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ORIGINAL PAPER

Apolipoprotein A-I attenuates renal ischemia/reperfusion injury in rats

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Abstract Apolipoprotein A-I (ApoA-I), the major protein component of serum high-density lipoprotein (HDL), exhibits its anti-inflammatory activity in inflammatory responses. As renal inflammation plays an important role in ischemia/reperfusion (I/R) injury of the kidney, the aim of this study was to investigate the beneficial effect of ApoA-I on renal I/R injury in rats and the underlined mechanism. Using rats subjected to renal I/R by occlusion of bilateral renal pedicles, we found that administration of ApoA-I significantly reduced serum creatinine levels, serum TNF-α and IL-1β levels as well as tissue myeloperoxidase (MPO) activity, compared with I/R controls. Moreover, ApoA-I treatment suppresses the expression of intercellular adhesion molecules-1 (ICAM-1) and P-selectin on endothelium, thus diminishing neutrophil adherence and the subsequent tissue injury. These results showed that ApoA-I reduced I/R-induced inflammatory responses, decreased renal microscopic damage and improved renal function. It seems likely that ApoA-I protects kidney from I/R injury by inhibiting inflammatory cytokines release and neutrophil infiltration and activation.

Keywords Apolipoprotein A-I · Ischemia/reperfusion · Myeloperoxidase · Neutrophil · Cytokines

Introduction

Renal ischemia, occurring during transplantation, shock, vascular surgery, is a major cause in the development of ischemic acute renal failure, which has been known to result in high morbidity and mortality [1]. Although the return of blood flow to ischemic tissue is vital for recovery of normal functions, the tissue may also be injured paradoxically during reperfusion (reperfusion-injury) [2]. The mechanisms underlying I/R damage to kidneys are multifactorial and interdependent, involving hypoxia, vascular endothelial injury, inflammatory responses, radical-induced damage, tubular obstruction, apoptosis and endothelial–epithelial cell dysfunction [3]. In recent studies, HDL has been introduced to solve the clinical problem mentioned above because of its role in endothelial protection, antioxidation, and anti-inflammation [4, 5]. There is emerging evidence that, Apolipoprotein A-I (ApoA-I), the major protein component of serum HDL, is the main factor in the anti-inflammatory actions of HDL [6] and can diminish neutrophil activation, an important event in renal I/R injury [7]. Our laboratory has also demonstrated that ApoA-I provides cardioprotection against I/R injury [8]. In this study, we extended our ApoA-I investigation into renal I/R injury in rats. We found that ApoA-I treatment reduces inflammatory cytokines release and neutrophil infiltration and activation, thus diminishing renal I/R injury.

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Materials and methods

Materials

Sprague Dawley rats were purchased from Shanghai Laboratory Animal Centre of Chinese Academy of Sciences, Shanghai, China; plasma precipitate IV was obtained from Shanghai Bioproduct Institute, Shanghai, China; primary monoclonal ICAM-1 and P-selectin antibody, secondary and nonspecific IgG antibodies, and the reagents for immunohistochemical analysis were purchased from Santa Cruz

Biotechnology, CA, USA; the ELISA kits for assay of TNF-α and IL-1β were from Pepro Tech. Inc., NJ, USA; the MPO activity assay kit was from Jiancheng Bioengineering Institute, Nanjing, China; sheep anti-human ApoA-I antibody used for western blot was from Sigma, USA.

ApoA-I preparation

Human plasma precipitate IV was utilized to isolate ApoA-I according to our modified Lerch's method [8, 9]. In brief, precipitate IV was suspended in 4 vol of a buffer (65% ethanol, 10 mM $NaHCO_3$ and 1 mM EDTA) for 2 h at −20°C, and the pH was adjusted to 7.3. After centrifugation for 30 min at 5,000 rpm at −4°C, the pH of the supernatant was adjusted to 5.5 and the resulting precipitate was collected by further centrifugation for 30 min at 5,000 rpm at −4°C. The pellet was then dissolved in a buffer containing 100 mM Tris–HCl and 6 M Urea (pH 8.6) and delipidated by adding equal volume of ethanol and chloroform (1:1 v/v) for 2 h at −20°C. After centrifugation for 30 min at 5,000 rpm at −4°C, the supernatant was then mixed with equal volume of ethanol and centrifuged for 30 min at 5,000 rpm at −4°C again. The final supernatant, which contained pure ApoA-I, was concentrated by polyethylene glycol (Mr: 20,000) and dialysed against phosphate buffer (150 mM; pH 7.4).

