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Knockdown of ANGPTL2 Protects Renal Tubular Epithelial Cells Against Hypoxia/Reoxygenation-Induced Injury via Suppressing TLR4/NF-κB Signaling Pathway and Activating Nrf2/HO-1 Signaling Pathway

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Abstract

Renal ischemia/reperfusion (I/R) injury is a particular threat faced by clinicians in kidney transplantation. Previous studies have confirmed the importance of oxidative stress and inflammation in the pathogenesis of I/R injury. Angiopoietin-like protein 2 (ANGPTL2) belongs to the angiopoietin-like family and has been found to be involved in the regulation of kidney function as well as oxidative and inflammatory response. In the present study, we aimed to evaluate the role of ANGPTL2 in renal I/R injury *in vitro*. The human proximal tubular epithelial cell line (HK-2 cells) was subjected to hypoxia/ reoxygenation (H/R) to mimic I/R injury *in vitro*. We found that the expression level of ANGPTL2 was markedly increased in H/R-induced HK-2 cells. Knockdown of ANGPTL2 improved the decreased cell viability of HK-2 cells in response to H/R stimulation. Knockdown of ANGPTL2 significantly inhibited the H/R-caused increase in levels of reactive oxygen species, malondialdehyde, and proinflammatory cytokines, including interleukin (IL)-6, IL-1β, and tumor necrosis factor-alpha, as well as a decrease in superoxide dismutase activity in the HK-2 cells. Besides, the increased bax expression and caspase-3 activity and decreased bcl-2 expression in H/R-induced HK-2 cells were also attenuated by knockdown of ANGPTL2. Moreover, ANGPTL2 overexpression showed the opposite effects. Further mechanism investigations proved that the activation of Nrf2/HO-1 signaling pathway and the inhibition of toll-like receptor 4/nuclear factor kappa-light-chain-enhancer of activated B cells signaling pathway were both implicated in the renal-protective effects of ANGPTL2 knockdown on H/R-induced HK-2 cells. Collectively, these findings suggested that ANGPTL2 might be a new possible target for the treatment and prevention of renal I/R injury.

Keywords

renal ischemia/reperfusion (I/R) injury, angiopoietin-like protein 2 (ANGPTL2), oxidative stress, inflammation, TLR4/NF-κB signaling pathway, Nrf2/HO-1 signaling pathway

Introduction

Kidney transplantation is an important treatment option for end-stage renal disease[1]. In clinic, renal ischemia/reperfusion (I/R) injury is a particular threat faced by the clinicians perioperatively in kidney transplantation[2]. Renal I/R injury leads to loss of tubular epithelial cells function, thereby contributing to the development of acute kidney injury, delayed graft function, and acute and chronic organ rejection[3]. Thus, strategies to prevent I/R injury after transplantation may improve graft outcomes.

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Researches during the past decade have improved our understanding of the molecular mechanisms that contribute to renal I/R injury[2]. The molecular and cellular events that occur in renal I/R injury are complex, involving oxidative and inflammatory damage[4,5]. Long exposure to ischemia and the following reperfusion result in excessive production of reactive oxygen species (ROS), including superoxide, hydrogen peroxide, and hydroxyl radicals, which play a central role in the pathogenic process of I/R injury[6]. Excessive ROS causes oxidative damage, as well as induce the secretion of chemokines and cytokines together with other factors, which promote the inflammatory response[7]. Therefore, it is of particular importance to prevent ROS production and ROS-mediated oxidative and inflammatory damage.

Angiopoietin-like protein 2 (ANGPTL2) belongs to the angiopoietin-like family, which contains eight members (ANGPTL1-8) of glycoproteins[8]. Different studies proved that ANGPTL2 maintains tissue homeostasis by promoting adaptive inflammation and subsequent tissue reconstruction[9]. However, excess activation of ANGPTL2 promotes the breakdown of tissue homeostasis due to chronic inflammation and irreversible tissue remodeling[10]. Many clinical researches reported that ANGPTL2 levels are associated with the diagnosis and/or prognosis of various diseases, such as diabetes, cardiovascular diseases, chronic kidney disease, and cancers[11–15]. Additionally, ANGPTL2 deficiency has been demonstrated to possess antioxidative and renal-protective effects[16–18]. However, the effect of ANGPTL2 on renal I/R injury remains unclear. We hypothesized that knockdown of ANGPTL2 protects renal tubular epithelial cells against hypoxia/reoxygenation (H/R)-induced injury. Thus, in this study, we examined the effect of ANGPTL2 on renal I/R injury using an *in vitro* H/R model.

Materials and Methods

Cell Culture

The human proximal tubular epithelial cell line (HK-2 cells) purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle medium (DMEM)-F12 medium (Gibco Laboratories, Grand Island, NY, USA). The medium was supplemented with 10% fetal bovine serum (FBS; Gibco Laboratories), 5 ng/ml human recombinant epidermal growth factor (hEGF; Gibco Laboratories), 100 IU/ml penicillin, and 100 μg/ml streptomycin. The HK-2 cells were maintained in a humidified atmosphere of 5% carbon dioxide (CO_2) and 95% oxygen (O_2) at 37 °C.

H/R Model

To mimic I/R injury *in vitro*, the HK-2 cells grown to 80% confluence were cultured for 12 h under hypoxic condition with 1% O_2, 94% nitrogen (N_2), and 5% CO_2 in glucose-free and serum-free medium to induce hypoxic injury. Afterward, the medium was replaced by a regular culture medium, and the cells were incubated with 21% oxygen for 2 h for reoxygenation. HK-2 cells in the control group were incubated in complete culture medium under normal conditions (5% CO_2 and 95% air).

Cell Transfection

Small interfering RNA (siRNA) targeting ANGPTL2 (si-ANGPTL2), Nrf2 (si-Nrf2), and negative scramble control siRNA (si-NC) were purchased from GenePharma Co. (Shanghai, China). The full length of ANGPTL2 or toll-like receptor 4 (TLR4) was ligated into a pcDNA3.1 plasmid, and the recombined plasmids were referred to as pcDNA3.1-ANGPTL2 and pcDNA3.1-TLR4. Transfection was performed when the HK-2 cells reached 70% confluence using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The experiment was performed in triplicate.

