

Berberine prevents the apoptosis of mouse podocytes induced by TRAF5 overexpression by suppressing NF- κ B activation

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Abstract. Berberine (BBR) has previously been found to exert beneficial effects on renal injury in experimental rats. However, the mechanisms underlying these effects are not yet fully understood. Tumor necrosis factor (TNF) receptor-associated factor 5 (TRAF5) has been demonstrated to mediate the activation of nuclear factor- κ B (NF- κ B), which has been implicated in the pathogenesis of chronic kidney disease (CKD). The aim of this study was to investigate the effects of BBR on kidney injury and the activation of the NF- κ B signaling pathway in mouse podocytes. TRAF5 was found to be overexpressed in patients with CKD and chronic renal failure (CRF) (data obtained from the dataset GSE48944, as well as from patients at Shuguang Hospital). TRAF5 overexpression significantly inhibited cell viability and induced the apoptosis of mouse podocytes. However, BBR prevented the decrease in cell viability and the apoptosis induced by TRAF5 overexpression. The NF- κ B inhibitor, caffeic acid phenethyl ester (CAPE), mimicked the protective effects of BBR, as evidenced by the increased expression of nephrin and podocin, and the decreased expression of caspase-3 and the ratio of Bax/Bcl-2. Moreover, BBR prevented the decrease in cell viability decrease and the apoptosis induced by TNF- α , interleukin (IL)-6 and lipopolysaccharide (LPS). Taken together, our data indicate that BBR exerts protective effects against CRF partly through the TRAF5-mediated activation of the NF- κ B signaling pathway in mouse podocytes.

Introduction

Chronic renal failure (CRF) is considered the most severe outcome of chronic kidney disease (CKD) and is defined

by a glomerular filtration rate (GFR) persistently below 15 ml/min/1.73 m², and represents the end-stage of CKD requiring treatments, such as dialysis or transplantation. Cardiovascular disease and infection are two most frequent causes of death in patients with CRF (1). There are also other causes of death for such patients, which vary and these include cancer, cachexia, death attributable to social factors and other unknown causes (1-3). Kidney failure results in a decline in renal function, as evidenced by neurohumoral and metabolic abnormalities and the accumulation of damaging molecules, metabolic acidosis, electrolyte abnormalities and volume overload. Large observational databases have identified many hypothesis-generating risk factors for mortality in CRF (1,4). Despite some novel biomarkers which have been implicated in the risk of mortality (5), their effects on outcomes when used for therapeutic decisions have been insufficiently identified.

Berberine (BBR), the major pharmacological constituent of *Coptis chinensis*, is a type of isoquinoline alkaloid used as a therapeutic agent in the treatment of cancer, bacterial infections, diabetes, and cardiovascular and inflammatory diseases (6-8). Furthermore, accumulating evidence suggests that BBR can effectively inhibit cell proliferation and induce apoptosis, and that it has antioxidant properties (9,10). Therefore, the present study aimed to examine the effects of BBR on the proliferation and apoptosis of mouse podocytes.

Tumor necrosis factor (TNF) receptor (TNFR)-associated factors (TRAFs) were originally identified as signal-transducing molecules for TNFR, but have also been linked to downstream signaling via other receptors, such as interleukin (IL)-1 receptor (11,12). To date, 7 members of the TRAF family have been described. TRAF2, TRAF5 and TRAF6 have been demonstrated to mediate the activation of nuclear factor- κ B (NF- κ B) by interacting with the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK1/2) pathway, the TNF- β -activated kinase, or the atypical protein, thus being implicated in the regulation of cell death and cellular responses to stress (13,14). The TNF- α -induced activation of NF- κ B has been shown to be severely inhibited in murine embryonic fibroblasts (MEFs) derived from TRAF5 knockout mice (15). Moreover, it has been reported that the TRAF5-induced activation of NF- κ B is involved in glioma cell migration and invasion (16). However, whether the TRAF5-

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induced NF- κ B activation is involved in proliferation and apoptosis remains unknown.

In the present study, we examined the effects of BBR on mouse podocyte viability and apoptosis. We found that BBR prevented the induction of cell apoptosis induced by TRAF5 overexpression in mouse podocytes by suppressing NF- κ B activation. Therefore, our results suggest that BBR plays an important role in the proliferation and apoptosis of mouse podocytes, and thus TRAF5 may be a potential therapeutic target in CKD.

Materials and methods

Bioinformatics analysis. The array expression data of TRAF5 for 13 patients with CKD and 12 healthy controls were downloaded from the NCBI Gene Expression Omnibus (GEO, <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series accession no. GSE48944 (17), following the approval of this project by the consortium.

Patient samples. Serum samples were obtained from 30 patients with CRF and 30 healthy controls admitted to Shuguang Hospital, Shanghai, China. Ethical approval for the study was provided by the independent ethics committee, Shuguang Hospital of Shanghai University of Traditional Chinese Medicine. Written informed consent was obtained from all participants in this study. None of these patients had received radiotherapy or chemotherapy prior to obtaining the samples.

Cell culture. Mouse podocytes were obtained from the the BeNa Culture Collection (cat. no. BNCC100668; Beijing, China) and cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 10% penicillin-streptomycin solution and 10 U/ml interferon- γ (IFN- γ), and incubated in a humidified atmosphere at 33°C with 5% CO₂. Following culture for a period of time, the podocytes were cultured in the above-mentioned medium without 10 U/ml IFN- γ and incubated in a humidified atmosphere at 37°C with 5% CO₂ for 10-14 days.