The purity of the yielded ApoA-I was confirmed by SDS-PAGE and Western blot.

Renal ischemia/reperfusion (I/R) model

Adult male Sprague Dawley rats (220–300 g) were anesthetized with urethane (1.2 g/kg, intraperitoneal). Both renal pedicles were occluded with vascular clamps while the animal was kept at a constant temperature and well hydrated. The clamps were removed after 45 min to allow reperfusion of the kidneys. Saline (I/R group) or ApoA-I (25 mg/kg) (ApoA-I group) was administered intravenously 30 min before occlusion of the renal pedicles. After surgery, the wounds were sutured and blood samples were collected at 6 h of reperfusion, the serum was stored in −70°C after isolation. Animals were then sacrificed. Harvested kidneys were stored in −70°C or fixed in 10% neutral formaldehyde immediately. Rats in sham group were subjected to the same surgical procedures as I/R rats except that the renal pedicles were not occluded.

All animals received humane care as described in the *Principles of Laboratory Animal Care* formulated by Fudan University. Animals received food and water on an ad libitum basis, and lighting was maintained on a 12-h cycle.

Histologic evaluation

Kidneys were fixed in 10% (w/v) neutral buffered formaldehyde at room temperature for 1 day; after ethanol dehydration, pieces of kidney were embedded in paraffin and cut into 4 μm sections and mounted on glass slides; sections were then deparaffinized, counterstained with hematoxylin-eosin (HE staining), and viewed under a light microscope.

Immunohistochemical analysis of ICAM-1 and P-selectin

The processes of mounting sections on glass slides were same as above. Sections were baked at 80°C for 3 h, and deparaffinized, and incubated with protein block reagent (10% BSA) for 5 min to minimize nonspecific adsorption. Sections were incubated with monoclonal antibodies against ICAM-1 or P-selectin at a dilution of 1:100 for 30 min, and then incubated with horseradish peroxidase (HRP)-anti IgG for an additional 30 min. Antibody-binding sites were visualized with a HRP complex immunoperoxidase technique with the use of diaminobenzidine. All steps were performed at room temperature (25°C).

Measurement of serum creatinine, TNF-α and IL-1β

Serum creatinine levels were determined by a kit according to the manufacturer's protocol. Serum TNF-α and IL-1β levels were measured by two ELISA kits according to the manufacturer's instructions and guidelines.

Measurement of myeloperoxidase (MPO) activity in kidney homogenates

At the end of the experiments, kidney was weighed and homogenized in a buffer containing 50 mM Tris–HCl, 1 mM EDTA and 5% Sucrose (pH 7.4). The detailed steps were in accordance with manufacturer's protocol of the kit. MPO activity was defined as the amount of enzyme required to degrade 1 μmol of hydrogen peroxide at 37°C and was expressed as U/g wet tissue.

Statistical analysis

Data are expressed as Mean ± SD. Data among multiple groups were analyzed by ANOVA followed by LSD test. The P value of less than 0.05 was considered significant.