Cell Viability Assay

Cell viability of HK-2 cells was assessed using the methylthiotetrazole (MTT) assay kit (Beyotime Biotechnology, Shanghai, China) following the manufacturer's instructions. Briefly, HK-2 cells (at a density of 1×10^4 cells/well) were incubated for 24 h and then administered with 20 μl MTT solution (5 mg/ml) for 4 h, followed by incubation with 10 μl formazan solution for 4 h at 37 °C. The optical density (OD) values at 490 nm were measured using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA). The experiment was performed in triplicate.

Measurement of ROS Production

Intracellular ROS level in HK-2 cells was detected using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). Briefly, HK-2 cells were incubated with 25 μM DCFH-DA in DMEM for 20 min at 37 °C and then washed thrice with PBS. HK-2 cells were collected and the fluorescence intensity of 2′,7′-dichlorofluorescein was observed with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at ex/em wavelengths of 488/525 nm. The experiment was performed in triplicate.

Detection of Malondialdehyde (MDA) Level and Superoxide Dismutase (SOD) Activity

HK-2 cells homogenate (10%, w/v in saline) was prepared and centrifuged at 4 °C and 4000 × *g* for 30 min. Then the supernatants were collected for the detection of MDA level and SOD activity by using the corresponding assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions. The experiment was performed in triplicate.

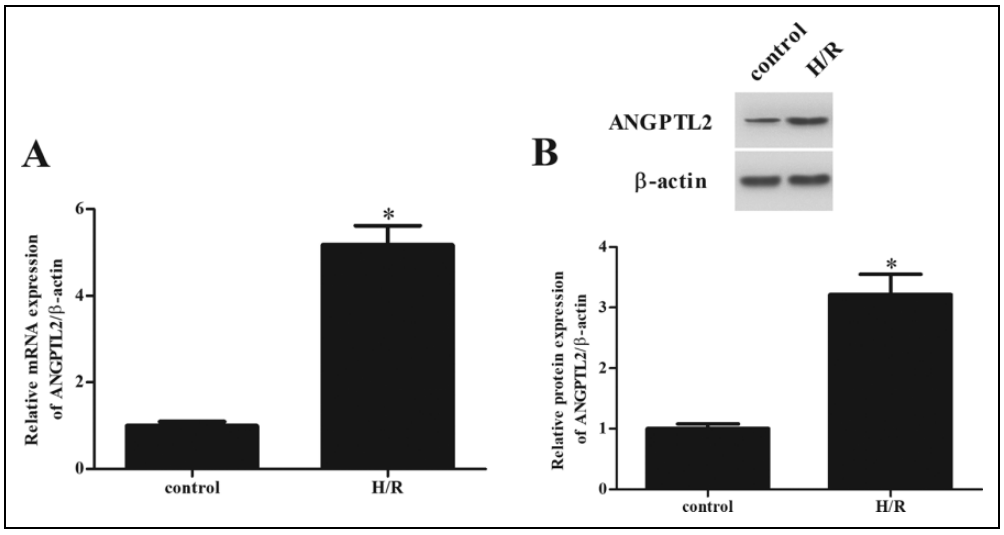

Figure 1. Increased expression levels of ANGPTL2 in HK-2 cells exposed to H/R. HK-2 cells were exposed to H/R, and then the mRNA and protein levels of ANGPTL2 were measured using qualitative real-time-PCR (A) and western blot (B). $n = 5$. *$P < 0.05$. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation.

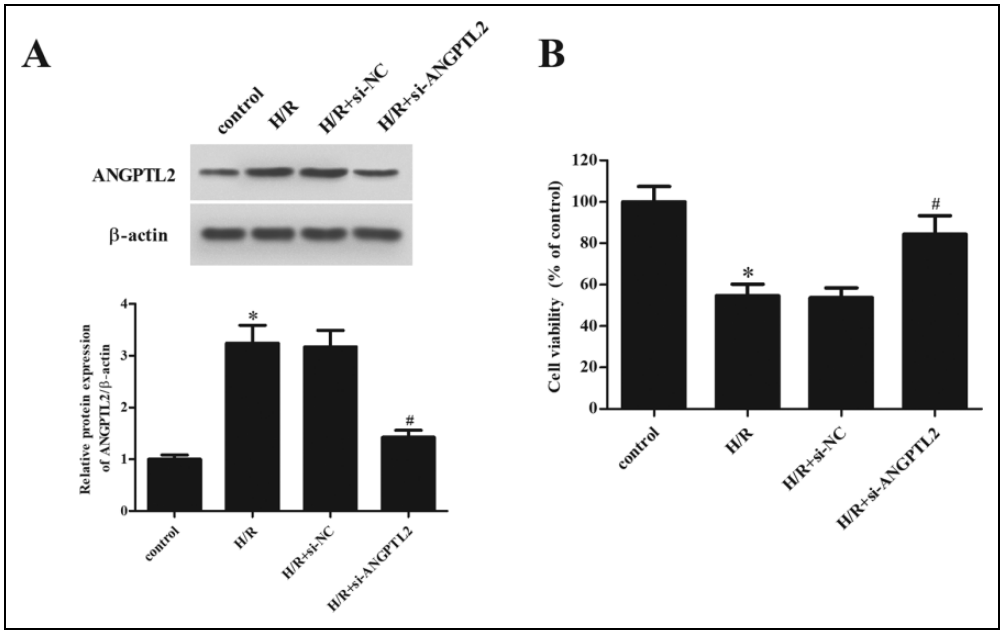

Figure 2. Improved cell viability of HK-2 cells after transfection with si-ANGPTL2. (A) Western blot was performed to detect the protein levels of ANGPTL2 after transfection with si-ANGPTL2 or si-NC in H/R-induced HK-2 cells. (B) Methylthiotetrazole assay was performed to examine the cell viability of HK-2 cells. $n = 6$. *$P < 0.05$ versus control, $^{\#}P < 0.05$ versus si-NC + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA from HK-2 cells was extracted using the RNAiso plus Total RNA extraction reagent (Takara, Dalian, China) according to the manufacturer's instructions. Subsequently, the cDNA was then synthesized using the total RNA with a reverse transcription system kit (Takara). qRT-PCR was performed using SYBR Premix Ex Taq (Takara) on an ABI PRISM 7300 real-time PCR System (Applied Biosystems, Foster, CA, USA). Primer sequences were used as follows: ANGPTL2, forward 5′- GCCACCAAGTGTCAGCCTCA-3′, reverse 5′- TGGACAGTACCAAACATCCAACATC-3′; β-actin, forward 5′-GATCATTGCTCCTCCTGAGC-3′

and reverse 5′-ACTCCTGCTTGCTGATCCAC-3′. The relative mRNA levels of ANGPTL2 were analyzed using the comparative cycle threshold (ΔΔCt) method. The experiment was performed in triplicate.

