN rats.

Triptolide protected podocytes against C5b-9 induced injury in vitro

To unravel the mechanisms underlying the protective effect of triptolide on podocytes, we established an in vitro podocytes injury model induced by C5b-9. The effects of triptolide on the expression of synaptopodin, desmin cleav-

Triptolide reduces podocytes injury in PHN model

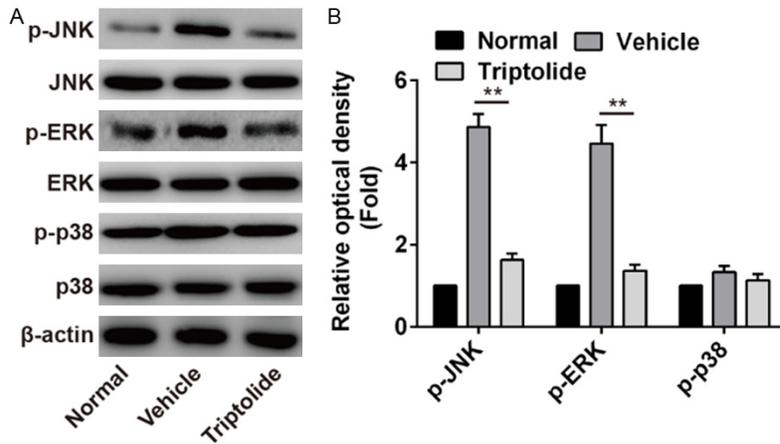


Figure 4. Triptolide inhibited C5b-9-induced MAPK activation. A. Western blot was used to examine total and phosphorylated expression of the MAPK family members (JNK, p38, ERK, p-JNK, p-p38, p-ERK). B. The optical densities of the bands were measured using Image-Pro Plus software. Data are presented as means \pm S.D. ** $P < 0.01$ vs vehicle group.

ed-caspase-3 and cleaved PARP were further confirmed in cultured podocytes. As shown in **Figure 3A, 3B**, the expression of synaptopodin (a podocyte marker) was increased, whereas the expression of desmin (marker of podocyte injury) was decreased after triptolide treatment. Moreover, the rate of apoptosis measured by flow cytometry was markedly decreased in triptolide treatment group compared with vehicle group (**Figure 3C**). A similar finding was obtained using the TUNEL assay for apoptosis in PHN rats treated with triptolide. These results showed that triptolide treatment efficiently protected the podocyte from injuries in cultured podocytes.

Triptolide inhibited C5b-9-induced MAPK activation

To characterize the intracellular signaling pathway associated with the protective effects of triptolide in podocytes, phosphorylation of p38, JNK and ERK mitogen-activated protein kinase (MAPK) was analyzed. As shown in **Figure 4A, 4B**, p-JNK and p-ERK expression were dramatically decreased after triptolide treatment, which indicated the inhibition of JNK and ERK cell signal pathway activation. Triptolide did not affect basal phosphorylation of p38 MAPK. In previous study, Chen et al. found that triptolide could inhibit the phosphorylation of p38 [6], which was different from our study of triptolide, and its blocking effect of JNK was consistent

with the study in neuron [15]. So we believed triptolide protected podocytes from complement-mediated injury partly by inhibiting the activation of MAPK.

Discussion

In the present study, we demonstrated that triptolide effectively ameliorated the symptoms in PHN rats by reducing podocyte injuries. In addition, we found that triptolide inhibited podocytes apoptosis by caspase-dependent process as evidenced by the reduced level of cleaved-caspase-3 and cleaved-PARP in PHN rats.

We also provided evidence that triptolide ameliorated C5b-9 induced injury in podocyte cells in vitro through blocking ERK and JNK pathways. And, our studies indicate that triptolide could be a novel agent for treatment of MN.

Recent study has demonstrated that the extracts of TWHF showed very dramatic effects on decreasing proteinuria in patients with membranous nephropathy [16]. Triptolide, one of the major active components of TWHF, has been proved to be effective in experiments of kidney diseases [6, 17-19]. And, a study from Ma RX et al. showed that triptolide could effectively reduce proteinuria and remarkably improve podocyte injuries in PHD rats [20], which implicated the potential therapeutic effects of triptolide on proteinuria. In addition, triptolide showed direct protective effect on podocyte injury in rats with PAN-induced nephrosis [7]. But little is known about the effects of triptolide in MN. Zhao-Hong Chen et al. found that triptolide reduced established heavy proteinuria and podocyte injuries in rats with passive Heymann nephritis, and protects podocytes from C5b-9-mediated injury in vitro [6]. Our data reinforce this renoprotection of triptolide by showing that the ability of triptolide reduced proteinuria as well as the titer of circulating rat anti-rabbit IgG antibodies and increased the level of serum albumin in PHN rats. This improvement was paralleled by promotion of synaptopodin, nephrin and podocin expression

and reduction of desmin expression. In addition, podocyte apoptosis was significantly decreased after triptolide treatment.

Recent experimental studies have revealed that the podocyte-protective effects of triptolide by acting on apoptotic [7]. To further clarify the mechanism of its anti-apoptotic action, we examined the influence of triptolide on the expression of caspase-3 and PARP. As a major executor of apoptosis, caspase-3 is considered to be the final downstream protein required for apoptosis. The activation of caspase-3 mediates cleavage of various substrates to trigger apoptosis. PARP is a downstream target of caspase-3, which is a useful hallmark of cell death [21, 22]. Therefore, inhibiting extensive activation of caspase-3 and PARP may be the key to decreasing podocyte cell apoptosis. We observed that the upregulated protein levels of caspase-3, cleaved caspase-3, PARP, and cleaved PARP were significantly inhibited by triptolide treatment in PHN rats and in a C5b-9 injury model of podocytes. This effect may be an important anti-apoptotic mechanism for triptolide.

Several studies have implicated a critical role of the MAPK in pathological conditions including nephropathy. Extracellular signal-regulated kinases (ERKs), c-Jun N-terminal kinases (JNKs) and p38 constitute conventional MAPKs, each of which contributes differentially to pro- and anti-apoptotic pathways [23, 24]. A study performed by Ming-Hua Zhang et al. showed that ginsenoside-Rg1 protected podocytes from complement-mediated injury partly by inhibiting the activation of MAPK. And Chen et al. found that triptolide prevented podocyte from damage by inhibiting p38 MAPK phosphorylation [6]. Interestingly, we found that C5b-9 stimulated a strong increase in phosphorylated ERK and JNK in MPCs, and triptolide could protect MPCs through effective inhibition of this activation. In contrast, triptolide treatment had no significant effect on phosphorylated p38. While the JNK and p38 pathways are often associated with induction of apoptosis, the ERK pathway signaling is thought to protect cells from apoptosis [25-27]. ERK promotes cell survival during development and tissue homeostasis by phosphorylation and inhibition of caspase-3 activation [28]. So we believed the inhibition of ERK and JNK pathways is pro-

TECTIVE AGAINST complement-mediated injury induced podocytes apoptosis.

In conclusion, this study demonstrates that triptolide has beneficial effects on PHN rats as it improves kidney function and attenuates cellular apoptosis in the kidneys. Furthermore, triptolide ameliorates C5b-9-induced apoptosis in podocytes by inhibiting MAPK activation in vitro. This renal-protective effect of triptolide is associated with the inactivation of ERK and JNK signaling pathways. Thus, our data implicate triptolide might be a promising drug against podocytes injury in MN.

Disclosure of conflict of interest

None.

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