

Original Article

Sesamin protects against renal ischemia reperfusion injury by promoting CD39-adenosine-A₂AR signal pathway in mice

Ke Li^{1,2*}, Xia Gong^{3*}, Ge Kuang⁴, Rong Jiang⁵, Jingyuan Wan⁴, Bin Wang^{1,2}

¹Department of Orthopaedic Surgery, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China; ²Department of Anesthesiology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China; ³Department of Anatomy, Chongqing Medical University, Chongqing 400016, China; ⁴Chongqing Key Laboratory of Biochemistry and Molecular Pharmacology, Chongqing Medical University, Chongqing 400016, China; ⁵Laboratory of Stem Cell and Tissue Engineering, Chongqing Medical University, Chongqing 400016, China. *Equal contributors.

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Abstract: Ischemia reperfusion injury (IRI) is a leading cause of acute kidney injury with high morbidity and mortality due to limited therapy. Here, we examine whether sesamin attenuates renal IRI in an animal model and explore the underlying mechanisms. Male mice were subjected to right renal ischemia for 30 min followed by reperfusion for 24 h with sesamin (100 mg/kg) during which the left kidney was removed. Renal damage and function were assessed subsequently. The results showed that sesamin reduced kidney ischemia reperfusion injury, as assessed by decreased serum creatinine (Scr) and Blood urea nitrogen (BUN), alleviated tubular damage and apoptosis. In addition, sesamin inhibited neutrophils infiltration and pro-inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-1 β production in IR-preformed kidney. Notably, sesamin promoted the expression of CD39, A₂A adenosine receptor (A₂AAR), and A₂BAR mRNA and protein as well as adenosine production. Furthermore, CD39 inhibitor or A₂AR antagonist abolished partly the protection of sesamin in kidney IRI. In conclusion, sesamin could effectively protect kidney from IRI by inhibiting inflammatory responses, which might be associated with promoting the adenosine-CD39-A₂AR signaling pathway.

Keywords: Renal ischemia reperfusion injury, sesamin, adenosine, CD39, A₂ adenosine receptors (A₂AR)

Introduction

Acute kidney injury (AKI) is a clinical challenge often leading to multiple organ dysfunctions, collapse of the circulatory system and death [1, 2]. Renal ischemia reperfusion injury (IRI) is one of the leading cause of AKI occurred in many clinical settings, including renal transplantation, shock, and cardiothoracic and vascular surgery [3, 4]. It is manifested by a local inflammatory response caused by an initial ischemia and followed by reperfusion, which elicits to rapid neutrophil infiltration, pro-inflammatory cytokines release, oxidative stress generation, and subsequent proximal tubule apoptosis/necrosis [3-5]. Due to limited therapeutic modalities available, renal IRI is associated with extremely high morbidity and mortality [4]. Therefore, novel and effective therapeutic strategies are required desperately.

In recent some years, the biologically active substances derived from natural plants have been paid attentions on the prevention and treatment of acute and chronic diseases. Sesamin (**Figure 1A**), the major lignan extracted from sesame seed and oil, has been found to exert various pharmaceutical functions, including anti-hypertensive, hypocholesterolemic, neuroprotective, anti-fibrotic, anti-oxidative, anti-tumor, and anti-inflammatory actions [6-12]. In fact, our and other studies have shown that sesamin reduced production of inflammatory mediators, alleviated inflammatory responses, and improved survival in various inflammatory cells and animal models [13-16]. In addition, some previous studies have indicated that sesamin protected from brain and hepatic ischemia-reperfusion injury [17, 18]. Furthermore, Wu et al reported that sesamin exerted renoprotective effects in renovascular

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hypertensive rats [6]. Thus, we hypothesized that sesamin might be a possible candidate for treating acute renal IRI.

Here, the aims of the present study were to examine whether sesamin protects against acute renal IRI in a mouse model and, if so, to identify its underlying mechanisms. The results showed that sesamin protected kidneys from IRI by inhibiting inflammation and inducing the endogenous production of adenosine.

Materials and methods

Reagents

Sesamin (C₂₀H₁₅O₆, FW=354.34, purity ≥98%) was purchased from Aladdin Industrial Corporation (Shanghai, China). POM-1 (the CD39 specific inhibitor) was purchased from Santa Cruz Biotechnology (CA, USA). SCH-58261 (the A2AAR antagonist), PSB1115 (the A2BAR antagonist) was purchased from Sigma-Aldrich (USA). Mouse tumor necrosis factor (TNF)-α and interleukin (IL)-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from Bender MedSystems (Vienna, Austria). Blood urea nitrogen (BUN), creatinine, and myeloperoxidase (MPO) detection kits were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The caspase 3 colorimetric assay kit was from Beyotime institute of biotechnology (Nanjing, China). In Situ Cell Death Detection Kit was from Roche Applied Science (Basel, Switzerland). The bicinchoninic acid (BCA) protein assay kit was purchased from Pierce (Rockford, IL, USA). Anti-CD73, anti-CD39, anti-A2AAR, and anti-A2BAR and anti-GAPDH antibodies were purchased from Abcam (Cambridge, MA, UK).