Western Blotting

The protein lysates from the HK-2 cells were prepared, and the protein content was determined using the bicinchoninic acid protein assay kit (Nanjing KeyGen Biotech, Nanjing, China). The nuclear protein was extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). The protein samples (40 μg/per lane) were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA) using a wet transfer system. Membranes were then blocked in 5% bovine serum albumin in Tris-buffered saline with Tween 20 (TBS-T) buffer for 1 h at room temperature, followed by incubation with primary antibodies against ANGPTL2 (1:1000), bcl-2 (1:1500), bax (1:1500), TLR4 (1:800), p65 (1:1000), p-p65 (1:2000), Nrf2 (1:1500), lamin B1 (1:800), HO-1 (1:1000), and β-actin (1:1500; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4 °C. Subsequently, membranes were washed by TBS-T buffer for three times and incubated with secondary horseradish peroxidase-conjugated antibodies (1:2000; Santa Cruz Biotechnology) for 2 h at room temperature. The blots were detected using an ECL advanced system (GE Healthcare, Piscataway, NJ, USA), and the signals were analyzed by ImageJ software (National Institutes of Health, NIH, Bethesda, MD, USA). The experiment was performed in triplicate.

Cell Apoptosis Assay

Caspase 3 activity in HK-2 cells was tested using Caspase-3 Activity Assay Kit for (Beyotime) according to the manual. The HK-2 cells were lysed by lysis buffer and centrifuged at 600 × *g* for 5 min. Next, the cells were added with 2 mM Ac-DEVD-pNA for 1 h at 37 °C, and the absorbance was detected at 405 nm to calculate caspase 3 activity. The experiment was performed in triplicate.

ELISA

Interleukin (IL)-6, IL-1β, and tumor necrosis factor-alpha (TNF-α) contents in cell-free supernatants of HK-2 cells were measured using relevant commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) and performed according to the manufacturer's instructions. The experiment was performed in triplicate.

Statistical Analysis

All data were expressed as the mean ± standard deviation. Data were analyzed using SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA). The statistical difference was analyzed using Student's *t*-test between two groups and one-way analysis of variance followed by Tukey's post hoc test among multiple groups. Differences with *P* values less than 0.05 were considered statistically significant.

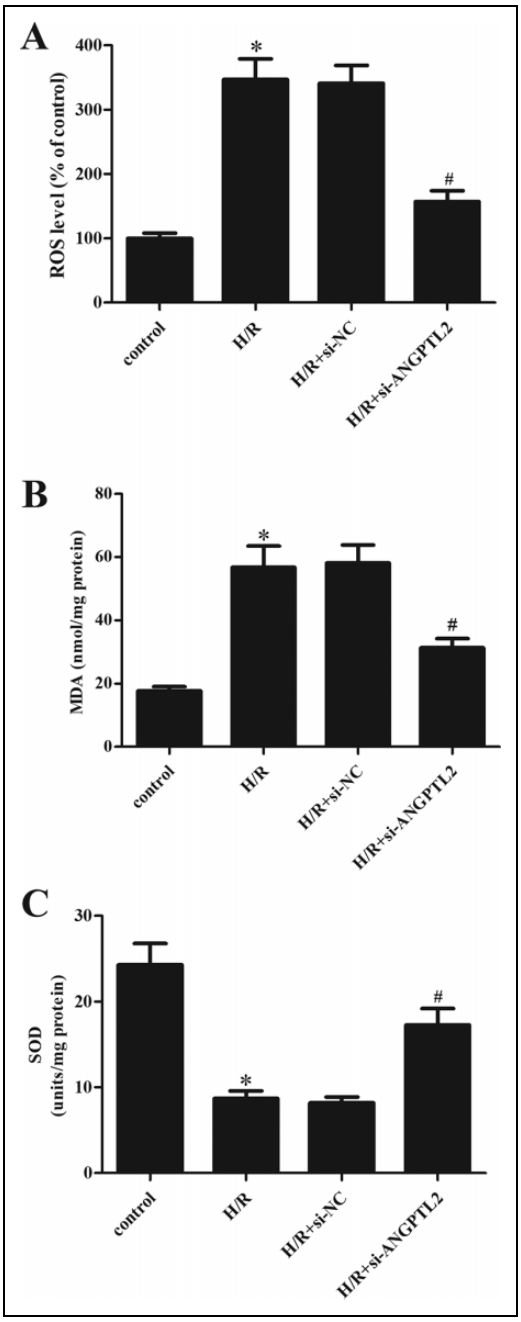

Figure 3. Decreased oxidative stress in HK-2 cells transfected with si-ANGPTL2. After transfection with si-ANGPTL2 or si-NC, the ROS production (A), MDA level (B), and SOD activity (C) were detected for the assessment of oxidative stress in HK-2 cells. $n = 5$. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus si-NC + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation; MDA: malondialdehyde; ROS: reactive oxygen species; SOD: superoxide dismutase.