Results

ApoA-I isolation and identification

We isolated and purified ApoA-I from human plasma precipitate IV according to our modified Lerch's method [8, 9]. As shown on SDS-PAGE and Western blot, ApoA-I

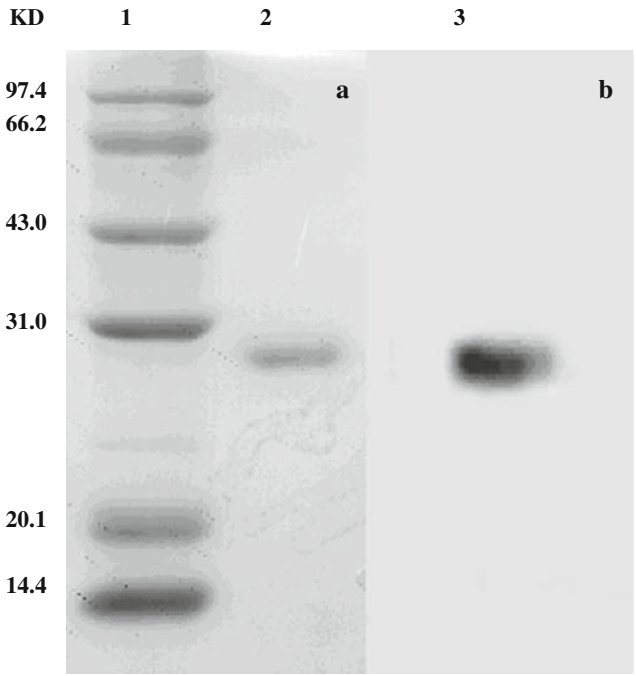

Fig. 1 SDS-PAGE (**a**) and Western blot (**b**) of purified ApoA-I. (**a**) lane 1: marker; lane 2: purified ApoA-I. (**b**) lane 3: purified ApoA-I

(Mr: 28,300) was purified (Fig. 1), and the SDS-PAGE scanning showed that the purity of ApoA-I was 98%. The purified ApoA-I was dissolved in PBS (pH 7.2) and used in the following experiments.

ApoA-I reduces serum creatinine, TNF-α and IL-1β levels in I/R rats

Rats that underwent renal I/R exhibited significant increases in the serum creatinine levels, an important parameter for renal (glomerular) damage [10], compared with sham group (172.78 ± 19.15 μM vs. 73.75 ± 9.16 μM, $P < 0.01$). ApoA-I (25 mg/kg) treatment significantly reduced the serum creatinine levels in I/R group (114.50 ± 7.30 μM) ($P < 0.05$) (Fig. 2), suggesting the improvement in renal function.

We next sought to measure serum TNF-α and IL-1β levels, two well-known factors mediating tissue damage [11], and found that both were significantly increased in I/R animals compared with sham group (10.95 ± 3.63 pg/ml vs. 2.14 ± 1.36 pg/ml and 355.00 ± 52.62 pg/ml vs. 110.42 ± 20.96 pg/ml, $P < 0.01$, respectively), while administration of ApoA-I (25 mg/kg) markedly reduced serum TNF-α and IL-1β levels in I/R rats (6.43 ± 1.43 pg/ml and 208.75 ± 37.76 pg/ml, respectively, $P < 0.05$ and $P < 0.01$, respectively) (Fig. 3). These results showed that ApoA-I treatment has anti-inflammatory effect on I/R injury.

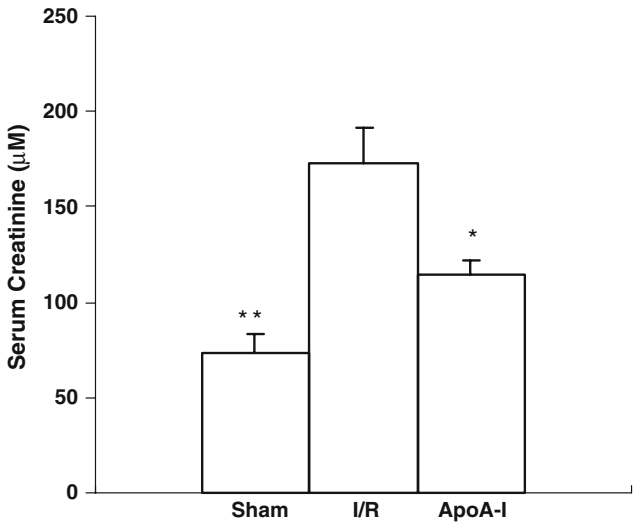

Fig. 2 Serum creatinine levels of sham, I/R and ApoA-I groups. Values are means ± SD ($n = 7$). * $P < 0.05$, ** $P < 0.01$ vs. I/R group

