

## Original Article

# AS-IV protects against kidney IRI through inhibition of NF- $\kappa$ B activity and PUMA upregulation

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**Abstract:** Objective: To determine and explore the effect of Astragalus saponin IV (AS-IV) on ischemia/reperfusion (IR)-induced renal injury and its mechanisms. Methods: Experimental model of renal I/R was induced in rats by bilateral renal artery clamp for 45 min followed by reperfusion of 6 h. Rats were divided into three groups: ① sham ② IRI ③ IRI/AS-IV. In IRI/AS-IV groups, AS-IV was orally administered once a day to rats at 2 mg·kg<sup>-1</sup>·d<sup>-1</sup> for 7 days prior to ischemia. At 6 h after reperfusion, the inflammatory cytokines and renal function was assessed and NF- $\kappa$ B activity and PUMA expression was detected. Apoptotic cells was detected by TUNEL assay. Results: AS-IV significantly decreased serum and tissue levels of IL-6 and TNF- $\alpha$ , and reduced apoptotic cell counts and histological damage. AS-IV down-regulated the phosphorylation of p65 subunit of NF- $\kappa$ B (NF- $\kappa$ B p65) and PUMA expression, and the NF- $\kappa$ B activity compared to the I/R groups. Conclusions: AS-IV provided protection against IRI-induced renal injury by reducing apoptosis and inflammation through inhibition of NF- $\kappa$ B activity and PUMA expression. AS-IV pre-treatment ameliorated tubular damage and suppressed the NF- $\kappa$ B p65 expression.

**Keywords:** Kidney, ischemia reperfusion injury (IRI), NF- $\kappa$ B, p53 upregulated modulator of apoptosis (PUMA), apoptosis, inflammatory genes, astragaloside IV (AS-IV)

## Introduction

Ischemia/reperfusion injury (IRI) is a leading cause of acute renal failure (ARF). It is a common renal disease that is still associated with high mortality, despite significant advances in the healthcare system [1]. IRI is caused by a suddenly transient drop in blood flow associated with a robust inflammatory and oxidative stress response to hypoxia and reperfusion, frequently occurring during shock, sepsis and transplantation [2]. Although important findings have been made in the definition of the cell biologic consequences of IRI [3, 4], there are still few therapies available for this clinical problem [5]. Thus, there is an urgent need for the development of novel and effective therapeutic approaches to prevent IRI.

Inflammatory response is now believed to play a central role in the pathophysiology of IRI. IRI-induced inflammatory responses make the inflammatory cells extravasulate from the blood stream and attract to the kidney tissues

[6, 7]. Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and IL-6 is a pleiotropic cytokine that has an important role in a variety of physiological and pathological processes such as immune and inflammatory [8, 9]. Numerous studies have demonstrated that TNF- $\alpha$  and IL-6 play an important role in the pathophysiology of IRI [10, 11]. Therefore, inflammation is now believed to have a central role in the pathogenesis of IRI. There is mounting evidence indicating a critical role of Nuclear factor- $\kappa$ B (NF- $\kappa$ B) in the pathogenesis of renal IRI. Activation of NF- $\kappa$ B has a major role in the pathophysiology of experimental ischemic IRI [12]. NF- $\kappa$ B is the best-known mediator of TNF- $\alpha$  and IL-6-associated cellular response. We suggested NF- $\kappa$ B-mediated inflammatory processes represent an important mechanism leading to IRI. Therefore, anti-inflammatory strategies that targeting NF- $\kappa$ B pathway may effectively prevent IRI.

Since investigations of the mechanism of the renal dysfunction and injury in IRI have also revealed involvement of apoptosis except for

inflammation [13]. Apoptotic cell death has been documented in experimental animal models and humans post-renal ischemia, and inhibition of apoptotic cell death is shown to ameliorate the injury and inflammation [14, 15].