Animals

Male C57/BL6-mice (6-8 weeks old, weight 18-22 g) were obtained from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). Mice were housed in a specific pathogen-free (SPF) laboratory under optimum conditions (25 ± 2°C, 55% humidity, and a 12 h light/dark cycle) and fed a standard laboratory diet and water. Mice were acclimatized for at least 1 week before use. All experimental procedures involving animals were approved by the Animal Care and Use Committee of Chongqing Medical University.

Experimental protocol

Renal IRI was induced by performing a left nephrectomy followed by ischemic to the remaining right kidney. Briefly, the mice were anesthetized by intraperitoneal injection of a mixture of ketamine and xylazine (45 mg/kg and 8 mg/kg, respectively) and placed on a temperature-controlled heating table. A flank incision was performed using a coagulation electrode to prevent bleeding. The left kidney was removed without interfering with the adrenal vessels and the right renal pedicle was then clamped for 30 min. Subsequently, the clamp was released and the kidney was monitored for color changes to confirm blood reflow before the incision was closed. Sham control animals were subjected to the same procedure without clamping of the renal pedicle.

Mice were give oral gavage of sesamin (100 mg/kg, dissolved in 0.5% carboxymethylcellulose sodium salt in 0.9% normal saline, respectively) every 8 h one time for three times within 24 h before IR. The dose of sesamin (100 mg/kg) was chosen for our previous preliminary experiment and study [13]. To inhibit or antagonize CD39 or A2AAR, A2BAR, POM-1 (5 mg/kg) or SCH-58261 (10 mg/kg), PSB115 (10 mg/kg) was administered intraperitoneally 30 min before IR, respectively. Each experiment group had 6 mice. After surgery, the mice were kept on a warming blanket for 24 h and allowed food and water *ad libitum*. At the end of the 24 h reperfusion period, all animals were sacrificed by injecting a high dose of pentobarbital sodium. The blood and kidney tissues were then harvested.

Measurement of creatinine and BUN

After reperfusion for 24 h, blood samples were collected and renal function was monitored by measuring the concentration of creatinine and BUN in the serum using the commercial kits according to the manufacturer's suggestions.

Hematoxylin and eosin (HE) staining

Kidneys were harvested and fixed with 4% formaldehyde prior to paraffin embedding. Paraffin-embedded tissues were sectioned (5 μm thick) and stained with hematoxylin and eosin (H&E). The degree of kidney injury was scored by a pathologist in a blinded fashion on a 5-point scale: 0 = no damage, 1 ≤ 10%, 2 =

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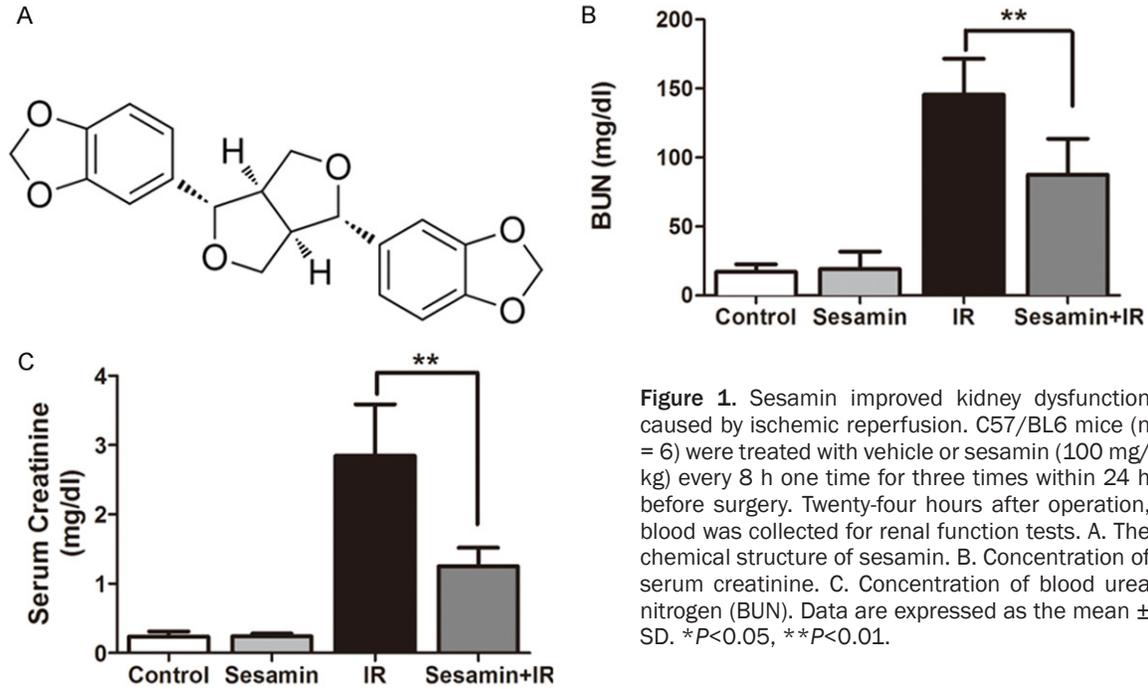