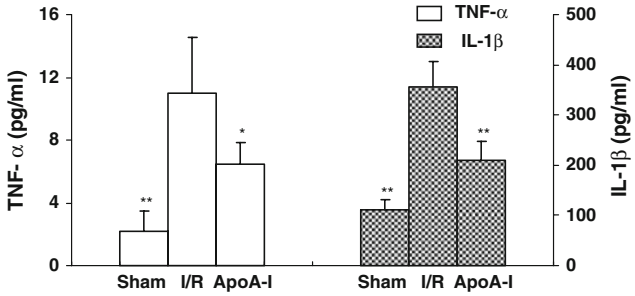

Fig. 3 Serum TNF-α and IL-1β levels of sham, I/R and ApoA-I groups. Values are means ± SD ($n = 7$). * $P < 0.05$, ** $P < 0.01$ vs. I/R group

ApoA-I decreases MPO activity in I/R rat kidneys

MPO activity, which is regarded as an indicator of neutrophil infiltration and activation [4], was significantly higher in the kidney tissue of I/R group (6.14 ± 0.58 U/g wet tissue) than sham group (1.22 ± 0.15 U/g wet tissue) ($P < 0.01$). ApoA-I treatment (25 mg/kg) in I/R group significantly reduced renal tissue MPO activity (3.03 ± 0.51 U/g wet tissue) ($P < 0.01$) (Fig. 4).

ApoA-I diminishes ICAM-1 and P-selectin expression in I/R rat kidneys

Increase of ICAM-1 and P-selectin expression is a well-known phenomenon in I/R injury [12, 13]. To evaluate the effect of ApoA-I treatment on ICAM-1 expression in I/R injury, we used an immunohistochemical approach. After ischemia followed by 6 h of reperfusion, the staining intensity was greatly increased on endothelial cells

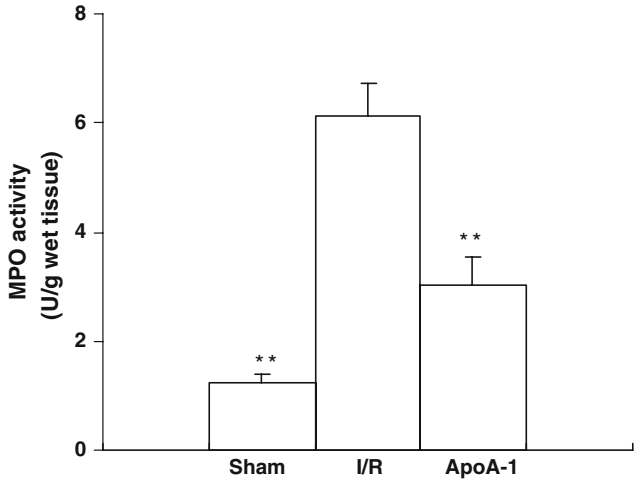

Fig. 4 Kidney tissue MPO activity of sham, I/R and ApoA-I groups. Values are means ± SD ($n = 7$). ** $P < 0.01$ vs. I/R group

(Fig. 5b) compared with sham group (Fig. 5a). However, after ApoA-I treatment, such staining was greatly diminished (Fig. 5c).

We next sought to measure P-selectin expression using same approach. The similar tendency was also observed in P-selectin expression (Fig. 6a, b, c).

Histological analysis

The renal histology of rats from sham group (Fig. 7a) showed a regular renal tissue with normal morphology, while rat kidneys that underwent ischemia followed by 6 h of reperfusion demonstrated increased infiltration of neutrophils and presented with severe tubulointerstitial damage and interstitial hemorrhage surrounding both the glomeruli and the tubuli (Fig. 7b). In contrast, renal sections obtained from ApoA-I group demonstrated less neutrophil infiltration and interstitial hemorrhage and tubular degeneration than those obtained from I/R rats (Fig. 7c).