A large body of evidence has demonstrated a protective role of NF- $\kappa$ B in TNF- $\alpha$ -induced apoptosis in most tissues and cell types. For example, deletion of NF- $\kappa$ B p65 (p65) in mice leads to increased apoptosis in several tissues [16]. The protection by NF- $\kappa$ B is due to transcriptional activation of a number of antiapoptotic proteins, such as c-FLIP, Bcl-2, Bcl-XL, cIAP2, and A1/Bfl-2 [17]. Conversely, NF- $\kappa$ B has been found to induce apoptosis under certain conditions by activating proapoptotic proteins, such as p53, Fas, Fas ligand and death receptor [18]. However, the mechanisms and physiological significance of NF- $\kappa$ B in apoptosis regulation remain controversial and poorly understood.

p53 upregulated modulator of apoptosis (PUMA) is a downstream target of p53 and a BH3-only Bcl-2 family member [19, 20]. It is induced by p53 following exposure to DNA-damaging agents, such as  $\gamma$ -irradiation and commonly used chemotherapeutic drugs [19, 20]. It is also activated by a variety of nongenotoxic stimuli independent of p53, such as serum starvation, kinase inhibitors, glucocorticoids, endoplasmic reticulum stress, and ischemia/reperfusion [21].

Recently, it has been found that PUMA is a direct target of NF- $\kappa$ B and mediates TNF- $\alpha$ -induced apoptosis *in vitro* and *in vivo* [22]. Qiu et al. found that the induction of PUMA was p53-independent but required NF- $\kappa$ B [23]. Therefore, anti-apoptotic strategies that targeting NF- $\kappa$ B pathway may effectively prevent IRI.

Astragaloside IV (AS-IV) is one of the main active ingredients of *A. membranaceus*. Chemically, it is a cycloartane triterpene saponin with a clear formula and definite molecular weight [24, 25]. It was reported that AS-IV ameliorated IRI in heart and brain [26, 27]. It has demonstrated the anti-inflammatory effects of AS-IV through inhibition of NF- $\kappa$ B mediated inflammatory genes expression *in vitro* [28, 29].

It has recently reported pretreatment with AS-IV significantly decreased blood urea nitrogen,

serum creatinine, cystatin C as well as urinary kidney injury molecule-1 level and tubular injury. AS-IV also reduced tubular cell apoptosis [30]. Therefore, AS-IV can be developed as a novel therapeutic approach to prevent IRI through targeting apoptotic pathways.

In the present study, we adopted the murine model of renal IRI to test the hypothesis that AS-IV prevents inflammation and apoptosis in renal IRI by suppressing NF- $\kappa$ B activity and PUMA expression, and provide new insights into the field of renal IRI therapy.

### Materials and methods

#### *Drug preparation*

AS-IV (99.2% purity) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Shanghai, People's Republic of China). AS-IV was suspended in 1% carboxymethyl cellulose (CMC) solution as a vehicle for its administration and was administered once a day to rats by oral gavage. The dosage of AS-IV used in this study was chosen as 2 mg·kg<sup>-1</sup>·d<sup>-1</sup> for 7 days.

#### *Mice*

Isogenic male C57BL/6 mice, age 8-12 wks (25-28 g), were purchased from SPF Ltd. Com, Beijing, China. All animals were housed in individual and standard cages and had free access to water and food. All procedures were previously reviewed and approved by the Ethics Committee of the Qingdao University (document number 17/2014).

#### *Experimental model of renal IRI*

Animals were further subdivided into the following groups (n = 8 per subgroup): ① sham-operated mice pretreated with normal saline (Sham), ② IRI mice pretreated with CMC vehicle alone served as control (Veh), ③ IRI rats pretreated with AS-IV at dose of 2 mg/kg (AS-IV). AS-IV was orally administered once a day to rats for 7 days prior to ischemia. Surgery was performed as previously described [31]. Briefly, mice were anesthetized with Ketamine-Xylazine, a midline incision was made and both renal pedicles were cross-clamped for 45 minutes. During the procedure, animals were kept well hydrated with saline and at a constant temperature (37°C) through a heating pad device.

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45 min after ischemia, microsurgery clamps were removed. The mice were sacrificed after 6 hours of reperfusion.

