Original Article

Sinomenine protects mice against ischemia reperfusion induced renal injury by attenuating inflammatory response and tubular cell apoptosis

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Abstract: Sinomenine (SIN) is a purified alkaloid from the Chinese herb Sinomenium acutum. Previous studies demonstrated that SIN possesses anti-inflammatory and anti-apoptotic properties. We thus in the present report conducted studies to examine its impact on ischemia reperfusion (IR) induced renal injury. Precondition of mice with 200 mg/kg of SIN provided significant protection for mice against IR-induced renal injury as manifested by the attenuated serum creatinine (Cre) and blood urea nitrogen (BUN) along with less severity for histological changes and tubular cell apoptosis. In line with these results, treatment of mice with SIN suppressed IR-induced inflammatory infiltration and the expression of chemokine CXCL-10, adhesion molecule ICAM-1, and cytokines TNF-α/IL-6. Mechanistic studies revealed that SIN inhibits NF-κB transcriptional activity to suppress IR-induced inflammatory response in the kidney, while it attenuates MAP kinase signaling to prevent tubular cells undergoing apoptosis after IR insult. Altogether, our data support that SIN could be a useful therapeutic agent for prevention and treatment of IR-induced renal injury in the clinical settings.

Keywords: Sinomenine, reperfusion injury, inflammation, apoptosis

Introduction

Renal ischemia reperfusion (IR) injury often arises from shock and various surgical procedures such as kidney transplantation, tumor resection and trauma, it acts as a major cause of morbidity and mortality in clinical settings [1, 2]. Interruption of blood flow to the kidney along with subsequent reperfusion leads to an acute inflammatory response including the activation and infiltration of macrophage and neutrophil, the release of pro-inflammatory cytokines and chemokines, and the enhanced expression of adhesion molecules [3]. Research into the pathogenesis of renal IR injury has demonstrated that acute inflammatory response plays a key role in inducing kidney damage [4, 5]. More recently, tubular cell apoptosis has also been recognized contributing to the pathoetiology of renal IR injury [6, 7]. Therefore, therapeutic approaches aimed at suppressing inflammatory response and tubular apoptosis were recognized effective against renal injury along with better prognosis after an episode of IR insult [8, 9].

Sinomenine or SIN (7, 8-didehydro-4-hydroxy-3, 7-dimethoxy-17-methylmorphinan-6-one) is a purified alkaloid extracted from the Chinese herb Sinomenium acutum, which has been employed to treat various rheumatic diseases in China and Japan for over 2000 years [10]. There is compelling evidence indicating that SIN possesses anti-inflammatory characteristics. For example, in a model of collagen-induced arthritis (CIA), SIN was found to markedly reduce CIA incidence along with improved disease symptoms such as paw swelling and arthritis scores [11]. SIN has also been found...
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Table 1. Primers used for real time PCR analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Sequence</th>
<th>Reverse Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>5'-TAC TGA ACT TCG GGG TGA TTG GTCC-3'</td>
<td>5'-CAG CCT TGT CCC TTG AAG AGA ACC-3'</td>
</tr>
<tr>
<td>CXCL-10</td>
<td>5'-CCT ATC CTG CCC ACC TGT TG-3'</td>
<td>5'-CGC ACC TCC ACA TAG CTT ACA-3'</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>5'-TGT TTC CTG CCT CTG AAG C-3'</td>
<td>5'-CTT CGT TTG TGA TCC TCC G-3'</td>
</tr>
<tr>
<td>TNF-α</td>
<td>5'-TAC TGA ACT TCG GGG TGA TTG GTCC-3'</td>
<td>5'-CAG CCT TGT CCC TTG AAG AGA ACC-3'</td>
</tr>
<tr>
<td>IL-6</td>
<td>5'-CAC GAT TTC CCA GAG AAC ATG TG-3'</td>
<td>5'-GTC TGT ATT CCC CTC CAT CG-3'</td>
</tr>
<tr>
<td>β-actin</td>
<td>5'-CCA GTT GGT AAC AAT GCC ATG T-3'</td>
<td>5'-CCA GTT GGT AAC AAT GCC ATG T-3'</td>
</tr>
</tbody>
</table>

*CXCL-10, CXC chemokine ligand-10; ICAM-1, intercellular adhesion molecule-1.

