ROCK2 knockdown alleviates LPS-induced inflammatory injury and apoptosis of renal tubular epithelial cells via the NF-κB/NLRP3 signaling pathway

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Received December 14, 2021; Accepted June 30, 2022

DOI: 10.3892/etm.2022.11540

Abstract. Rho-associated protein kinase 2 (ROCK2) is an important regulator of the inflammatory response and has been reported to serve a role in sepsis. The present study aimed to investigate whether ROCK2 served a role in sepsis-associated acute kidney injury (S-AKI). HK-2 cells were stimulated with lipopolysaccharide (LPS) to simulate S-AKI *in vitro*. Subsequently, the change in ROCK2 expression levels were determined. ROCK2 in LPS-induced HK-2 cells was knocked down using short hairpin RNA-ROCK2, in the absence or presence of phorbol 12-myristate 13-acetate (PMA), an activator of NF-κB. Cell viability, cytotoxicity, inflammation and apoptosis were assessed using MTT, lactate dehydrogenase (LDH) release, reverse transcription-quantitative PCR, ELISA, TUNEL and western blotting assays. The protein expression levels of proteins involved in the NF-κB/NLR family pyrin domain containing 3 (NLRP3) signaling pathway were also assessed using western blotting. The results demonstrated that ROCK2 was upregulated in HK-2 cells upon LPS treatment. LPS also reduced cell viability, promoted LDH activity and increased TNF-α, IL-6 and IL-1β mRNA expression levels and concentrations. Apoptosis was also induced by LPS as indicated by an increase in the proportion of TUNEL-positive cells, decreased Bcl-2 protein expression levels and increased cleaved caspase-3 and cleaved poly (ADP-ribose) polymerase protein expression levels. However, ROCK2 knockdown in LPS-induced HK-2 cells reversed cell viability damage and inhibited LDH activity, the generation of pro-inflammatory cytokines and apoptosis caused by LPS. Furthermore, ROCK2 knockdown inhibited the LPS-induced expression of phosphorylated-NF-κB p65, NLRP3, apoptosis-associated speck-like protein containing a CARD and caspase-1 p20. PMA treatment reversed all the aforementioned effects of ROCK2 knockdown on LPS-treated HK-2 cells. Therefore, ROCK2 knockdown may alleviate LPS-induced HK-2 cell injury via the inactivation of the NF-κB/NLRP3 signaling pathway.

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Key words: acute kidney injury, NF-κB, Rho-associated protein kinase 2, sepsis

Introduction

Sepsis is a life-threatening immune response caused by a dysregulated reaction to infection, and can lead to organ dysfunction (1). The kidney is one of the most commonly affected organs during sepsis, which can result in sepsis-associated acute kidney injury (S-AKI). This condition contributes to the morbidity and the high mortality rate of sepsis, which is close to 50% within 90 days of intensive care admission (2,3). Although the pathophysiological mechanisms underlying S-AKI remain to be fully elucidated, it is widely accepted that deleterious inflammatory cascade responses during sepsis can contribute to AKI (1,4). Moreover, innate immunity-mediated inflammation and the consequent oxidative stress and apoptosis are closely associated with AKI damage of renal tubular epithelial cells (RTECs) (1,4).

Toll-like receptors (TLRs), which are activated following lipopolysaccharide (LPS) binding, induce downstream inflammatory signaling cascades and therefore serve a key role in innate immunity (5). Previous studies have demonstrated that LPS significantly enhances the expression of Rho-associated protein kinase (ROCK)1 and ROCK2 in human umbilical vein endothelial cells (6) and retinal pigment epithelium (7). ROCK2 is an important regulator of the inflammatory response and serves a role in sepsis. For example, TNF and heterogeneous nuclear ribonucleoprotein L-related immunoregulatory long non-coding RNA aggravates sepsis-induced acute lung injury by regulating ROCK2 expression (8). Another study reported that ROCK inhibitors can potentially be used to overcome sepsis-induced deleterious effects in the brain (9). In a mouse model of sepsis, ROCK2 was involved in the effect of heparin sodium on endotoxin-induced lung vascular leakages (10). However, whether ROCK2 serves a role in S-AKI is unclear.

The NF-κB signaling pathway has long been considered a prototypical proinflammatory signaling pathway and it serves an important role in the pathogenesis of organ injury

induced by sepsis (11). Furthermore, ROCK2 exhibits its inducible effect on the inflammatory response via NF-κB signaling pathway activation (12). In the present study, the role of ROCK2 in LPS-induced RTEC inflammation and apoptosis as well as the underlying mechanisms were investigated.

Materials and methods

Cell culture and treatment. The human RTEC HK-2 cell line was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in Keratinocyte Serum-Free medium (K-SFM; cat. no. 17005-042; Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and gentamicin/amphotericin solution (500X; Gibco; Thermo Fisher Scientific, Inc.) in a 37˚C humidified atmosphere with 5% CO_2 and 95% air.

For establishment of the S-AKI cell model, HK-2 cells were stimulated with 10 μg/ml LPS (Sigma-Aldrich; Merck KGaA) for 24 h at 37˚C (13). Phorbol 12-myristate 13-acetate (PMA; DC Chemicals) is an activator of NF-κB (14). To confirm whether PMA induces cytotoxicity to HK-2 cells, the cell culture medium of HK-2 cells was replaced with fresh complete culture medium containing different concentrations (5, 10, 20, 40 and 80 ng/ml) of PMA for 24 h at 37˚C. For further NF-κB activation, the cell culture medium of LPS-induced HK-2 cells was replaced with fresh medium containing 20 ng/ml PMA for 24 h at 37˚C (15). The cell culture medium of LPS-induced HK-2 cells was replaced with fresh medium containing 0.1% DMSO as a solvent control for 24 h at 37˚C.

Cell transfection. pGPU6 plasmids (cat. no. C02001; Shanghai GenePharma Co., Ltd.) containing short hairpin (sh)RNA-ROCK2#1 (5'-ATCAGACAGCATCCTTTCT-3') and #2 (5'-GCAAATCTGTTAATACTCG-3') or the scrambled shRNA-negative control (shRNA-NC; 5'-GGACTACTCTAG ACGTATA-3') were designed by Shanghai GenePharma Co., Ltd. All plasmids (50 nM) were transfected into cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) for 24 h at 37˚C. Transfection efficiency was evaluated after 48 h of transfection at 37˚C via reverse transcription-quantitative PCR (RT-qPCR) and western blotting.

