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Dongcheng Feng, Jihong Yao, Guangzhi Wang, Zhenlu Li, Guo Zu, Yang Li, Fuwen Luo, Shili Ning, Wasim Qasim, Zhao Chen, Xiaofeng Tian

Intestinal epithelial oxidative stress and apoptosis constitute key pathogenic mechanisms underlying intestinal ischemia/reperfusion (I/R) injury. We previously reported that the adaptor p66Shc-mediated pro-apoptotic pathway was activated after intestinal I/R. However, the upstream regulators of the p66Shc pathway involved in intestinal I/R remain to be fully identified. Here, we focused on the role of a prolyl-isomerase, Pin1, in the regulation of p66Shc activity during intestinal I/R. Intestinal I/R was induced in rats by superior mesenteric artery (SMA) occlusion.Juglone (Pin1 inhibitor) or vehicle was injected intraperitoneally before I/R challenge. Caco-2 cells were exposed to hypoxia/reoxygenation (H/R) *in vitro* to simulate an *in vivo* I/R model. We found that p66Shc was significantly up-regulated in the I/R intestine and that this up-regulation resulted in the accumulation of intestinal mitochondrial reactive oxygen species (ROS) and massive epithelial apoptosis. Moreover, intestinal I/R resulted in elevated protein expression and enzyme activity of Pin1 as well as increased interaction between Pin1 and p66Shc. This Pin1 activation was responsible for the translocation of p66Shc to the mitochondria during intestinal I/R, as Pin1 suppression by juglone or siRNA markedly blunted p66Shc mitochondrial translocation and the subsequent ROS generation and cellular apoptosis. Additionally, Pin1 inhibition alleviated gut damage and secondary lung injury, leading to improvement of survival after I/R. Collectively, our findings demonstrate for the first time that Pin1 inhibition protects against intestinal I/R injury, which could be partially attributed to the p66Shc-mediated mitochondrial apoptosis pathway. This may represent a novel prophylactic target for intestinal I/R injury.

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Title page

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Dongcheng Feng[1], Jihong Yao[2], Guangzhi Wang[1], Zhenlu Li[1], Guo Zu[1], Yang Li[1], Fuwen Luo[1], Shili Ning[1], Wasim Qasim[1], Zhao Chen[1], Xiaofeng Tian[1,*]

[1]Department of General Surgery, The Second Affiliated Hospital of Dalian Medical University, Dalian 116023, China

[2]Department of Pharmacology, Dalian Medical University, Dalian 116044, China

[*]**Corresponding author**

Department of General Surgery, The Second Affiliated Hospital of Dalian Medical University, Dalian 116023, China

Tel: +86-0411-86110010. Fax: +86-0411-86110010

E-mail: txfdl@dmu.edu.cn

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Abstract

Intestinal epithelial oxidative stress and apoptosis constitute key pathogenic mechanisms underlying intestinal ischemia/reperfusion (I/R) injury. We previously reported that the adaptor p66Shc-mediated pro-apoptotic pathway was activated after intestinal I/R. However, the upstream regulators of the p66Shc pathway involved in intestinal I/R remain to be fully identified. Here, we focused on the role of a prolyl-isomerase, Pin1, in the regulation of p66Shc activity during intestinal I/R. Intestinal I/R was induced in rats by superior mesenteric artery (SMA) occlusion. Juglone (Pin1 inhibitor) or vehicle was injected intraperitoneally before I/R challenge. Caco-2 cells were exposed to hypoxia/reoxygenation (H/R) *in vitro* to simulate an *in vivo* I/R model. We found that p66Shc was significantly up-regulated in the I/R intestine and that this up-regulation resulted in the accumulation of intestinal mitochondrial reactive oxygen species (ROS) and massive epithelial apoptosis. Moreover, intestinal I/R resulted in elevated protein expression and enzyme activity of Pin1 as well as increased interaction between Pin1 and p66Shc. This Pin1 activation was responsible for the translocation of p66Shc to the mitochondria during intestinal I/R, as Pin1 suppression by juglone or siRNA markedly blunted p66Shc mitochondrial translocation and the subsequent ROS generation and cellular apoptosis. Additionally, Pin1 inhibition alleviated gut damage and secondary lung injury, leading to improvement of survival after I/R. Collectively, our findings demonstrate for the first time that Pin1 inhibition protects against intestinal I/R injury, which could be partially attributed to the p66Shc-mediated mitochondrial apoptosis pathway. This may represent a novel prophylactic target for intestinal I/R injury.

