

Original Research

Inhibition of TRAF1 protects renal tubular epithelial cells against hypoxia/reoxygenation injury

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Abstract

Background and objective: This study aimed to explore the expression of TRAF1 *in vitro* kidney injury model, and the function mechanism of TRAF1 in the model growth and apoptosis.

Methods: After transfecting HK2 cells with short hair RNA (shRNA), shTRAF1 gene silencing model was established. The cells were divided into shRNA group and shNC group. For kidney injury model, we used hypoxia/reoxygenation to establish H/R cell lines. MTT assay was used to determine cell viability. PI/FITC staining was used to determine cell apoptosis. The genes expressions were determined by RT-qPCR and western blotting, respectively. The concentration of MDA, SOD, iNOS and LDH was determined by ELISA.

Results: The results of RT-qPCR and western blotting assay revealed that TRAF1 upregulated expression in AKI model cells. The results of MTT assay revealed that shRNA group exhibited significantly higher cell viability and lower cell apoptosis compared with the control group in H/R HK2 cells. In addition, TRAF1 downregulated expression inhibits oxidative stress response in H/R treated HK2 cell. Mechanically, TRAF1 deficiency protects HK2 cell via inhibiting p38-MAPK pathway.

Conclusions: Our study suggests that TRAF1 could be a target in kidney injury treatment.

Keywords

TRAF1; AKI; Cell apoptosis; H/R injury; p38 MAPK pathway

1. Introduction

Acute kidney injury (AKI) is a common clinical syndrome, including contrast-induced nephropathy [1], kidney transplantation [2], sepsis [3] and cardiovascular surgery [4]. AKI, as a common disease, is divided into stage I, stage II, and stage III according to its progress. Moreover, severe AKI is associated with poor survival of patients [5]. At the cellular level, renal tubular cells are the main targets of AKI, and studies have found that there are a large number of renal tubular deaths in the tissues of AKI patients [6]. In addition, oxidative stress of renal tubular cells, impaired mitochondrial energy metabolism, inflammation, endoplasmic reticulum stress, autophagy and pyrolysis are the main pathological re-

actions of acute kidney injury [7–10]. Therefore, protecting renal tubular epithelial cells from damage by external factors is one of the ways to prevent AKI.

Excessive reactive oxygen species (ROS) is one of the characteristics of AKI. The excessive production of ROS directly stimulates the oxidation of biological macromolecules (such as DNA, proteins and lipids) and regulates many signal pathways, especially the signal involved in mitogen-activated protein kinase (MAPK) pathway, eventually leading to cell death [11]. MAPKs are members of serine/threonine protein kinases family, and are mainly composed of extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK). A large number

studies showed that the MAPK pathway is over-activated in AKI tissues, leading to increased apoptosis and fibrosis [12, 13]. Moreover, the process of hypoxia/reoxygenation (H/R) can also cause renal tubular cell apoptosis, which is related to the activation of MAPK [14–16]. The above studies suggest that inhibiting the activity of MAPK can protect renal tubular cells from damage from external adverse factors.

Mechanically, tumor necrosis factor receptor associated factor 1 (TRAF1) as one of the 7 members of the TRAF family, exerts crucial role in cell progression through regulating of various signaling pathways, such as NF- κ B/p65, p38 and JNK. For example, TRAF protein can act as intracellular signal transduction by binding to tumor necrosis factor (TNF) receptor and IL-1 receptor superfamily to cause the activation of nuclear factor κ B (NF- κ B) and c-Jun N-terminal kinase (JNK) [17]. TRAF1 promotes the inflammatory response and apoptosis of cardiomyocytes induced by H/R by up-regulating ASK1-JNK/p38 signal, and aggravates myocardial ischemia-reperfusion injury [18]. In a LPS-induced lung injury mouse model, knocking out TRAF1 can inhibit the activity of JNK, and down-regulate LPS-induced ROS, inflammatory factors, and apoptosis levels, thereby alleviating acute lung injury [19]. By inhibiting the activity of TRAF1/ASK1/JNK, H/R induced the apoptosis of hepatocytes can also be inhibited [20]. In addition, TRAF1 participates in the regulation of apoptosis, proliferation, differentiation and stress responses in several malignancies. TRAF1 deficiency decreases apoptosis and increases the proliferative activity of ACHN RCC cells treated with radiation and interferon-alpha *in vivo* [21, 22].