Cell viability assay. Cell viability was quantified using an MTT Assay kit (cat. no. ab211091; Abcam). HK-2 cells were seeded into 96-well plates at a density of $1x10^5$ cells/well and then cultured in complete K-SFM with LPS and PMA or DMSO. After incubation for 24 h at 37˚C, the medium was replaced with 50 μl serum-free K-SFM and 50 μl MTT reagent was added. After incubation for 3 h at 37˚C, 150 μl DMSO was added to each well and the cells were incubated at 37˚C for a further 15 min on an orbital shaker. Subsequently, the optical density at 590 nm was determined using a microplate reader (Multiskan FC Microplate Photometer; cat. no. 51119180; Thermo Fisher Scientific, Inc.).

Lactate dehydrogenase (LDH) assay. An LDH Assay kit (cat. no. ab65393; Abcam) was used to assess the level of cell plasma membrane damage or cytotoxicity. Briefly, HK-2 cells were seeded into 96-well plates at a density of $5x10^4$ cells/well and were then cultured in complete K-SFM with LPS and PMA or DMSO. Following incubation for 24 h at 37˚C, the cell culture medium was centrifuged at 600 x g at 4˚C for 10 min and the supernatant was transferred into another 96-well plate. Subsequently, 100 μl LDH reaction mix was added to each well and incubated at room temperature for 30 min. The optical density at 490 nm was determined using a microplate reader (Multiskan FC Microplate Photometer; Thermo Fisher Scientific, Inc.).

ELISA. Inflammatory cytokine levels, including TNF-α, IL-6 and IL-1β in the cell supernatant were quantified using ELISA kits for TNF-α (cat. no. ab181421; Abcam), IL-6 (cat. no. ab178013; Abcam) and IL-1β (cat. no. ab100562; Abcam) according to the manufacturer's protocol.

Western blotting. Total protein was extracted from cells using RIPA lysis buffer (Beyotime Institute of Biotechnology) and was subsequently quantified using a BCA Protein Concentration Assay kit (Beyotime Institute of Biotechnology). Equal quantities of total protein (50 μg) were separated via SDS-PAGE on 10-12% gels. Separated proteins were electro-transferred onto a PVDF membrane (MilliporeSigma). Subsequently, membranes were blocked with 5% non-fat milk at room temperature for 2 h and were then incubated with primary antibodies overnight at 4˚C. Following the primary incubation, membranes were incubated with an HRP goat anti-rabbit IgG secondary antibody (cat. no. ab6721; 1:10,000; Abcam) at 37˚C for 1 h. Proteins were visualized using an ECL reagent kit (Shanghai Yeasen Biotech Co., Ltd.) and were semi-quantified using ImageJ software (1.46r; National Institutes of Health). The rabbit primary antibodies used were as follows: ROCK2 (cat. no. ab125025; 1:10,000), Bcl-2 (cat. no. ab32124; 1:1,000), Bax (cat. no. ab32503; 1:1,000), cleaved caspase 3 (cat. no. ab32042; 1:500), caspase 3 (cat. no. ab32351; 1:5,000), cleaved poly (ADP-ribose) polymerase (PARP; cat. no. ab32064; 1:1,000), PARP (cat. no. ab191217; 1:1,000), phosphorylated (p)-NF-κB p65 (cat. no. ab76302; 1:1,000), NF-κB p65 (cat. no. ab32536; 1:1,000), NLR family pyrin domain containing 3 (NLRP3; cat. no. ab263899; 1:1,000), apoptosis-associated speck-like protein containing a CARD (ASC; cat. no. ab283684; 1:1,000) (all from Abcam), caspase 1 p20 (cat. no. 22915-1-AP; 1:1,000; ProteinTech Group, Inc.), procaspase 1 (cat. no. ab207802; 1:1,000), kidney injury molecule-1 (KIM-1; cat. no. ab228973), neutrophil gelatinase-associated lipocalin (NGAL; cat. no. ab125075; 1:1,000) and GAPDH (cat. no. ab9485; 1:2,500) (all from Abcam).

RT-qPCR. Total RNA was extracted from cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Reverse transcription was performed using a 5X All-In-One RT Master Mix first strand cDNA synthesis kit (Applied Biological Materials, Inc.) according to the manufacturer's protocol. qPCR was performed using a QuantiTect SYBR Green PCR Kit (Qiagen AB; 10 μl reaction volume) and the Roche LightCycler® 480 System (Roche Diagnostics GmbH). The following thermocycling conditions were used: Pre-denaturation at 95˚C for 10 sec; followed by 40 cycles of denaturation at 95˚C for 15 sec and annealing at 75˚C for 20 sec; and a final extension of 10 min at 72˚C. The mRNA

expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (16) and normalized to GAPDH. The following primers were used: ROCK2 forward (F), 5'-TCCCGATAACCACCCCTCTT-3' and reverse (R), 5'-CCAAGGAATTTAAGCCATCCACT-3'; TNF-α F, 5'-GCTGCACTTTGGAGTGATCG-3' and R, 5'-CTTGTCACTCGGGGTTCGAG-3'; IL-6 F, 5'-AGTGAGGAACAAGCCAGAGC-3' and R, 5'-AGCTGCGCAGAATGAGATGA-3'; IL-1β F, 5'-CAGAAGTACCTGAGCTCGCC-3' and R, 5'-AGATTCGTAGCTGGATGCCG-3'; and GAPDH F, 5'-AATTCCATGGCACCGTCAAG-3' and R, 5'-TGGACTCCACGACGTACTC-3'.

TUNEL assay. Apoptosis was detected using an *In Situ* Cell Death Detection kit (cat no. 11684817910; Roche Diagnostics GmbH) according to the manufacturer's protocol. Briefly, cultured cells were fixed with 4% formaldehyde at room temperature for 30 min. Next, the cells were incubated with 1% Triton X-100. Subsequently, the cells were mixed with 50 μl TUNEL reaction mixture containing biotin-11-dUTP and TdT Enzyme, and then with streptavidin fluorescein at 37˚C for 30 min. The nuclei were counterstained using 4',6-diamidino-2-phenylindole at room temperature for 10 min in the dark. Slides were mounted using glycerol. TUNEL-positive cells were determined in three random fields of view using an Olympus BX60 fluorescence microscope (Olympus Corporation) equipped with a digital charge-coupled device. Apoptosis (%) was quantified as follows: Number of TUNEL-positive nuclei/total number of cells x100.