Effective against 2, 4, 6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice as manifested by the improved weight loss and histological scores [12]. Typically, SIN is capable of inhibiting lymphocyte proliferation, macrophage activation and migration [13, 14]. Other than its anti-inflammatory property, a recent study further demonstrated that SIN could induce heme oxygenase-1 expression, and by which it prevents IR-induced hepatocellular apoptosis [15]. Based on these discoveries, we thus assumed that SIN could be effective against IR-induced renal injury as well; particularly, SIN could be potent to protect tubular cells undergoing apoptosis after IR insult.

Materials and methods

Animals and drugs

Male C57BL/6 mice (H-2b, 22-25 g, Joint Venture SIPPR BK Experimental Animal Co., Shanghai, China) were housed in a specific pathogen-free environment and fed with laboratory chow and ad libitum. All studies were approved by the Animal Care Committee of School of Medicine Shanghai Jiao Tong University, and were conducted in accordance with our institutional guidelines. SIN (98% purity verified by HPLC) was purchased from SIGMA Co., Ltd (USA), and was freshly dissolved in normal saline (NS) for intravenous (i.v.) injection.

IR induction in the kidneys

Mice were anesthetized with intraperitoneal (i.p.) injection of pentobarbital (45-50 mg/kg) and placed supine on a heating pad to maintain their body temperature during surgery. Bilateral flank incisions were made and the left kidney was subjected to 30 min of ischemia with a microvascular clamp after right nephrectomy. The time of ischemia (i.e., 30 min) was chosen on the basis of preliminary studies, in which a reproducible renal IR injury along with a minimum of mortality rate can be achieved. In sham-operated (SO) group, the mice were undergone anesthesia, bilateral flank incisions and right nephrectomy alone. In SIN group, mice were injected with sinomenine (200 mg/kg) by tail vein 1 h before ischemia [12, 15]. In vehicle-treated (Saline) group, the mice were administered with same volume of saline. Mice from each group (6 mice/group) were sacrificed 6 h and 24 h after reperfusion to obtain blood and kidney samples, respectively.

Assessment of kidney function

Renal functions were assessed by blood urea nitrogen (BUN) and serum creatinine (Cr) levels at the core laboratory of Shanghai Jiao Tong University School of Medicine.

Histological and immunohistochemical analysis

Renal samples were fixed with 10% formalin, embedded in paraffin, and cut into 4 μm sections. The sections were stained with hematoxylin-eosin (H-E), followed by score for the severity of IR injury as described by Jablonski and colleagues by assessing the necrosis of the proximal tubules [16], in which 4 scales were employed: 0, absence of necrosis; 1, presence of necrosis of individual cells; 2, presence of necrosis of all cells in adjacent proximal convoluted tubule (PCT) with survival of surrounding tubules; 3, presence of necrosis confined to distal third of PCT with band(s) of necrosis extending across inner cortex; and 4, presence of necrosis of all 3 segments of PCT.

For immunostaining of macrophage infiltration, the sections (5 μm) were fixed in acetone, followed by blockade of endogenous peroxidase activity with 0.2% H2O2. The sections were next incubated with a rat anti-mouse F4/80 antibody (Santa Cruz Biotechnology, CA, USA), and...
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Figure 1. SIN provides protection for mice against IR-induced renal injury. The mice were first subjected to 30 min of renal ischemia followed by reperfusion to induce IR injury. Blood samples were next collected 6 h and 24 h after reperfusion to determine serum levels for creatinine (A) and blood urea nitrogen (B), respectively. Mice treated with SIN showed significantly lower levels of serum creatinine and blood urea nitrogen. ##, *P < 0.01 (SIN vs. SO group); *, *P < 0.05, **, *P < 0.01 (SIN vs. Saline group). Six mice were included in each study group.