Induction of TRAF5 overexpression in mouse podocytes. pLV-IRES-eGFP, psPAX2 and pMD2G were obtained from Addgene (Cambridge, MA, USA). Commercial TRAF5 expression vectors were obtained from Genewiz Biotechnology (Suzhou, China). The TRAF5 expression sequence was cloned into the pLV-IRES-eGFP lentiviral vector. The blank pLV-IRES-eGFP lentiviral vector used as the negative control (NC). 293T cells (ATCC, Manassas, VA, USA) were seeded in 60 mm culture dishes, and after 24 h, they were co-transfected with 2 μ g of the plasmid vector, 1 μ g pLV-IRES-eGFP-TRAF5/pLV-IRES-eGFP, 0.1 μ g psPAX2 and 0.9 μ g pMD2G using Lipofectamine 2000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. The recombinant lentivirus was collected 48 h after transfection and used to infect the mouse podocytes.

Cell treatment. Following the induction of TRAF5 overexpression in mouse podocytes, the mouse podocytes were treated with various concentrations of BBR (10, 30 and 90 μ M; Sigma-Aldrich, St. Louis, MO, USA) and cell viability was measured by CCK-8 assay to obtain the optimal concentration of BBR. To examine the effects of BBR and NF- κ B on cell viability, apop-

toxis and related protein expression, the mouse podocytes were treated with 30 μ M BBR or 25 μ g/ml of the NF- κ B inhibitor, caffeic acid phenethyl ester (CAPE; Selleck, Shanghai, China) for 0, 24, 48 and 72 h (for cell viability assay) or for 48 h (for cell apoptosis assay). To examine the effects of TNF- α , IL-6 and LPS on cell viability, apoptosis and related protein expression, the mouse podocytes were treated with 100 ng/ml TNF- α (PeproTech, Rocky Hill, NJ, USA), 100 U/ml IL-6 (PeproTech) or 100 ng/ml LPS (Sigma-Aldrich) for 0, 24, 48 and 72 h (for cell viability assay) or for 48 h (for cell apoptosis assay) in the absence or presence of 30 μ M BBR treatment.

Reverse transcription and real-time PCR. Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies, Carlsbad, CA, USA) as previously described (18). Complementary DNA was synthesized using a cDNA synthesis kit (Thermo Fisher Scientific Inc.). Real-time PCR was performed using a standard SYBR-Green PCR kit (Takara Biotechnology Co., Ltd., Dalian, China) and an ABI 7500 (Applied Biosystem Life Technologies, Foster City, CA, USA) thermal cycler. The primers used were as follows: TRAF5 forward, 5'-CACTCCGTGCTTCACAAC-3' and reverse, 5'-AAGGTGGTCCTGGAATCG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward, 5'-ATCACTGCCACCCAGAAG-3' and reverse, 5'-TCCACGACGGACACATTG-3'. GAPDH was used as an internal control for normalization. The gene expression was calculated using the 2^{- $\Delta\Delta C_q$} method, as previously described (19).

Cell counting kit-8 (CCK-8) assay. Mouse podocytes infected with pLV-IRES-eGFP-TRAF5 (3x10³/well) were plated in 96-well plates. Following treatment as indicated for 0, 24, 48 and 72 h, 10% of CCK-8 solution (Dojindo Molecular Technologies, Kumamoto, Japan) diluted in serum-free RPMI-1640 was mixed in each well for a further 1 h. The optical density 450 nm value in each well was determined by a microplate reader (SM600 LabSystem; Shanghai Utrao Medical Instrument Co., Ltd., Shanghai, China).

Apoptosis assay. Mouse podocytes infected with pLV-IRES-eGFP-TRAF5 infection (5x10⁵/well) were plated in 6-well plates. Following treatment as indicated for 48 h, the mouse podocytes were collected and incubated with 195 μ l Annexin V-fluorescein isothiocyanate (FITC) and 5 μ l propidium iodide (PI) for 15 min in the dark at 4°C, prior to analysis by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. Mouse podocytes were seeded at a density of 5x10⁵ cells/well in 6-well plates, cultured overnight and then treated as indicated for 3 or 48 h. Total proteins were isolated from the mouse podocytes and were subjected to 12% glyceraldehyde 3-phosphate dehydrogenase (SDS-PAGE) and electroblotted onto polyvinylidene fluoride membranes (Roche Diagnostics, Mannheim, Germany). The membranes were first incubated with rabbit polyclonal anti-Bax (1:300; Sc-493, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-caspase-3 (1:500; ab44976, Abcam, Cambridge, MA, USA) and anti-nephrin (1:500; ab58968, Abcam) antibodies; rabbit monoclonal anti-TRAF5 (1:1,000; ab137763, Abcam), anti-p-NF- κ B p65 (1:1,000; #3033), anti-NF- κ B p65 (1:1,000; #8242) (both from Cell Signaling Technology, Danvers, MA, USA), anti-