Statistical analysis. Experiments were performed in triplicate at minimum. Data are presented as the mean ± SD and were analyzed using GraphPad Prism 8.0 (GraphPad Software, Inc.). For statistical analysis, pairwise comparisons between two groups were analyzed using the unpaired Student's t-test. One-way ANOVA followed by Tukey's post hoc test was used for comparisons between >2 groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

ROCK2 knockdown protects HK-2 cells against LPS-induced damage. ROCK2 expression levels in HK-2 cells following LPS stimulation were investigated. As presented in Fig. 1A and B, both ROCK2 mRNA and protein expression levels in HK-2 cells were significantly increased by LPS, which indicated the potential role of ROCK2 in LPS-induced HK-2 injury. Subsequently, HK-2 cells and LPS-induced HK-2 cells were all respectively transfected with shRNA-ROCK2 to silence ROCK2 expression. The transfection efficiency was determined at the mRNA and protein expression levels and shRNA-ROCK2#2 was selected for subsequent experiments based on its greater efficiency compared with shRNA-ROCK2#1, as demonstrated in Fig. 1C-F. Moreover, cell viability was determined. The results presented in Fig. 1G and H demonstrated that LPS resulted in a significant decrease in cell viability and significant increase in LDH activity compared with the control, which suggested that LPS may be cytotoxic to HK-2 cells. However, LPS-treated HK-2 cells that were transfected with shRNA-ROCK2 exhibited significantly increased cell viability along with significantly decreased LDH activity compared with the LPS + shRNA-NC group. These results indicated the inhibitory effect of ROCK2 silencing on LPS cytotoxicity on HK-2 cells.

ROCK2 knockdown protects HK-2 cells against LPS-induced inflammation and apoptosis. As observed in Fig. 2A and B, both concentrations and mRNA expression levels of pro-inflammatory factors TNF-α, IL-6 and IL-1β were significantly enhanced when treated with LPS compared with control HK-2 cells. However, this increase was significantly inhibited by ROCK2 knockdown. Furthermore, as presented in Fig. 2C, LPS treatment also resulted in a significantly increased apoptosis rate in HK-2 cells compared with the control; whereas ROCK2 knockdown significantly reduced apoptosis caused by LPS in the LPS + shRNA-ROCK2 group compared with the LPS + shRNA-NC group. Similar results are demonstrated in Fig. 3, whereby LPS treatment resulted in a significant reduction in the protein expression levels of the anti-apoptotic protein Bcl-2, but a significant increase in the expression levels of the proapoptotic proteins Bax, cleaved caspase-3 and cleaved PARP compared with the control. However, ROCK2 knockdown in LPS-induced HK-2 cells significantly increased Bcl-2 protein expression levels and reduced Bax, cleaved caspase-3 and cleaved PARP protein expression levels compared with the LPS + shRNA-NC group. These results indicated that ROCK2 knockdown may suppress LPS-induced inflammation and apoptosis in HK-2 cells.

ROCK2 knockdown inhibits the activation of NF-κB/NLRP3 signaling induced by LPS in HK-2 cells. To investigate whether ROCK2 knockdown exerted the aforementioned effects on LPS-induced HK-2 cells by regulating the NF-κB signaling pathway, the protein expression levels of NF-κB and its associated downstream proteins were analyzed. As presented in Fig. 4A, the results from the western blotting analysis demonstrated that the protein expression levels of p-NF-κB p65, NLRP3, ASC and caspase-1 p20 were significantly increased when treated with LPS compared with the control, which indicated the activation of the NF-κB/NLRP3 signaling pathway. Subsequently, an NF-κB activator, PMA, was used to treat HK-2 cells at different concentrations and the results demonstrated that PMA inhibited the HK-2 cells viability from 20 to 80 ng/ml while the inhibition effect of 20 ng/ml PMA on cell viability was the smallest (Fig. S1). To avoid significant damage to HK-2 cells, 20 ng/ml PMA was used to treat LPS-induced ROCK2 knocked-down HK-2 cells. As presented in Fig. 4B, compared with the LPS + shRNA-ROCK2 group and DMSO + LPS + shRNA-ROCK2 group, PMA treatment significantly increased the protein expression levels of p-NF-κB p65, NLRP3, ASC and caspase-1 p20. This result indicated that the inhibitory effect of ROCK2 knockdown on LPS-induced NF-κB/NLRP3 signaling pathway activation may be partially reversed by PMA.

Activation of NF-κB reverses the effect of ROCK2 knockdown on LPS-treated HK-2 cells. The role of PMA in ROCK2 knockdown on LPS-induced HK-2 cell injury was further explored. The results in Fig. 5A and B demonstrated that PMA led to a significant decrease in cell viability and an increase in LDH activity in the PMA + LPS + shRNA-ROCK2 group compared with the DMSO + LPS + shRNA-ROCK2 group.