Discussion

In previous studies, we have demonstrated that ApoA-I, isolated from human plasma HDL, has anti-inflammatory activity [6, 14]. However, classical procedures (ultracentrifugation, delipidation and size exclusion chromatography) for ApoA-I isolation from plasma HDL [15, 16] are unsuitable for large-scale production. To solve the problem, in the present research, we obtained ApoA-I from plasma precipitate IV with purity of 98% by our modified Lerch's method [8, 9].

Although there are some research papers exploring various methods to protect kidney tissue from ischemia/reperfusion injury in different animal models [4, 10, 17, 18], the ability of ApoA-I to reduce renal injury caused by I/R has not been investigated so far. In this study, we

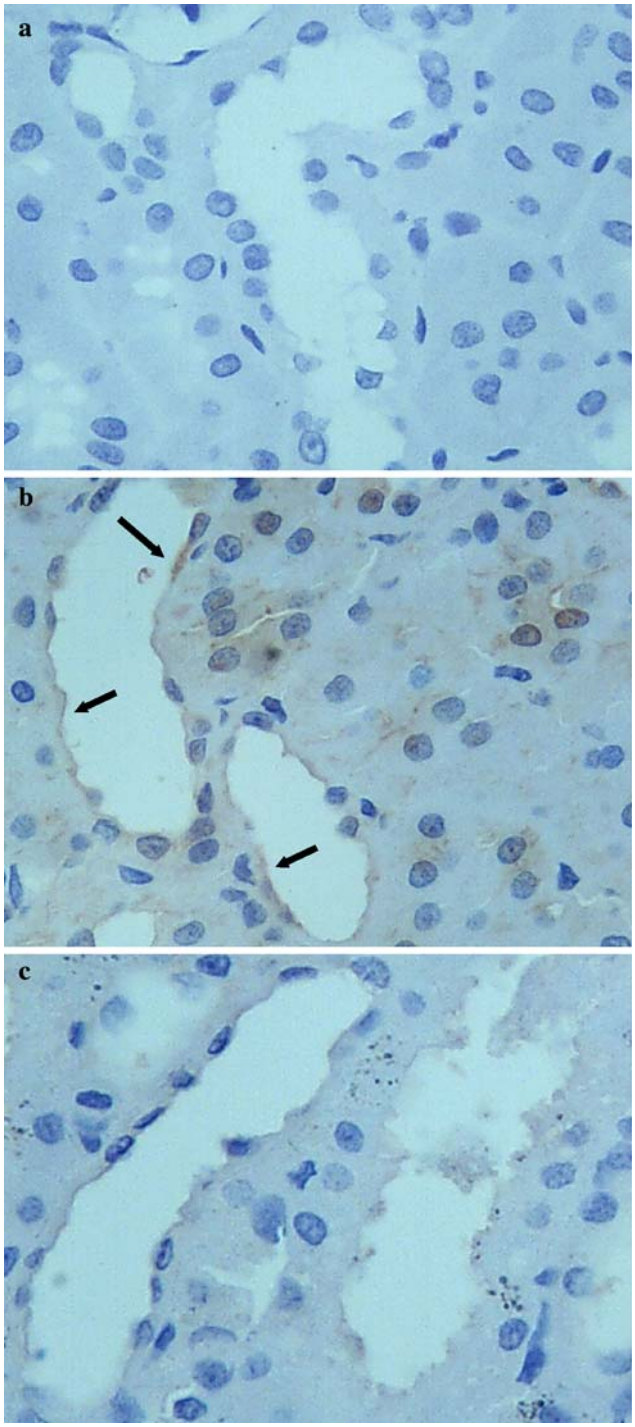

Fig. 5 Photomicrographs of immunohistochemical staining for ICAM-1 in renal sections (400×). Marked staining for ICAM-1 on endothelium of blood vessels is found in I/R group (**b**, *arrows*) compared with sham group (**a**), suggesting upregulation of ICAM-1 expression during I/R. ApoA-I group (**c**) demonstrates reduced staining for ICAM-1, suggesting a reduction in ICAM-1 expression after ApoA-I treatment. This is the representative of three independent experiments