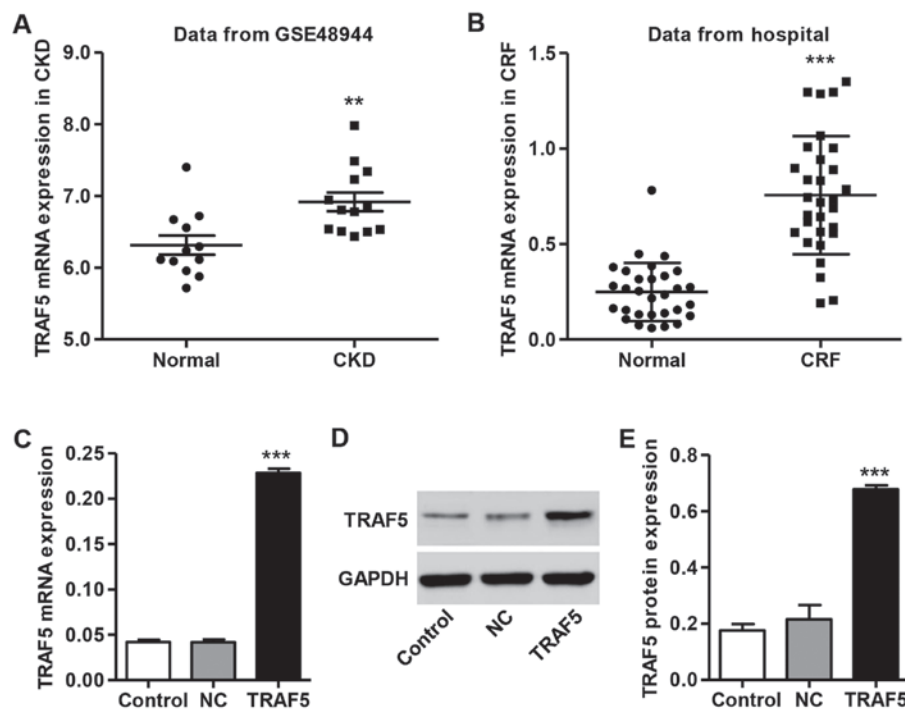


Figure 1. TNF receptor-associated factor 5 (TRAF5) levels in peripheral blood of patients with chronic kidney disease (CKD) and chronic renal failure (CRF). (A) The TRAF5 levels in peripheral blood of patients with CKD (n=13) and healthy controls (n=12) were measured by bioinformatics analysis. (B) The TRAF5 levels in peripheral blood of patients with CRF (n=30) and healthy controls (n=30) were measured by real-time PCR. Mouse podocytes were infected with pLV-IRES-eGFP-TRAF5 for 48 h. (C) The mRNA level of TRAF5 was measured by real-time PCR. (D and E) The protein level of TRAF5 was measured by western blot analysis. **P<0.01 and ***P<0.001 vs. normal and negative control (NC).

podocin (1:10,000; ab181143, Abcam) and anti-GAPDH (1:1500; #5174; Cell Signaling Technology) antibodies; and mouse monoclonal anti-Bcl-2 (1:400; ab117115, Abcam) antibody. The blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody (1:1,000; A0208 and A0216, Beyotime Institute of Biotechnology, Haimen, China) and visualized using enhanced chemiluminescence (ECL; Thermo Fisher Scientific). GAPDH antibody was used as an internal control. The blotting bands were quantified with ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All data are expressed as the means \pm SD and representative of experiments were carried out in triplicate analyzed with SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The unpaired, two-tailed Student's t-test and ANOVA followed by Tukey's post hoc test were used to analyze the significance of differences between groups. Differences were considered significant if the probability (P)-value was <0.05.

Results

TRAF5 levels in the peripheral blood of patients with CKD and CRF. In order to examine the role of TRAF5 in kidney disease, we first measured the levels of TRAF5 in the peripheral blood of patients with CKD (n=13) and healthy controls (n=12) using the data from the GSE48944 database. As shown in Fig. 1, TRAF5 mRNA expression was significantly increased in patients with CKD compared with the healthy controls. Furthermore, we also detected the levels of TRAF5 in the peripheral blood of patients with CRF (n=30) and healthy controls (n=30) from the

Shuguang Hospital database. As shown in Fig. 1B, similar to the data from the GSE48944 database, the mRNA expression of TRAF5 was significantly increased in patients with CRF (average, 0.756 and median, 0.726) compared with the healthy controls (average, 0.249 and median, 0.244).

To further examine the effects of TRAF5 on kidney function *in vitro*, mouse podocytes were infected with the TRAF5 overexpression vector pLV-IRES-eGFP-TRAF5. As shown in Fig. 1C-E, the expression of TRAF5 was markedly increased at both the mRNA and protein level in the mouse podocytes infected with pLV-IRES-eGFP-TRAF5 compared with the controls. However, the podocytes infected with the blank pLV-IRES-eGFP (NC) vector exhibited no changes in TRAF5 expression.

TRAF5 overexpression inhibits cell viability and induces the apoptosis of mouse podocytes. Following infection with pLV-IRES-eGFP-TRAF5, the mouse podocytes exhibited a significant decrease in cell viability in a time-dependent manner (Fig. 2A). After 72 h of incubation, the viability of the mouse podocytes infected with the TRAF5 overexpression vector was suppressed by $47.03 \pm 0.11\%$ compared with the control group. However, the mouse podocytes infected with the blank pLV-IRES-eGFP (NC) vector exhibited no change in viability compared with the control. Furthermore, we also investigated the role of TRAF5 in the apoptosis of mouse podocytes. As shown in Fig. 2B and C, infection with the pLV-IRES-eGFP-TRAF5 vector increased the apoptosis ($35.9 \pm 0.8\%$) of mouse podocytes compared with the control group ($2.05 \pm 0.6\%$). However, infection of the mouse podocytes with the blank pLV-IRES-eGFP (NC) did not affect