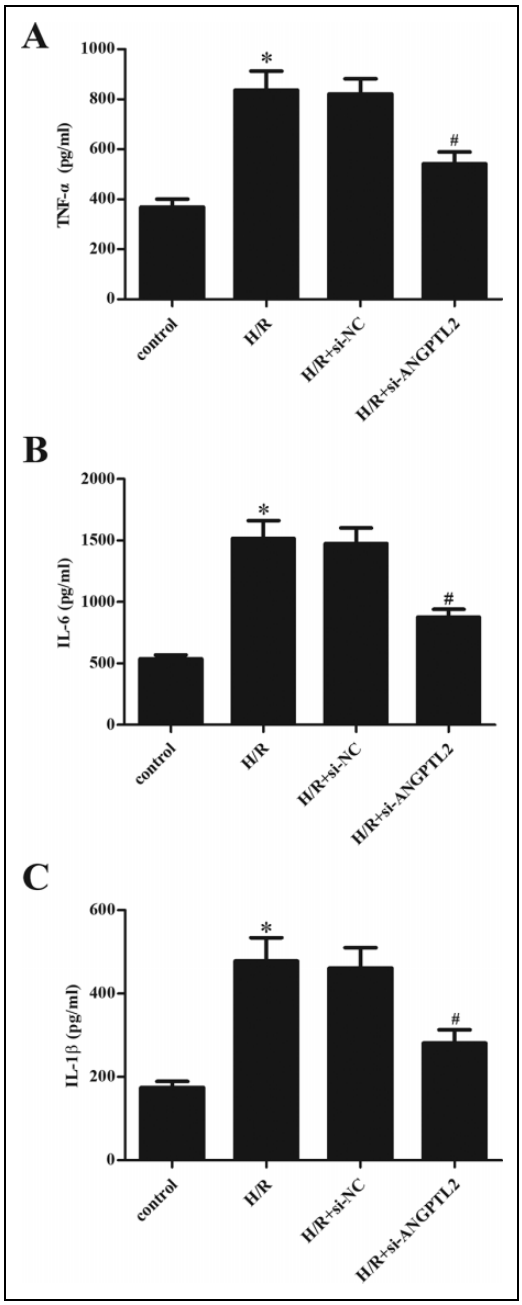

Figure 4. Decreased inflammatory response in HK-2 cells transfected with si-ANGPTL2. The secretion levels of proinflammatory cytokines, including TNF-α (A), IL-6 (B), and IL-1β (C) in culture supernatants were determined to assess inflammation in HK-2 cells. $n = 6$. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus si-NC + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation; IL: interleukin; TNF-α: tumor necrosis factor-alpha.

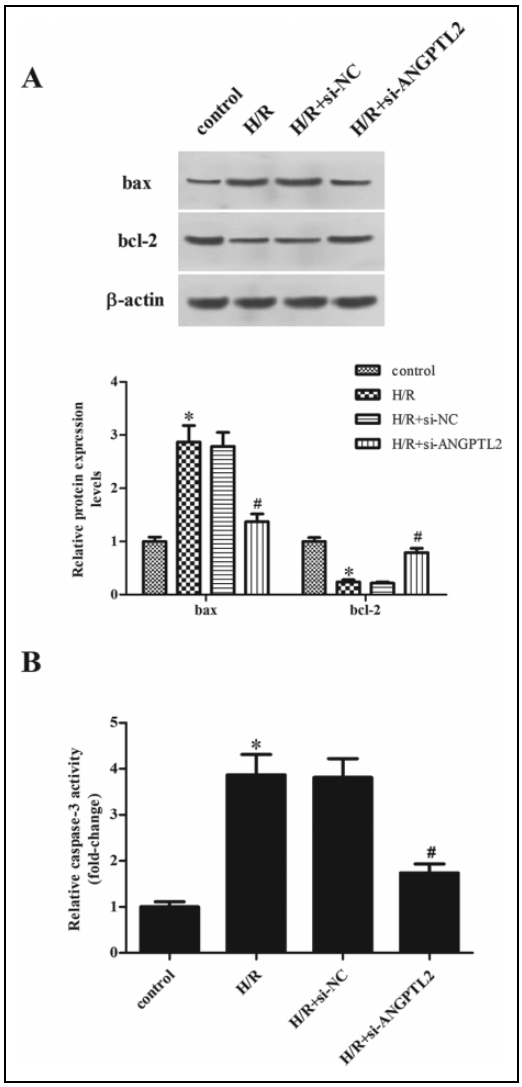

Figure 5. Decreased cell apoptosis in HK-2 cells transfected with si-ANGPTL2. (A) Western blot was performed to detect the protein levels of bax and bcl-2 after transfection with si-ANGPTL2 or si-NC. (B) Caspase-3 activity in HK-2 cells was determined. $n = 4$. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus si-NC + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation.

Results

ANGPTL2 was Significantly Upregulated in HK-2 Cells Exposed to H/R

HK-2 cells were exposed to H/R, followed by the detection of mRNA and protein levels of ANGPTL2 using qRT-PCR and western blot. Figure 1A showed that exposure to H/R caused a significant increase in the mRNA level of ANGPTL2 in HK-2 cells. Similarly, the protein level of ANGPTL2 was also markedly increased in HK-2 cells exposed to H/R (Fig. 1B).

Knockdown of ANGPTL2 Improved the Viability of HK-2 in Response to H/R

To uncover the precise regulatory effect of ANGPTL2 in H/R-induced HK-2 cells, we performed loss-of-function experiments of ANGPTL2 by transfecting ANGPTL2 siRNA into HK-2 cells. The results of western blot showed that the protein