Figure 2. SIN treatment protects kidneys from IR-induced damage. Renal sections were prepared from mice 6 h and 24 h after reperfusion and then subjected to HE staining. A: Representative HE staining results (magnification 200x). B: Degree of renal damage graded using the Jablonski's criteria. ##, *P < 0.01 (SIN vs. SO group); *, *P < 0.05, **, *P < 0.01 (SIN vs. Saline group). Six mice were examined for each group.
then with a horseradish peroxidase-conjugated rabbit anti-rat IgG (Dako, Carpinteria, CA, USA) as reported [17].

Detection of apoptosis

Paraffin embedded renal sections were stained for apoptotic cells by the terminal transferase mediated dUTP nick end-labeling (TUNEL) using an Apop Tag Peroxidase In Situ Apoptosis Detection Kit (Chemicon International Inc., Billerica, Ma, USA) as reported [18]. The results were scored semiquantitatively by averaging the number of apoptotic cells/field at 400× magnification. Five fields were evaluated per tissue sample, and the results were presented as means ± SD.

Assessment of myeloperoxidase activity

Myeloperoxidase (MPO) activity in the renal samples was measured with a commercial kit (NJJC Bio Inc., Nanjing) according to the manufacturer’s instructions.

SYBR green real-time RT-PCR

Total RNA was extracted from renal tissue samples using the TRizol reagent (Invitrogen, USA) as instructed. After removal of potentially contaminated DNA with DNase I (Invitrogen, USA), the samples were subjected to reverse transcription using a kit from Mbi Fermentas Inc. (Burlington, USA). The resulting cDNA was next used for real time PCR analysis of relative expression levels of target genes using the primers listed in Table 1. PCR was carried out at 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 15 s and 60°C for 30 s on an ABI StepOneTM real-time TR-PCR system (Applied Biosystems, USA). The amount of mRNA for each gene was normalized by β-actin, and the relative expression levels were calcu-
lated using the \(2^{-\Delta\Delta CT}\) method [19]. Relative mRNA levels of each gene in the SO group samples were adjusted to 1 and served as a calibrator, while the corresponding mRNA levels in the experimental group were expressed as fold changes as compared with that of SO samples.

**Western blot analysis**

Total proteins from renal lysates (30 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gels electrophoresis (SDS-PAGE), and the separated proteins were next electro-transferred onto nitrocellulose membranes using a transblot system (Bio-Rad, Hercules, CA, USA). The membranes were first blocked with 5% non-fat milk for 2 h at room temperature, followed by incubating with indicated primary antibodies (IκB-α, C-Jun NH2-terminal kinase (JNK), p38 mitogen-activated protein (MAP), kinase, extracellular signal-regulated kinase (ERK), phospho-IKK-β, phospho-JNK, phospho-p38 MAPK, phospho-ERK and β-actin, Cell Signaling, MA, USA) at 4°C overnight, respectively. After washes, the reactive bands were detected by a horseradish peroxidase-conjugated anti-rabbit or anti-rat antibody. The intensity of each reactive band was quantified by densitometric analysis [20].

**Statistical analysis**

All data were expressed as mean ± SD. Results were analyzed by one-way ANOVA, and Student’s t test was employed for assessing statistical significance if differences were established. In all cases, \(P\) value < 0.05 was considered with statistical significance.