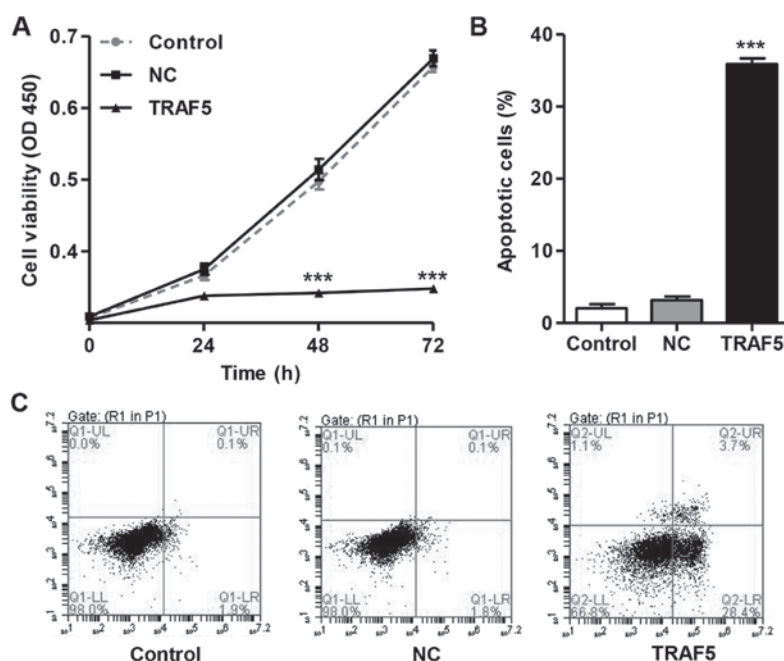


Figure 2. Effect of TNF receptor-associated factor 5 (TRAF5) overexpression on the viability and apoptosis of mouse podocytes. (A) The viability of mouse podocytes was measured by cell counting kit-8 (CCK-8) assay at the indicated time points. (B and C) The apoptosis of mouse podocytes was measured by flow cytometry. *** $P < 0.001$ vs. negative control (NC).

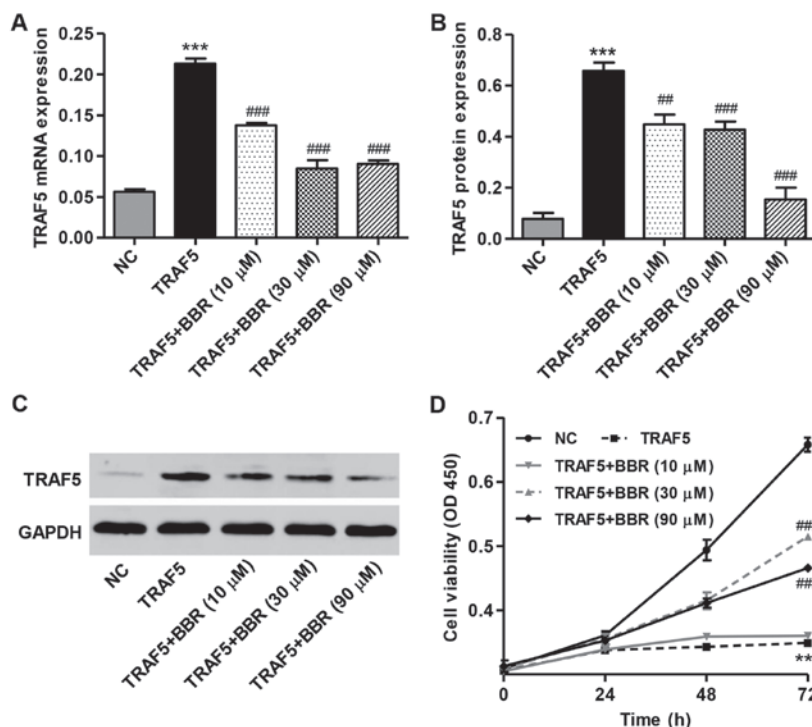


Figure 3. Berberine (BBR) suppresses the inhibition of cell viability and apoptosis induced by TNF receptor-associated factor 5 (TRAF5) overexpression. After the infection of mouse podocytes with pLV-IRES-eGFP-TRAF5, the cells were treated with BBR at the indicated concentrations. (A) The mRNA level of TRAF5 was measured by real-time PCR. (B and C) The protein level of TRAF5 was measured by western blot analysis. (D) The viability of mouse podocytes was measured by cell counting kit-8 (CCK-8) assay at the indicated time points. *** $P < 0.001$ vs. negative control (NC); ** $P < 0.01$ and *** $P < 0.001$ vs. TRAF5.

cell apoptosis compared with the control. These results indicate that TRAF5 overexpression is implicated in the inhibition of the viability and the apoptosis of mouse podocytes.

BBR suppresses TRAF5 expression and prevents the inhibition of cell viability induced by TRAF5. Considering the role

of BBR in renal injury in experimental rats (20), and as the mechanisms underlying the effects of BBR are not yet fully understood, we wished to determine whether BBR also possesses an effect on TRAF5 overexpression in podocytes. As shown in Fig. 3A, BBR (10, 30 and 90 μ M) treatment significantly decreased the expression of TRAF5 at both the