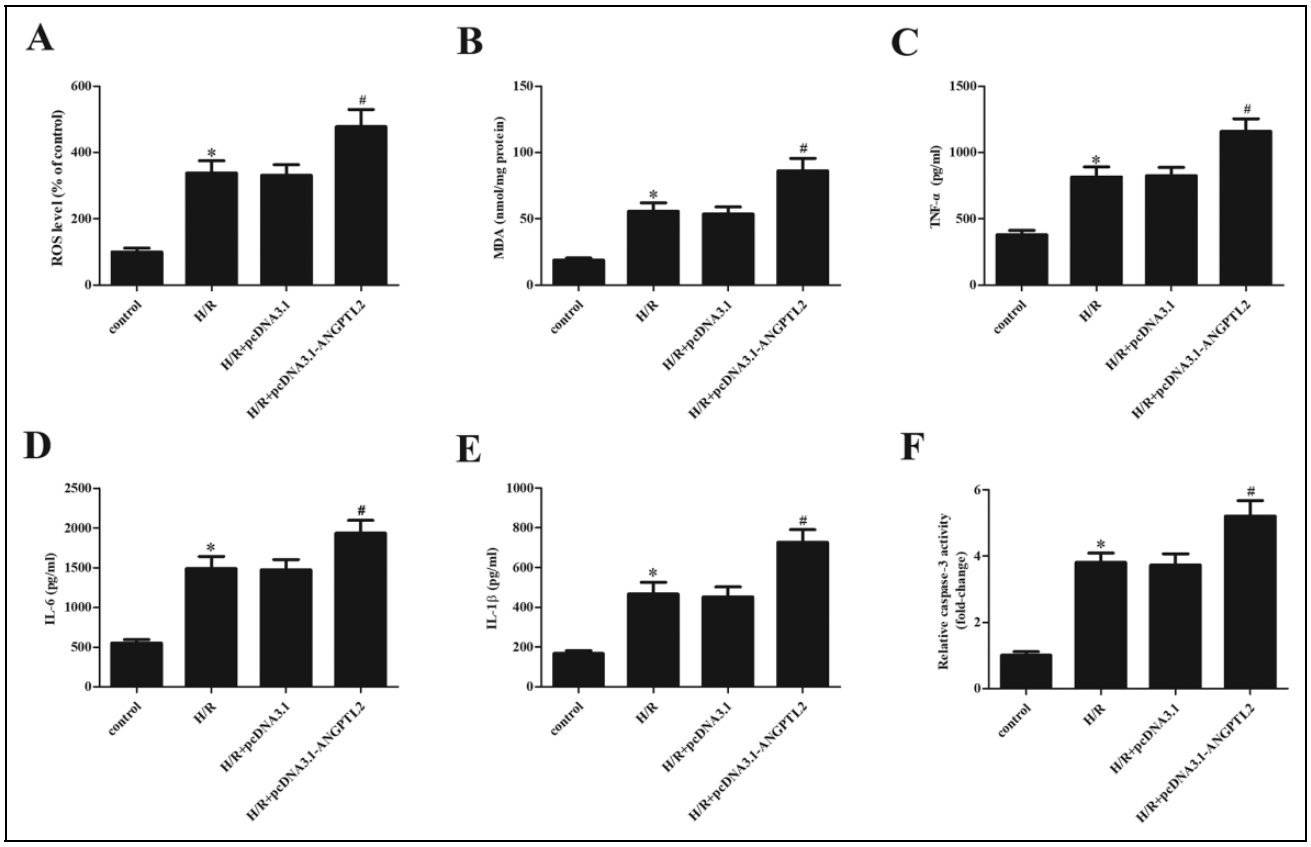

Figure 6. Enhanced oxidative stress, inflammatory response, and apoptosis in HK-2 cells transfected with pcDNA3.1-ANGPTL2. (A and B) ROS and MDA productions were detected for the assessment of oxidative stress in HK-2 cells. (C–E) Levels of TNF-α, IL-6, and IL-1β in culture supernatants were determined to assess inflammation in HK-2 cells. (F) Caspase-3 activity in HK-2 cells was determined. $n = 4$. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus pcDNA3.1 + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation; IL: interleukin; MDA: malondialdehyde; ROS: reactive oxygen species; TNF-α: tumor necrosis factor-alpha.

level of ANGPTL2 was markedly decreased in HK-2 cells transfected with si-ANGPTL2 under H/R condition, as compared with HK-2 cells transfected with si-NC under H/R condition (Fig. 2A). In addition, MTT assay showed that HK-2 cells underwent H/R had significantly decreased cell viability compared with those cells under normoxia. This reduction was reversed by the transfection with si-ANGPTL2 (Fig. 2B).

Knockdown of ANGPTL2 Inhibited Oxidative Stress in H/R-Stimulated HK-2 Cells

HK-2 cells under H/R exposure showed increased production of ROS, whereas cells transfected with si-ANGPTL2 showed reduced intracellular ROS level (Fig. 3A). Compared with normoxia, H/R caused a significant increase in MDA level and a decrease in SOD activity. However, knockdown of ANGPTL2 prevented the H/R-caused changes in MDA level and SOD activity (Fig. 3B, C).

Knockdown of ANGPTL2 Inhibited the Secretion of Proinflammatory Cytokines in H/R-Stimulated HK-2 Cells

Next, we examined the effect of ANGPTL2 knockdown on the secretion of proinflammatory cytokines, including TNF-α, IL-6, and IL-1β. HK-2 cells exposed to H/R exhibited significant increases in the levels of TNF-α, IL-6, and IL-1β, compared with normoxic controls. Knockdown of ANGPTL2 resulted in obvious inhibitory effects on H/R-stimulated secretion of TNF-α, IL-6, and IL-1β (Fig. 4A–C).

Knockdown of ANGPTL2 Suppressed Cell Apoptosis Induced by H/R in HK-2 Cells

Compared with control HK-2 cells, cultures exposed to H/R had obvious increase in bax expression and decrease in bcl-2 expression, while H/R-induced changes in the expression levels of bax and bcl-2 were prevented by knockdown of ANGPTL2 (Fig. 5A). Moreover, caspase-3 activity was obviously increased in H/R-stimulated HK-2 cells, which was inhibited by knockdown of ANGPTL2 (Fig. 5B).

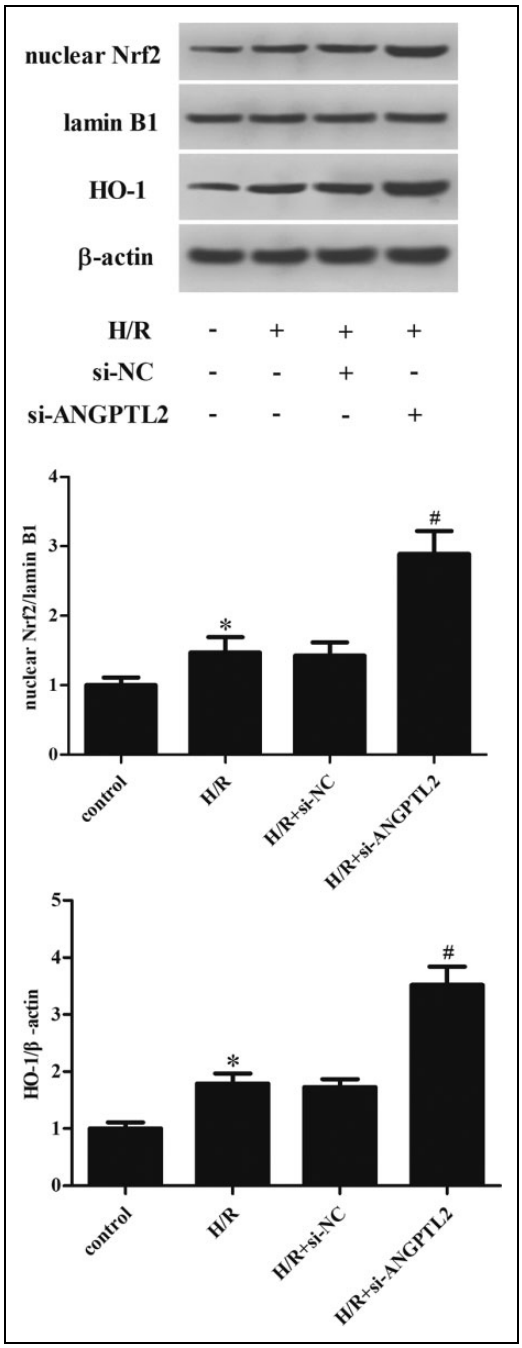

Figure 7. Induction of Nrf2/HO-1 pathway in HK-2 cells by transfection with si-ANGPTL2. Western blot was performed to detect the expression levels of nuclear Nrf2 and HO-1 in HK-2 cells after transfection with si-ANGPTL2 or si-NC. $n = 3$. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus si-NC + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation.