### *Analysis of renal function*

Serum Cr was used for evaluation of renal function. Blood samples were collected at 6 hours post reperfusion from the abdominal inferior cava vein immediately before induced death. Serum samples were analyzed.

### *Histology assay*

4% paraformaldehyde-fixed, paraffin-embedded 5 mm kidney sections were stained with PAS stain using standard methods. Histological examination was performed by a renal pathologist in a blinded fashion. Histological injury was scored based on the percentage of tubular cell necrosis, dilation, and cell detachment. In brief, the following criteria were used: 0, no abnormality; 1+, changes affecting less than 25% of sample; 2+, changes affecting 25%-50%; 3+, changes affecting 50%-75%; and 4+, changes affecting 75% of the sample.

### *TUNEL assay*

A transferase-mediated dUTP nick-end labeling (TUNEL) assay was performed to detect apoptotic nuclei in kidney sections [32]. TUNEL assay was conducted using a TUNEL detection kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instruction. Apoptosis was measured by the pathologist who was blinded to the treatment animals had received. A minimum of five fields per slide and six slides per group were counted by this method. Positive cells were counted as podocytes when residing on the outer surface of GBM. Cells residing in areas circumscribed by GBM were counted as endocapillary or mesangial cells. Apoptotic cells with nuclei staining dark brown were counted by light microscopy. For quantitative analysis, TUNEL-positive cells were quantified by counting five different fields at 200 magnification with two independent investigators who was blinded to the treatment that the animals had received.

### *RT-PCR*

Total RNA from kidney cortex was isolated by the Trizol procedure (Invitrogen, Carlsbad, CA, USA), and subsequently converted into

cDNA templates by reverse transcription using SuperScript II RT kit and random hexamer primers (Invitrogen, USA). The intracellular mRNAs encoding P65, PUMA, TNF- $\alpha$ , IL-6 and  $\beta$ -actin were determined by PCR technique with AccuPrime™ Taq DNA polymerase (Invitrogen) and specific primers: P65: 5'-GCG AGA GGA GCA CAG ATA CC-3' and 5'-CTG ATA GCC TGC TCC AGG TC-3'. PUMA: 5'-CGACCTCAACGCACAGTACGA-3' and 5'-AGGCACCTAATTGGGCTCCAT-3'; TNF- $\alpha$  5'-CCTCACACTCAGATCATCTTC-3' and 5'-CGGCTGGCACCCTAGTTG-3'; IL-6: 5'-GCCTTCCTACTTCAACAAGT-3' and 5'-GAATTGCCATTGCAACTCT-3';  $\beta$ -actin: 5'-GGCACCACCTTCTCAATGA-3' and 5'-GGAGTTGAAGGTAGTTTCGTGGA-3'. The resultant PCR products were analyzed by 1.2% agarose gel electrophoresis. Relative mRNA levels were normalized to those of  $\beta$ -actin.

