Protective Effects of SP600125 on Renal Ischemia-Reperfusion Injury in Rats


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Background. Ischemia/reperfusion injury (IRI) has a negative effect on renal allograft survival. Using a rat model of kidney IRI in this study, we investigated the overall effect of selective c-Jun N-terminal kinase (JNK) inhibitor SP600125 on renal IRI events.

Methods. All 45 Fisher rats were anesthetized and renal IRI model was established by 45 min clamp of bilateral renal pedicles and 24 h reperfusion. Vehicle solution or SP600125 solution was intraperitoneally injected 45 min before ischemia, respectively. Analysis of renal histology, function, reactive oxygen species (ROS) expression, JNK phosphorylation status, as well as intra-renal pro-inflammatory cytokines expression was evaluated in this study.

Results. After IRI, the levels of blood urea nitrogen, creatinine, tissue malondialdehyde, TNF-α, IL-1β, IL-6 were all elevated significantly, while superoxide dismutase, catalase activity were decreased. Histologic findings showed severe devastating lesions and increased rodent cell apoptosis; SP600125 effectively improved morphologic features, reversed above-mentioned parameters, and significantly attenuated c-Jun phosphorylation, as well as intra-renal pro-inflammatory cytokines expression compared with vehicle-treated group.

Conclusion. These data demonstrate that inhibition of c-Jun with SP600125 is capable of attenuating renal IRI, which might be a novel therapy target.

Key Words: ischemia; reperfusion; JNK; kidney.

INTRODUCTION

Chronic allograft nephropathy (CAN) is the leading cause of renal allograft loss, which remains a great challenge for transplant clinicians. The precise cause of CAN following renal allograft remains unclear, but appears to be related to pathologic changes due to organ preservation techniques and ischemia-reperfusion (I/R) injury prior to and after organ implantation [1, 2]. During I/R, a robust inflammatory cascade occurs, which includes cell-surface immune receptor activation, subsequent up-regulation of a series of proinflammatory cytokines and chemokines, etc. [3]. It was established that reactive oxygen species (ROS) and reactive nitrogen species (RNS) increase in the areas of ischemia and reperfusion, which are responsible for renal injury. Meanwhile, the above mentioned pro-inflammatory factors also play important roles in pathogenesis of renal I/R injury, through leukocyte activation and expression of adhesion molecules and cytokines [4, 5].

c-Jun N-terminal kinase (JNK) is a member of the mitogen-activated protein kinase family, and its function is critical for signal transduction in a variety of endothelial and epithelial cells. JNK is a serine/threonine protein kinase that phosphorylates c-Jun, a component of the activator protein-1 transcription factor complex. Recently described JNK inhibitor SP600125 [anthra[1,9-cd]pyrazol-6(2H)-one] is a novel synthetic anthrapyrazole inhibitor that is a reversible, ATP-competitive inhibitor of JNK1/2, the chemical structure of which is demonstrated in the Figure 1. SP600125 exhibited broad-based antiproliferative activity in human endothelial and tumor cell lines. SP600125 affects proliferation by arresting cells in the G2/M phase of the cell.
cycle. SP600125 also acts to inhibit endothelial cell migration. Recently, it has been demonstrated that SP600125 have some protective effects against transient brain ischemia/reperfusion-induced neuronal death in rat hippocampal CA1 region, however, the potential effect of SP600125 on renal I/R injury has not been well investigated [6, 7]. Thus, in the present study, we examined the effects of SP600125 on renal function and the expression levels of proteins affecting renal function during the process of renal I/R injury.

MATERIALS AND METHODS

Animals, Surgery, and Treatment Design

All 45 adult male Fisher rats weighing 200–230 g were fed with standard chow and water ad libitum and provided by the experimental animal center in Tongji University. All procedures were performed in accordance with the National Institutes of Health (NIH, Bethesda, MD) guidelines for the use of experimental animals.