Figure 1. Sesamin improved kidney dysfunction caused by ischemic reperfusion. C57/BL6 mice ($n = 6$) were treated with vehicle or sesamin (100 mg/kg) every 8 h one time for three times within 24 h before surgery. Twenty-four hours after operation, blood was collected for renal function tests. A. The chemical structure of sesamin. B. Concentration of serum creatinine. C. Concentration of blood urea nitrogen (BUN). Data are expressed as the mean \pm SD. * $P < 0.05$, ** $P < 0.01$.

10-25%, 3 = 25-50%, 4 = 50-75%, 5 = more than 75% of the corticomedullary junction injured, as described by Jablonski et al [19].

TUNEL assay

Deparaffinized and dehydrated sections were incubated for 15 min at 37°C with proteinase K. The sections were rinsed twice (5 min/each) with PBS, and the area around the sections was dried. TUNEL reaction mixture (50 μ l) was added to the samples, which were then incubated for 60 min at 37°C in a humidified atmosphere. The sections were rinsed three times with PBS. Horseradish-peroxidase-labeled streptavidin was used to bind the biotinylated nucleotides. The sections were then colored with DAB and counterstained with hematoxylin.

Immunofluorescence

Neutrophils were identified by immunofluorescence using a rat anti-mouse Ly6G Ab followed by AlexaFluora 488-labelled goat anti-rat IgG. Goat IgG was used as an isotype control.

Measurement of caspase-3 activity

Caspase-3 activity in the liver tissue was measured using a caspase-3 colorimetric assay kit, according to the manufacturer's instructions. Briefly, after homogenization of whole liver tissue in cell lysis buffer, homogenates were cen-

trifuged for 1 min at 10,000 g, and the supernatant (100 mg protein) was incubated with Ac-DEVD-pNA substrate for caspase 3, and reaction buffer for 90 min at 37°C. Absorbance was measured at 405 nm as caspase protease activity.

MPO assays

Kidney tissues were thawed and homogenized in phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide. MPO activity was measured using a commercial kit, according to the manufacturer's instructions.

Measurement of TNF- α and IL-1 β production

Mouse kidneys were homogenized in phosphate buffer. TNF- α and IL-1 β were measured by commercial ELISA kits as manufacturer described.

Measurement of adenosine concentration

After renal ischemic and reperfusion, mouse kidneys were pulverized in liquid nitrogen and then sonicated in 0.6 N ice-cold perchloric acid to extract the proteins. The extracts were then neutralized by addition of 0.6 M potassium phosphate tribasic. Tissue adenosine levels were determined by high performance liquid chromatography-ultraviolet (HPLC-UV) as previously described [20].

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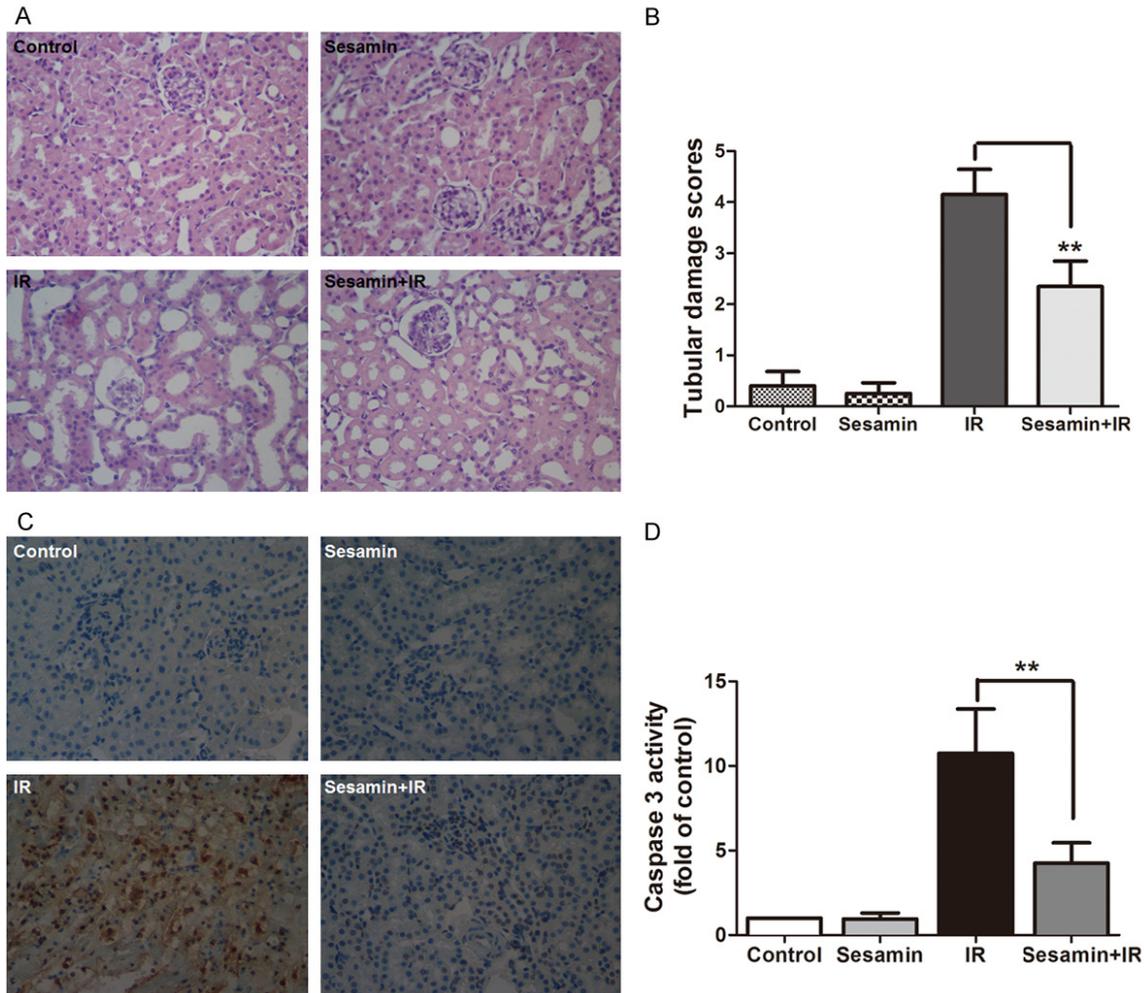


