Original Article
Role of miR-146a in astragaloside IV ameliorated ischemic acute renal injury by regulating NF-κB signaling activity

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Abstract: Acute kidney injury (AKI) has received increasing attention in recent years. Our previous study has revealed that Astragaloside IV (AS-IV) ameliorated ischemic AKI by inhibiting the inflammatory response, which might be implicated in the aberrant expression of miR-146a. Therefore, in the present study, we aimed to elucidate the role of miR-146a in the renal protection of AS-IV. Rats were orally administered 15 mg·kg^{-1} AS-IV for 7 days prior to ischemic-reperfusion (I/R), and were sacrificed for the evaluation of injury and inflammation at 12 h, 24 h, 48 h and 72 h after I/R. We found that pretreated with AS-IV significantly attenuated the functional deterioration and histological damage induced by AKI, as well as decreased the inflammatory mediators, such as myeloperoxidase (MPO), tumor necrosis factor α (TNF-α), monocyte chemo-attractant protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1). In addition, AS-IV pretreatment significantly elevated the expression of miR-146a, which had a lower expression in AKI rats and in injured rat renal epithelial cell line (NRK-52E), originally. Moreover, up-regulation of miR-146a in injured NRK-52E cells could deregulate the levels of MCP-1, ICAM-1 and TNF-α. Furthermore, in the protein levels, interleukin-1-receptor-associated kinase 1 (IRAK-1), C-X-C motif chemokine ligand 2 (CXCL-2), hypoxia inducible factor-1α (HIF-1α) and the phosphorylated p65 subunit of NF-κB (p-p65) were also greatly decreased. Taken together, our results provide important evidences that AS-IV pretreatment ameliorated ischemic AKI and inhibited the inflammatory response by up regulating the expression of miR-146a, implying that miR-146a might be serve as a potential therapeutic target against AKI.

Keywords: Acute kidney injury, astragaloside IV, miR-146a, NF-κB signaling, ischemia reperfusion injury

Introduction

Acute kidney injury (AKI), the clinical manifestation of several disorders that affect the kidney acutely, is common in hospital patients, especially in critically ill patients [1]. According to statistics, about one third of AKI is caused by direct or indirect nephrotoxicity, but two thirds are caused by ischemic/reperfusion (I/R) injury or sepsis [2, 3]. Although the pathophysiological mechanisms of AKI have been described in several studies, therapeutic options are still limited, resulting in its high mortality and poor prognosis. Therefore, it is imperative to accelerate the development of new and more effective strategies for AKI treatment.

Astragaloside IV (AS-IV) is one of the main active ingredients of A. membranaceous. Che-

mically, it is a cycloartanetriterpenesaponin with a clear formula and definite molecular weight [4, 5]. It has received more attention from the field of drug research due to its multiple properties, like anti-viral, immune modulatory, pro-angiogenesis and anti-inflammation [6, 7]. More importantly, AS-IV has been widely used for the cardiovascular diseases (CVDs) treatment in China [4]. Our previous study has revealed that AS-IV had positive effects on ischemic AKI by inhibiting the expression of inflammatory mediators [8]. However, the molecular pathogenesis by which AS-IV protected ischemic AKI and inhibited inflammation remains to be explored.

In recent years, emerging evidence has indicated the critical role of microRNAs (miRNA) in the regulation of various biological and pathologic

processes [9, 10]. These small, non-coding molecules elicit their regulatory effects by imperfectly binding to the 3' untranslated region of target mRNA, causing either degradation of mRNA or inhibition of their translation to functional proteins [11-13]. Recent evidence has indicated that miR-146a plays a key role in the development of AKI. In particular, the deregulated expression of miR-146 has also been observed in kidneys subjected to I/R injury [14, 15]. Moreover, further studies showed that over expression of miR-146a could suppress the inflammatory response through down regulation of the NF-κB and the NF-κB regulatory kinase interleukin-1-receptor-associated kinase 1 (IRAK-1) [16]. These results compelled us to assume that the protective effects of AS-IV on I/R injury by inhibiting the inflammation were associated with miR-146a expression. Thus, in the current study, we adopted the I/R injury model in vivo and injured NRK52E cells in vitro to investigate the role of miR-146a in renal protection mediated by AS-IV, which will provide new insights into the field of AKI therapy.