Summary statement:

Blockade of p66Shc mitochondrial translocation by Pin1 inhibition ameliorates I/R-induced mitochondrial oxidative stress and apoptosis in the intestine and improves local and systemic injuries. Targeting Pin1 may serve as a promising therapeutic strategy for intestinal I/R injury.

Short title: Targeting Pin1 reduces gut ischemia reperfusion injury

Keywords: p66Shc; Pin1; mitochondrial oxidative stress; apoptosis; intestinal ischemia reperfusion

Abbreviations list:

I/R, ischemia/reperfusion; H/R, hypoxia/reoxygenation; p66Shc, 66 kDa isoform of the adaptor molecule ShcA; Pin1, peptidyl-prolyl cis/trans isomerase; J, juglone; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; TNF-α, tumor necrosis factor-α; IL-6, interleukin 6; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; MPO, myeloperoxidase; VDAC, voltage-dependent anion channels; siRNA, small interfering RNA; H&E, hematoxylin and eosin; ROS, reactive oxygen species; SD, standard deviation

Introduction

Intestinal ischemia/reperfusion (I/R) injury is a frequent and life-threatening complication seen in a broad range of clinical settings, such as acute mesenteric ischemia (AMI), small intestine volvulus, trauma, shock, cardiopulmonary disease and some surgical procedures, including small bowel transplantation and abdominal vascular surgery [1-3]. Intestinal I/R leads to loss of the mucosal barrier with

increased permeability and bacterial translocation, which can often result in the development of systemic inflammation and multiple organ dysfunction [4]. It has been demonstrated that restoration of the blood supply to the intestine (the reperfusion period) unexpectedly exacerbates the damaging effects of tissue ischemic injury, in part owing to a burst of reactive oxygen species (ROS) [5]. Excessive ROS levels damage cellular proteins, lipids and DNA, disrupt intestinal epithelial homeostasis and, ultimately, induce apoptosis [6, 7]. Given that mitochondria contribute to the major portion of endogenous ROS production [8], a better understanding of the mitochondrial oxidative stress signaling pathway involved in intestinal I/R injury may provide a basis for novel therapeutic strategies to improve the poor prognosis of critically ill patients.

P66Shc, an isoform of the ShcA adaptor protein family, has been characterized as a crucial mediator of mitochondrial ROS generation due to the presence of an additional domain at the N terminus (the CH2 domain) containing a critical serine in position 36 (Ser36) that differs from the other two isoforms, p52Shc and p46Shc [9, 10]. The phosphorylation of p66Shc at Ser36 in response to several stress stimuli and its consequent transfer from the cytosol to the mitochondria are required for the "pro-oxidative" and "pro-apoptotic" properties of the protein [11]. P66Shc-derived ROS trigger the mitochondrial route of apoptosis, which is involved in many pathological conditions and disease states, including several organ I/R injuries [12-17]. Indeed, mice with a genetic deletion of p66Shc display increased resistance to oxidative stress and apoptosis [18] and are also protected against severe tissue injury caused by hind limb [19], brain [13], and *ex vivo* heart I/R [20]. Additionally, we previously reported that p66Shc activation is associated with gut injury after intestinal I/R [21, 22]. These findings suggest that p66Shc may be a promising candidate for therapeutic intervention. However, the modulation of the p66Shc pathway is a complicated process, and no specific p66Shc inhibitors are currently available. Therefore, the development of a new therapeutic target controlling p66Shc function may offer effective approaches to combat intestinal I/R injury.