In this study, we provided evidence that inhibition of TRAF1 by regulating the p38 MAPK pathway can inhibit H/R-induced apoptosis and oxidative stress of renal tubular epithelial cells, improve the survival rate of renal tubular epithelial cells, and protect them from H/R injury.

2. Materials and methods

2.1 Cell culture and shRNA transfection

Renal tubular epithelial cell HK2 cells were acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and the cells were cultured in Dulbecco's modified Eagle's medium (DMEM, purchased from Procell Company, Wuhan, China) containing of 10% FBS (gibco) and 100 U/mL penicillin/streptomycin (purchased from Sigma (St. Louis, MO, USA)). All the cell lines were maintained in 37 °C, 5% CO₂ incubator. The experiments in this study were performed following previous report [23]. Induction of *in vitro* kidney injury model by H/R was established as previously depicted [14]. For hypoxia induction, HK-2 cells were cultured in serum-free DMEM, and incubated in 95% N₂ and 5% CO₂ at 37 °C hypoxia chamber for 24 h. After exposure to hypoxic conditions, cells were changed with fresh oxygenated DMEM and cells were reoxygenated for 12 h in normoxic conditions (5% CO₂) at 37 °C. The experiment in this study is conducted in the Chongqing General Hospital's laboratory

during 2018–2019.

2.2 RNA isolation and RT-qPCR

Total RNAs were extracted by TRIzol reagent (Ambion, CA, USA). A total of 1 μ g of RNA was reverse-transcribed using the ImProm-IITM Reverse Transcription System (Promega, WI, USA). Quantitative real-time RT-PCR was carried out using SYBR GREEN qPCR Super Mix (Invitrogen, CA, USA). A standard amplification protocol was used according to the supplier's directions. Primers were listed as following.

TRAF1 forward: 5'-TCCTGTGGAAGATCACCAATGT-3';

TRAF1 reverse: 5'-GCAGGCACAACCTTGTAGCC-3';

GAPDH forward: 5'-AGACAGCCGCATCTTCTTGT-3';

GAPDH reverse: 5'-CTTGCCGTGGGTAGAGTCAT-3'.

2.3 Western blotting analysis

Western blotting assay was conducted as previously depicted [24]. And the extracted proteins were immunoblotted with the following antibodies: anti-mouse TRAF1 (1 : 1000, Santa, sc-6253, USA), anti-mouse Bax (1 : 1000, Santa, sc-20067, USA), anti-mouse Bcl-2 (1 : 1000, Santa, sc-73822, USA), anti-rabbit Cleaved Caspase-3 (1 : 1000, abcam, ab32042, England), anti-rabbit c-IAP1 (1 : 1000, abcam, ab108361, England), anti-mouse p-ERK (1 : 1000, Santa, sc-7383, USA), anti-mouse ERK (1 : 1000, Santa, sc-271270, USA), anti-mouse p-P38 (1 : 1000, Santa, sc-166182, USA), anti-mouse P38 (1 : 1000, Santa, sc-398305, USA), anti-mouse p-JNK (1 : 1000, Santa, sc-6254, USA), anti-mouse JNK (1 : 1000, Santa, sc-7345, USA), and anti-mouse β -actin (1 : 1000, Santa, sc-8432, USA). Then, the PVDF membranes were washed and secondary antibodies were applied 1 : 5000 for 1 h at room temperature, the immunoreactions were visualized with chemiluminescent ECL reagent.

2.4 Enzyme-linked immunosorbent assay (ELISA)

4,000 cells of each cell lines were plated into 96-well plates with complete medium for 24 h. Then the supernatant was tested using the MDA, SOD, and iNOS ELISA kit (R&D Systems, Inc., Minneapolis, MN) according following the manufacturer's protocols. Then, the absorbance value was detected using a microplate reader (BioTek, Vermont, USA). The levels of cytokines were calculated according to the standard curves.