Materials and methods

Cell culture and drugs preparation

Rat renal tubular epithelial cells NRK-52E were maintained in DMEM/F 12 (Gibco) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 atmosphere at 37°C. AS-IV, purchased from Xi'an Sobeo Pharmaceutical Technology Company, Limited (purity above 98%, Xi'an, China), was suspended in 1% carboxymethyl cellulose (CMC) solution and was administered once a day to rats by oral gavage as described in previous studies [8, 17]. Cobalt (II) chloride hexahydrate ($CoCl_2$) purchased from Sigma (Sigma-Aldrich, Shanghai, China), was dissolved in ddH_2O, and cells were treated at a final concentration of 800 nM for 24 h.

Rat model of I/R injury

This study was approved by the Animal Ethics Committee of Zhejiang Academy of Medical Sciences. All animal procedures were performed in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

160 male Sprague-Dawley (SD) rats of 7-week old, purchased from the Experimental Animal Center, Zhejiang academy of medical sciences, Hangzhou, China, were adopted as the subjects of this study. Animals were randomly divided into four groups according to the duration of reperfusion: 12 h, 24 h, 48 h, and 72 h. In each group, animals were further subdivided into the following groups (n = 8 per subgroup): (1) sham-operated rats pretreated with normal saline (Sham); (2) I/R rats pretreated with CMC vehicle alone served as control (Veh); (3) I/R rats pretreated with AS-IV at dose of 10 mg·kg^{-1} (L-AS); (4) I/R rats pretreated with AS-IV at dose of 15 mg·kg^{-1} (M-AS); and (5) I/R rats pretreated with AS-IV at dose of 20 mg·kg^{-1} (H-AS). AS-IV was orally administered once a day to rats for 7 days prior to ischemia. Rats were then anesthetized with pentobarbital sodium (50 mg·kg^{-1} ip) and placed on a homoeothermic table to maintain core body temperature at 37°C. Rat ischemic AKI model was established by bilateral clamping of the renal arteries for 45 min, followed by reperfusion for 12 h, 24 h, 48 h, and 72 h, respectively. Rats in the sham group underwent identical procedure but without bilateral renal artery clamping. At 12 h, 24 h, 48 h and 72 h after reperfusion, blood samples were collected from the abdominal inferior cava vein and the kidneys were then removed and bisected in the equatorial plane. The left kidney was snap-frozen in liquid nitrogen, and the right kidney was fixed in 10% buffered formalin and prepared for routine histological examinations. Blood samples and the left kidney were stored at -80°C for analysis.

Evaluation of renal function and histological damage

Serum creatinine, BUN and cystatin C were used to evaluate renal function. Levels of creatinine and BUN were measured by ELISA kit (CUSABIO, Wuhan, China). Levels of Cystatin C were measured by particle-enhanced turbid metric immunoassay (PETIA) in accordance with the manufacturer's instructions.