The peptidyl-prolyl cis-trans isomerase Pin1 is a highly conserved enzyme that can

only specifically recognize phosphorylated (p-) Ser/Thr-Pro peptide bonds of the substrate proteins and induce their conformational changes with high efficiency [23, 24]. Such Pin1-catalyzed isomerization alters the functional activities of many target phosphoproteins, thereby controlling an array of various signaling pathways, ranging from gene transcription, tumor development, and redox balance to apoptosis [25]. Recent data have shown that Pin1 inhibition can prevent oxidative stress and mitochondrial injury in human endothelial cells as well as mice under hyperglycemic conditions [26]. Moreover, Pin1 plays a major role in oxidative stress-induced neuronal apoptosis [27]. These findings may suggest a key role for Pin1 in other oxidative stress- and apoptosis-related pathological processes, such as I/R injury, a relationship not previously fully characterized. It has been reported that Pin1 regulates the p66Shc pathway through its prolyl-isomerase activity by directly binding to p66Shc and inducing the mitochondrial import of p66Shc [28]. The interaction between Pin1 and the p66Shc pathway as well as the well-established role of p66Shc in I/R injury are consistent with the possible involvement of Pin1 in intestinal I/R injury via interaction with p66Shc signaling.

Based on the above findings, we hypothesize that the Pin1/p66Shc signaling pathway may participate in the pathogenesis of intestinal I/R and that Pin1 inhibition may suppress the mitochondrial translocation of p66Shc and subsequent mitochondrial ROS generation, thus preventing the apoptosis and tissue damage induced by intestinal I/R. In the present study, we demonstrate that rats treated with a Pin1 inhibitor exhibited reduced oxidative stress and apoptosis and improved gut and lung injury in a model of intestinal I/R, which may provide novel therapeutic insights into intestinal I/R injury.

Materials and Methods

Experimental animals and treatment.

Adult male Sprague Dawley rats (aged 6 weeks) weighing 200±20 g were obtained from the Animal Center of Dalian Medical University (Dalian, China) and were kept

under pathogen-free conditions and fed a standard laboratory rat chow diet. The animals were housed in a temperature-controlled room on a 12 h light-to-dark cycle and were acclimated for 1 week before experimentation. Rats were randomly assigned to four groups (eight rats per group): Sham group, I/R group, Sham+juglone-treated group and I/R+juglone-treated group. Rats were fasted overnight with free access to water before surgery. An intestinal I/R model was established by superior mesenteric artery (SMA) occlusion, as we described previously [21]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg body weight, i.p.). After midline laparotomy, the SMA was isolated and clamped with an atraumatic microvascular clip for 60 min. On completion of 60 min of ischemia, the clamp was removed to allow for reperfusion. Sham animals underwent the same surgical procedure with the exception of the SMA occlusion. In each of the juglone-treated group, rats received an intraperitoneal injection of juglone (1 mg/kg body weight/d; Sigma-Aldrich, St. Louis, MO, USA) for 3 consecutive days prior to I/R surgery. Juglone was dissolved in 100% ethanol (2.44 mg/ml stock solution) and was diluted with normal saline to obtain a dose of 1 mg/kg body weight in a final volume of 5 ml per injection. Control animals received weight-appropriate volumes of 100% ethanol in 5 ml of normal saline. After the indicated periods of reperfusion, animals were anesthetized again and blood was collected via exsanguination. Then the small intestine and lung tissue samples were harvested and frozen immediately in liquid nitrogen, and stored at –80°C until analysis. Additional experiments for observation of survival over the course of 24 h were performed in vehicle and juglone-treated groups. For this survival study, rats were administrated with buprenorphine (0.1 mg/kg body weight, i.p.) after the incision was closed to minimize pain, distress or discomfort. All procedures were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Ethics Committee of Dalian Medical University (Dalian, China).