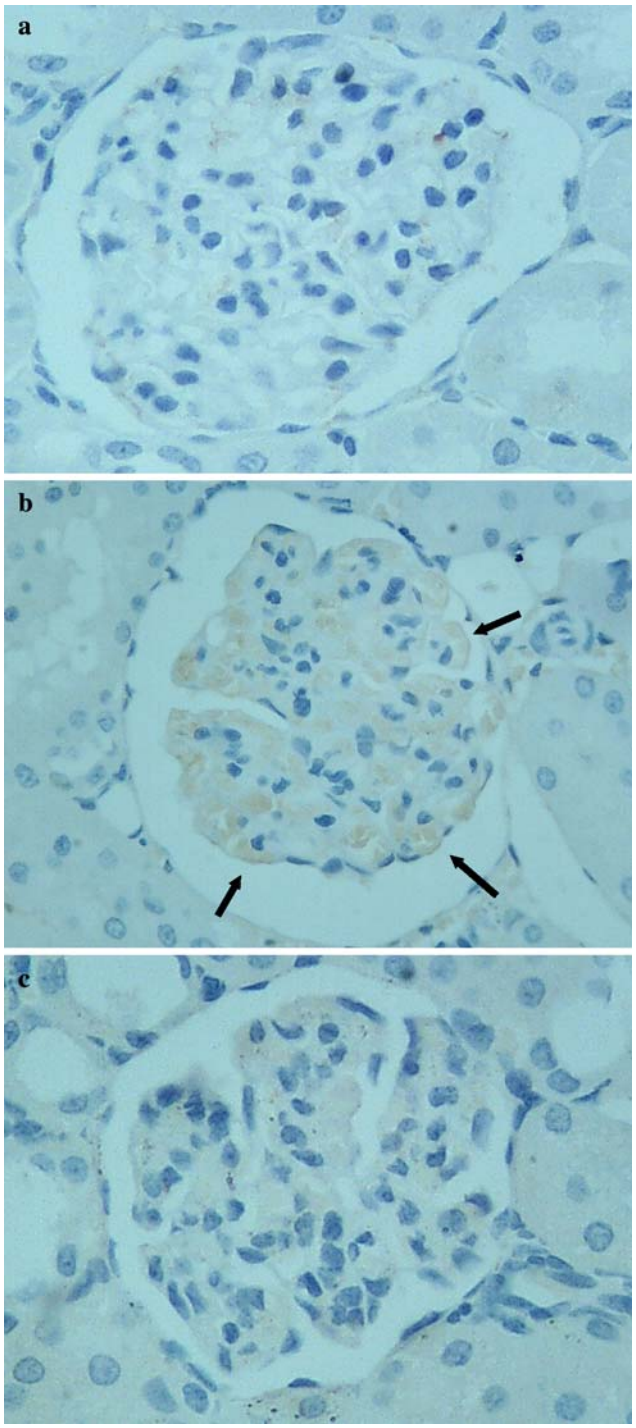

Fig. 6 Photomicrographs of immunohistochemical staining for P-selectin in renal sections (400×). Marked staining for P-selectin on endothelium of blood vessels is found in I/R group (**b**, *arrows*) compared with sham group (**a**), suggesting upregulation of P-selectin expression during I/R. ApoA-I group (**c**) demonstrates reduced staining for P-selectin, suggesting a reduction in P-selectin expression after ApoA-I treatment. This is the representative of three independent experiments