### *Western blot analysis*

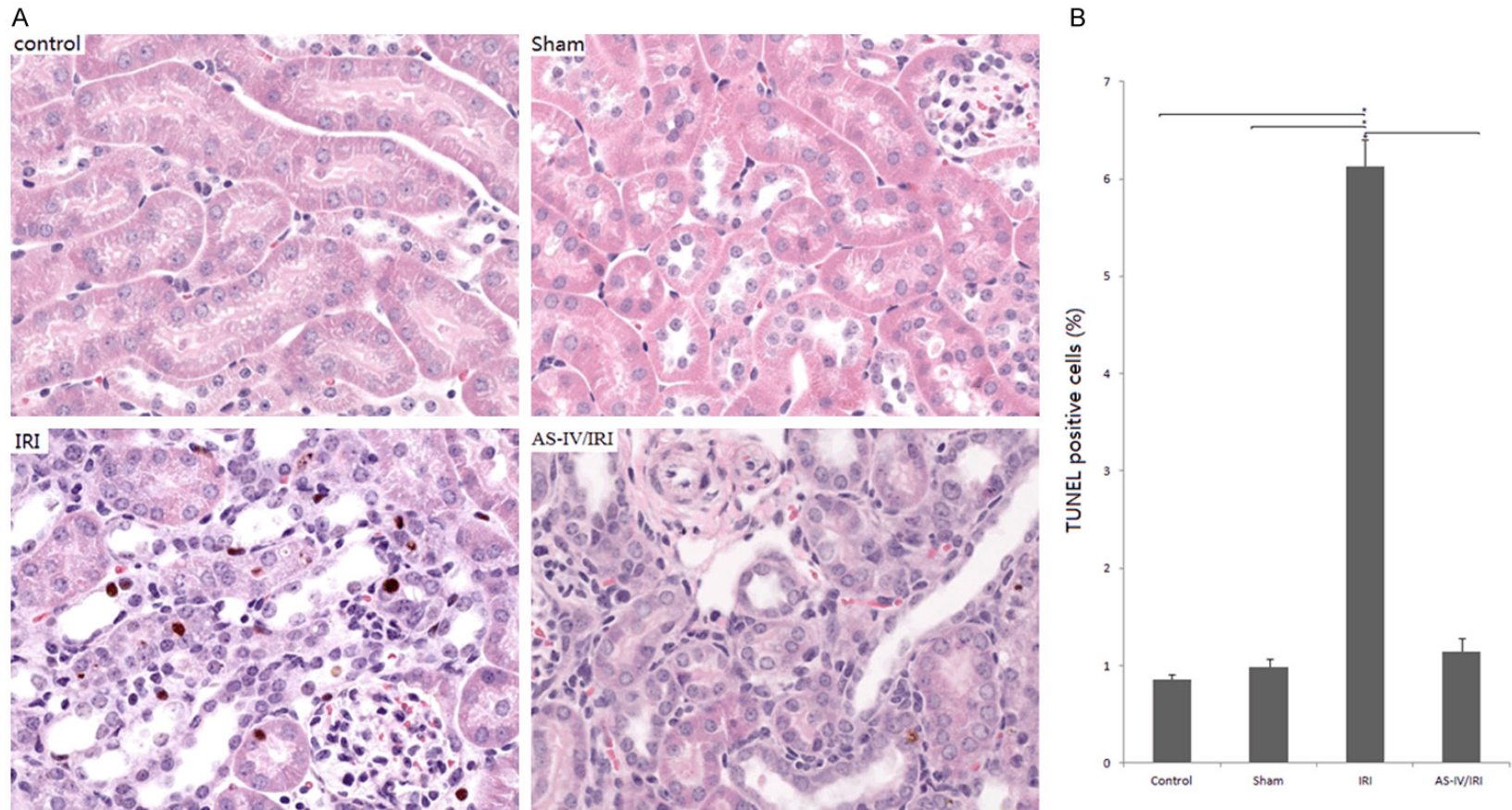
The kidney cortex were homogenized in 500  $\mu$ l of lysis buffer [1% Triton X-100, 0.1% SDS, 50 mM Tris (pH 8.0), 150 mM NaCl, 1 mM PMSF, 0.1 mM NaVO<sub>4</sub>, 0.1 mM benzamidine, 5  $\mu$ g/ml leupeptin, and 5  $\mu$ g/ml aprotinin]. Homogenates were clarified by centrifugation at 15,000 rpm for 15 min at 4°C. 30  $\mu$ g of proteins were resolved by 12% SDS-PAGE, and were subsequently transferred onto PVDF transfer membrane (GE Healthcare Life Sciences). Membranes were blocked with 5% non-fat milk in TBST (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, and 0.1% Tween-20) at 4°C overnight. The membranes were then probed with the antibodies specific for the indicated proteins (P65, 1:100 dilution; PUMA 1:200 dilution; TNF- $\alpha$  1:200 dilution; IL-6 1:200 dilution; and  $\beta$ -actin 1:1000 dilution) at room temperature for 1 h. The bound antibodies were detected by horseradish peroxidase (HRP)-conjugated secondary antibody (1:1,500 dilution) at room temperature for 1 h and then visualized by enhanced chemiluminescence (ECL) reaction reagents (GE Healthcare Life Sciences). Protein loading was normalized by re-probing the same membranes with  $\beta$ -actin specific antibodies.