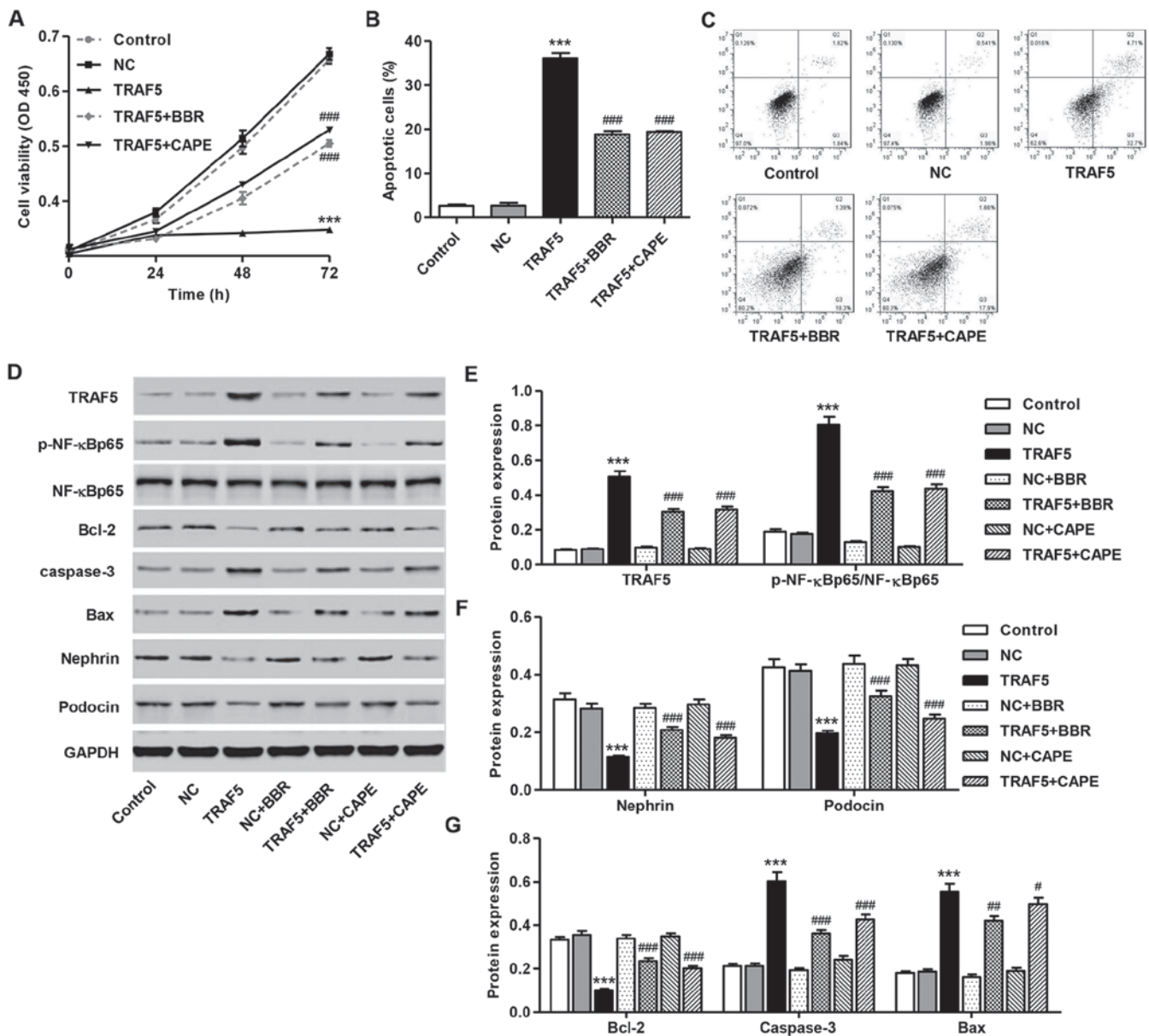


Figure 4. Berberine (BBR) suppresses nuclear factor- κ B (NF- κ B) activation induced by TNF receptor-associated factor 5 (TRAF5) overexpression. (A) The viability of mouse podocytes was measured by cell counting kit-8 (CCK-8) assay at the indicated time points. (B and C) The apoptosis of mouse podocytes was measured by flow cytometry. (D-G) The protein levels of TRAF5, p-NF- κ B p65, NF- κ B p65, nephrin, podocin, Bax, Bcl-2 and caspase-3 were measured by western blot analysis. ***P<0.001 vs. negative control (NC); #P<0.05, ##P<0.01 and ###P<0.001 vs. TRAF5.

mRNA (Fig. 3A) and protein level (Fig. 3B and C) compared with the mouse podocytes infected with the TRAF5 overexpression vector and not treated with BBR. Compared with the podocytes infected with the TRAF5 overexpression vector and not treated with BBR, treatment of the mouse podocytes with various concentrations of BBR for 48 and 72 h increased cell viability in a time-dependent manner (Fig. 3D). After 72 h of incubation, the viability of the mouse podocytes treated with with BBR (30 and 90 μ M) was increased by 47.5 ± 2.6 and $33.5 \pm 1.2\%$, respectively compared with the podocytes infected with the TRAF5 overexpression vector and not treated with BBR. These findings suggest that the downregulation of TRAF5 is involved in the BBR-induced increase in podocyte viability.

BBR suppresses the NF- κ B activation induced by TRAF5 overexpression. Following treatment with 30 μ M BBR or 25 μ g/

ml CAPE for 72 h, the viability of the mouse podocytes was significantly increased by 47.9 ± 2.8 and $52.3 \pm 3.5\%$, respectively compared with the untreated podocytes infected with the TRAF5 overexpression vector (Fig. 4A). Treatment with BBR or CAPE treatment for 48 h also decreased the apoptosis of the mouse podocytes by 47.8 ± 3.9 and $46.1 \pm 2.5\%$, respectively compared with the untreated podocytes infected with the TRAF5 overexpression vector (Fig. 4B and C).