Human intestinal tissue samples.

Samples of human ischemic intestinal tissues were collected from patients who underwent surgery for acute mesenteric arterial embolism, strangulated intestinal

obstruction or incarcerated hernia. For comparison, the corresponding non-ischemic tissues from the margin of resected intestinal segments were defined as normal intestinal tissues. Written informed consent was obtained from the family of surgical patients. Intestinal samples were obtained with the approval of the institutional ethical committees of Dalian Medical University.

Histological analysis and *in situ* TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining.

Morphological alterations in the intestine and lungs were examined by light microscopy. Briefly, paraffin-embedded tissue blocks were sectioned at a thickness of 4 μm and were then stained with hematoxylin and eosin (H&E). The histopathological scores of the intestine and lung were graded based on a Chiu's [29] and Mikawa's [30] method, respectively, in a blinded manner by two independent experienced pathologists. A minimum of six randomly chosen fields from each rat were evaluated and averaged to determine the degree of organ injury, and the results of two pathologists were averaged. Intestinal TUNEL staining was performed using an *in situ* cell death detection kit (Roche, Branchburg, NJ, USA) according to the manufacturer's instructions.

Pin1 activity assay.

An isolated intestinal segment was placed on ice in a reaction buffer containing 100 mM NaCl, 50 mM HEPES, pH 7, 2 mM dithiothreitol (DTT) and 0.04 mg/ml bovine serum albumin (BSA). The tissues were homogenized and the supernatant cleared by centrifugation at 12 000 *g* for 10 min at 4°C. Pin1 activity was measured using equal amounts of intestinal cytoplasmic lysates and α-chymotrypsin using a synthetic tetrapeptide substrate Suc-Ala-Glu-Pro-Phe-pNa (Peptides International, Louisville, KY, USA). Absorption at 390 nm was measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

Cell culture and hypoxia/reoxygenation incubation.

Caco-2 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1% non-essential amino acids, 1% glutamine and penicillin/streptomycin. Cells were

maintained in a humidified atmosphere containing 5% CO_2 at 37°C. To create a hypoxic condition, cells were incubated in a microaerophilic system (Thermo Scientific) with 5% CO_2 and 1% O_2 balanced with 94% N_2 for 12 h. The cells were then cultured under normoxic conditions for 6 h to achieve reoxygenation.

Cell viability assay.

Cell viability was measured by the CCK-8 (Dojindo, Tokyo, Japan) assay according to the manufacturer's protocols. Briefly, after Caco-2 cells were seeded in 96-well plates and received appropriate treatments, 10 μl of CCK-8 reaction solution was added to each well at a 1/10 dilution. After 1 h of incubation at 37°C, the absorbance of each individual well was measured at 450 nm by a microplate reader (Biotec, USA).

Enterocyte apoptotic cell death and caspase-3 activity assay.

In vitro apoptotic cell death was performed using a TUNEL Apoptosis Detection Kit from Roche. Briefly, Caco-2 cells were fixed in 4% paraformaldehyde (PFA) for 30 min at room temperature. Then, the fixed cells were incubated with 0.1% Triton X-100 for 10 min. After washing cells with phosphate-buffered saline (PBS), 50 μl of TUNEL reaction mixture was added, and cells were incubated at 37°C for 1 h. Cells were then washed and labeled with 4',6-diamidino-2-phenylindole (DAPI) to counterstain nuclei. The green (TUNEL)/blue (DAPI) fluorescence ratios were calculated by counting a minimum of 10 randomly selected fields per group under a fluorescence microscope (Olympus BX 51, Tokyo, Japan). Caspase-3 activity in cell lysates was measured using a caspase-3 activity assay kit (Beyotime Institute of Biotechnology, Jiangsu, China) according to the manufacturer's protocol.

RNA interference.