### *ELISA for detection of serum levels of TNF- $\alpha$ and IL-6*

Rat serum was collected as described above. ELISA was used to detect the serum levels of TNF- $\alpha$  and IL-6 in strict accordance with



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**Figure 1.** AS-IV significantly inhibits apoptosis in mice with IRI. Graph depicting the number of apoptotic nuclei (TdT-mediated dUTP nick end labeling) per high-powered field ( $\times 200$ ) in each treatment group.  $*P < 0.05$ . A. Cell apoptosis was detected in renal tissue with ISI by TUNEL (original magnification, 400). B. The histogram shows the average apoptotic rate ( $P < 0.05$ ).

the manufacturer's instructions. A Synergy HT Microplate Reader was used to read the optical density at 450 nm, and the concentration of the sample was determined using a standard curve. The lower detection limits of ELISA kit for IL-6 were 16 pg/mL and 31 pg/mL for TNF- $\alpha$ .

### *ELISA for detection of NF- $\kappa$ B activity*

The cells of kidney cortex were then harvested, and the nuclear extracts were collected by the Nuclear Extract kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. The DNA binding activity of the NF- $\kappa$ B subunit p65 was assessed by the TransAM NF- $\kappa$ B kit (Active Motif). Briefly, 4 mg nuclear extract was incubated in wells coated with oligonucleotides with the NF- $\kappa$ B consensus sequence for 1 h. After washing, an anti-p65-specific Ab was added to each well and incubated for 1 h. The secondary HRP conjugated Ab was then added and incubated for another hour. After the colorimetric reaction with the substrate, the absorbance was measured at 450 nm.

### *Statistical analysis*

Statistics were conducted by SPSS 17.0 software. All data were expressed as mean  $\pm$  standard error. One-way ANOVA was used for comparisons between groups; the t-test was used to compare the mean values of the samples from different groups; A value of  $P < 0.05$  was considered statistically significant.

## **Results**

### *AS-IV significantly ameliorated renal dysfunction in mice with IRI*

Blood samples were collected at 6 hours post reperfusion from the abdominal inferior cava vein immediately before induced death. Renal function was determined by measurements of blood Cr levels. Serum Cr levels were significantly decreased in the AS-IV/IRI group ( $0.42 \pm 0.02$ ) mg/DL compared with the IRI alone groups ( $1.87 \pm 0.13$ ) mg/DL ( $P = 0.018$ ).

### *AS-IV significantly inhibits apoptosis in mice with IRI*

We quantified cells undergoing apoptosis in kidney sections by the transferase mediated

deoxyuridine triphosphate (dUTP)-digoxigenin nick-end labeling (TUNEL) assay. The apoptotic cells were exclusively limited to the proximal tubules. There were significantly less TUNEL-positive cells in the AS-IV-pretreated rats ( $0.85 \pm 0.16$ ) % as compared to the vehicle-treated ( $5.77 \pm 0.2$ ) % (**Figure 1A and 1B**,  $P = 0.042$ ).

### *AS-IV protects renal structure*

Many proximal tubules from the deep cortex had significant injury in vehicle-treated groups; in contrast, AS-IV-pretreated rats had minimal injury (**Figure 2**). There was significantly less injury in the renal cortex in the AS-IV-treated rats as compared to the vehicle-treated groups.

### *The effect of AS-IV-pretreatment on NF- $\kappa$ B p65 expression and activity in renal tissues*

Western blot analysis showed that the expression levels of NF- $\kappa$ B p65 in vehicle-treated groups was higher than that in AS-IV-pretreated groups. After AS-IV-pretreatment, the expression levels of NF- $\kappa$ B p65 was significantly lower than that in vehicle-treated and sham-treated groups (**Figure 2A**). RT-PCR assay for NF- $\kappa$ B p65 mRNA has the same results as Western blot assay (**Figure 2B**). As shown in **Figure 2C**, vehicle-treated groups showed increased NF- $\kappa$ B promoter activity by  $\sim 6$ -fold; however, AS-IV-pretreatment significantly decreased the induction of NF- $\kappa$ B activity in mice with IRI.