2.5 Cell viability

MTT assay was performed following the previous report [25]. Briefly, 1×10^4 cells were plated into a 96-well-plate in triplicate and the colorimetric MTT Assay kit (keygenbio) was used to detect the cell viability rate after cell cultured for 96 hours.

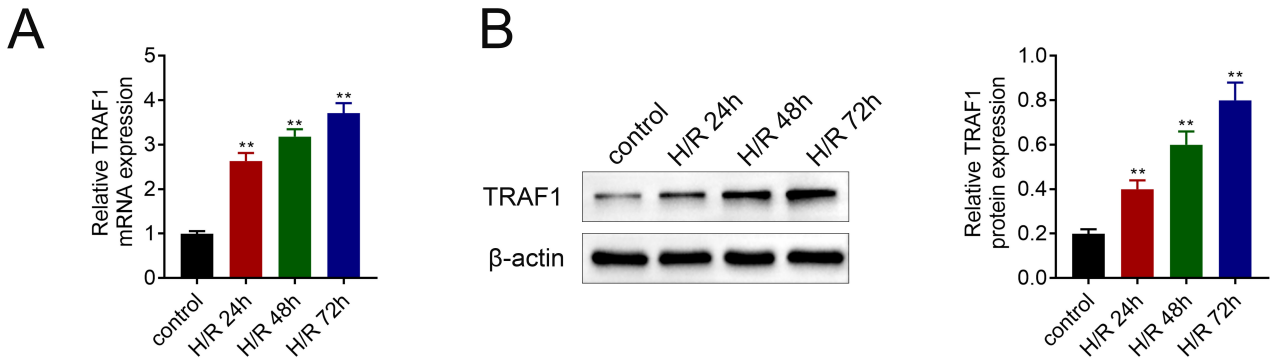


FIG. 1. TRAF1 is elevated in renal tubular cells induced by hypoxia/reoxygenation. (A&B) RT-qPCR and western blotting analysis the expression of TRAF1 in hypoxia/reoxygenation injury HK2 cell. Error bars represent data from three independent experiments (mean \pm SD). ** $P < 0.01$.