Caco-2 cells were cultured and transfected with a specific p66Shc siRNA or Pin1 siRNA (50 nM) or with a negative control (NC) siRNA (50 nM) using Lipofectamine 2000 (Invitrogen) for 48 h according to the manufacturer's instructions. The p66Shc siRNA sequences were sense: 5′-AUGAGUCUCUGUCAUCGCUdTdT-3′; and antisense: 5'-AGCGAUGACAGAGACUCAUdTdT-3'. The Pin1 siRNA sequences were sense: 5′-CGGGAGAGGAGGACUUUGAdTdT-3′; and antisense:

5′-UCAAAGUCCUCCUCUCCCGdTdT-3′ (GenePharma, Shanghai, China).

Protein extraction and western blot analysis.

Cytosolic and mitochondrial protein extracts or total protein extracts from intestinal tissues were individually prepared with the use of a commercial protein isolation kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's instructions. Equivalent amounts of samples (30 μg) were subjected to 10–15% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). After blocking membranes with 5% non-fat milk or 5% BSA in TBS-Tween buffer (0.1% Tween-20; pH 7.5) for 60 min at 37°C, membranes were incubated with the respective primary antibodies against total p66Shc, Ser36 phosphorylated p66Shc (Abcam, Cambridge, UK), Pin1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), caspase-3, cytochrome *c* and voltage-dependent ion channels (VDAC) (Proteintech, Wuhan, China), and β-actin (ZSGB-BIO, Beijing, China) overnight at 4°C. After washing, the membranes were then incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at room temperature. The blots were visualized using enhanced chemiluminescence-plus reagents (Advansta Inc., K-12043). Spectrophotometric analysis was performed with a BioSpectrum-510 multispectral imaging system (UVP, Upland, CA, USA), and gels were analyzed with Gel-Pro Analyzer Version 4.0 (Media Cybernetics, MD, USA).

Co-immunoprecipitation.

Total proteins were extracted in IP lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, pH 7.5). For immunoprecipitation, precleared lysates were incubated with equal amounts of anti-Pin1 antibody and Protein A+G Agarose beads (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Normal IgG (Beyotime Institute of Biotechnology) was used as a negative control. The precipitate was washed 5 times with ice-cold PBS. After washing, immunoprecipitated proteins were eluted from the beads by boiling for 5 min in 2× loading buffer and were immunoblotted with anti-p66Shc and anti-Pin1 antibodies according to the manufacturer's instructions.

Immunofluorescence staining.

For cell staining, Caco-2 cells were seeded on glass slides. Cells were treated with 200 nM MitoTracker Red CMXRos (Invitrogen) for 30 min at 37°C in the dark and then washed with PBS, fixed in 4% PFA for 30 min at room temperature and permeabilized using 0.1% Triton X-100 in PBS for 10 min. Cells were rinsed with PBS, blocked with 1% BSA in PBS for 1 h at 37°C, and then incubated with anti-p66Shc antibody overnight at 4°C. The cells were then washed with PBS and incubated with Alexa Fluor 488-conjugated secondary antibody (Proteintech) for 1 h at room temperature. Subsequently, the cells were washed with PBS and treated with DAPI solution for 5 min at room temperature to stain nuclei. The cells were washed twice with PBS and examined under an Olympus fluorescence microscope.

Lung MPO activity and serum levels of TNF-α, IL-6, ALT, AST and LDH.

The levels of serum tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) were measured using an enzyme-linked immunosorbent assay (ELISA) kit specifically for rat TNF-α or IL-6 (R&D systems), according to the manufacturer's instructions. The serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and lactate dehydrogenase (LDH) levels and myeloperoxidase (MPO) activity in the lung were determined using commercial assay kits (Nanjing Jiancheng Corp, Nanjing, China) according to the manufacturer's protocols.

Determination of mitochondrial ROS production.