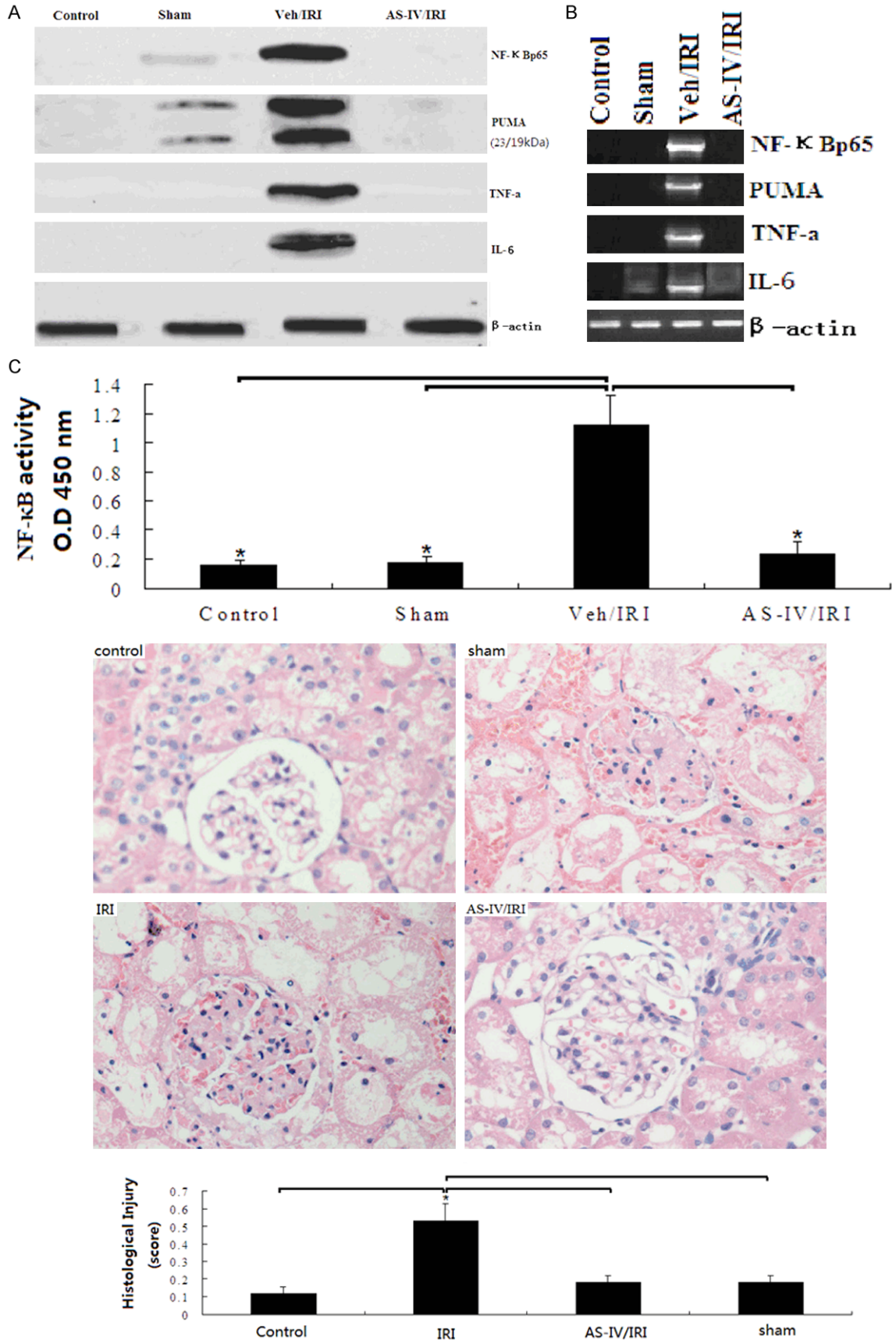
### *Changes of the serum levels of the inflammatory mediators TNF- $\alpha$ and IL-6*