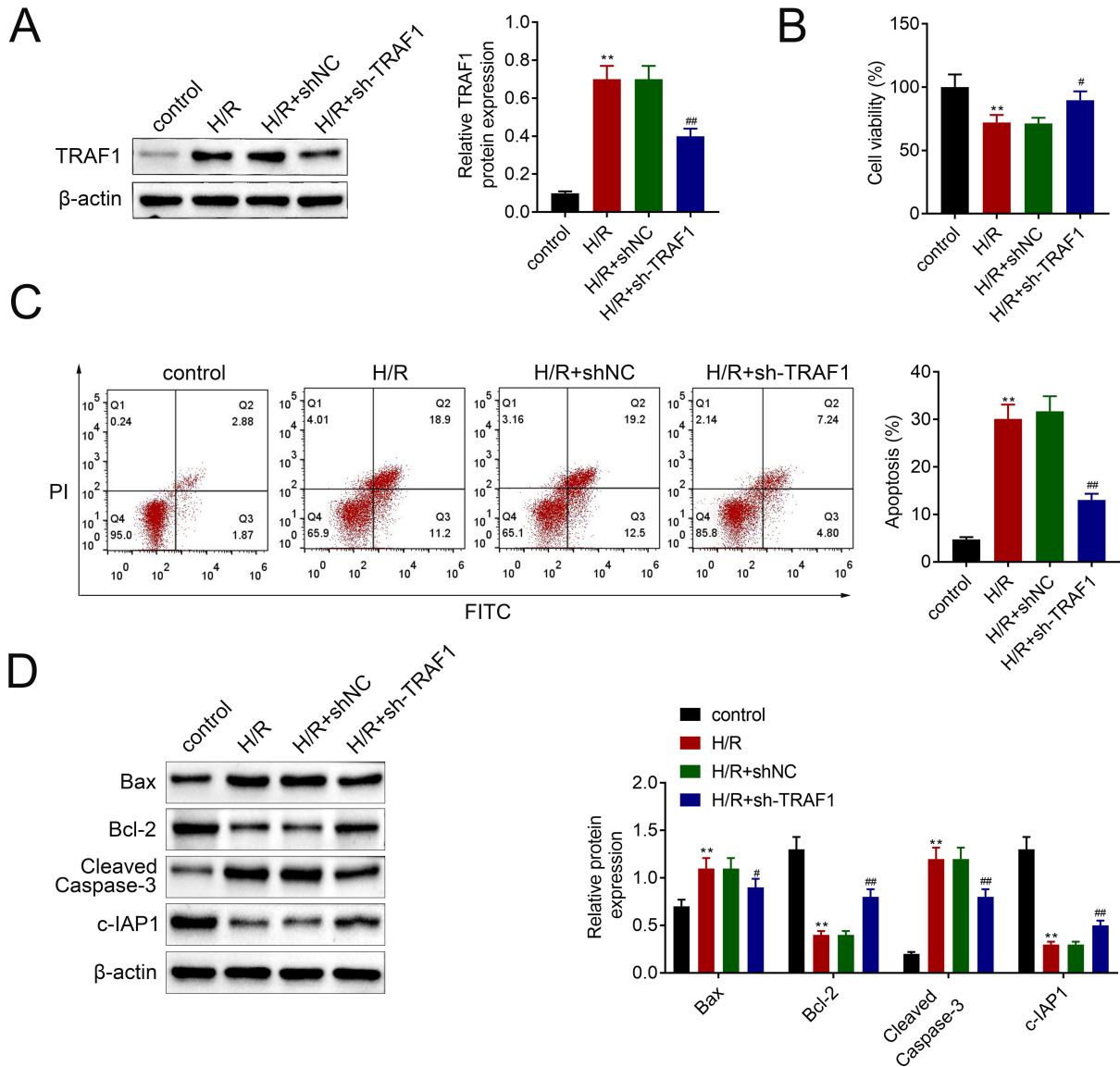


FIG. 2. Knockdown of TRAF1 improves the survival rate of renal tubular cells induced by hypoxia/reoxygenation and inhibits apoptosis. (A) HK2 Cell was divided into four groups: control, H/R, H/R + shNC and H/R + sh-TRAF1. Western blotting analysis the expression of TRAF1 in these cells. (B) Cell viability was measured by MTT assay. (C) Cell apoptosis was determined by flow cytometry. (D) Western blotting analysis the expression of cell apoptosis related genes. Error bars represent data from three independent experiments (mean \pm SD). ** $P < 0.01$, # $P < 0.05$, ## $P < 0.01$.

2.6 Flow cytometry analysis

Cells were seeded into six-well plate before analysis, then collected, fixed, and incubated at -20°C overnight. Cells were digested with 10 mg/mL RNase for 30 min and stained with 5 mg/mL of propidium iodide or/and FITC, and then subjected to cell apoptosis analysis by a flow cytometer (Becton Dickinson, CA).

2.7 Statistical analysis

Statistical analyses were performed using Graphpad prism 5 through One-way ANOVA, Newman-Keuls Multiple Comparison Test (Graphpad Software, San Diego, CA). Data were shown as means \pm SEM of at least three independent experiments. A *P*-value of 0.05 or less was considered to be statistical significant.

3. Results

3.1 TRAF1 is overexpressed in H/R induced HK2 cell

We first clarified the important role of TRAF1 in HK2 cell, by analyzing the expression of TRAF1 in H/R induced HK2 cells. As shown in Fig. 1A–B, the mRNA and protein expression of TRAF1 was increased with the prolongation of H/R treatment time.