The kidneys, fixed in a 10% neutral buffered formalin solution, were embedded in paraffin, cut into 4 μm sections and then stained with hematoxylin and eosin. The sections were then examined by light microscopy. Histopathology scoring was assessed by grading tubular necro-
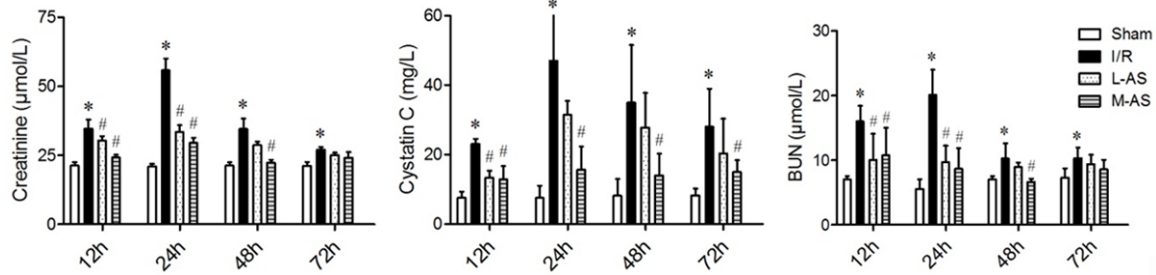
Figure 1. Protective effects of AS-IV on renal dysfunction at 12 h, 24 h, 48 h and 72 h of reperfusion. Serum creatinine, cystatin C and BUN were measured by ELISA. Sham, sham-operated rats treated with normal saline; I/R, I/R rats pretreated with carboxymethyl cellulose vehicle alone served as control; L-AS, I/R rats pretreated with AS-IV (10 mg·kg^{-1}·d^{-1}); M-AS, I/R rats pretreated with AS-IV (15 mg·kg^{-1}·d^{-1}). Results are expressed as mean ± SD (n = 8). $*P <$ 0.05 versus Sham group; $\#P <$ 0.05 versus I/R group. Data were analysis by ANOVA and Dunnett's multiple range test. Abbreviations: I/R, ischemia-reperfusion; BUN, blood urea nitrogen; AS-IV, Astragaloside IV.

sis, loss of brush border, cast formation, and tubular dilatation in 10 randomly chosen, no overlapping fields. The degree of kidney damage was estimated by the following criteria: 0, none; 1, ≤ 10%; 2, 10.1-25%; 3, 25.1-45%; 4, 45.1-75%; and 5, ≥ 75.1% [6]. The morphologic assessment was performed by a renal pathologist without knowledge of treatment.

Detection of inflammatory mediators in serum and cell culture supernatants

Inflammatory mediators like cytokines, chemokines, neutrophils, lymphocytes, NK cells, and macrophages are involved in the pathogenesis of AKI. Serum levels of myeloperoxidase (MPO) in rats subjected to I/R injury were measured by commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). In rats with I/R injury, levels of MCP-1 (Abcam), ICAM-1 (Abcam), and TNF-α (CUSABIO, Wuhan, China) in serum and cell culture supernatants were determined using commercial ELISA kits. All ELISAs were performed according to the manufacturer's instructions. Cell cultures supernatants was collected and centrifuge for 20 minutes to remove insoluble impurity and cell debris at 1000 × g at 2-8°C. The supernatants were then stored at -80°C until the ELISA assays could be performed.

Transient transfection of miR-146a mimic or inhibitor

MicrONTM rno-miR-146a mimic, micrOFFTM rno-miR-146a inhibitor, micrONTM mimic negative control and micrOFFTM negative control were obtained from RiboBio Co. Transfection

was performed using HiperFect Transfection Reagent (QIAGEN). Twenty-four hours before CoCl$_2$ treatment, cells were seeded in the six-well plate and transfected with miR-146a mimic and inhibitor at a final concentration of 50 nM and 100 nM respectively. The negative control used the same concentration as the miR-146a mimic and inhibitor used, respectively. Total RNA and protein were extracted from each sample for analysis.

Quantitative real time polymerase chain reaction

Total RNA was extracted from tissues and cells by using the Trizol reagent (Invitrogen) via the manufacturer's instructions. For miRNA detection, reverse transcription was carried out using high capacity RNA-to-cDNA Kit (Applied Biosystems) for 60 min at 37°C [6].

Quantitative real time polymerase chain reaction (PCR) analyses were carried out by SYBR Green Master Mix (Applied Biosystems) on an Applied Biosystems 7500 Real Time PCR System. For determination of miR-146a expression, we used QuantiTectprimer assays (QIAGEN). Each reaction was amplified in triplicate, and ratio results were calculated based on the 2$^{-\Delta\Delta CT}$ method. Values were normalized to Rnu6B expression.