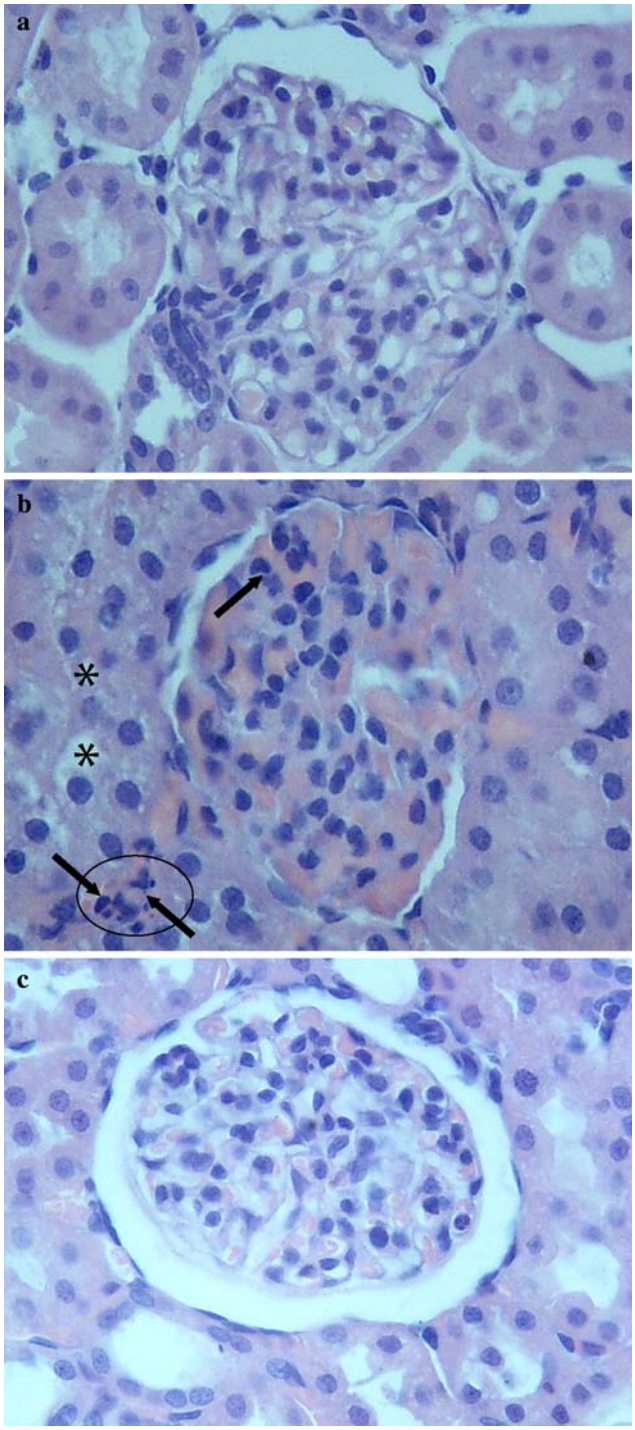

Fig. 7 Photomicrographs of renal tissues HE staining (400×). I/R group (**b**) shows obvious neutrophil accumulation (*arrows*), interstitial hemorrhage (*circle*) and tubular degeneration (*), compared with sham group (**a**). Whereas in ApoA-I group (**c**), there is reduced neutrophil infiltration, interstitial hemorrhage and tubular degeneration. This is the representative of three independent experiments

demonstrated that ApoA-I treatment attenuates I/R injury in rat kidney in vivo and improves renal function, as evidenced by decreasing serum creatinine, TNF-α and IL-1β levels, suppressing the expression of ICAM-1 and P-selectin, and reducing neutrophil infiltration and activation (reduced MPO activity). Renal I/R induces an

inflammatory reaction within the renal parenchyma and inflammatory cytokines release [19]. These cytokines, in turn, cause cellular and organ inflammatory injury and dysfunction. The present study provided further evidence that ApoA-I can reduce serum TNF-α and IL-1β levels in renal I/R injury, consistent with our previous studies that the ApoA-I decreased mortality and increased survival time of endotoxemia mice, and attenuated LPS-induced acute lung injury and inflammation [14]. NFκB (nuclear factor-κB) activation is necessary for cytokines gene expression and production [20]. We found recently that ApoA-I treatment can effectively inhibit NFκB activation [21].