3.2 Knockdown of TRAF1 promotes the proliferation and decreases cell apoptosis of H/R induced HK2 cell

Then we divided the HK2 cell into four groups: control, H/R group, H/R + shNC group and H/R + shTRAF1 group. As shown in Fig. 2A, TRAF1 was upregulated in H/R treated HK2 cell. Next, we analyzed the viability and apoptosis of these cells. As shown in Fig. 2B, H/R treatment significantly reduced cell viability, whereas knockdown of TRAF1 in H/R treated HK2 cell antagonized this malignant phenotype. Similarly, H/R treatment remarkably increased cell apoptosis while TRAF1 deficiency slightly decreased the increased apoptosis rate in H/R treated HK2 cells (Fig. 2C). In addition, apoptosis inducer genes Bax and cleaved-caspase3 were upregulated while suppressor genes Bcl2 and cleaved-IAP1 were inhibited in H/R treated HK2 cells. Furthermore, TRAF1 deficiency with sh-TRAF1 treatment in H/R treated HK2 cells reduced the expression of Bax and c-caspase3, while increased Bcl2 and c-IAP1 expression compared with the H/R treated HK2 cells. Therefore, these results indicated that TRAF1 deficiency improves the survival rate of renal tubular cells induced by H/R.

3.3 The oxidative stress response of renal tubular cells induced by H/R

Next, we wanted to know how H/R induces oxidative stress responses of renal tubular cells, we analyzed the concentration of MDA, SOD, iNOS and LDH in HK2 cells under different treatments. As shown in Fig. 3A, H/R treatment

induced the release of MDA and iNOS and suppressed SOD concentration. TRAF1 knockdown inhibited the release of MDA and iNOS and increased SOD concentration in H/R treated HK2 cells. Moreover, the release of LDH was up-regulated in H/R HK2 cells while knocking down of TRAF1 inhibited the release of LDH in H/R cells (Fig. 3B).

3.4 TRAF1 deficiency suppresses p38-MAPK pathway

Numerous studies have found that the MAPK pathway is over-activated in AKI tissues, causing increased apoptosis and fibrosis [12, 13]. Therefore, we analyzed the expression of p38-MAPK pathway proteins. As shown in Fig. 4, the expression of p-ERK, p-P38 and p-JUN were all increased in H/R treated HK2 cells, whereas TRAF1 deficiency downregulated the expression of p-ERK, p-P38 and p-JUN in H/R treated HK2 cells. Taken together, our data depicted that TRAF1 protects the cells from AKI via inhibition of p38-MAPK pathway.

4. Discussion

AKI is a common disease in clinical diagnosis, with pathological characteristics, such as oxidative stress response, inflammatory response, ER stress, impair of mitochondrial energy metabolism, autophagy and pyroptosis. In this study, we constructed an AKI model in HK2 cells with H/R treatment. We found that TRAF1 was overexpressed in H/R stimulated HK2 cells in a time-dependent manner. Knockdown of TRAF1 facilitated cell viability and decreased cell apoptosis in H/R stimulated HK2 cells. Based on this data, we speculated that TRAF1 deficiency may be a protecting approach for the cell model against H/R injury.

TRAFs were originally identified as TNF receptor family binding proteins, which are important intracellular signaling components involved in apoptosis and immune response pathways [17]. Seven TRAF proteins, TRAF1-TRAF7, are known to exhibit unique functions in the regulation of immune responses and in key cellular signaling events including NF- κ B, JNK, and apoptosis signaling pathways [26]. Previous study demonstrated that TNFR associated factor proteins are key adaptor molecules involved in immune cell signaling. In general, TRAF1 inhibited apoptosis which was induced by activation of the TNF receptor or the T-cell receptor, or by recruiting FADD (Fas-associated protein with death domain) [27]. TRAF1 can activate the pro-death pathway and inhibit protein kinase cell survival pathway, which promotes apoptosis of neurons in the brain I/R [28]. TRAF1 can also activate the ASK1 (apoptotic signal-regulated kinase 1) pathway to promote liver damage. In hepatocytes, TRAF1 lacks the way to suppress inflammation controlled by NF- κ B and cell death [29]. In NSCLC, TRAF1 expression affected TRAF2-mediated BRAF Lys48-linked ubiquitination, which subsequently inhibits the growth and differentiation, and the increases death in lung cancer cells [30]. Here, we demonstrated the same function role of TRAF1 in H/R HK2 cells. Additionally, miR-483 inhibits apoptosis of GES-1