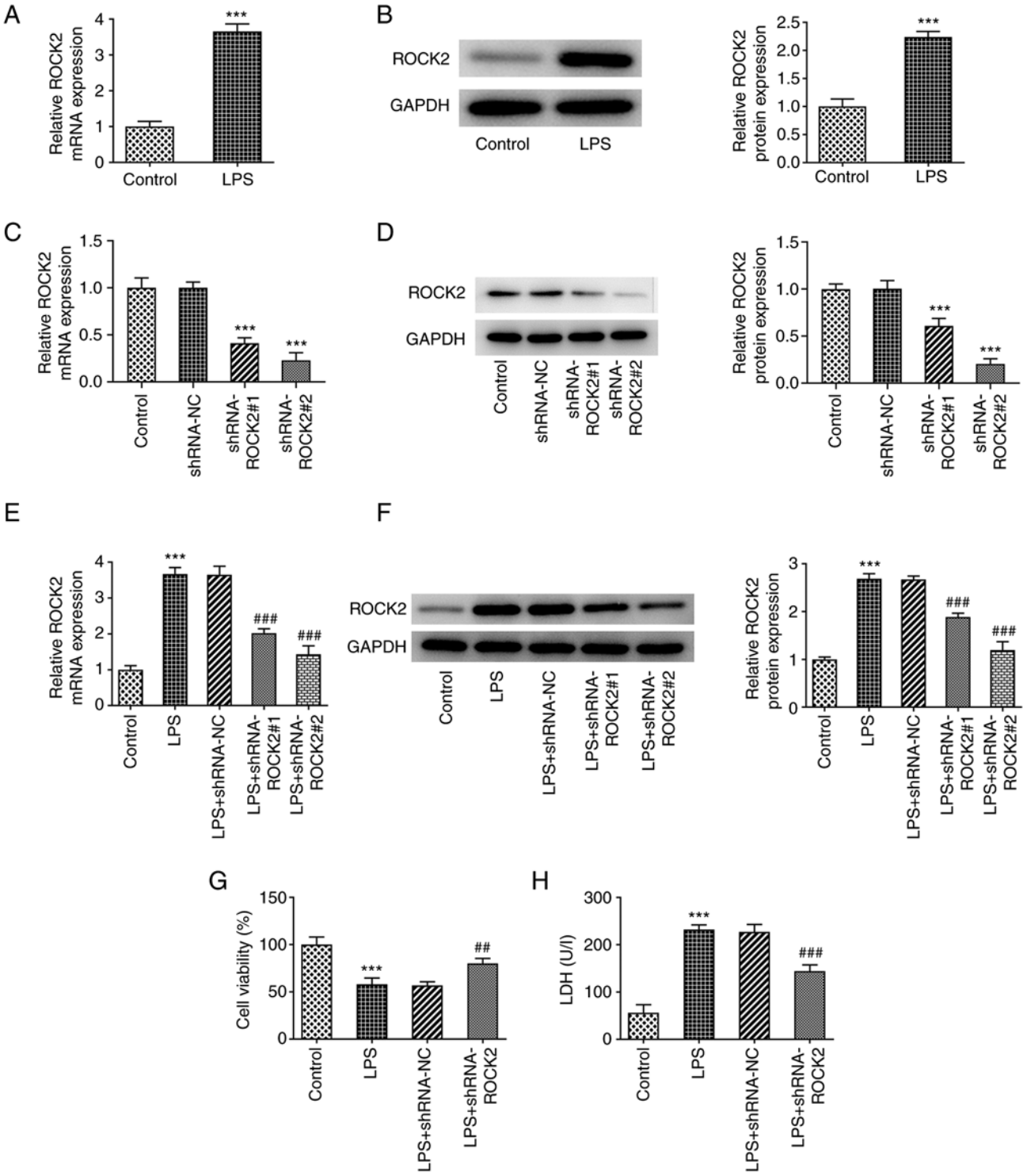

Figure 1. Effect of ROCK2 silencing on LPS-induced HK-2 cytotoxicity. (A) mRNA and (B) protein expression levels of ROCK2 in control and LPS-induced HK-2 cells were measured using RT-qPCR and western blot assays, respectively. (C) mRNA and (D) protein expression levels of ROCK2 in control and HK-2 cells transfected with indicated shRNAs were measured using RT-qPCR and western blot assays, respectively. The (E) mRNA and (F) protein expression levels of ROCK2 in control and LPS-induced HK-2 cells transfected with indicated shRNAs were measured using RT-qPCR and western blot assays, respectively. (G) Cell viability was measured using an MTT assay. (H) LDH production was measured using a commercially available LDH release assay kit. ***P<0.001 vs. control; $^{##}$P<0.01 and $^{###}$P<0.001 vs. LPS + shRNA-NC. ROCK2, Rho-associated protein kinase 2; LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative PCR; sh, short hairpin; LDH, lactate dehydrogenase; NC, negative control.

Moreover, as presented in Fig. 5C and D, the LPS-stimulated increase in the concentration and mRNA expression levels of TNF-α, IL-6 and IL-1β in HK-2 cells, which were reduced by shRNA-ROCK2, were subsequently significantly enhanced by PMA treatment. Furthermore, PMA significantly enhanced the apoptotic rate of HK-2 cells in the PMA + LPS + shRNA-ROCK2 group compared with the DMSO + LPS + shRNA-ROCK2 group (Fig. 5E). The effect of ROCK2 knockdown on the protein expression levels of apoptosis-associated proteins, including Bcl-2, Bax, cleaved caspase-3 and cleaved