Figure 2. Sesamin reduced kidney damage and apoptosis induced by ischemic reperfusion. C57/BL6 mice (n = 6) were treated with vehicle or sesamin (100 mg/kg) every 8 h one time for three times within 24 h before surgery. Twenty-four hours after reperfusion, kidneys were collected for histological examination. A. Representative images of damages in the kidney (H&E staining, $\times 200$). B. Statistical analysis of pathological damage scores. C. Representative images of apoptosis in the kidney (TUNEL staining, $\times 200$). D. The activity of caspase-3 in the kidney. Each value is mean \pm SD. * $P < 0.05$, ** $P < 0.01$ compared with ischemic reperfusion.

Real-time reverse transcriptase PCR

Total RNA was extracted from cells and tissues using Trizol (Invitrogen, CA). RNA samples were reverse transcribed into complementary DNA (cDNA) using a PrimeScript RT reagent kit (Takara, Dalian, China). The transcripts were amplified from the cDNA with PCR. Real-time PCR was performed with SYBR Premix Ex Taq (Takara). The specific primers used were as follows: GAPDH 5'-AGGTCGGTGTG AACGGATTG-3' (sense), 5'-TGTAGACCATGTAGTTGAGGTCA-3' (antisense); CD73 5'-GGACATTTGACCTCGTCCAAT-3' (sense), 5'-GGGCACTCGACACTTG-GTG-3' (antisense); CD39 5'-AAGGTGAAGAGATTTGCTCAA-3' (sense), 5'-TTTGTCTGGGTCA-

GTCCAC-3' (antisense); A2AAR 5'-GCCATCCCATT CGCCATCA-3' (sense), 5'-GCAATAGCCAAGAGGCTGAAGA-3' (antisense); A2BAR 5'-AGCTAGAGACGCAAGACGC-3' (sense), 5'-GTGGGGTCTGT AATGCACT-3' (antisense). The relative levels of expression were quantified and analyzed by the heat map.

Western blot analysis

Protein extracts were prepared according to the method described in the protein extract kit (Pierce Biotechnology, Rockford, USA). Protein concentrations were measured using the BCA Protein assay kit. 40 μ g proteins were separated by sodium dodecyl sulfate-polyacrylamide