Mitochondria from intestinal tissues were isolated using a commercial kit (TransGen Biotech, Beijing, China) according to the manufacturer's protocol. Isolated mitochondria were resuspended in storage buffer, and protein concentration was quantified by the BCA method. The mitochondrial H_2O_2 levels were determined using a commercial assay kit (Nanjing Jiancheng Corp) according to the manufacturer's instructions. Mitochondrial O_2^- production was measured by fluorescence microscopy with the use of a chemical probe MitoSOX Red (Invitrogen). Briefly, Caco-2 cells were plated on coverslips and incubated with 5 μM MitoSOX Red for 10 min at 37°C in the dark. MitoSOX red fluorescence image was visualized using a fluorescence microscope (Olympus BX 51). After staining with MitoSOX Red, the mitochondrial

O_2^- was also detected by flow cytometry analysis using a BD FACSCalibur flow cytometer.

Statistical analysis.

All values are presented as the means ± SD. Data with normal distributions were compared using one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) test. A two-tailed Student's *t*-test was used to compare means between two groups. The survival study was analyzed using the Kaplan-Meier method and compared by the log-rank test. All experimental results shown represent at least three independent experiments. All statistical analyses were performed using GraphPad Prism 5.0 (GraphPad Prism Software, La Jolla, CA). *P*-values less than 0.05 were considered statistically significant.

Results

1. p66Shc plays an essential role in mediating intestinal I/R injury.

Although our previous study observed p66Shc overexpression and phosphorylation at Ser36 during acute intestinal I/R [22], the functional relevance of I/R-responsive p66Shc activation has not been fully clarified. Here, we first determined the expression level of p66Shc during the reperfusion phase after intestinal ischemia in rats. As shown in Figure 1A, a time-dependent increase in p66Shc expression was detected after 60 min of ischemia followed by 2-8 h of reperfusion. Similarly, the p66Shc protein level was markedly increased in human ischemic intestinal tissues compared with that in normal intestines (Figure 1B). To elucidate the potential role of p66Shc in regulating intestinal I/R, we mimicked *in vivo* intestinal I/R conditions in human Caco-2 cells by exposing them to hypoxia/reoxygenation (H/R) and selectively silenced p66Shc using small interfering RNA (siRNA) (Figure S1A). Consistent with the *in vivo* findings, the expression levels of p66Shc were significantly upregulated in Caco-2 cells under H/R condition and p66Shc-specific siRNA efficiently attenuated the elevations of p66Shc induced by H/R (Figure 1C). As expected, exposure of Caco-2 cells to H/R led to a significant decrease in cell viability. However, p66Shc

knockdown by siRNA improved cell survival upon H/R insult (Figure 1D). In addition, the silencing of p66Shc also attenuated mitochondrial superoxide anion (O_2^-) levels (Figures 1E and 1F), which was paralleled by a reduction in apoptosis, as indicated by reduced TUNEL-positive cells and caspase-3 activation, in comparison with the H/R group (Figures 1G-I). Thus, these results demonstrate that p66Shc-mediated mitochondrial oxidative stress and apoptosis are critically involved in intestinal I/R injury.

2. Intestinal I/R stimulates the activation and physical interaction of Pin1 with p66Shc.

The pro-oxidant and pro-apoptotic activities of p66Shc mainly depend on its migration into mitochondria, which has been shown to be regulated by Pin1 [31]. We first evaluated the mitochondrial p66Shc expression level during intestinal I/R. As shown in Figure 2A, the mitochondrial p66Shc level was significantly increased at 2 h of reperfusion and then progressively decreased after 4-8 h of reperfusion. To investigate whether intestinal I/R affects Pin1 protein expression, rats were subjected to 60 min of ischemia followed by reperfusion for 2, 4, or 8 h. Compared to the sham group, a significant induction of Pin1 expression was observed after 2-4 h of reperfusion, but it recovered and reached a nearly normal level at 8 h of reperfusion (Figure 2B). Moreover, intestinal I/R increased Pin1 enzyme activity (Figure 2C), implying that Pin1 function is activated by I/R. We next performed pull-down experiments to evaluate the direct physical interaction between the isomerase Pin1 and p66Shc in this model. As shown in Figure 2D, intestinal I/R resulted in increased binding of the Pin1 protein to p66Shc in the intestine, as revealed by immunoprecipitation using an anti-Pin1 antibody, followed by immunoblotting with an anti-p66Shc antibody. The blot was also probed with an anti-Pin1 antibody to ensure equal immunoprecipitation. Collectively, these findings indicate that the activation of Pin1 and enhanced interaction of Pin1 with p66Shc occurred in response to intestinal I/R, which may be correlated with the promotion of p66Shc mitochondrial translocation.