**Results**

**Administration of SIN protects mice from IR-induced renal injury**

We first sought to demonstrate the impact of SIN on IR-induced renal injury, and for this purpose, we examined serum levels for Cr and BUN 6 h and 24 h after reperfusion. As compared with those mice from SO group, mice in Saline group displayed severe renal injury as manifested by tubular necrosis, vacuolization, loss of brush border, cast formation, tubules dilation, and edema. Remarkably, a significant attenuation for renal injury was noted in mice administered with SIN as characterized by less severity of edema, cast formation and tubular necrosis as compared with that of mice from Saline group (Figure 2A). To further confirm this observation, we performed quantitative analysis of multiple sections by scoring the severity of renal injury as described earlier. In line with the initial observation, mice from SIN group exhibited significantly lower scores as compared with that of mice from Saline group at all time points examined (Figure 2B).

**SIN treatment attenuates IR-induced tubular cell apoptosis**

Given that tubular cell apoptosis is a characteristic feature relevant to IR-induced renal injury, we next examined tubular cell apoptosis by TUNEL assay. Indeed, IR insult induced tubular cells undergoing massive apoptosis as manifested by the positive TUNEL staining of renal sections from mice in Saline group (Figure 3A). In line with our expectation, administration of SIN significantly protected mice from IR-induced tubular apoptosis as characterized by the reduction of TUNEL positive tubular cells (Figure 3A). Quantitative analysis of sections from multiple mice further confirmed these results as shown in Figure 3B (6 h, 35.6 ± 5.2/hpf vs. 20.7 ± 3.75.2/hpf; 24 h, 46.7 ± 7.2/hpf vs. 23.6 ± 4.35.2/hpf, \(P < 0.01\)). We further examined the expression of pro-apoptotic molecule, Caspase-3, by Western blotting 24 h after reperfusion. In consistent with the TUNEL results, significantly lower levels of Caspase 3 were noted in mice administered with SIN as compared with that of mice administered with control vehicle (Figure 3C, 3D). Together, our data support that SIN protects mice against
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Figure 4. Analysis of macrophage and neutrophil infiltration. Renal sections from mice 6 h and 24 h after reperfusion were subjected to immunostaining of F4/80 antigen for assessing macrophage infiltration. A: Representative immunostaining results of renal sections (magnification 400x). B: Average results for F4/80 positive cells in all mice examined. C: Results for MPO activity for assessing neutrophil infiltration. D: Real time PCR results for CD11b mRNA levels in renal tissues after IR insult. All data are expressed as mean ± SD, and 6 mice were analyzed in each study group. ##, \( P < 0.01 \) (SIN vs. SO group); *, \( P < 0.05 \), **, \( P < 0.01 \) (SIN vs. Saline group).

Figure 5. Real time PCR analysis of mRNA levels for the inflammatory mediators after renal IR insult. Real time PCR was employed to assess the impact of SIN treatment on the expression of inflammatory mediators following renal IR insult. Precondition of mice with SIN significantly attenuated the expression of chemokine CXCL-10 (A), adhesion molecule ICAM-1 (B), and inflammatory cytokines TNF-\( \alpha \) (C) and IL-6 (D). Results are expressed as mean ± SD of 4 mice examined for each group. ##, \( P < 0.01 \) (SIN vs. SO group); *, \( P < 0.05 \), **, \( P < 0.01 \) (SIN vs. Saline group).
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Next, we analyzed inflammatory infiltration in renal sections 6 h and 24 h after IR insult. We first examined macrophage infiltration by immunostaining of F4/80 expressions. Prominent interstitial macrophage infiltration was noted in mice from Saline group 6 h after reperfusion, and the infiltration was even higher 24 h after reperfusion. However, administration of SIN significantly suppressed macrophage infiltration both at 6 h and 24 h time points (Figure 4A, 4B). To examine neutrophil infiltration, we measured MPO activity in renal tissues. As shown in Figure 4C, MPO activity in renal lysates of mice from Saline group was significantly higher than that in mice from SO group. In contrast, SIN treated mice displayed significantly lower levels of MPO activity, suggesting less severe neutrophil infiltration. To further confirm these results, we examined mRNA levels for CD11b, a surface marker prominently expressed in macrophages and neutrophils by real time RT-PCR. Indeed, significantly lower levels of mRNA for CD11b was noted in mice treated with SIN as compared with that of control mice (Figure 4D). Collectively, our data suggest that SIN protects mice against IR-induced renal injury also involving suppression of inflammatory infiltration.