Animals were anesthetized by intraperitoneal injection of sodium pentobarbital (30 mg/kg) and placed in a supine position. A midline abdominal incision was made, and body temperature was maintained between 36 and 37 °C. After laparotomy and dissection of both renal pedicles, bilateral ischemia was induced by occluding the renal pedicles with atraumatic microvascular clamp for 45 min; then reperfusion was initiated by removal of the clamp. After clamps were released, the incision was closed in two layers with 2-0 sutures. Sham-operated animals underwent anesthesia, laparotomy, and renal pedicle dissection only. The immediate color change of the kidneys signifying the stoppage of blood flow indicates successful occlusion. During reperfusion, clamps were removed and the blood flow to the kidneys was re-established with visual verification of blood return. All animal procedures were followed in accordance with our institutional guidelines for animal care.

Animals were divided into three groups consisting of 15 rats each: (1) sham-operated plus PPCEs vehicle (30% PEG-400/20% polypropylene glycol/15%/Crephor EL/5%/ethanol/30%/saline) treatment; (2) rats subjected to renal I/R plus PPCEs vehicle treatment; (3) rats subjected to renal I/R plus SP600125 (15 mg/kg, intraperitoneally) treatment. Sham-operated animals received the same surgical procedures except that the bilateral renal pedicles were not clamped. PPCEs vehicle or SP600125 was intraperitoneally injected at 45 min before the ischemia, respectively. Drug treatment protocol was performed with some modifications of previous procedure described by Bennett et al. [8]. Twenty-four hours after reperfusion initiation, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg) and placed on a homeothermic table. Catheters were placed in the left carotid artery for blood sampling, and the left kidney was removed and quick-frozen for the next histologic studies. Harvested kidney was obtained and perfused with phosphate buffered saline (PBS) to remove all blood, then weighed and stored at –80 °C until assayed. Blood samples were spun at 1000 rpm for 15 min and serum samples were collected.

Assessment of Renal Function

Serum blood urea nitrogen (BUN) and creatinine (Cr) was used for the evaluation of renal function. The samples were measured with an autoanalyzer (Technicon RA-1000; Bayer, Tarrytown, NY).

Histology and Morphology

Harvested renal tissue was appropriately dehydrated and paraffin embedded and sectioned into 4-µm slides formalin-fixed, and then sections were stained using the periodic acid Schiff (PAS) reaction, which permits assessment of tubular integrity. Ten subcortical and juxtamedullary fields were recorded from each kidney slide by using a digital camera incorporated in a Nikon microscope (TMD300, Tokyo, Japan). The affected tubular area was analyzed blindly by an expert pathologist. Tubular damage was characterized by a loss of brush border, lumen dilatation or collapse, and detachment from basement membrane. Digital microphotographs were recorded for each rat to assess by morphometric analysis the total tubular area (excluding luminal, interstitial, and glomerular areas) and damaged tubular area, delimited by using eclipse net software (magnification ×400). The damaged tubular area was expressed as a proportion of the affected tubular area and total tubular area.

Renal Lipid Peroxidation

Malondialdehyde (MDA), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reactive substances (TBARS) as previously reported [9]. Briefly, after homogenization of the tissue, the reaction was performed in an 0.8% aqueous solution of thiobarbituric acid in 15% TCA and heated at 95 °C for 45 min, and the mixtures were centrifuged at 3000 g for 15 min. Supernatant absorbance was read at 532 nm. TBARS were quantified using an extinction coefficient of 1.56 × 10^5 M/cm and expressed as nanomoles of TBARS per milligram of protein. The tissue protein was estimated using the Bradford method.

In Situ Apoptosis Assay

Apoptosis in kidney sections was determined by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay using an ApoTag in situ apoptosis detection kit (S7101; Chemicon International, Temecula, CA). Slides were prepared by following the procedures previously described [10]. A minimum of 10 subcortical and 10 juxtamedullary fields (magnification ×400) per kidney were evaluated in all kidney tissues, and the images were recorded and analyzed blindly. Only tubular cells that contained TUNEL-positive nuclei with the characteristic morphology of apoptosis, including nuclear fragmentation and nuclear condensation, were quantified. TUNEL-positive cells were counted, and the results were expressed as the number of TUNEL-positive nuclei per square millimeter.