3. Pin1-mediated isomerization is indispensable for mitochondrial translocation

of p66Shc after intestinal I/R.

Next, we used juglone, a specific pharmacological inhibitor of Pin1, to clarify its role in intestinal I/R-induced p66Shc mitochondrial translocation. Juglone covalently binds to and irreversibly inhibits the enzymatic activity of Pin1 and accelerates its degradation by the proteasome [32, 33]. As shown in Figure 3A, juglone significantly decreased Pin1 expression after I/R. Consistent with the data shown in Figure 2D, a significant translocation of p66Shc to the mitochondria was observed after intestinal I/R, while juglone administration suppressed the I/R-induced mitochondrial translocation of p66Shc (Figure 3B). Moreover, treatment with juglone maintained the level of p-p66Shc at Ser36 in the cytoplasm under both sham and I/R conditions (Figure 3C), possibly because Pin1 inhibition affected the subsequent dephosphorylation of isomerized p-p66Shc by phosphatase 2A (PP2A).

To corroborate the Pin1 inhibitor data, we employed a specific Pin1 siRNA in Caco-2 cells upon H/R. The mitochondrial translocation of p66Shc was evaluated by fluorescence microscopy. Pin1 siRNA efficiently inhibited the overexpression of Pin1 in Caco-2 cells under H/R condition (Figure 3D and Figure S1B). Caco-2 cells exposed to H/R exhibited increased mitochondrial localization of p66Shc, as evidenced by the appearance of a yellow-orange color due to the merging of MitoTracker-associated red fluorescence and p66Shc-associated green fluorescence, which was rare in the control cells. However, the intensity of H/R-induced yellow-orange color was obviously lower in the Pin1 siRNA-transfected cells after exposure to H/R (Figure 3E). Taken together, our study suggests that intestinal I/R-induced p66Shc mitochondrial translocation is Pin1 dependent.

4. Pin1 inhibition attenuates intestinal I/R injury and improves survival.

To test the effects of Pin1 inhibition on intestinal I/R, juglone or vehicle was given as a pretreatment before surgery. A histopathological analysis of gut injury revealed that juglone administration markedly improved the histological injury in the intestine when compared to the I/R group (Figures 4A and 4B). It is widely acknowledged that intestinal I/R not only causes the intestine injury but also damages other organs [34]. Blood indicators of remote organ injury were significantly increased after intestinal

I/R, including ALT, AST and LDH, compared with sham-operated animals. Treatment with juglone dramatically ameliorated these organ injury indexes (Figure 4C-E). In addition, we examined the effect of juglone treatment on systemic levels of pro-inflammatory cytokines. As shown in Figure 4F and 4G, intestinal I/R-induced increases in serum TNF-α and IL-6 concentrations were almost abrogated by juglone. We further performed a 24-hour survival study to evaluate the long-term protective effects of Pin1 inhibition in rats after intestinal I/R. As shown in Figure 4H, the overall survival rate in the juglone-treated group was significantly higher than that in the vehicle-treated group. Collectively, these results demonstrate that Pin1 inhibition reduces organ injury and pro-inflammatory responses after intestinal I/R, ultimately improving the survival rate.