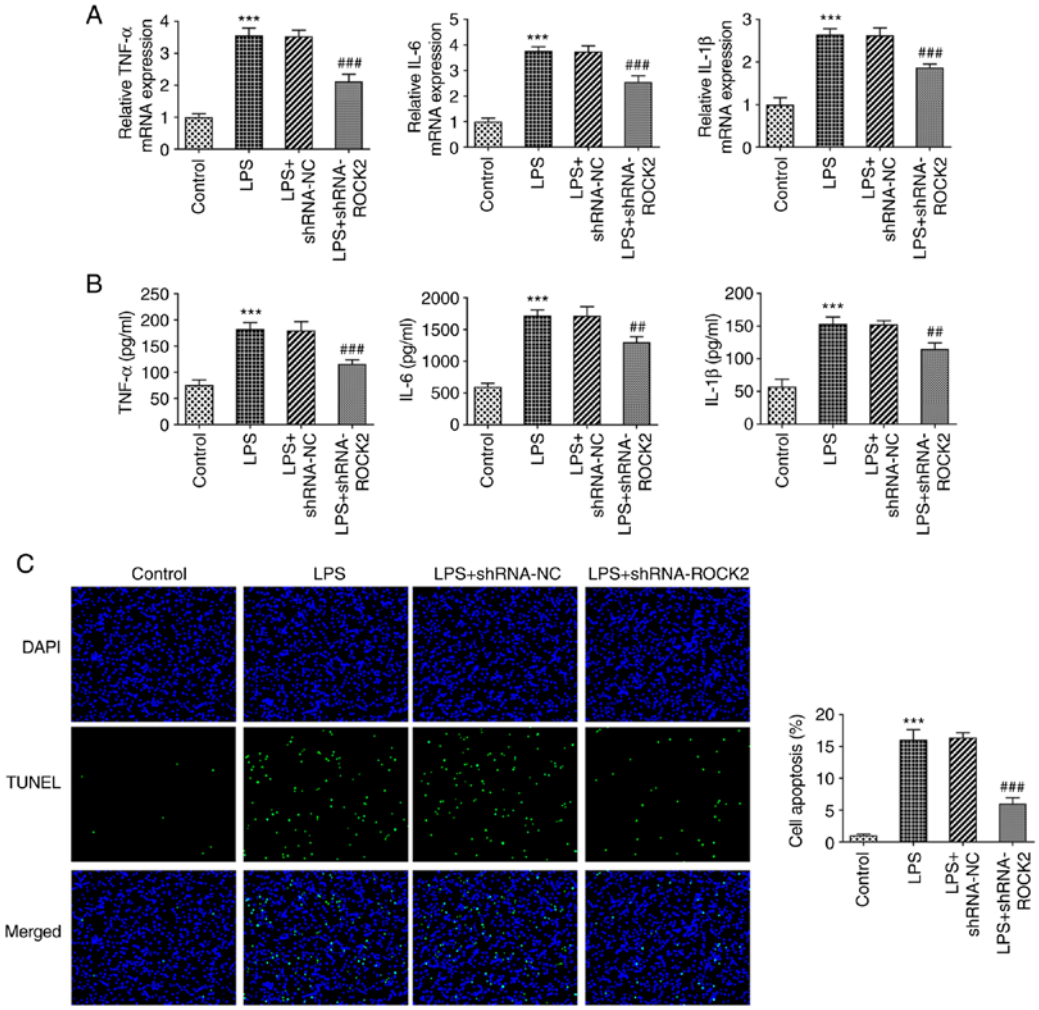

Figure 2. Effect of ROCK2 silencing on LPS-induced inflammation and apoptosis in HK-2 cells. (A) Reverse transcription-quantitative PCR was utilized to measure the mRNA level of TNF-α, IL-6 and IL-1β in HK-2 cells. (B) ELISA was employed to determine the production of TNF-α, IL-6 and IL-1β. (C) TUNEL staining was utilized to measure HK-2 apoptosis. ***P<0.001 vs. control; ##P<0.01 and ###P<0.001 vs. LPS + shRNA-NC. ROCK2, Rho-associated protein kinase 2; LPS, lipopolysaccharide; sh, short hairpin; NC, negative control.

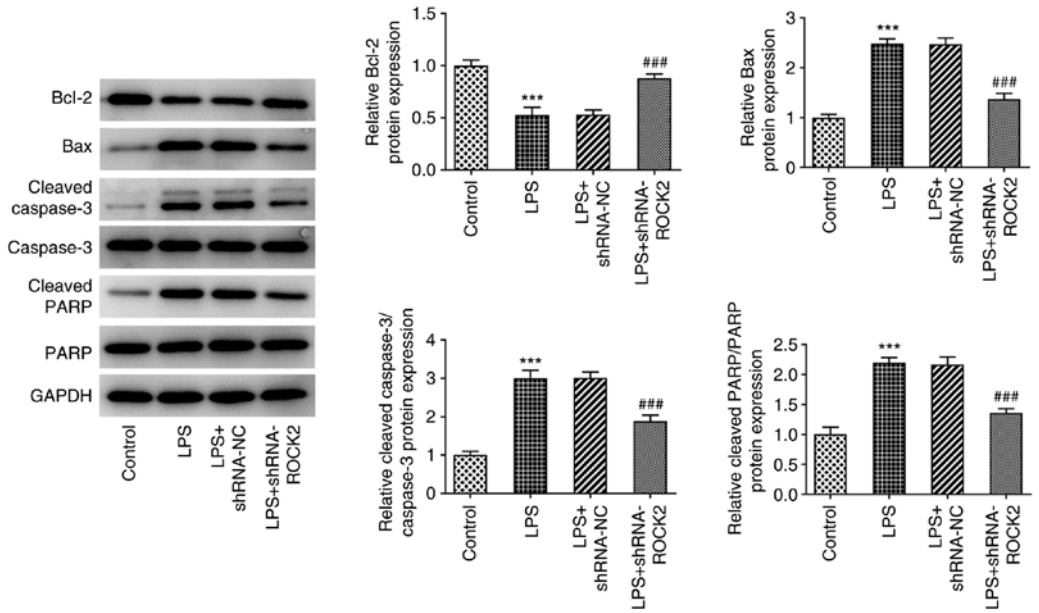

Figure 3. Effect of ROCK2 silencing on apoptosis-associated protein expression in LPS-induced HK-2 cells. The protein expression of Bcl2, Bax, cleaved caspase 3/caspase 3 and cleaved PARP/PARP was measured and semi-quantified using western blotting. ***P<0.001 vs. control; ###P<0.001 vs. LPS + shRNA-NC. ROCK2, Rho-associated protein kinase 2; LPS, lipopolysaccharide; sh, short hairpin; NC, negative control; PARP, poly (ADP-ribose) polymerase.