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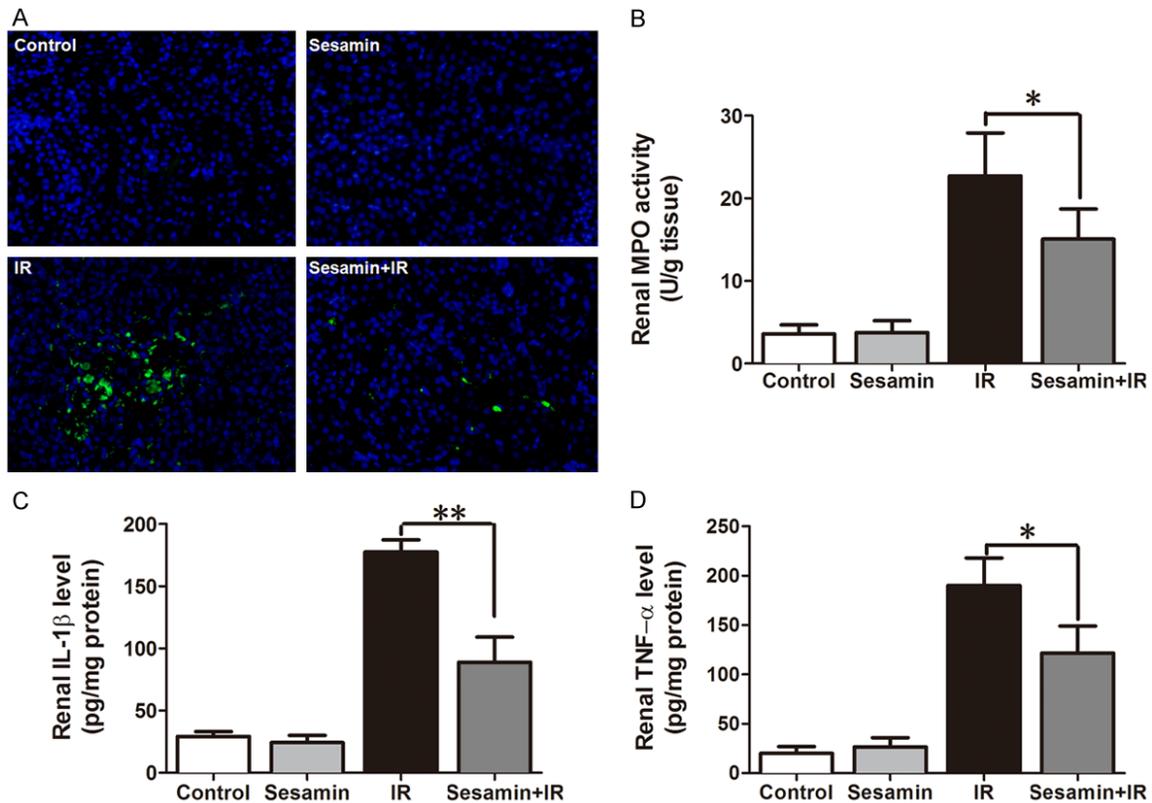


Figure 3. Sesamin alleviated neutrophil infiltration and proinflammatory cytokines production in the kidney of mice operated by ischemia reperfusion. C57/BL6 mice ($n = 6$) were pretreated with vehicle or sesamin (100 mg/kg) every 8 h one time for three times within 24 h before surgery. Twenty-four hours after reperfusion, kidneys were collected for Ly6G staining, MPO activity, and cytokines production. A. Representative images of Ly6G⁺ neutrophil in the kidney (immunofluorescence staining, $\times 200$). B. MPO activity in the kidney tissues. C. The level of IL-1 β in the kidney. D. The level of TNF- α in the kidney. Each value is mean \pm SD. * $P < 0.05$, ** $P < 0.01$ compared with ischemic reperfusion.

gel (SDS-PAGE) electrophoresis and transferred to nitrocellulose membrane. The membranes were incubated with primary antibody at 4°C overnight. HRP-conjugated secondary antibody was added and the blots were developed with the ECL plus kit.

Statistical analysis

All data were analyzed using Student's *t* test or analysis of variance (ANOVA) as appropriate. Results were expressed as the mean \pm S.D. *P* values ≤ 0.05 were considered significant.

Results

Sesamin attenuated renal damage and dysfunction after kidney ischemia-reperfusion

In order to evaluate the effect of sesamin on renal IRI, C57/BL6 mice were subjected to sham operation or warm ischemia (renal artery

occlusion for 30 minutes) followed by a 24 h reperfusion after treatment with either PBS or sesamin (100 mg/kg). As expected, compared with control, IR induced significantly acute kidney injury, characterized by marked increases in serum creatinine (Scr) and BUN. Pretreatment with sesamin in IR-operated mice led to a remarkable reduction in the levels of Scr and BUN compared with the IR model group (**Figure 1B** and **1C**).

Sesamin ameliorated renal damage and apoptosis after kidney ischemia-reperfusion

Kidney tissue morphology (H&E stain) was shown in **Figure 2A**. In the IR model, tubular dilatation, necrotic cells and brush border loss were clearly seen. However, pretreatment with sesamin obviously alleviated these pathological changes (**Figure 2B**). TUNEL staining and caspase 3 activity analysis showed that a significantly higher proportion of TUNEL-positive