Western blot

Total protein extracted from rat renal tissues and NRK-52E cells following the protocol as usual [6, 8]. Western blot analyses of protein expression carried out by routine procedures,
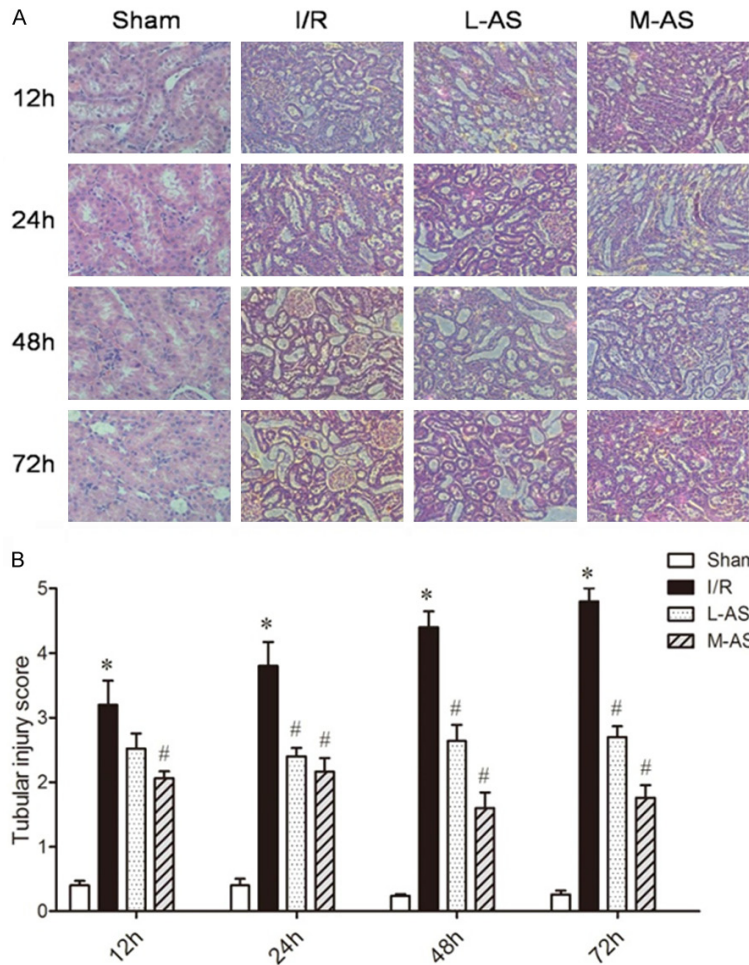
Figure 2. AS-IV protected rat from histology damage induced by ischemic AKI. Representative hematoxylin and eosin (H&E) stained kidney sections from Sham, I/R, or AS-IV pretreated rats at 12 h, 24 h, 48 h and 72 h of reperfusion (A) and the statistical results were showed by bar graphs (B). Sham, sham-operated rats treated with normal saline; I/R, I/R rats pretreated with carboxymethyl cellulose vehicle alone served as control; L-AS, I/R rats pretreated with AS-IV (10 mg·kg^{-1}·d^{-1}); M-AS, I/R rats pretreated with AS-IV (15 mg·kg^{-1}·d^{-1}). Images were taken at 200 × magnification. *$P < 0.05$, versus Sham group; #$P < 0.05$ versus I/R group. Data were analysis by ANOVA and Dunnett's multiple range test. Abbreviations: I/R, ischemia-reperfusion; AS-IV, Astragaloside IV.

as described previously [8]. The primary antibodies were obtained from following sources: Anti-HIF-1a (Abcam), anti-IRAK-1 (Santa Cruz Biotechnology), anti-CXCL-2 (Abcam), anti-NF-κB p65 (Cell Signaling), anti-p65 (Cell Signaling), and GAPDH (Cell Signaling) was used for loading controls.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). The significance of differences among experimental groups was determined by ANOVA analysis. When a significant difference

was detected, the data were further analyzed by Dunnett's multiple range test. A value of $P < 0.05$ was considered statistically significant.