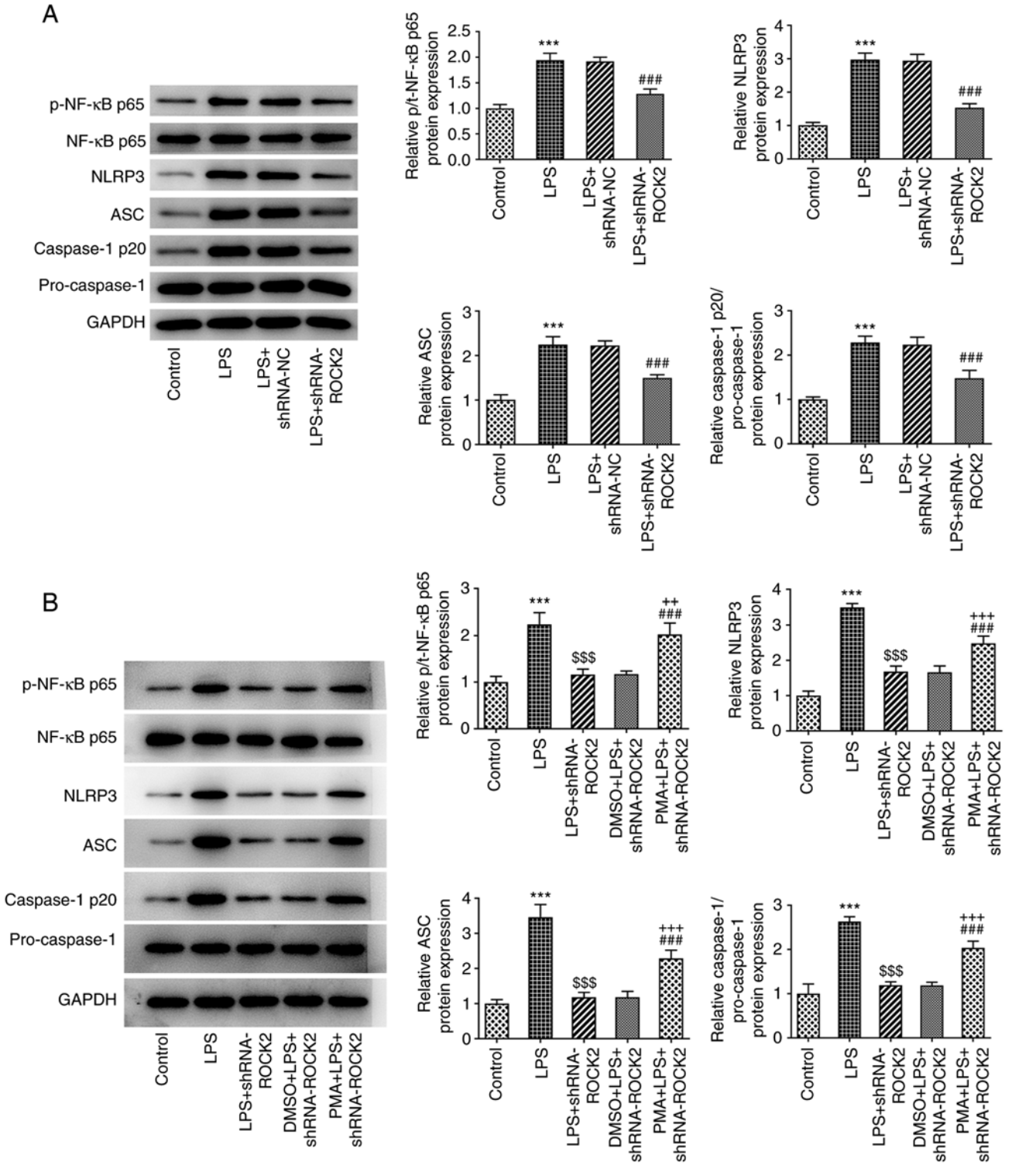

Figure 4. Effect of ROCK2 silencing on the NF-κB/NLRP3 pathway in LPS-induced HK-2 cells. (A) The protein expression of p-NF-κB p65/NF-κB p65, NLRP3, ASC, caspase-1 p20/pro-caspase-1 in HK-2 cells in different groups was measured using western blotting. (B) PMA was used to treat LPS-induced, shRNA-ROCK2-transfected HK-2 cells or untransfected cells, then the protein expression levels of p-NF-κB p65/NF-κB p65, NLRP3, ASC, caspase-1 p20/pro-caspase-1 in HK-2 cells in different groups were measured using western blotting. ***P<0.001 vs. control; $^{\$\$\$}$P<0.001 vs. LPS; $^{\#\#\#}$P<0.001 vs. LPS + shRNA-ROCK2 or LPS + shRNA-NC; $^{++}$P<0.01 and $^{+++}$P<0.001 vs. DMSO + LPS + shRNA-ROCK2. ROCK2, Rho-associated protein kinase 2; LPS, lipopolysaccharide; sh, short hairpin; NC, negative control; NLRP3, NLR family pyrin domain containing 3; ASC, apoptosis-associated speck-like protein containing a CARD; PMA, phorbol 12-myristate 13-acetate; p, phosphorylated; t, total.

PARP in LPS-induced HK-2 cells, was significantly reversed by PMA (Fig. 6A). The expression levels of KIM-1 and NGAL were significantly increased in LPS-induced HK-2 cells, which was lessened by ROCK2 depletion, and PMA reduced the aforementioned protein expression compared with the DMSO + LPS + shRNA-ROCK2 group (Fig. 6B). These results indicated that PMA may inhibit the protective effect of ROCK2 knockdown on LPS-induced HK-2 cell injury.