Overexpression of ANGPTL2 Enhanced H/R-Induced Oxidative Stress, Inflammatory Response, and Apoptosis in H/R-Stimulated HK-2 Cells

Then, we examined the effects of ANGPTL2 overexpression on oxidative stress, inflammatory response, and apoptosis in H/R-induced HK-2 cells. The results indicated that the H/R-induced increased levels of ROS, MDA, TNF-α, IL-6, and IL-1β, as well as caspase-3 activity in HK-2 cells, were enhanced by ANGPTL2 overexpression (Fig. 6A–F).

Knockdown of ANGPTL2 Induced the Activation of Nrf2/HO-1 Pathway in H/R-Stimulated HK-2 Cells

To elucidate the molecular mechanism underlying the effects of ANGPTL2, we detected the effect of ANGPTL2 knockdown on the expression levels of nuclear Nrf2 and HO-1. Interestingly, we found that knockdown of ANGPTL2 caused significant increases in the expression levels of nuclear Nrf2 and HO-1 in H/R-induced HK-2 cells (Fig. 7).

Knockdown of Nrf2 Abrogated the Renal-Protective Effect of ANGPTL2 Knockdown in H/R-Stimulated HK-2 Cells

To further confirm the role of Nrf2/HO-1, HK-2 cells were transfected with si-Nrf2 to knockdown Nrf2. Results from western blot analysis showed that expression levels of Nrf2 were markedly reduced after transfection with si-Nrf2 (Fig. 8A, B). The inhibitory effects of ANGPTL2 knockdown on the ROS production and caspase-3 activity were abrogated by knockdown of Nrf2 (Fig. 8C, D). The results indicated that Nrf2/HO-1 signaling pathway was involved in the antioxidative and antiapoptotic activities of si-ANGPTL2.

Knockdown of ANGPTL2 Decreased TLR4/NF-κB Activation in H/R-Stimulated HK-2 Cells

To elucidate the role of TLR4/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway in the effect of ANGPTL2, we detected the expression levels of TLR4, p65, and p-p65. As expected, we found that knockdown of ANGPTL2 significantly suppressed the expression levels of TLR4 and p-p65 in H/R-stimulated HK-2 cells (Fig. 9).

TLR4 Reversed the Anti-Inflammatory Effect of ANGPTL2 Knockdown in H/R-Stimulated HK-2 Cells

To further confirm whether TLR4/NF-κB is associated with the anti-inflammatory effect of ANGPTL2, HK-2 cells were transfected with pcDNA3.1-TLR4 to overexpress TLR4. The results of western blot analysis demonstrated that TLR4 expression levels were dramatically increased in TLR4-overexpressing HK-2 cells (Fig. 10A). Overexpression of TLR4 caused a significant increase in the levels of TNF-α, IL-6, and IL-1β in si-ANGPTL2 transfected HK-2 cells (Fig. 10B–D), which implied that TLR4/NF-κB signaling

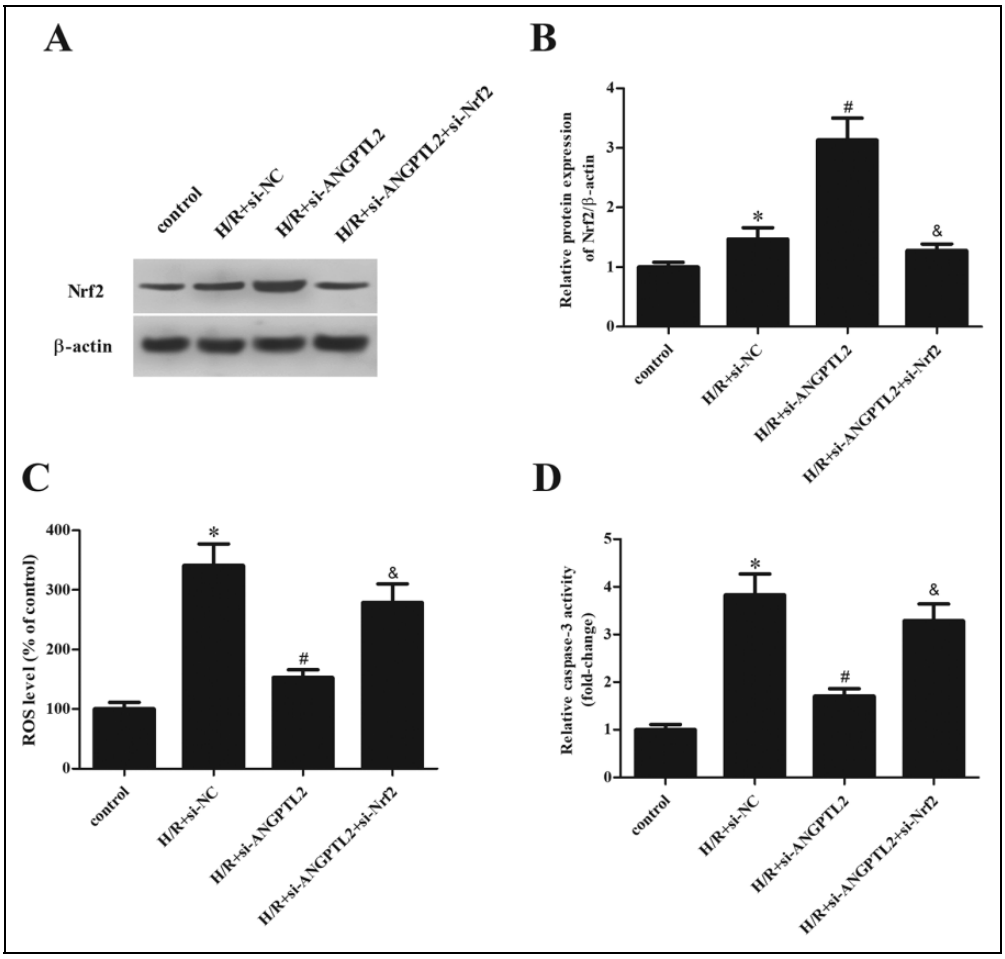

Figure 8. Prevention of the anti-oxidative and anti-apoptotic activities of ANGPTL2 knockdown by transfection with si-Nrf2. HK-2 cells were transfected with si-ANGPTL2 and/or si-Nrf2 under H/R condition. (A) Western blot were performed to detect the protein level of Nrf2. (B) Quantification analysis of Nrf2. (C and D) ROS production and caspase-3 activity in HK-2 cells. $n = 4$. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus si-NC + H/R, $^{\&}P < 0.05$ versus si-ANGPTL2 + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation; ROS: reactive oxygen species.

pathway was implicated in the anti-inflammatory effect of ANGPTL2 knockdown.