Results

AS-IV ameliorated renal dysfunction and protected AKI rats from kidney histological damage induced by I/R

In our model of I/R injury, serum creatinine, cystatin C and blood urea nitrogen (BUN) levels were significantly elevated compared with the sham-operated group at 12 h, 24 h, 48 h and 72 h of reperfusion. However, AS-IV pretreatment resulted in lower levels of serum creatinine, cystatin C and BUN at 12 h, 24 h, 48 h and 72 h of reperfusion (**Figure 1**). These results indicated that pretreated rats with AS-IV at 15 mg·kg^{-1}·d^{-1} significantly ameliorated renal dysfunction induced by reperfusion injury at 12 h, 24 h, 48 h and 72 h.

In addition, the kidneys from the vehicle group had various degree of severe tubular damage, as evidenced by tubular necrosis and tubular cell detachment at 12 h, 24 h, 48 h and 72 h of reperfusion. In contrast, AS-IV administered 7 days before I/R resulted in a significant lower level in the histopathology damage to tubules at 12 h, 24 h, 48 h and 72 h of reperfusion, respectively (**Figure 2A** and **2B**). Taken together, these findings revealed that AS-IV pretreatment prevented rats from renal dysfunction and histological damage induced by ischemic AKI.

AS-IV inhibited the inflammatory response in rats with AKI induced by I/R

Systemic inflammation is also of great importance in the pathogenesis of AKI, so we examined the effects of AS-IV on the serum levels of pro-inflammatory factors. As shown in **Figure 3**,
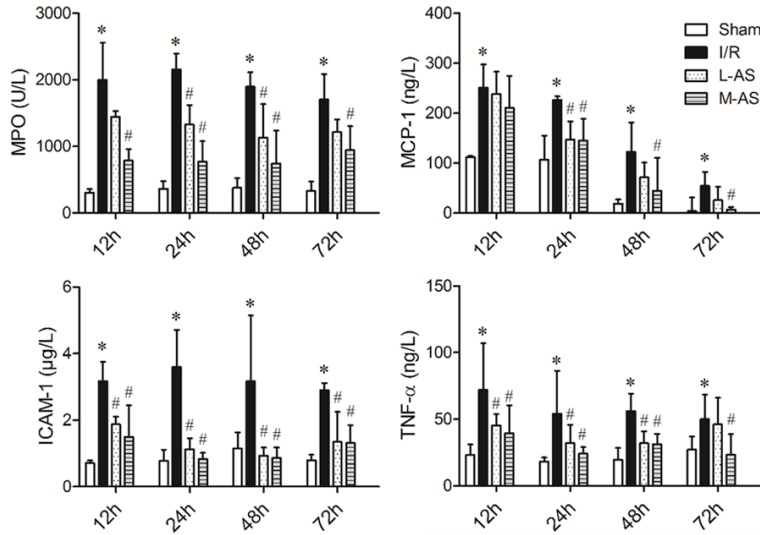
Figure 3. AS-IV pretreatment suppressed the inflammatory response induced by ischemic AKI. Sham, sham-operated rats treated with normal saline; I/R, ischemia-reperfusion (I/R) rats pretreated with carboxymethyl cellulose vehicle alone served as control; L-AS, I/R rats pretreated with AS-IV (10 mg·kg^{-1}·d^{-1}); M-AS, I/R rats pretreated with AS-IV (15 mg·kg^{-1}·d^{-1}). *P < 0.05, versus Sham or control group; #P < 0.05 versus I/R group. Data were analysis by ANOVA and Dunnett's multiple range test. Abbreviations: I/R, ischemia-reperfusion; MPO, myeloperoxidase; MCP-1, monocyte chemoattractant protein-1; ICAM-1, intercellular adhesion molecule-1; TNF-α, tumor necrosis factor α; AS-IV, Astragaloside IV.
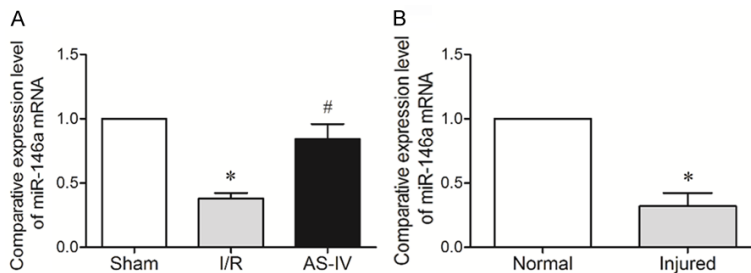