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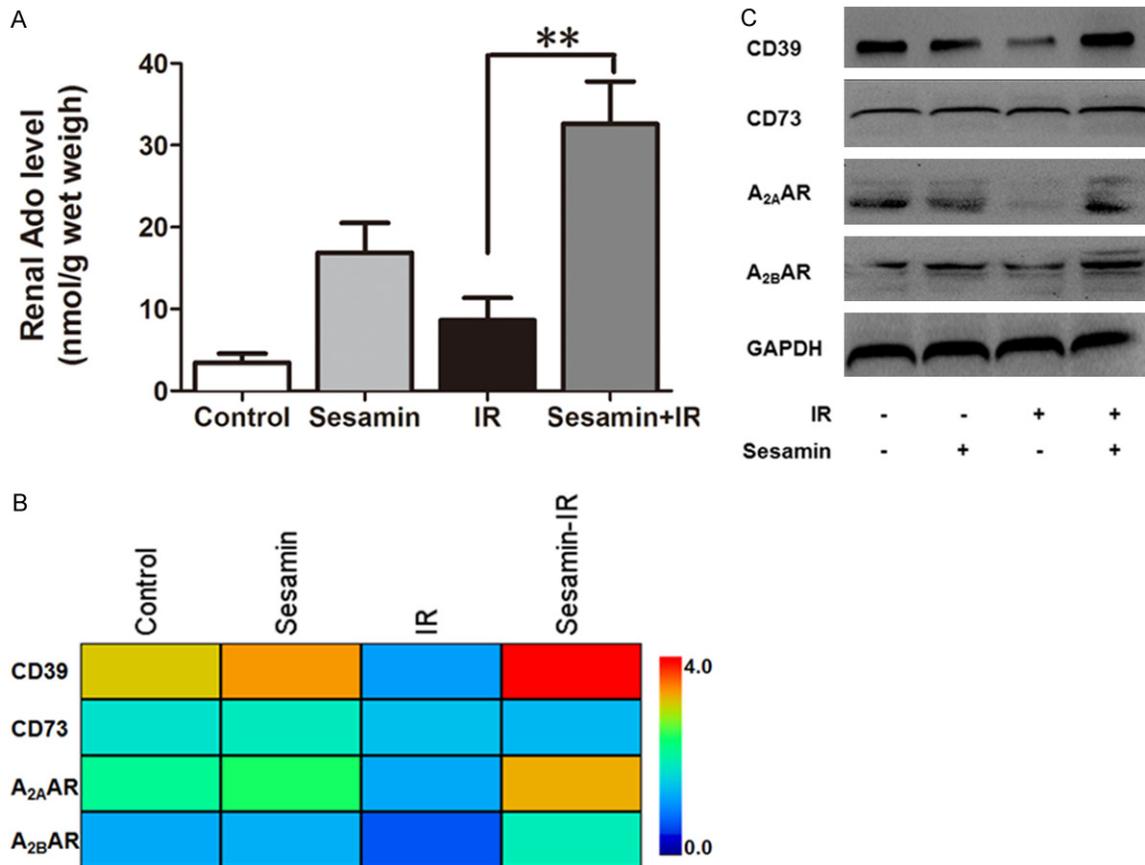


Figure 4. Sesamin promoted adenosine production and upregulated the expression of CD39 and A2AR in the kidney after ischemic reperfusion. C57/BL6 mice ($n = 6$) were pretreated with vehicle or sesamin (100 mg/kg) every 8 h one time for three times within 24 h before surgery. Twenty-four hours after reperfusion, kidney tissues were collected for next analysis. **A.** The concentration of adenosine in the kidney was measured by HPLC. **B.** The mRNA expression of CD39, CD73 and A2AR in the kidney was evaluated by qRT-PCR. **C.** The protein expression of CD39, CD73 and A2AR in the kidney was detected by western blotting. Data are expressed as the mean \pm SD. * $P < 0.05$; ** $P < 0.01$.

cells and increased caspase 3 activity were observed in the kidney tissues of IR-operated mice compared with control sham mice, whereas little TUNEL-positive cells and lower caspase 3 activity were detected in the kidney tissues of sesamin-treated mice after IR operation (**Figure 2C** and **2D**).

Sesamin reduced renal inflammation after kidney ischemia-reperfusion

Because renal inflammation contributes to kidney IRI, which involves in excessive neutrophil infiltration and pro-inflammatory cytokine production, we assessed renal neutrophils and cytokines in each experimental group. Immunofluorescence with Ly6G antibody showed that massive Ly6G⁺ neutrophils appeared in the kidney tissues of mice performed by IR.

However, sesamin pretreatment decreased the IR-induced infiltration of Ly6G⁺ neutrophils (**Figure 3A**). In addition, the MPO activity, the other marker of neutrophil, was also detected. It was markedly higher in IR group than that in the control group. Pretreatment with sesamin reduced the IRI-induced MPO activation (**Figure 3B**). Further, using the ELISA method, we evaluated the levels of TNF- α and IL-1 β in the kidney tissues. As shown in **Figure 3C** and **3D**, IR induced the significant increases in the levels of TNF- α and IL-1 β in the kidney, which were markedly inhibited by sesamin.