Discussion

In this study, we found that the expression level of ANGPTL2 was markedly increased in H/R-induced HK-2 cells. In addition, knockdown of ANGPTL2 significantly inhibited the H/R-induced oxidant stress, inflammatory response, and apoptosis in HK-2 cells. Furthermore, knockdown of ANGPTL2 induced the activation of the Nrf2/HO-1 pathway and decreased TLR4/NF-κB activation in H/R-stimulated HK-2 cells.

ANGPTL2 has received much attention as a circulating proinflammatory and pro-oxidative protein. Yang et al. reported that ANGPTL2 is involved in the inhibitory effects of forkhead box protein C2 on oxidized low-density lipoprotein -induced lipid accumulation, inflammation, and apoptosis of macrophage[19]. ANGPTL2 deficiency attenuates paraquat-induced lung injury progression with reduced inflammation cells in bronchoalveolar lavage fluid, as well as enhanced SOD activity and reduced MDA levels in serum or lung tissue samples[16]. Knockdown of ANGPTL2 protects against angiotensin II-induced cerebral endothelial dysfunction in mice, which is attributed to the regulation of production of inflammatory mediator nitric oxide[20]. ANGPTL2 knockout mice show significantly decreased oxidative stress in skin tissue as well as a lower incidence of squamous cell carcinoma compared with wild-type mice, which strongly suggests that the inflammatory mediator ANGPTL2 accelerates carcinogenesis by activating oxidative stress in skin tissue[17].

I/R injury permeates a variety of diseases and remains a ubiquitous concern in every transplantation proceeding,

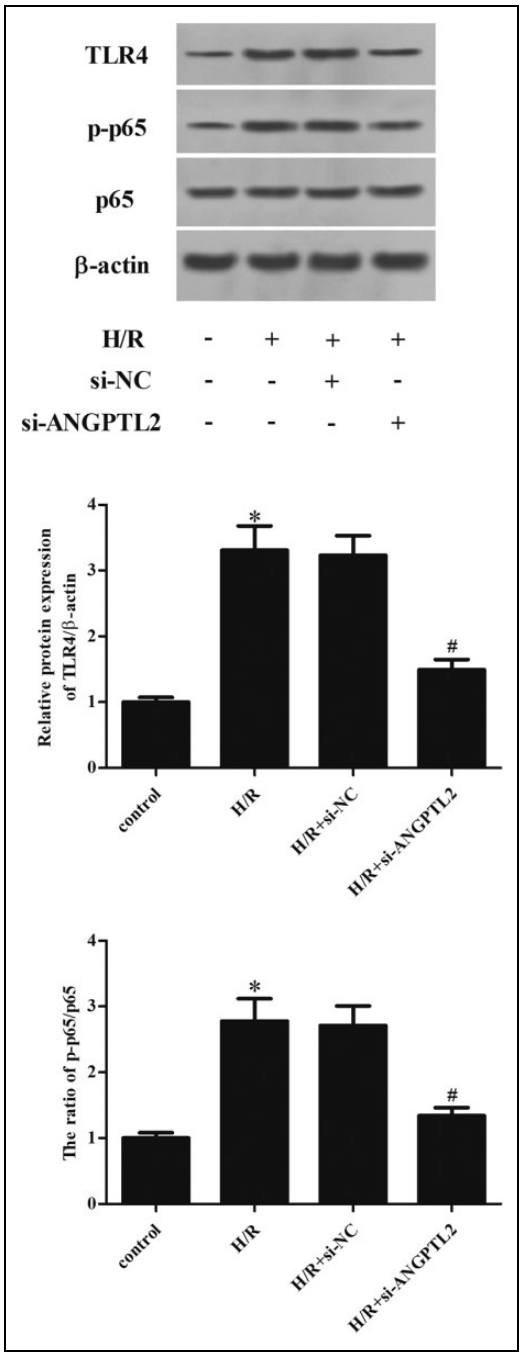

Figure 9. Inhibition of TLR4/NF-κB activation in HK-2 cells by transfection with si-ANGPTL2. Western blot was performed to detect the expression levels of TLR4, p65, and p-p65 in HK-2 cells after transfection with si-ANGPTL2 or si-NC. $n = 4$. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus si-NC + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells; TLR4: toll-like receptor 4.

from whole organs to modest grafts[21]. In ischemic kidney and the subsequent reoxygenation, the generation of ROS at the reperfusion phase initiates a cascade of deleterious cellular responses, which lead to oxidative stress, inflammation, cell death, and acute kidney failure[22]. In addition to the regulation of oxidative stress and inflammation, ANGPTL2 has been found to regulate renal function[23]. It was found that ANGPTL2 levels are positively associated with aortic stiffness and mortality after kidney transplantation[24]. Huang et al. demonstrated that ANGPTL2 regulates autophagy and affects the progression of renal fibrosis in diabetic nephropathy through the MEK/ERK/Nrf-1 pathway[18]. ANGPTL2 increases renal fibrosis by accelerating transforming growth factor-β signaling in chronic kidney disease[25]. Therefore, we speculated that ANGPTL2 might be involved in the renal I/R injury. The current study investigated the role of ANGPTL2 in H/R-stimulated HK-2 cells. The results showed that ANGPTL2 expression was induced by H/R in HK-2 cells. Knockdown of ANGPTL2 improved the viability of HK-2 in response to H/R and attenuated H/R-induced oxidative stress, inflammatory response, and apoptosis. Overexpression of ANGPTL2 in HK-2 cells exhibited contrary effects with ANGPTL2 knockdown.