Figure 4. Changed of miR-146a expression at mRNA level in vivo and in vitro. A. mRNA expression of miR-146a in AKI rat kidney. Sham, sham-operated rats treated with normal saline; I/R, I/R rats pretreated with carboxyl methylcellulose vehicle alone served as control; AS-IV, Rats pretreated with 15 mg·kg^{-1}·d^{-1} AS-IV suffered to I/R for 48 h. Results are expressed as mean ± SD (n = 8). B. mRNA expression of miR-146a in NRK-52E cells treated with CoCl$_2$ at the concentration of 800 μM for 24 h. All experiments were repeated three times. *P < 0.05, versus Sham or normal group; #P < 0.05 versus I/R group. Data were analysis by ANOVA and Dunnett's multiple range test. Abbreviations: I/R, ischemia-reperfusion; AS-IV, Astragaloside IV.

after reperfusion, the serum levels of tumor necrosis factor α (TNF-α), monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule-1 (ICAM-1) and the kidney myeloperoxidase (MPO) activity were greatly increased in the vehicle group compared with the sham-operated group at 12 h, 24 h, 48 h and

72 h of reperfusion. However, AS-IV pretreatment group showed much lower levels of TNF- α, MCP-1, ICAM-1 and MPO than the vehicle group at 12 h, 24 h, 48 h and 72 h of reperfusion. These results suggested that AS-IV pretreatment protected rats from I/R injury in the kidney and inhibited inflammation response induced by I/R.

AS-IV elevated the mRNA expression of miR-146a in the kidney of AKI rats and the injured renal tubular epithelial cells

We have reported that AS-IV pretreatment greatly decreased the expression of NF-κB in the kidney of rats subjected to I/R [8]. Furthermore, a number of studies have demonstrated that miR-146a suppresses the expression of NF-κB by targeting IRAK-1 [18, 19]. By quantitative real time PCR analysis, we found that the expression of miR-146a was much lower in the kidney of rats subjected to 24 h of reperfusion in comparison to the sham-operated group. However, AS-IV pretreatment significantly increase its expression in comparison to the vehicle group (**Figure 4A**), suggesting a key role of miR-146a in renal protection mediated by AS-IV.

Up-regulation of miR-146a inhibited inflammatory response stimulated by CoCl$_2$ treatment in NRK-52E cells