**SIN attenuates the expression of inflammatory mediators**

The above results prompted us to examine the impact of SIN on the production of inflammatory mediators that are implicated in IR-induced tissue damage [21]. To this end, we selectively examined CXCL-10, ICAM-1, TNF-α and IL-6 by real time PCR. It was noted that mRNA levels for CXCL-10 (Figure 5A) and ICAM-1 (Figure 5B) were markedly decreased in mice from SIN group as compared with that of mice from Saline group 6 h and 24 h after reperfusion, and similar results were obtained for mRNA levels for TNF-α (Figure 5C) and IL-6 (Figure 5D). Altogether, our data indicate that administration of SIN attenuated IR-induced expression of inflammatory mediators.

**SIN acts as a negative regulator for NF-κB signaling and MAP kinase activation**

To dissect the mechanisms underlying SIN suppression of inflammatory response, we examined its impact on NF-κB signaling. To this end, we examined IKK-β phosphorylation and IκB-α degradation by Western blot analysis of renal lysates. Indeed, IR insult induced a significant increase for the phosphorylated IKK-β (p-IKK-β) along with enhanced IκB-α degradation, suggesting an increased NF-κB activity. Remarkably, administration of SIN significantly attenuated IKK-β activity, and by which it prevented IR-induced IκB-α degradation (Figure 6). Together, our data support that SIN is a negative regulator for NF-κB signaling.

Next, we examined MAP kinase signaling to demonstrate the mechanisms for SIN protecting tubular cells against IR-induced apoptosis. SIN did not show a perceptible effect on the expression of total ERK1/2 (Figure 7A), JNK (Figure 7B) and p38 (Figure 7C); however, it
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Figure 7. SIN possesses high potency to attenuate IR-induced MAP kinase signaling. Similarly, renal lysates were prepared from mice 24 h after reperfusion and then subjected to Western blot analysis of total ERK1/2 (p-44/42) and phosphorylated ERK1/2 (p-p44/42) (A), total JNK and phosphorylated JNK (p-JNK) (B), total p-38 and phosphorylated (p-p38) (C). Four mice were analyzed for each group and the relative expression levels for each target were determined by densitometric analysis. ##, $P < 0.01$ (SIN vs. SO group); *, $P < 0.05$, **, $P < 0.01$ (SIN vs. Saline group).

Discussion

In the present report, we conducted studies to address the effect of SIN on IR-induced renal injury in a mouse model. To our knowledge, this is the first report demonstrating that precondition of mice with SIN ameliorates IR-induced renal injury, which implicates suppression of inflammatory response and protection of tubular cells undergoing apoptosis. Our data support that SIN could be a novel therapeutic agent for prevention of IR-induced renal injury in clinical settings. Indeed, oral SIN has been approved for treating inflammatory diseases such as rheumatism and arthritis without apparent hepatic or renal toxicities. Our studies in animals in the current report employed a dose of 200 mg/kg without perceptible renal and hepatic toxicity; rather we observed improved renal function after IR insult, indicating that SIN possesses a safe range of dosage in the setting for treatment of IR injury.

There is compelling evidence that renal tubular cell apoptosis plays an essential role in IR-induced injury and contributes to acute renal failure [22, 23]. Apoptosis can be triggered by diverse stimuli leading to the activation of extrinsic and intrinsic pathways. In the extrinsic pathway (also called death receptor pathway), apoptotic signaling is mediated mainly through stimulation of the TNF-α receptor family members that mediates death signals through caspase-3 activation [24, 25]. In the intrinsic pathway (also called mitochondrial pathway), cytochrome c is released from mitochondria into
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the cytosol which then promotes the formation of apoptosome along with Caspase-3 activation, leading to DNA fragmentation [26, 27]. However, these two pathways are ultimately mediated by means of Caspase-3 activation. Based on these observations, we thus examined the impact of SIN on IR-induced apoptosis in tubular cells. Both TUNEL assay and Caspase-3 analysis demonstrated that precondition of mice with SIN markedly protected tubular cells from IR-induced apoptosis.