The serum concentrations of TNF- $\alpha$  and IL-6 in IRI/Veh groups was ( $312.4 \pm 18.6$ ) pg/ml and ( $173 \pm 20.4$ ) pg/ml, which was significantly higher than TNF- $\alpha$  ( $112.5 \pm 10.2$ ) pg/ml and IL-6 ( $54.2 \pm 8.3$ ) pg/ml in groups of IRI/AS-IV ( $P < 0.01$ , respectively; **Figure 3**), no significant change was found in the sham groups (data not shown), suggesting IRI could induce inflammatory response in the kidney, and AS-IV-treatment could reverse IRI-induced increase of inflammatory mediators.

## **Discussion**

Astragaloside IV (As-IV) is 3-O-beta-D-xylopyranosyl-6-O-beta-D-glucopyranosyl-cycloastragenol, a lanolin alcohol-shaped tetracyclic triterpenoid saponin with high polarity, and its

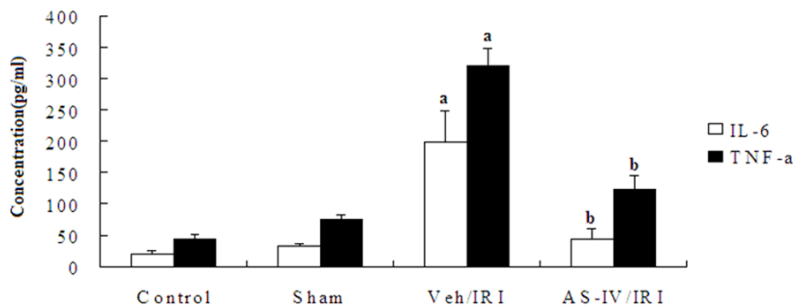
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**Figure 2.** Effect of AS-IV-pretreatment on NF- $\kappa$ B p65, PUMA, TNF- $\alpha$  and IL-6 expression. A. Total cellular and nuclear protein were subjected to Western blotting for NF- $\kappa$ B p65, PUMA, TNF- $\alpha$ , IL-6, and  $\beta$ -actin. B. The isolated RNA samples were analyzed by RT-PCR for NF- $\kappa$ B p65, PUMA, TNF- $\alpha$ , IL-6, and  $\beta$ -actin. C. The nuclear extract was prepared and used to measure the DNA-binding activity of p65 by an ELISA assay. The result is shown as the OD readout of the samples with the blank subtracted. One representative result from three independent experiments is shown ( $^*P < 0.05$ ). Protection against IRI-induced histological damage by AS-IV. Animals that received AS-IV as compared to controls, showed significantly less damage in the deep cortex. Results are expressed as mean  $\pm$  standard deviation of 8 different animals in each group.  $^*P < 0.01$ .



**Figure 3.** Serum concentration of TNF- $\alpha$  and IL-6. Note: <sup>a</sup> $P < 0.01$  compared to control or Sham; <sup>b</sup> $P < 0.01$  compared to group<sup>a</sup>.

molecular formula is  $C_{14}H_{68}O_{14}$ . It has been reported that AS-IV has an antioxidant effect and can ameliorate ischemia reperfusion (IR)-induced injury in brain and heart [33, 34]. In the guinea pig cochlea, AS-IV inhibits apoptotic cell death in the guinea pig cochlea exposed to impulse noise [35]. Research studies into the biological properties of AS-IV have revealed strong anti-inflammatory activity, which involves inhibition of NF- $\kappa$ B activation and downregulation of adhesion molecule expression [36]. Gui et al. has recently reported AS-IV prevents Glucose-Induced podocyte apoptosis partly through restoring the balance of Bax and Bcl-2 expression and inhibiting caspase-3 activation [37]. However, the precise mechanisms by which AS-IV inhibits apoptosis and inflammation remain to be elucidated.