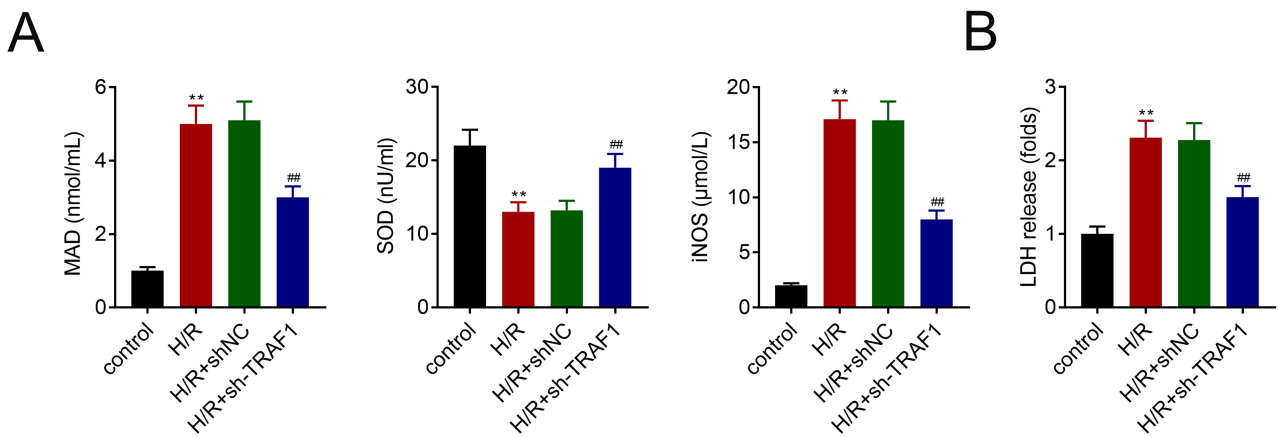


FIG. 3. Knock down TRAF1 inhibits the oxidative stress response of renal tubular cells induced by hypoxia/reoxygenation. (A) The concentration of MDA, SOD and iNOS was measured by ELISA assay. (B) Lactate dehydrogenase assay was used to analysis the release of LDH in HK2 cell. Error bars represent data from three independent experiments (mean \pm SD). ** $P < 0.01$, ## $P < 0.01$.

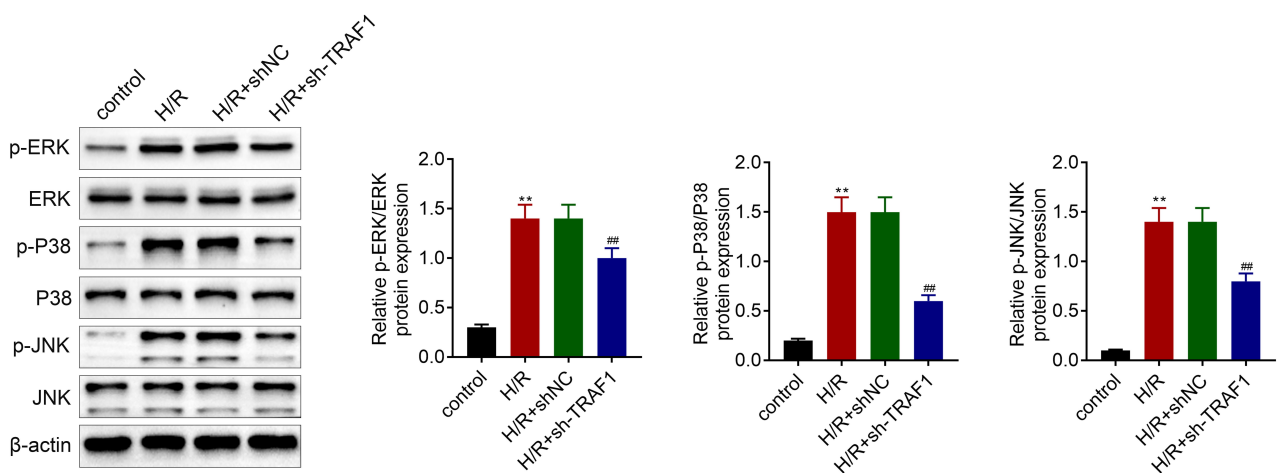


FIG. 4. TRAF1 Knockdown inhibits p38-MAPK pathway. The expression of P38-MAPK signal pathway proteins was measured by western blotting. Error bars represent data from three independent experiments (mean \pm SD). ** $P < 0.01$, ## $P < 0.01$.