Western Blot Analysis

Proteins were extracted from kidneys as previously described [11]. Samples were eluted by boiling at 100 °C for 5 min in SDS-PAGE sample buffer. Samples (100 µg/lane) were separated on either a 10% or 12% SDS-polyacrylamide gel and electrotransferred onto a nitrocellulose membrane (Amersham, Buckinghamshire, UK). Membranes were blocked for 3 h in Tris-buffered saline with 0.1% Tween20 (TBST) and 3% bovine serum albumin (BSA). Membranes were incubated with anti-p-c-Jun (Ser63/Ser731:1:100), anti-c-Jun (1:500) overnight at 4 °C. Membranes were washed and incubated with anti-rabbit (1:2000) and anti-mouse (1:2000) horseradish peroxidase (HRP)-conjugated secondary antibodies. Membranes were then developed with Supersignal West Dura (Pierce) according to the manufacturer’s instructions.
FIG. 2. Renal functional parameters in sham, untreated ischemia-reperfusion (I/R), and SP600125-treated groups after 45 min of ischemia and 24 h of reperfusion (mean ± SD). I/R-induced renal failure was evidenced by a significant increase in BUN (A) and serum creatinine (B), renal dysfunction was completely prevented in I/R SP600125-pretreated rats. *P < 0.05 versus sham; **P < 0.01 versus I/R group.

FIG. 3. Histologic evaluation of harvested renal tissue. Periodic acid-Schiff staining from groups studied as stated. Lower-power (×100; (A)–(C)) microphotographs are shown. Detachment from basement membrane, tubular dilation, loss of brush border existed in I/R group, whereas practically absent in SP600125 pretreatment group. Morphometric quantification of affected tubular area was performed in Figure 3D. * P < 0.01 versus sham; **P < 0.01 versus I/R group.
with alkaline-phosphatase-conjugated secondary antibodies in TBST for 2h and developed using NBT/BCIP color substrate (Promega, Madison, WI). The density of the bands on the membrane was scanned and analyzed with an image analyzer (LabWorks Software; UVP Upland, CA).

Determination of TNF-α, IL-1β, IL-6 Levels in the Renal Tissues

The supernatant from kidney homogenate was prepared for detecting the levels of TNF-α, IL-1β, and IL-6 with a commercial enzyme linked immunosorbent assay (ELISA) kit following the instructions of the manufacturer. The absorbance was read on a microplate reader and the concentrations were calculated according to the standard curve. Protein content in the sample was determined by Coomassie blue assay and the results were corrected per microgram of protein.

Statistical Analysis

Quantitative data were presented as means ± SD. Significance of the differences among groups was tested by ANOVA using Bonferroni’s correction for multiple comparisons. All comparisons passed the normality test. A P value <0.05 was defined as statistically significant.

RESULTS

SP600125 Treatment Improved Renal Function

After 24 h reperfusion, rats that underwent renal ischemia developed marked renal dysfunction with elevated serum BUN and Cr levels; in the SP600125 pretreated group, serum BUN and Cr levels were significantly reduced, which is demonstrated in Figure 2.

SP600125 Treatment Improved Histological Lesions

Rats of I/R group revealed severe tubular damage characterized by loss of brush border, lumen dilatation or collapse, and cellular detachment from tubular basement membranes both in cortex and medulla area, which is demonstrated in Figure 3 A–C. These lesions were markedly attenuated by SP600125 treatment. We quantitatively investigated the percentage of injured tubular areas, and morphometric quantification of affected tubular area was performed as shown in

FIG. 4. Effect of renal I/R and SP600125 on renal lipoperoxidation and antioxidant enzymes mRNA levels. (A) SP600125 prevented the kidney lipoperoxidation induced by I/R (malondialdehyde; MDA). Associated with increased mRNA levels of SOD (B) and glutathione peroxidase (C), with no marked changes in catalase (D). *P < 0.01 versus sham; **P < 0.01 versus I/R group.
Figure 3D, and SP600125 demonstrated its tubule-protective effect (Fig. 3 D).