During renal reperfusion, expression of adhesion molecules such as ICAM-1 and P-selectin in endothelial cells is a fundamental requirement for the recruitment of neutrophils into renal tissue [12, 13]. Neutrophil recruitment begins with interaction of neutrophils with the endothelium through rolling along the endothelial surface, which is mediated by P-selectin on endothelium and sialylated glycoprotein on neutrophils [22, 23]. This loose adherence is necessary for later firm adherence mediated by the CD11/CD18 complex and ICAM-1, leading to transendothelial migration into the renal parenchyma and their physiological sequellae. Kirstan et al. reported that TNF-α could recruit neutrophils to kidney and induce the expression of ICAM-1 on the endothelial cells [24]. Kelly also showed that, in vitro, TNF-α and IL-1β could upregulate adhesion receptors such as ICAM-1 on endothelium [25]. These lines of evidence revealed that adhesion molecules and neutrophils are interlaced with each other in renal I/R injury.

The present results demonstrate that administration of ApoA-I can suppress the expression of ICAM-1 and P-selectin, and significantly reduce renal I/R injury. Histological analysis of our experiments revealed that there was decreased neutrophil infiltration in ApoA-I treated I/R animals. Neutrophils can directly injure parenchymal cells through release of proteolytic enzymes, reactive oxygen species as well as MPO [26]. Our previous study also showed that ApoA-I could diminish the neutrophil adhesion and activation [7]. In the present study, elevated activity of MPO, which is an index of tissue neutrophil infiltration and activation [4], was inhibited by ApoA-I treatment, indicating the beneficial effect of ApoA-I on renal I/R injury.

Recent studies revealed that HDL could also reduce renal ischemia/reperfusion injury in rats via modulation of the expression of adhesion molecules, resulting in reduction of neutrophil infiltration and oxidative stress [4]. Human HDL plays an important role against the development of atherosclerosis and cardiovascular diseases, and high plasma levels of HDL are inversely correlated with the incidence of these pathologies [27, 28]. ApoA-I, as a major protein component of HDL, is mainly synthesized by hepatocytes. It is secreted in a lipid-poor/free form and then immediately recruits additional phospholipids and free cholesterol via the ABCA1 (the ATP binding cassette transporter A1), forming nascent HDL [29, 30], and the nascent HDL acquires more lipids from other peripheral tissues or from lipoproteins, forming mature HDL [31]. Exogenous ApoA-I infused into the body could mimic the process of lipidation of lipid-poor/lipid-free ApoA-I secreted by hepatocytes to form HDL. Thus, ApoA-I performs its beneficial effect on I/R injury, at least in part, through the conversion of ApoA-I into HDL, which was confirmed by the findings that infused ApoA-I was incorporated into alpha-HDL or prebeta-HDL in vivo [32, 33].

Both ApoA-I and HDL have beneficial effect on I/R injury, however, it seems impossible to use plasma-derived HDL in clinical application because of shortage of plasma supplement. Meanwhile, reconstituted HDL (rHDL), prepared with ApoA-I and phospholipids, was reported to have the detrimental effects on livers [34, 35]. In contrast, during or after ApoA-I treatment, no adverse clinical effects were observed, and no ApoA-I appeared in the urine [32]. Furthermore, preparation of ApoA-I is much easier than that of rHDL. In view of aspects mentioned above, ApoA-I could be a better choice than HDL for treatment of I/R injury.

In summary, it seems likely that ApoA-I attenuates renal I/R injury. This protective effect can be attributed, at least in part, to its ability to inhibit neutrophil infiltration and activation, suppress inflammatory cytokines release and expression of adhesion molecules. These results suggest that ApoA-I could have promising clinical value for the treatment of I/R injury.

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