To investigate the biological mechanisms by which miR-146a contribute to the protective effects of AS-IV in ischemic AKI, we established an injured renal tubular epithelial model by treating NRK-52E cells with CoCl$_2$. As shown in **Figure 4B**, the expression of miR-146a at mRNA levels significantly decreased after CoCl$_2$ treat-
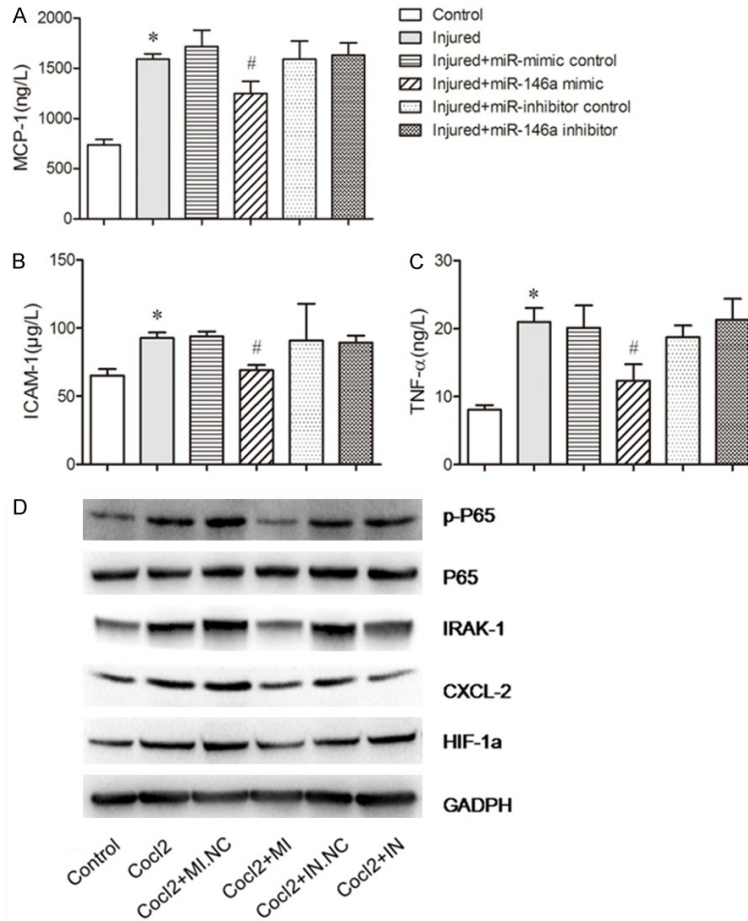
Figure 5. Ffects of miR-146a on inflammatory cytokines and related proteins expression in vitro. Levels of MCP-1 (A), ICAM-1 (B), and TNF-α (C) in the culture supernatants of NRK-52E measured by ELISA. The protein expression of HIF-1α, CXCL-2, IRAK-1, and the p-p65 in injured NRK-52E cells in the condition with transferring miR-146a mimic, inhibitor and control were detected by western blot (D). Results are expressed as mean ± SD (n = 8). *$P < 0.05$ versus control group; #$P < 0.05$ versus CoCl$_2$ treatment group. Data were analysis by ANOVA and Dunnett's multiple range test. Abbreviations: IRAK-1, interleukin-1-receptor-associated kinase 1; CXCL-2, chemokine ligand 2; HIF-1α, hypoxia inducible factor 1 α; p-p65, the phosphorylated p65 subunit of NF-κB.

the inflammatory response induced by CoCl$_2$ treatment, indicating that the renal protection effects of AS-IV could be mediated through the up-expression of miR-146a.

MiR-146a protects renal injury by repressing IRAK-1 and subsequently inhibiting NF-κB activity

The NF-κB signaling pathway is important in inflammation and cell survival. To elucidate the molecular mechanisms of miR-146a, we detected the expression of related proteins by western blot. As shown in **Figure 5D**, CoCl$_2$ treatment significantly increased the phosphorylated p65 subunit of NF-κB(p-p65) compared to the control group, whereas the levels of total NF-κB have no significant changed after CoCl$_2$ treatment. The levels of hypoxia inducible factor-1α (HIF-1α), C-X-C motif chemokine ligand 2 (CXCL-2), and IRAK-1 also significantly increased in cells after CoCl$_2$ treatment in comparison to that of the control group. However, cells transferred miR-146a mimic before CoCl$_2$ treatment showed much lower levels of HIF-1α, CXCL-2, IRAK-1 and the p-p65 than CoCl$_2$ treated alone. In contrast, cells transferring miR-146a inhibitor or negative control showed no statistical changes in the expression of HIF-1α, CXCL-2, IRAK-1 and the p-p65 compared to the group CoCl$_2$ treated alone. These results suggested that AS-IV ameliorated renal dysfunction and inhibited the inflammatory response by up regulating expression of miR-146a, which suppressed NF-κB activity and its regulatory mediator IRAK-1.

ment, which was consistent with the results obtained from the kidneys of rats with AKI. We also examined the influence of miR-146a on level of inflammatory factors in NRK-52E cells. As shown in **Figure 5A-C**, after CoCl$_2$ treatment, the levels of MCP-1, ICAM-1 and TNF-α markedly increased in comparison to that of the control group. However, cells transfected with miR-146a mimic before CoCl$_2$ treatment showed a significantly lower levels of these inflammatory factors than the CoCl$_2$ treated alone group. Transfection of miR-146a inhibitor or negative control to cells did not change the levels of MCP-1, ICAM-1 and TNF-α. These results suggested that up-regulation of miR-146a inhibited