SP600125 Treatment Reduced Renal Lipoperoxidation

Lipoperoxidation caused by reactive oxygen species (ROS) is a critical mechanism involved in cellular damage during I/R injury; therefore, renal lipoperoxidation and the mRNA levels of antioxidant enzymes were evaluated. There was a significant increase in kidney lipoperoxidation in I/R group, which was prevented by the prophylactic treatment with JNK inhibitor SP600125 (Fig. 4A). The reduction of lipoperoxidation observed in the SP600125-treated groups was accompanied by a significant increase in SOD and glutathione peroxidase as antioxidant enzymes, while the change of catalase mRNA levels was not statistically different (Fig. 4 B–D).

SP600125 Treatment Reduced I/R Injury Related Apoptosis Cell Death

In situ labeling of cell nuclei by the TUNEL showed that I/R injury induced a significant increase in apoptosis cell death measured by positive nuclei stain per square milliliter in juxtamedullary areas as well as subcortical sections, while in rats subjected to SP600125 pretreatment, the extent of apoptosis was significantly different from the untreated I/R group, which demonstrated the protective effects of SP600125 in renal I/R injury (Fig. 5A, B).

SP600125 Inhibited Activation and Expression of c-Jun During I/R Injury

As reported in literature, JNK activation phosphorylates nuclear substrates, c-Jun was closely related to cell death, and after activation, it could induce neuronal cell death by promoting its transcriptional activity. In our study, the effects of SP600125 on activation and expression of c-Jun were investigated [12]. The pre-administration of SP600125 (15 mg/kg) 45 min

![Figure 5](image)

**FIG. 5.** Effect of renal I/R and SP600125 on I/R injury related apoptosis cell death: the percentage of TUNEL positive staining cells both in subcortical (A) and juxtamedullary areas (B) was significantly inhibited by SP600125 treatment compared with I/R group. *$P < 0.01$ versus sham; **$P < 0.01$ versus I/R group.

![Figure 6](image)

**FIG. 6.** Effect of SP600125 on I/R induced activation and expression of c-Jun. Bands stand for phosphorylated c-Jun (p-c-Jun) and c-Jun that were scanned and the intensities are represented as folds versus sham group. Significant inhibition of I/R-induced up-regulation of p-c-Jun and c-Jun were found in I/R plus SP600125 treatment group; *$p < 0.05$ versus sham; **$p < 0.05$ versus I/R group.
before ischemia led to a significant down-regulation of phosphorylated c-Jun, whereas the expression of c-Jun was not affected compared with vehicle-treated I/R rats, as demonstrated in Figure 6.

**SP600125 Treatment Attenuated Expression of Pro-inflammatory Cytokines**

By enzyme-linked immunosorbent assay (ELISA), renal expression of TNF-α, IL-1β, and IL-6 exhibited a more than 2-fold increase in I/R vehicle treated group versus sham group, however, significant inhibition of these pro-inflammatory cytokines were found in I/R plus SP600125 treatment group (Fig. 7 A–C).

**DISCUSSION**

In 2007, Wang *et al.* proposed the reno-protective effect of SP600125; however, the limitation in his study was that only anti-apoptosis effect was mentioned, in fact, therapeutic effects of SP600125 contain pleiotropic consequence in the process of IRI, as described in our study, that the inhibition of the JNK signaling pathway by intraperitoneal injection of SP600125 not only attenuated I/R induced cell apoptosis, but also inhibited MPO, MDA accumulation, the inflammatory cascades were suppressed as well. In fact, inflammatory accentuation was recognized as one of the main causes of renal allograft dysfunction post-kidney transplantation [13]. The data presented in our study demonstrated that SP600125 administration could significantly improve renal function, ameliorate morphologic features of I/R, inhibit roles of I/R related inflammatory mediators, and generally protect kidney from I/R injury.