cells by overexpression of TRAF1/4-1BB. TNF-receptor I-induced apoptosis is thought to occur through recruitment of the FADD and Caspase-8 receptor complex [31]. TRAF1 was a downstream gene of TNFR1. Similarly, we chose BAX, Bcl2, c-caspase 3 and c-IAP1 to analysis the role of TRAF1 by western blotting. We found that TRAF1 acts as an inhibitor gene in cell apoptosis in H/R treated HK2 cells, and TRAF1 regulates cell apoptosis via upregulation of Bcl2 and c-IAP1, and downregulation of c-caspase3 and Bax. Moreover, UCP2 decreases tubular epithelial cell apoptosis in lipopolysaccharide-induced AKI by reducing ROS production [8]. In this study, we found that TRAF1 suppresses tubular epithelial cell apoptosis in H/R-induced AKI by downregulating ROS production. However, further studies will be performed in the future to investigate the detail regulatory role of TRAF in ROS production in H.R-induced AKI.

Mechanically, previous studies reported that TRAF1 was associated with acute lung injury [19], myocardial ischemia reperfusion injury [18], renal ischemia and reperfusion injury [13], and acute kidney injury. Wan and his colleagues reported [19] that TRAF1 meditates lipopolysaccharide-

induced acute lung injury by up-regulating JNK activation, including downregulated expression of p-JNK and p-c-JUN. In arterial injury, CD4 acted as a crucial protein in the upregulation the expression of TRAF proteins and NF- κ B-dependent proinflammatory genes. TRAF1 deficiency inhibited inflammatory responses, and promoted hepatic injury through suppression of ASK1/JNK pathway [32]. TRAF1 also played critical role in cerebral ischaemia-reperfusion injury and neuronal death, and TRAF1 regulated apoptosis associated with the JNK pro-death pathway activity and inhibited the Akt cell survival pathway through suppressing of ASK1 [28]. In addition, overexpression of TRAF1 in primary cardiomyocytes promotes H/R induced inflammation and apoptosis *in vitro*, and in terms of mechanism, TRAF1 increased the myocardial I/R injury through enhancing the activation of ASK1-mediated JNK/p38 cascades [18]. Huang *et al.* [20] reported that microRNA214 positively regulated hepatocyte apoptosis after IR injury by inhibition of TRAF1/ASK1/JNK Pathway. In our study, we found that TRAF1 deficiency reduced the phosphorylation of ERK, P38 and JUN, which

are in accordance with previous studies. However, whether TRAF1 was targeted regulation of these genes expression needs further investigation.

One limitation of our study was that TRAF1 knockdown HK2 cells were used to investigate the function of TRAF1 in kidney H/R injury. Using TRAF1-KD HK2 cells, we proved that TRAF1 deficiency inhibits H/R-induced apoptosis, ROS response, and phosphorylation of ERK, p38 and JNK. However, the underlying functional regulatory mechanism of TRAF1 in H/R induced injury HK2 model is still unclear, and whether upregulation of TRAF1 in HK2 cells promotes oxidative stress and apoptosis need more studies.

In conclusion, knockdown of TRAF1 expression in HK-2 cells with H/R injury promoted cell viability, inhibited cell damage, decreased apoptotic index, decreased cleaved caspase-3, Bcl-2 expression and suppressed the expression of p-ERK, p-P38 and p-JNK. TRAF1 maybe serve as a biomarker in H/R injury AKI.

Author contributions

WY designed the study, supervised the data collection, QFM analyzed the data, interpreted the data, WY and QFM prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Acknowledgment

Thanks to all the peer reviewers for their opinions and suggestions.

Funding

This research received no external funding.

Conflict of interest

The authors declare no conflict of interest.

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