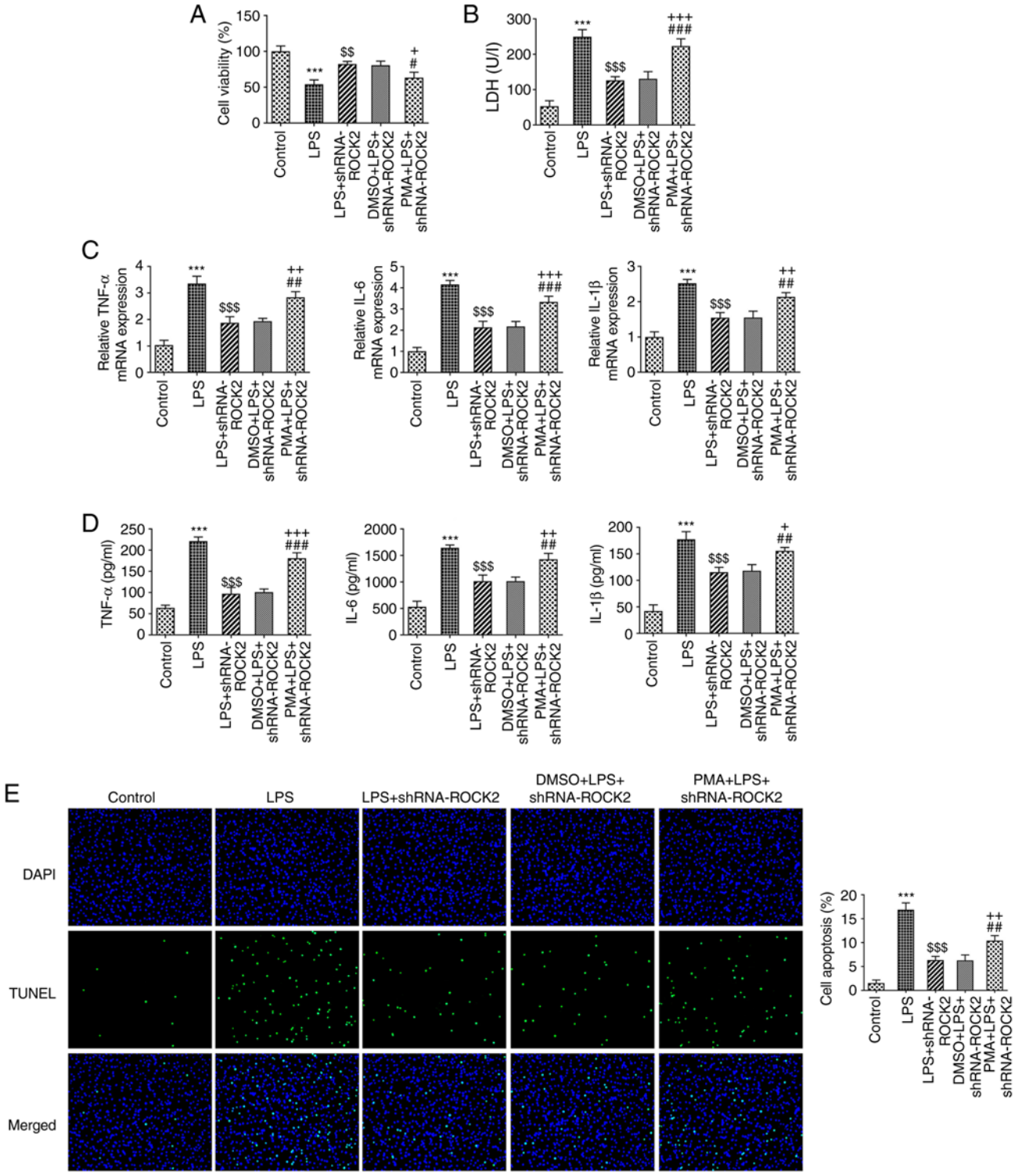

Figure 5. PMA reverses the effect of ROCK2 silencing on the viability, inflammation and apoptosis of LPS-induced HK-2 cells. (A) Cell viability was measured using MTT assay. (B) LDH production was measured using a commercially available LDH release assay kit. (C) Reverse transcription-quantitative PCR was utilized to measure the mRNA level of TNF-α, IL-6 and IL-1β in HK-2 cells. (D) ELISA was used to determine the production of TNF-α, IL-6 and IL-1β. (E) TUNEL staining was utilized to measure HK-2 apoptotic rate. ***P<0.001 vs. control; $^{\$\$}$P<0.01 and $^{\$\$\$}$P<0.001 vs. LPS; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS + shRNA-ROCK2; $^{+}$P<0.05, $^{++}$P<0.01 and $^{+++}$P<0.001 vs. DMSO + LPS + shRNA-ROCK2. ROCK2, Rho-associated protein kinase 2; LPS, lipopolysaccharide; sh, short hairpin; LDH, lactate dehydrogenase; PMA, phorbol 12-myristate 13-acetate.

Discussion

AKI is a common complication in patients in the intensive care unit (ICU) and ~50% of AKI cases can be attributed to sepsis, as observed in critically ill children in Southern India in a prospective observational study from June 2010 to March 2011 (17). Sepsis and AKI synergistically increase the morbidity and mortality of patients in the ICU. Therefore, a more complete molecular understanding of renal tubular injury is important for the discovery of novel S-AKI therapeutics. The results of