Sesamin promoted renal adenosine signaling after kidney ischemia-reperfusion

It has been reported that adenosine protects renal from IR injury. Thereby, we determined

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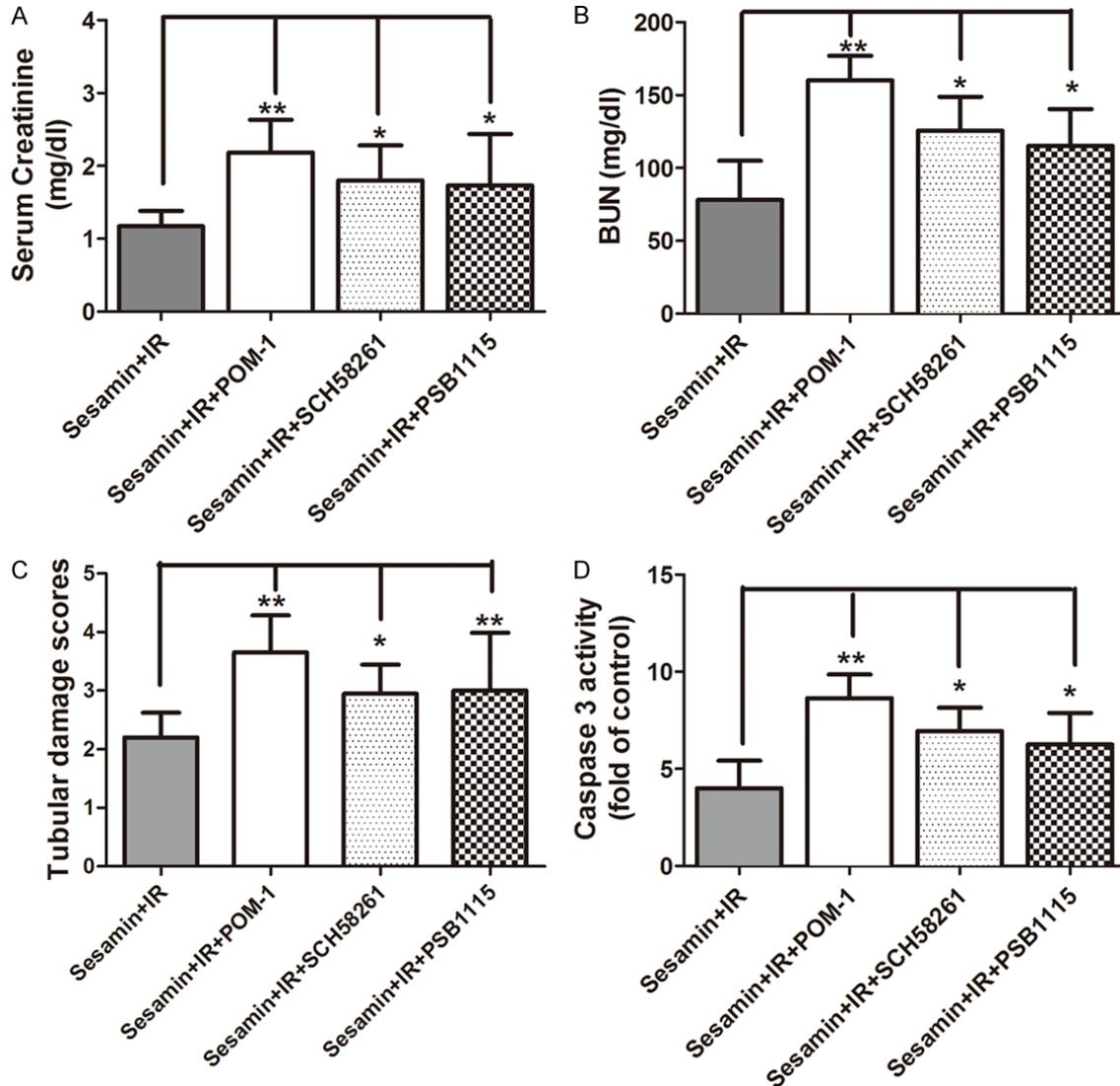


Figure 5. CD39 inhibitor or A2AR antagonist reversed the protective actions of sesamin in IR-operated mice. C57/BL6 mice ($n = 6$) were treated with sesamin (100 mg/kg) every 8 h one time for three times within 24 h before surgery with or without POM-1 (CD39 inhibitor, 5 mg/kg), SCH-58261 (the A2AAR antagonist, 10 mg/kg), PSB115 (A2BAR antagonist, 10 mg/kg) 30 min before IR. Twenty-four hours after IR, blood and kidney tissues were collected. A. Concentration of serum creatinine. B. Concentration of BUN. C. The tubular damage scores. D. The caspase 3 activity. Data are expressed as the mean \pm SD. * $P < 0.05$; ** $P < 0.01$.

adenosine level in kidney tissue by HPLC. Although ischemia-reperfusion slightly induced the production of adenosine, this induction was markedly increased by sesamin. The level of adenosine was also induced by sesamin in the sham-operated kidneys compared to control mice (Figure 4A). Meantime, these key limit enzymes of adenosine production, such as CD39 and CD73, and its receptors A2AR were determined by qRT-PCR and western blotting. The results displayed that IR inhibited the expression of CD39, A2AAR, and A2BAR mRNA

and protein, pretreatment with sesamin markedly promoted the expression of CD39, A2AAR, and A2BAR mRNA and protein in the kidney. However, there are no significant difference in the expression of CD73 mRNA and protein in four different groups (Figure 4B and 4C).