Our study is the first to demonstrate the preventive effect of AS-IV on apoptosis in renal IRI, which has potentially important clinical consequences. This antiapoptotic effect was shown by TUNEL assays. The studies demonstrated that pretreatment with AS-IV significantly ameliorated the renal histopathology. Moreover, IRI-induced apoptosis was associated with upregulation PUMA expression, which were completely restored by AS-IV treatment. The study also demonstrated that AS-IV ameliorated structural and biochemical abnormalities in a rat model of IRI through suppressing NF- $\kappa$ B activation

and its key downstream inflammatory mediators. Pretreatment with AS-IV apparently reduced inflammatory responses in ischemic rats, as evidenced by a significant decrease in kidney expression of inflammatory mediators.

We explored the possible mechanisms for the renoprotective effects of AS-IV, we investigated the effects

of AS-IV on the NF- $\kappa$ B activity and expression during IRI. NF- $\kappa$ B regulates expression of the inflammatory genes associated with many pathophysiological conditions, including renal IRI [38-40]. Upon stimulation, the inhibitor of NF- $\kappa$ B becomes degraded, and NF- $\kappa$ B releases and translocates into the nucleus where it induces the expression of target genes, most of which encode proteins involved in immune and inflammatory responses.

IL-6 and tumor necrosis factor (TNF- $\alpha$ ) was associated with delayed activation of nuclear factor kappa B (NF-kappaB) p65. The results presented in this study also demonstrated that renal IRI induced activation of NF- $\kappa$ B, which was consistent with previous study [39]. IRI resulted in the production of the proinflammatory cytokines TNF- $\alpha$  and IL-6. AS-IV has been reported to have anti-inflammatory activity through inhibiting NF- $\kappa$ B activation and adhesion molecule expression in lipopolysaccharide (LPS-) stimulated HUVECs [28]. Here, the increased expression of phosphorylated p65 subunit of NF- $\kappa$ B and NF- $\kappa$ B activity was observed in IRI rats. Furthermore, tissue TNF- $\alpha$  and IL-6, and serum TNF- $\alpha$  and IL-6 were also upregulated. However, AS-IV pretreatment apparently suppressed NF- $\kappa$ B activation, as evidenced by a decrease in activity and phosphorylation of NF- $\kappa$ B. TNF- $\alpha$  and IL-6 in serum and renal tissues were restored by AS-IV pretreat-

ment. AS-IV pretreatment also induced an apparent reduction in the gene expression of TNF- $\alpha$  and IL-6 in the renal tissue.

Recent study has shown simulated ischemia induces nuclear factor-kappaB intranuclear translocation and activation in renal tubular cells. Furthermore, nuclear factor-kappaB mediates ischemia induced renal tubular cell apoptosis [41]. It has found PUMA is a direct target of NF- $\kappa$ B and mediates TNF- $\alpha$ -induced apoptosis *in vitro* and *in vivo* [22]. Qiu et al. found the induction of PUMA was p53-independent but required NF- $\kappa$ B [23]. The results presented in this study demonstrated that renal IRI induced activation of NF- $\kappa$ B and increased PUMA expression, followed by increased apoptosis in the renal cortex. Pretreatment with AS-IV apparently reduced apoptotic cells in ischemic rats, as evidenced by a significant decrease in kidney expression of NF- $\kappa$ B and PUMA. We suggested PUMA upregulation may be dependant of activation of NF- $\kappa$ B. Pretreatment with AS-IV inhibits apoptosis by PUMA inhibition.

In summary, our results demonstrate that IRI activates NF- $\kappa$ B and induces renal tubular epithelial apoptosis and inflammation by increasing PUMA expression and inflammatory genes expression. AS-IV prevents renal tubular epithelial apoptosis and reduces inflammatory response. These findings strengthen the therapeutic rationale for AS-IV in the treatment of renal IRI.

### Disclosure of conflict of interest

None.

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