To clarify the effects of TRAF5 on NF- κ B p65 activation *in vitro*, western blot analysis was performed. As shown in Fig. 4D and E, the ratio of p-NF- κ B p65/NF- κ B p65 was significantly decreased following treatment with 30 μ M BBR compared with the untreated podocytes infected with the TRAF5 overexpression vector. Similarly, CAPE (25 μ g/ml), a potent and specific inhibitor of the activation of NF- κ B, mimicked the suppressive effects of BBR on TRAF5 overex-

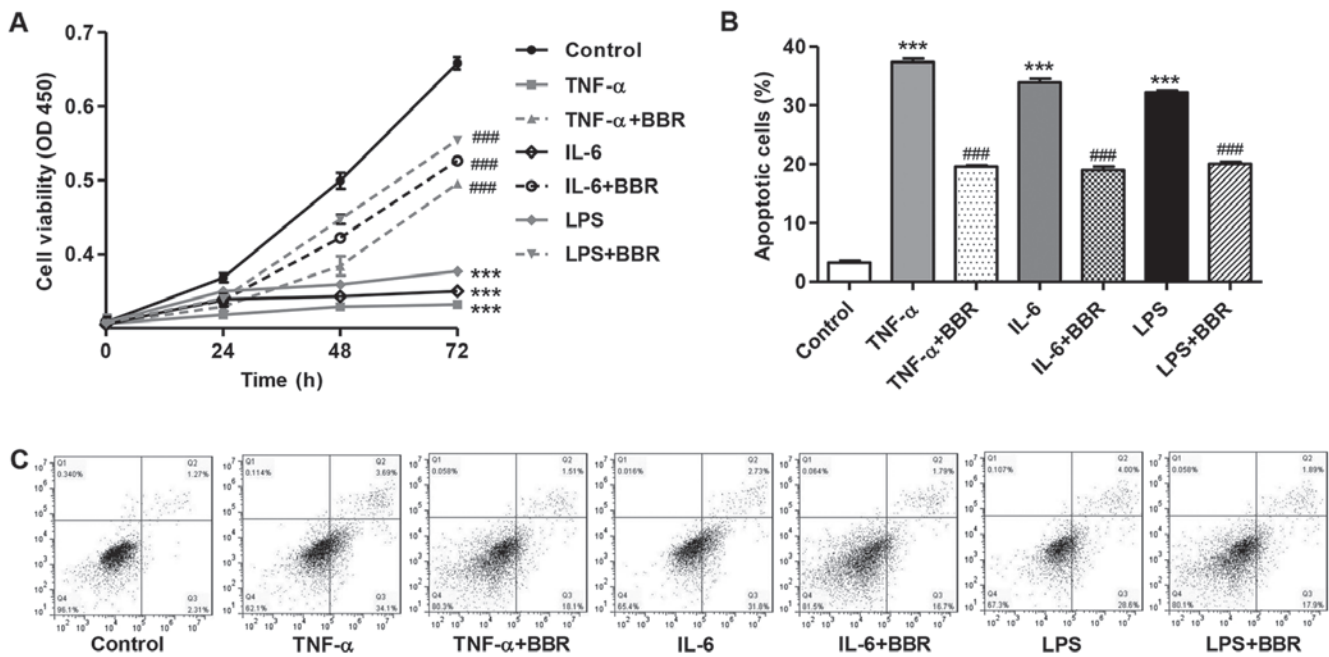


Figure 5. Berberine (BBR) suppresses the inhibition of cell viability and the apoptosis induced by tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and LPS. (A) The viability of mouse podocytes was measured by cell counting kit-8 (CCK-8) assay at the indicated time points. (B and C) The apoptosis of mouse podocytes was measured by flow cytometry. *** $P < 0.001$ vs. control; ### $P < 0.001$ vs. corresponding TNF- α , IL-6 and LPS treatment alone, respectively.

pression and NF- κ B p65 activation in mouse podocytes. These data demonstrate that the inactivation of NF- κ B may contribute to the BBR-induced protective effects on mouse podocytes.

Effect of BBR and CAPE on protein expression induced by TRAF5 overexpression. Following the infection of the mouse podocytes with pLV-IRES-eGFP-TRAF5 for 48 h, the expression of nephrin and podocin was significantly suppressed (Fig. 4D and F), while the Bax/Bcl-2 ratio and caspase-3 levels were significantly increased compared with the control group (Fig. 4D and G). However, treatment with 30 μ M BBR or 25 μ g/ml CAPE for 48 h suppressed the effects induced by TRAF5 overexpression on these protein expression levels in mouse podocytes (Fig. 4D-G).

BBR prevents the inhibition of cell viability and the apoptosis induced by TNF- α , IL-6 and LPS. Treatment with TNF- α (100 ng/ml), IL-6 (100 U/ml) or LPS (100 ng/ml) for 72 h significantly decreased cell viability by 48.0 ± 3.2 , 46.8 ± 2.7 and $45.1 \pm 2.9\%$, respectively compared with the control group (Fig. 5A). However, treatment with 30 μ M BBR markedly prevented the inhibition of cell viability induced by TNF- α , IL-6 or LPS in mouse podocytes. Moreover, treatment with 30 μ M BBR also suppressed the apoptosis induced by TNF- α , IL-6 or LPS by 47.6 ± 2.6 , 43.9 ± 3.3 and $37.7 \pm 1.9\%$, respectively compared with the cells exposed to TNF- α , IL-6 or LPS (Fig. 5B and C). These data suggest that BBR inhibits TNF- α , IL-6- or LPS-induced cytotoxicity in mouse podocytes.