CD39-Adenosine-A2AR contributed to the protective actions of sesamin on IRI

To examine whether adenosine signaling participated in the renalprotective action of sesamin,

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min in IR injury, we administered injection of the CD39 inhibitor or the A2AR antagonists, respectively, before IR surgery. Renal function analysis showed that these inhibitors or antagonists partly abrogated the beneficial actions of sesamin on the serum creatinine and BUN in IR-mice (**Figure 5A** and **5B**). Consistence with these results, the improvement of sesamin on the pathological damage scores and caspase 3 activity was also reversed partly by the CD39 inhibitor or the A2AR antagonists, respectively (**Figure 5C** and **5D**).

Discussion

IR-induced acute kidney injury (AKI) remains an unailing clinical problem associated with high health care costs as well as very high mortality and morbidity [3, 21]. There are no effective drugs or therapies for the treatment of AKI. Therefore, therapies to prevent or accelerate renal recovery during and after ischemic AKI are critically needed. The preliminary experiments suggested that sesamin is a promising preventive drug for IR-induced AKI. In this study, we found that sesamin significantly improved IR-induced kidney injury, as assessed by reduced Scr and BUN concentrations, ameliorated renal pathological damages, and alleviated tubular cell apoptosis, suggesting that sesamin may be a potential therapeutic agent for the treatment/prevention of acute renal IRI.

Renal IR injury leading to AKI involves a complex series of events, including renal tubular and endothelial necrosis and apoptosis, neutrophil infiltration, and pro-inflammatory cytokines release and production [3, 5, 22, 23]. It is well known that renal tubular cell death and inflammation are hallmark traits of IRI [3]. Thereby, we explored the effects of sesamin on tubular cell death and inflammatory response caused by IR. The results showed that sesamin significantly not only alleviated tubular cell necrosis and apoptosis, but also inhibited renal neutrophil infiltration and pro-inflammatory cytokines production, which is consistent with published reports describing that sesamin exerts cytoprotective, anti-apoptosis, and anti-inflammatory properties [8, 14, 24].

Recent studies suggest that adenosine, an endogenous signaling molecule, plays a key role in protecting the kidney from ischemia-induced damage [25, 26]. Adenosine is derived

from the phosphohydrolysis of adenosine triphosphate (ATP) and adenosine monophosphate (AMP) by ecto-nucleoside triphosphate diphosphohydrolase-1 (CD39) and ecto-5'-nucleotidase (CD73) [26-31]. Extracellular adenosine signals are activated through G-protein coupled purinergic adenosine receptors (ARs), which includes A1AR, A2AAR, A2BAR and A3AR [32, 33]. As a critical cytoprotective molecule, adenosine could inhibit renal tubular cell death including necrosis and apoptosis, and relieve inflammatory response in ischemic AKI. Previous studies have shown that ischemic AKI was worse in CD39, or CD73 deficient mice, alleviated in overexpression or activation of CD39 or CD73 [27, 28, 34], indicating that these key limit enzymes controlling adenosine production play important role in protecting from renal IRI. In addition, it has been found that A2AR could effectively alleviate inflammatory response and protect from tissues damages in some inflammatory diseases, and activation of A2AAR or A2BARs prevents against IR injury in several organs, including the kidney [35-38]. Thereby, the modulation of adenosine signal pathway might be major targets of novel drug for prophylactic or therapeutic intervention in IRI [26, 39]. In fact, a previous study has reported that sesamin exhibits anti-apoptosis effect via the mechanisms that involve adenosine signaling [24]. In this study, we intriguingly found that sesamin pretreatment increased both adenosine production and CD39 and A2AR expression. Moreover, inhibition or antagonism of CD39, A2AAR and A2BAR reversed the beneficial effects of sesamin on IR-induced acute kidney injury. Thus, it is reasonable to assume that sesamin prevents kidney IRI by promoting adenosine signaling pathway.

In conclusion, the current findings demonstrate that sesamin protect the kidney from IRI by inhibiting tubular cell death and inflammatory response, further confirming that upregulating CD39-adenosine-A2AR signal contributes to the protection of sesamin.

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Disclosure of conflict of interest

None.

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Address correspondence to: Bin Wang, Department of Anesthesiology, The First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China. E-mail: 13637815096@163.com; Jingyuan Wan, Chongqing Key Laboratory of Biochemistry and Molecular Pharmacology, Chongqing Medical University, Chongqing 400016, China. E-mail: jywan@cqmu.edu.cn

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