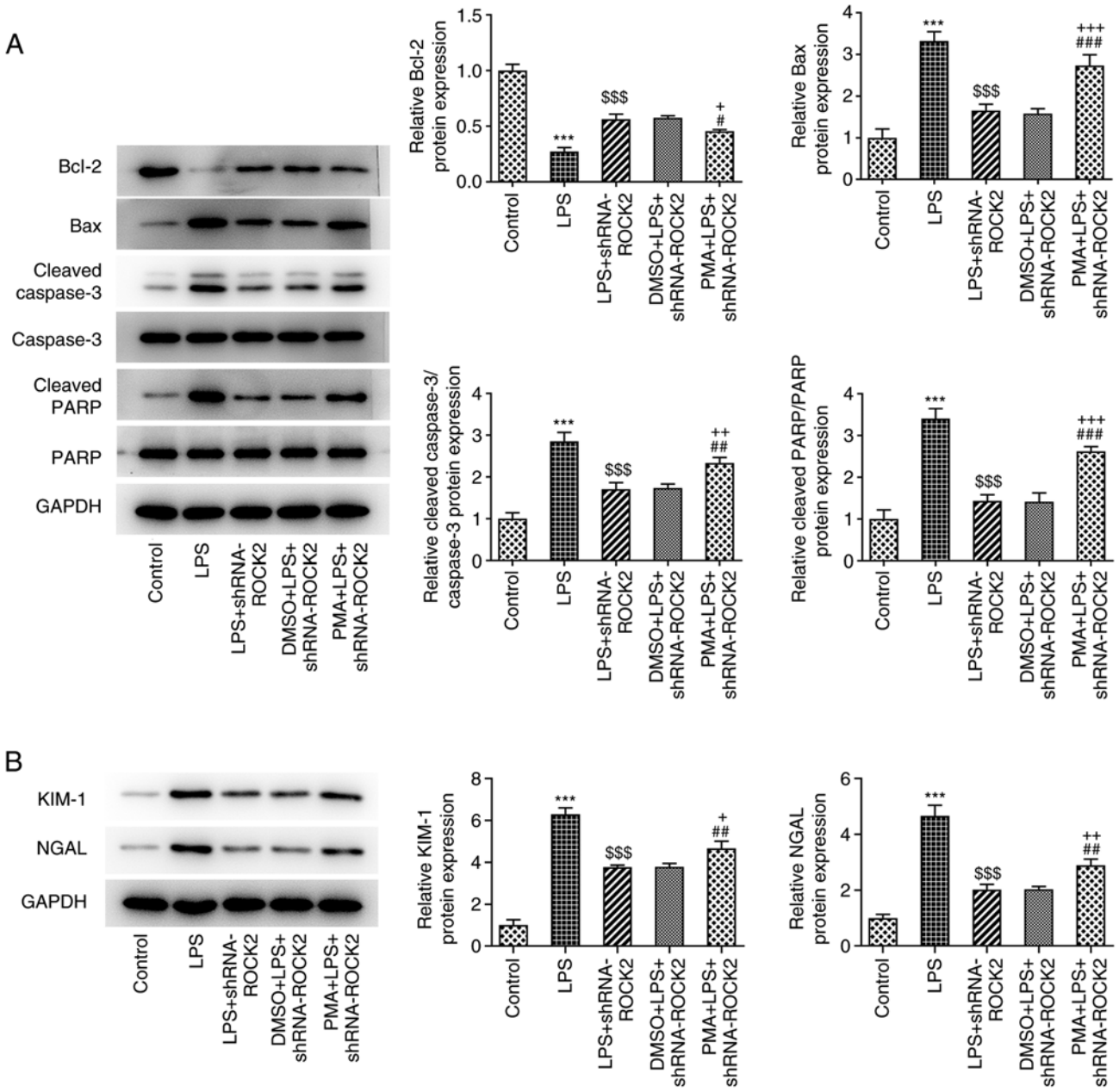

Figure 6. PMA reverses the effect of ROCK2 silencing on apoptosis related protein expression in LPS-induced HK-2 cells. (A) Protein expression of Bcl-2, Bax, cleaved caspase 3/caspase 3 and cleaved PARP/PARP was measured using western blotting. (B) Protein expression levels of KIM-1 and NGAL was measured using western blotting. ***P<0.001 vs. control; $^{\$\$\$}$P<0.001 vs. LPS; $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 vs. LPS + shRNA-ROCK2; $^{+}$P<0.05, $^{++}$P<0.01 and $^{+++}$P<0.001 vs. DMSO + LPS + shRNA-ROCK2. ROCK2, Rho-associated protein kinase 2; LPS, lipopolysaccharide; sh, short hairpin; PARP, poly (ADP-ribose) polymerase; KIM-1, kidney injury molecule-1; NGAL, neutrophil gelatinase-associated lipocalin; PMA, phorbol 12-myristate 13-acetate.

the present study demonstrated that ROCK2 knockdown markedly attenuated LPS-induced HK-2 cell injury. Furthermore, PMA, an activator of NF-κB, significantly reversed the effects of ROCK2 knockdown. Therefore, ROCK2 inhibition may serve as an important target for the prevention of S-AKI. A previous study indicated that Rho kinase inhibition attenuates renal injury in LPS-treated mice (18). However, Rho kinase includes ROCK1 and ROCK2, and isoform-specific regulation remains largely unknown (18). Nozaki *et al* (19) demonstrated that the Rho-kinase inhibitor fasudil alleviates cisplatin nephrotoxicity in the kidney; however, the disease model and interventions were all different compared with the present study in which cell model was constructed and ROCK2 was knocked down. The present study confirmed the regulatory role of ROCK2 in LPS-induced HK-2 cell injury.

LPS is a TLR4 agonist that can stimulate an immediate and robust inflammatory response, which can induce the activation of the innate immune system in human sepsis (5). LPS is widely used to induce cells to construct sepsis cell models (20). In the present study, it was demonstrated that LPS notably increased ROCK2 expression levels in HK-2 cells. LPS has previously been reported to upregulate ROCK2 expression levels in pulmonary microvascular endothelial cells (21) and brain microvascular endothelial cells (22). In the kidney tissues of sepsis animal models and LPS-induced HK-2 cells, ROCK2 expression is also enhanced (23).

Previous research has reported that the RhoA/ROCK signaling pathway serves an important role in the regulation of the inflammatory response (24). In sepsis, inhibition of the RhoA/ROCK2 signaling pathway is implicated in alleviating sepsis-associated injury (24-26). In the present study, it was hypothesized that the downregulation of ROCK2 would reduce LPS-induced HK-2 cell injury. Therefore, ROCK2 expression in HK-2 cells was inhibited using shRNA-ROCK2. The results demonstrated that ROCK2 knockdown significantly improved cell viability, in addition to reducing the levels of LDH activity and of pro-inflammatory cytokines, apoptosis rate and the expression levels of proapoptotic proteins in LPS-induced HK-2 cells, all of which indicated the potential suppression of HK-2 cell injury. Although the pathogenesis of AKI caused by sepsis still remains unclear, the theory of tubular apoptosis and inflammation has been widely accepted (27). Therefore, ROCK2 knockdown may serve as a potential therapeutic approach for alleviating S-AKI.

NF-κB regulates the inflammatory response and has been reported to serve an important role in the pathogenesis of organ injury induced by sepsis (11). The results of the present study demonstrated reduced protein expression levels of p-NF-κB p65, NLRP3, ASC and caspase-1 p20 upon ROCK2 knockdown in LPS-induced HK-2 cells. This result was similar to that of a previous study, which reported that the downregulation of ROCK2 alleviates ethanol-induced cerebral nerve injury partly through the suppression of the NF-κB signaling pathway (28). NLRP3 mediates the cleavage and maturation of proinflammatory cytokines, such as IL-1β, which results in a complex network of cellular responses that trigger local and systemic inflammatory reactions (29,30). NF-κB signaling is a prerequisite for the activation of the NLRP3 inflammasome (31). It has previously been reported that inhibition of the NF-κB signaling pathway contributes to the protective effect of dexmedetomidine on LPS-induced AKI *in vivo* (32). The present study therefore hypothesized that ROCK2 knockdown may exhibit a protective effect against S-AKI via the inhibition of NF-κB activation. Therefore, PMA, an activator of NF-κB, was introduced into cells, and the results demonstrated that PMA significantly reversed the effect of ROCK2 knockdown on HK-2 cells. These results demonstrated the role of NF-κB inactivation in ROCK2 knockdown-mediated S-AKI alleviation. Activation of the NLRP3 inflammasome in tubular cells has been associated with the pathogenesis of AKI (30,33). Renal ischemia/reperfusion (I/R) injury activates the NLRP3 and nuclear factor erythroid 2-related factor 2 (Nrf2) signaling pathways and MCC950 (an NLRP3 inhibitor) can suppress the NLRP3 signaling pathway and protect against I/R-induced renal injury (34). Propofol was indicated to increase Nrf2 expression and decrease NLRP3 expression in a ventilator-induced lung injury (VILI) mouse model, and activation of Nrf2 or inhibition of NLRP3 could reduce the pro-inflammatory factors in lung tissues in VILI mice (35). The present study indicated that LPS induction activated the NLRP3 pathway. ROCK2 knockdown could alleviate LPS-induced inflammatory injury and apoptosis of HK-2 cells by suppressing the NLRP3 pathway. Therefore, it was speculated that regulating the NLRP3 signaling pathway could affect the biological behaviors of HK-2 cells induced by LPS. In addition, an inhibitor of the NLRP3 pathway should be applied in further experiments.

In conclusion, the results of the present study demonstrated that ROCK2 knockdown inhibited tubular apoptosis and decreased inflammation by decreasing the NF-κB/NLRP3 activation in S-AKI. These results also indicated that pharmacological inhibition of ROCK2 may be a potential therapeutic approach for the treatment of S-AKI. However, further research is needed to investigate the effect of ROCK2 inhibition on S-AKI using other renal tubular epithelial cells (rat NRK-52E cells and mouse TCMK-1 cells) and animal models.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LY designed the study, performed the experiments, interpreted the data and critically revised the manuscript. XQ performed the experiments, analyzed the data and drafted the manuscript. LY and XQ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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