Effect of BBR on NF- κ B activation and protein expressions induced by TNF- α , IL-6 and LPS. As shown in Fig. 6, the ratio of p-NF- κ B p65/NF- κ B p65 and Bax/Bcl-2 and the expression levels of TRAF5 and caspase-3 were significantly increased

by TNF- α , IL-6 or LPS treatment compared with the controls. However, treatment with 30 μ M BBR markedly suppressed the effects of TNF- α , IL-6 or LPS on NF- κ B activation and on the expression levels of these proteins in mouse podocytes. These results indicate that TRAF5 downregulation is implicated in the protective effects of BBR against the effects of TNF- α , IL-6 or LPS in mouse podocytes.

Discussion

This study reports the novel finding that TRAF5 expression was increased in the peripheral blood of patients with CKD and CRF. *In vitro* experiments revealed that TRAF5 overexpression inhibited the viability and induced the apoptosis of mouse podocytes. We further demonstrated that BBR inhibited the negative effects of TRAF5 overexpression by suppressing NF- κ B activation in mouse podocytes. These data provide novel evidence (at least to the best of our knowledge) that BBR protects mouse podocytes from the suppressive effects of TRAF5 on viability and the promoting effects of TRAF5 on apoptosis via the NF- κ B signaling pathway.

It has previously been reported that soluble TRAF5 levels are increased in plasma and peripheral blood mononuclear cells from patients with Crohn's disease and ulcerative colitis (21). Other studies have implicated TRAF5 in carcinogenesis attributable to increased levels in splenic marginal zone lymphoma (22) and Hodgkin-Reed-Sternberg cells (23). Intriguingly, the TRAF5/GAPDH mRNA ratios have been shown to be significantly decreased in the blood of patients with chronic and acute coronary heart disease, supporting the notion that TRAF5 represents a protective marker in atherosclerosis (24). However, the expression of TRAF5 in patients with CKD has not yet been elucidated. To examine the hypothesis that TRAF5 may also be

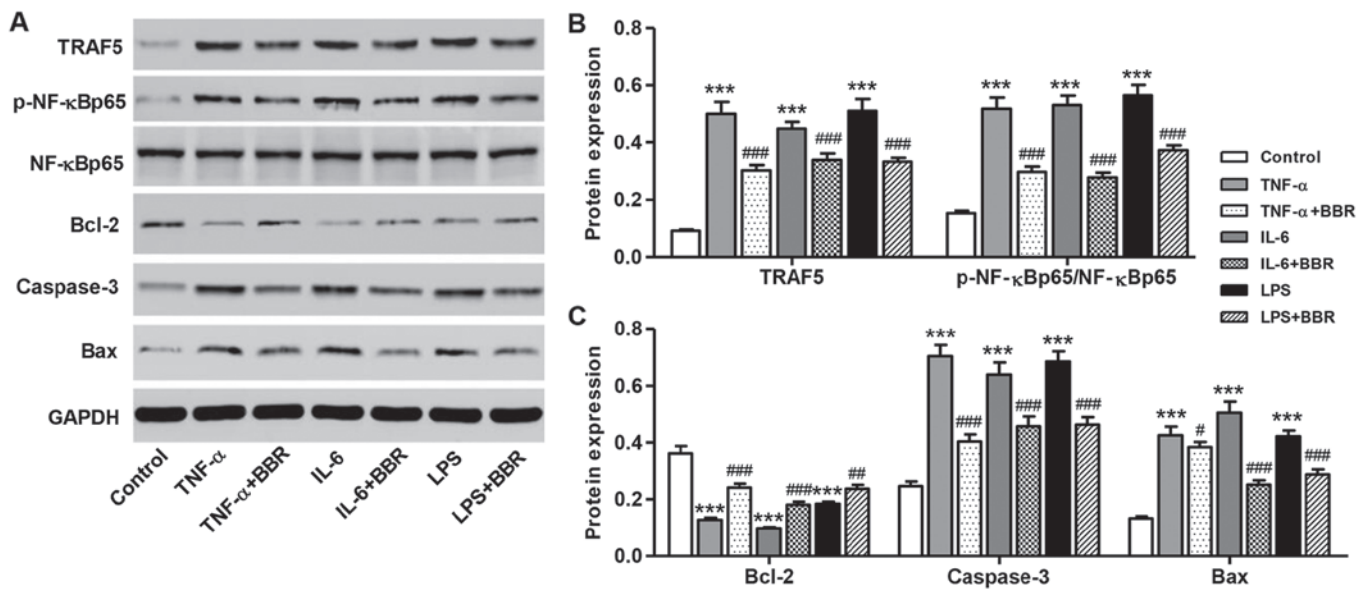


Figure 6. Effect of berberine (BBR) on nuclear factor- κ B (NF- κ B) activation and protein expressions induced by tumor necrosis factor- α (TNF- α), interleukin-6 (IL-6) and LPS. (A-C) The protein levels of TNF receptor-associated factor 5 (TRAF5), p-NF- κ B p65 and NF- κ B p65, Bax, Bcl-2 and caspase-3 were measured by western blot analysis. *** $P < 0.001$ vs. control; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. corresponding TNF- α , IL-6 and LPS treatment alone, respectively.

associated with clinical disease, we performed bioinformatics analysis and a pilot clinical study in patients with CKD and CRF. This study revealed increased peripheral blood levels of TRAF5 in patients with CKD and CRF compared with the healthy controls. As reported previously, TRAF5 knockout had no effect on the viability of MEFs, while MEFs from double TRAF2 and TRAF5 knockout mice exhibited significantly decreased cell viability compared with wild-type and single TRAF2 or TRAF5 knockout mice, suggesting a critical role of TRAF2 rather than TRAF5 in protection from cell death (15). However, as demonstrated in another study, the elimination of TRAF5 expression significantly decreased the migration and invasion of the glioma cells, and although the underlying mechanisms were not elucidated, this may have been due to the inhibition of cell viability and apoptosis induction (16). In the present study, to examine whether the podocyte apoptosis was mediated via TRAF5 overexpression, we infected mouse podocyte with a TRAF5 overexpression vector. We found that TRAF5 overexpression significantly induced mouse podocyte apoptosis.