Discussion

Inflammation has an important role in the initiation and extension phases of AKI. Therefore, the inhibition of an inflammatory response in

the kidney might be a novel strategy for protecting AKI [20-24]. We previously reported that AS-IV protected I/R injury by suppressing NF-κB activity [8], which is closely associated with inflammatory response. In the present study, we found that AS-IV pretreatment effectively protected rats from I/R injury and inhibited the inflammatory response, although we did not detected the expression of NF-κB in the kidney of rats subjected to I/R injury, the results has been established [8, 25, 26]. In currently study, we added two time points of reperfusion of 48 h and 72 h. The data obtained suggest that AS-IV play its protective effects continue to 72 h after I/R injury. In addition, our previous study demonstrated that the better protective effects of AS-IV were treated rats with 20 mg/kg. However, here we found that the effects of AS-IV pretreatment with 15 mg/kg were superior to 20 mg/kg (data not shown). The possible factors resulting in discrepancies between them might be caused by different batches of rat and AS-IV, and different experimenters, etc. Indeed, we also noticed a great reduction of creatintine, cystatin C, BUN and MCP-1 levels in I/R rats at 72 h of reperfusion with no treatment, but AS-IV pretreatment decreased the levels more quicker than I/R group. Furthermore, our results showed that AS-IV pretreatment significantly prevented renal histological damage from I/R injury. Taken together, it is reasonable to speculate that AS-IV had preventive benefits for AKI.

The molecular mechanisms involved in the AS-IV mediated protection of ischemic AKI and inhibition of NF-κB were still not fully understood. We found that miR-146a was lower expression in the kidney of rats subjected to I/R injury, but higher expression in the kidney of AS-IV pretreatment rats (**Figure 4A**). Several studies demonstrated that NF-κB could be activated by IRAK-1, a target of miR-146a [16, 27, 28]. Therefore, it's reasonable to specula that inflammatory inhibition mediated by miR-146a could be through the down-regulation of IRAK-1 and subsequent inactivation of NF-κB. Indeed, greatly decreased expression of the p-p65 and IRAK-1 were observed in this study (**Figure 5D**), as well as the levels of the inflammatory mediators including MCP-1, TNF-α, ICAM-1 and MPO (**Figure 5A-C**) after miR-146a mimic transfection, which were in line with the findings reported by other investigators showing that up-regu-

lation of miR-146a inhibited NF-κB activity by targeting IRAK-1, and that miR-146a controlled cytokine and toll like receptor (TLR) signaling through a negative feedback regulatory loop [27-31]. Moreover, the protective effects of miR-146a on I/R injury by inhibiting IRAK-1 were also reported in mouse and human small intestine [18]. Taken together, above results suggested that miR-146a and its targeted genes could be exploited for designing novel strategies for the treatment of ischemic AKI in the future. However, controversies still exist. For example, Joh et al reported the mRNA expression of IRAK-1 and TNF receptor associated factor 6 (TARF-6) was not affected by miR-146a [32]; moreover, the expression of miR-146a could enhance NF-κB activity [33]. The different samples adopted may be the main reason for the inconsistent experimental results.

There still exist some limits in our study. Firstly, the precise mechanism by which IRAK-1 mediates NF-κB remains to be elucidated. Although several studies reported that IRAK-1 could inhibit IκBα, which leads to the activation of NF-κB [16]. Secondly, experimental studies in AKI commonly have three models, I/R, sepsis-endotoxemia and nephrotoxic models [34]. We demonstrated the key role of miR-146a in a model of I/R injury, but its role in other models remain to be investigated. Furthermore, because the protective role of miR-146a on ischemic AKI was not validated in vivo experiment, further study using miR-146a knockdown mice should be required. Finally, the effects of AS-IV and miR-146a on ischemic AKI were also need to validate on larger animal sample, although it has established that AS-IV has no toxic side effects to the liver and renal function [26].

In conclusion, our results showed that AS-IV pretreatment ameliorated ischemic AKI dysfunction and inhibited an inflammatory response through up-regulation of miR-146a to repress NF-κB activation. The present study, together with our previous work [8], suggested that miR-146a might be used as a potential therapeutic target for AKI.

Acknowledgements

Disclosure of conflict of interest

None.

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