Macrophages and neutrophils are important components of the innate immune system, and play an important role in IR injury. The infiltrated macrophages and neutrophils can secrete large amount of inflammatory cytokines, reactive oxygen species, and a variety of proteolytic enzymes, which could induce cellular apoptosis and necrosis resulting in organ dysfunction. The above secreted inflammatory mediators would further promote the aggregation of inflammatory cells into damaged tissues and exacerbate inflammatory response. Furthermore, infiltration of inflammatory cells into renal mesenchymal and blood capillary would reduce renal blood flow and deteriorate microcirculatory failure [28-30]. We thus next determined the role of SIN in IR-induced inflammatory infiltration in the kidney. Both immunohistochemical staining and MPO activity assay revealed that treatment of mice with SIN significantly prevented IR-induced macrophage and neutrophil infiltration. Indeed, real-time RT-PCR analysis confirmed that mRNA levels for CXCL-10 and ICAM-1 relevant to those infiltrated immune cells are significantly lower in SIN-treated mice as compared with that of control mice. Similarly, inflammatory cytokines such as TNF-α and IL-6 have been proved to play key roles in the pathological processes of renal IR injury, and they serve as central propagating factors and promote inflammatory response primarily through signal transduction pathways leading to the induction of gene expression for secondary inflammatory mediators [29]. In line with the observation for CXCL-10 and ICAM-1, a significant reduction of mRNA levels for TNF-α and IL-6 was noted in mice treated with SIN as compared with that of control vehicle treated mice.

Given that transcription factor NF-κB is one of the most important inflammatory signaling molecules, we thus next embarked on NF-κB signaling to explore the mechanisms underlying SIN suppression of inflammatory response after IR insult. Due to the fact that NF-κB activation depends on the phosphorylation of its inhibitor, IκB, by the specific inhibitory κB kinase (IKK) subunit, IKK-β [31, 32], we therefore examined IKK-β phosphorylation and IκB-α degradation to assess NF-κB activity. As expected, SIN showed high potency to repress IKK-β activity, and through which it prevented IκB-α from IR-induced degradation, demonstrating that SIN suppresses IR-induced inflammatory response by attenuating NF-κB activity.

There is evidence that altered MAP kinase signaling implicates in IR-induced apoptosis. For example, in a rat myocardial IR model, C-phycocyanin treatment significantly decreased IR-induced p38 and ERK1/2 activation, and by which it provided protection for cardiomyocytes against IR-induced apoptosis [33]. Similarly, Ginkgo extract (ginaton) provides protection for renal tubular epithelial cells against IR-induced death by suppressing JNK activity [34]. We thus assumed that SIN protects tubular cells against IR-induced apoptosis also involving regulation of MAP kinase signaling. Indeed, Western blot analysis of renal lysates revealed that SIN strikingly inhibited JNK, p38 and ERK1/2 activation as manifested by the significantly lower levels of phosphorylated ERK1/2, p38 and JNK, suggesting that SIN attenuates IR-induced MAP kinase activity, and by which it prevents tubular cells undergoing apoptosis.

In summary, we demonstrated evidence supporting that precondition of SIN provides protection for mice against IR-induced renal injury as manifested by suppressed inflammatory response along with reduced tubular cell apoptosis. Our mechanistic studies revealed that SIN represses NF-κB activity to inhibit IR-induced inflammatory response, while it attenuates MAP kinase signaling to prevent tubular cells undergoing apoptosis after IR insult. Altogether, our data suggest that SIN could be a novel therapeutic agent for prevention of IR-induced renal injury in clinical settings.

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Disclosure of conflict of interest

The authors declare no competing financial interests.

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