c-Jun N-terminal Kinase (JNK), which was firstly discovered in 1991, was found to be activated by cellular stress such as heat, osmotic shock, UV light, endotoxins, and cytokines, hence the alternative name was stress-activated protein kinase (SAPK) [14]. The role of JNK in the setting of ischemia reperfusion injury has been controversial and a protective or a detrimental aspect to JNK activation remained debatable. However, JNK inhibitors for the prevention of cell death induced by ischemia and other stress-induced apoptotic responses have shown significant therapeutic potential, especially in the field of neurology [15]. More evidence demonstrated that JNK/SAPK inhibitors were protective in the pathogenesis of neuronal cell death, JNK was highly expressed in the fore- and hind-brain.
regions in the mouse, and the JNK−/− mice are resistant to excitotoxic agent-induced hippocampal neuronal death. A similar study in gerbils also proved that inhibition of JNK could reduce ischemia cell death, which is consistent with our study that demonstrated the anti-apoptotic effects of JNK inhibitor [16].

The involvement of JNK in the pathogenesis of hepatic ischemia reperfusion injury was well focused. It has been shown that the JNK inhibitor AS601245 decreases cardiomyocyte apoptosis and infarct size in a rat cardiac I/R model in rats [17]. JNK activation in this model occurs primarily in the reperfusion phase in response to the generation of reactive oxygen species (ROS). In our present study, the inhibition of JNK significantly alleviated ROS agent production, perhaps consisting of one aspect of its underlying reno-protection mechanism.

Similarly, in a transplantation of rat lung model, the JNK inhibitor SP600125 was shown to improve I/R injury. In this model, the author proposed that JNK mediates the release of pro-apoptotic factors from mitochondria, may directly phosphorylate pro-apoptotic Bcl family members such as Bak and Bid, and induce the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6 [18]. In the present study, we were able to confirm the proposed role of JNK in IRI by demonstrating that SP600125 inhibition rats were protected against kidney I/R injury, with significantly lower serum creatinine, less tubular damage, and less interstitial monocyte accumulation versus vehicle-treated controls.

SP600125 is a potent, cell-permeable, selective, and reversible inhibitor of JNK, functions as a reversible ATP competitive inhibitor of JNK and MAPKs, and its selectivity on JNK is more than 300-fold compared with the extracellular signal-regulated kinases (ERKs) and p38 MAPKs [19]. In our previous study, the inhibition of p38 MAPK could effectively protect kidney from ischemia-reperfusion injury and, interestingly, the mechanism of preconditioning-induced protection comprises several signal transduction pathways that involve protein kinases. Whether there exists a cross-linking of these kinase pathways is noteworthy of further investigation.

In addition, a third mechanism by which JNK signalling could promote renal I/R injury might be through the recruitment and activation of T cells. Recent studies have described the role of T cells in causing renal I/R injury, presumably as part of an innate immune response rather than an antigen-specific immune response. The primary effect of JNK blockade was in reducing T-cell infiltration, which is likely to be an indirect effect through preventing up-regulation or activation of leucocyte adhesion molecules, as illustrated by the effects on ICAM-1 and CD44 expression. However, there is also evidence that JNK signalling plays a regulatory role in the primary T-cell response, which could represent a direct mechanism targeted by JNK blockade in I/R injury [20]. Whether SP600125 influence on T cell interactions mediate renal I/R injury has yet to be proven.

In summary, the current study demonstrated that JNK inhibitor SP600125 has a general role in the response to ischemia and reperfusion in the kidney, which is consistent with novel report in a model of liver transplantation [21]. We further extended the studies and found that SP600125 could protect the kidney from I/R injury. Therefore, these finding elucidate the potential role of JNK inhibition as a novel and efficient strategy for I/R related kidney injury.

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