Recent work has demonstrated the key importance of several key signaling pathways in tubular epithelial cells exposed to I/R injury[3]. Nrf2/HO-1 is a pivotal antioxidant signaling pathway responsible for the defense of intracellular oxidative stress[26]. Importantly, the activation of Nrf2/HO-1 plays an important role in the inhibition of cellular damage and cell apoptosis by modulating oxidative stress in response to I/R injury[27,28]. Additionally, TLR4/NF-κB signaling is an important mediator of inflammation and was found to play a significant role in the inflammatory response during the I/R injury[29,30]. There is evidence showing that ANGPTL2 deficiency attenuates inflammation through the inactivation of the NF-κB pathway and mitigates oxidative stress by regulating the Nrf2 pathway in lung injury mice[16]. The results in the current study showed that knockdown of ANGPTL2 induced the activation of Nrf2/HO-1 and inhibited the activation of the TLR4/NF-κB signaling pathway. Knockdown of Nrf2 abrogated the antioxidative and antiapoptotic effects of ANGPTL2 knockdown in H/R-stimulated HK-2 cells. Besides, the restoration of TLR4 reversed the anti-inflammatory effect of ANGPTL2 knockdown in H/R-stimulated HK-2 cells. Collectively, the renal-protective effect of ANGPTL2 knockdown against H/R injury was mediated by Nrf2/HO-1 and TLR4/NF-κB signaling pathways.

To conclude, our results highlighted the importance of ANGPTL2 involvement in the H/R-stimulated HK-2 cells. Our data revealed the renal-protective effects of ANGPTL2 knockdown against H/R-induced oxidative stress, inflammation, and apoptosis in HK-2 cells, which were attributed to the regulation of Nrf2/HO-1 and TLR4/NF-κB signaling pathways. These findings suggested that ANGPTL2 might be a new possible target for the treatment and prevention of renal I/R injury.

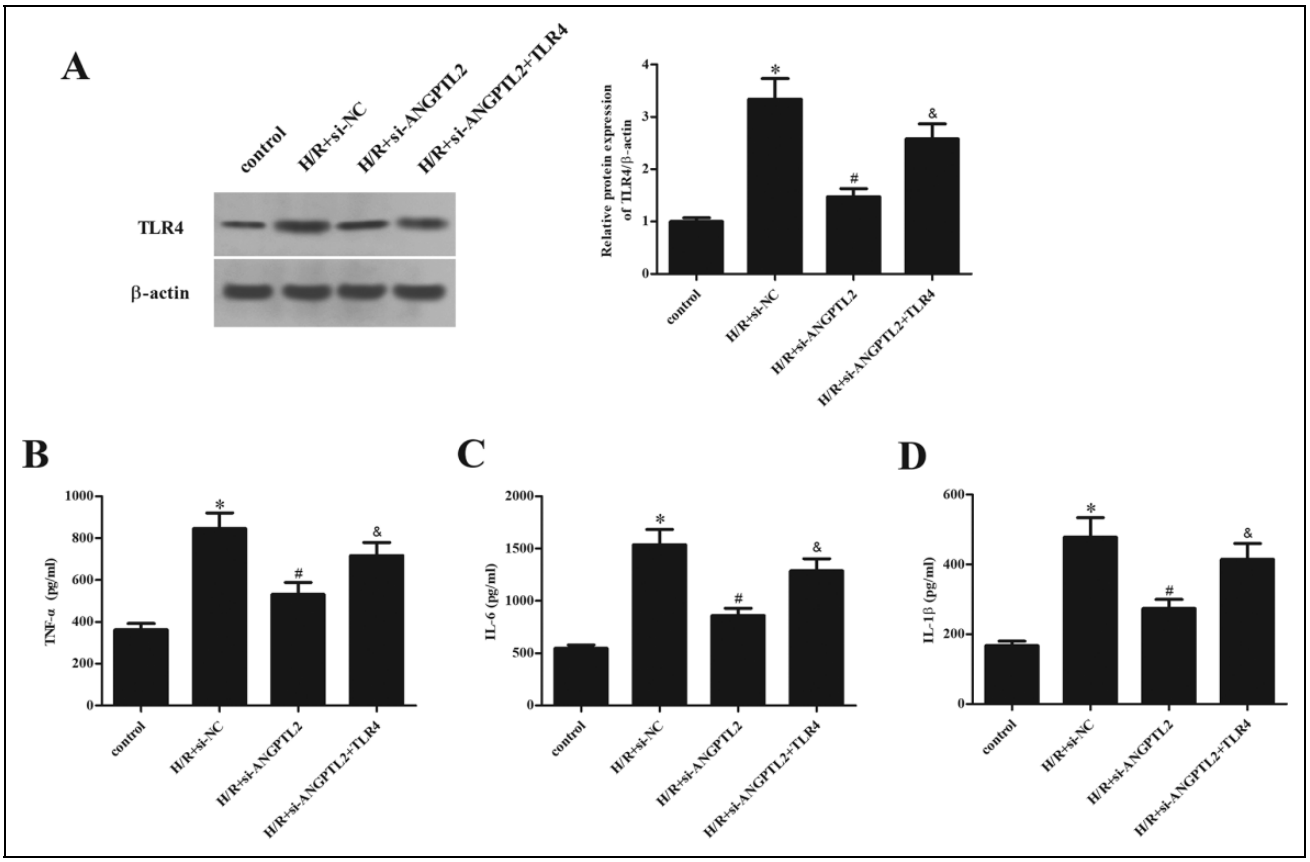

Figure 10. Prevention of the anti-inflammatory effect of ANGPTL2 knockdown by transfection with pcDNA3.1-TLR4. HK-2 cells were transfected with si-ANGPTL2 and/or TLR4 under H/R condition. (A) Western blot was performed to detect the protein levels of TLR4. (B–D) Levels of TNF-α, IL-6, and IL-1β in culture supernatants were determined. $n = 3$. $^{*}P < 0.05$ versus control, $^{\#}P < 0.05$ versus si-NC + H/R, $^{\&}P < 0.05$ versus si-ANGPTL2 + H/R. ANGPTL2: angiopoietin-like protein 2; H/R: hypoxia/reoxygenation; IL: interleukin; TNF- α: tumor necrosis factor-alpha.

Ethical Approval

This study was approved by our institutional review board.

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

Declaration of Conflicting Interests

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