TRAF5 was originally identified as an activator of interleukin-induced NF- κ B signal transduction via its TRAF domain. NF- κ B comprises a family of transcription factors involved in the regulation of a wide variety of biological responses. It is generally accepted that NF- κ B activation is responsible for apoptosis resistance (25,26). However, there is evidence to support a pro-apoptotic role for NF- κ B. It has been speculated that NF- κ B may have a dual function, either as an inhibitor or an activator of apoptotic cell death, depending on the levels of RelA and c-Rel (27). In this study, TRAF5 overexpression in mouse podocytes led to NF- κ B activation, accompanied by an increased expression of caspase-3 and an increased Bax/Bcl-2 ratio. Within cells, there is a machinery consisting of pro-apoptotic genes (Bax) that promote apoptosis and anti-apoptotic genes (Bcl-2) that function as suppressors of

apoptosis, and the balance between these genes may be a determinant of apoptosis or cell survival. Despite extensive studies in either field, there is only limited information on the role of Bcl-2 and Bax in CKD. *In vivo*, Bcl-2 and Bax proteins have not been detected in the kidneys during ischemia (28), whereas the overexpression of Bcl-2 can suppress the apoptosis of renal tubule cells induced by hypoxia/reoxygenation (29). Moreover, an increase in the Bax/Bcl-2 ratio by hypoxia/reoxygenation or ischemia/reperfusion injury triggers Bax translocation to the mitochondria and cytochrome *c* release to cytoplasm, and enhances caspase-3-mediated renal tubular apoptosis (30). Podocyte damage is a common feature in glomerular diseases with ultrastructural changes, with the reduced expression of components of the slit diaphragm, such as nephrin and podocin (31). The levels of nephrin and podocin have been shown to be significantly reduced in lupus nephritis, with these effects beginning from the earlier stages and becoming more pronounced at advanced histological forms (32). Our data reported that the expression of nephrin and podocin was significantly decreased in mouse podocytes overexpressing TRAF5.

NF- κ B is activated by inflammatory cytokines and cellular stresses, including TNF, IL-1, LPS, UV or γ -irradiation. Thus far, TRAF5-deficient mice do not show substantial defects in TNF- α -induced NF- κ B activation, suggesting that TRAF5 plays a redundant role in TNF- α -induced NF- κ B activation (15). By contrast, TRAF5 acts downstream of ILs, including IL-1 β and IL-6, and plays a key role in IL-1 β /IL-6-mediated NF- κ B activation during glioma migration and invasion (16). In the present study, we observed that the exposure of mouse podocytes to TNF- α , IL-6 and LPS significantly decreased cell viability and induced apoptosis. More importantly, the activation of NF- κ B and the increased Bax/Bcl-2 ratio and caspase-3 expression were also observed in the podocytes exposed to TNF- α , IL-6 and LPS. These findings suggest that TRAF5 induces podocyte

injury, to a certain extent, through NF- κ B activation induced by TNF- α , IL-6 and LPS.

Emerging evidence has indicated that BBR has multiple beneficial effects in the treatment of diabetes and cardiovascular diseases (33,34). However, the protective effects of BBR and its molecular mechanisms of action in CKD and chronic kidney injury remain to be determined. BBR has been shown to attenuate renal injury in diabetic C57BL/6 mice through the suppression of the SphK-S1P signaling pathway (35). Additionally, BBR has been shown to exert protective effects in the presence of high glucose related to the inhibition of glucose-induced apoptosis that in turn upregulates the expression of nephrin and podocin (36). In agreement with the previous study, our results demonstrated that BBR increases cell viability and inhibits apoptosis by upregulating the expression of nephrin and podocin, and downregulating the expression of caspase-3 and the Bax/Bcl-2 ratio. The NF- κ B inhibitor, CAPE, mimicked the protective effects of BBR. Moreover, the NF- κ B activation induced by TRAF5 overexpression and exposure to TNF- α , IL-6 or LPS was significantly inhibited by BBR, which is in line with the findings of a previous study that BBR ameliorates intrarenal inflammation and tubulointerstitial injury, at least in part, through the suppression of the NF- κ B signaling pathway (37).

In conclusion, in this study, we demonstrate that TRAF5 is overexpressed in CRF and inhibits the viability and induces the apoptosis of mouse podocytes. Treatment of TRAF5-overexpressing mouse podocytes with BBR suppressed the inhibition of viability, prevented apoptosis, decreased the Bax/Bcl-2 and caspase-3 expression, and increased the expression of nephrin and podocin. Such effects appear to be mediated by the inhibition of NF- κ B activation. Thus, BBR may play an important role in delaying the progression of chronic kidney injury by preserving renal structure and function in patients with CRF.

Acknowledgements

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