5. Pin1 inhibition prevents p66Shc-mediated mitochondrial ROS production after intestinal I/R.

Since p66Shc was shown to serve as a redox protein that catalyzed the formation of H_2O_2 in mitochondria [35], we hypothesized that Pin1 inhibition might affect p66Shc-dependent ROS generation. Indeed, the mitochondrial H_2O_2 content was significantly elevated in the I/R intestine, whereas juglone treatment markedly decreased the level of H_2O_2 compared to that of the I/R group (Figure 5A). This finding was also confirmed in Caco-2 cells under H/R conditions. As shown in Figure 5B and 5C, exposure to H/R resulted in increased mitochondrial O_2^- production, as assessed by staining the cells with MitoSOX Red (a dye probe that specifically detects superoxide within the mitochondria) and analysis with FACS, which was dramatically reduced by the transfection of Pin1 siRNA. Taken together, these data indicate that blockade of Pin1 is capable of preventing mitochondrial oxidative stress after intestinal I/R.

6. Pin1 inhibition ameliorates gut apoptosis after intestinal I/R.

Given the pivotal role of p66Shc-generated ROS in triggering the mitochondrial apoptotic pathway, we investigated whether Pin1 inhibition could affect I/R-induced cytochrome *c* release and, hence, cellular apoptosis *in vivo*. As expected, intestinal I/R caused a significant accumulation of cytochrome *c* in the cytosol compared with that

of the sham group, while juglone administration restored the mitochondrial localization of the protein (Figure 6A). In addition, the gut cleaved form of caspase-3 and TUNEL-positive staining results paralleled the changes in cytochrome *c* release after intestinal I/R. However, these effects were significantly blunted by juglone treatment (Figure 6B and 6C).

7. Pin1 inhibition attenuates acute lung injury after intestinal I/R.

It has been proposed that the local intestine damage after intestinal I/R may cause the development of acute lung injury, which could be a critical determinant of the prognosis [36]. We thus examined the status of lungs after intestinal I/R. As shown in Figure 7A and 7B, juglone administration reduced I/R-induced histopathological changes in the lung. Intestinal I/R-associated lung edema and pulmonary neutrophil infiltration (determined by MPO activity) were also significantly reduced with juglone treatment (Figure 7C and 7D). These results indicate that inhibition of Pin1 by juglone attenuates intestinal I/R-induced remote lung injury

Discussion

In the current study, we demonstrated that intestinal I/R-induced gut damage involves the Pin1/p66Shc signaling pathway. We made the following observations. (i) p66Shc played a vital role in I/R-induced mitochondrial ROS production and cell death in the intestine. (ii) Pin1 expression and activity were elevated during intestinal I/R. This activation of Pin1 enhanced the interaction with p66Shc and induced its mitochondrial translocation, contributing to gut injury. (iii) Finally, pharmacological inhibition of Pin1 by juglone reduced the primary intestinal I/R injury, further alleviated systemic injury and improved survival. To our knowledge, this study is the first to suggest that juglone ameliorates intestinal I/R injury via a Pin1-dependent mechanism and that the protective effect of juglone is achieved, at least in part, through a p66Shc-mediated mitochondrial apoptotic pathway.

Excessive ROS production is widely recognized as a key mediator of I/R-induced intestinal mucosal injury [5]. Accumulating evidence links p66Shc to oxidative stress

since the adaptor protein functions as a ROS producer within the mitochondria and translates oxidative signals into apoptosis [9, 17, 35]. In a previous study, we showed that intestinal I/R activated p66Shc via a protein kinase C (PKC) βII-dependent pathway, leading to gut oxidative stress and apoptosis in mice [22]. In this study, we validated the intestinal I/R-responsive overexpression of p66Shc in rat intestines. Furthermore, we observed increased p66Shc expression in ischemic human intestines, which was consistent with the data from our animal I/R model. This observation underscores the potential contribution of p66Shc to intestinal I/R injury. Next, using loss-of-function analyses, we found that p66Shc silencing significantly attenuated human Caco-2 cell injury induced by H/R, as indicated by improved cell viability, decreased mitochondrial superoxide levels and reduced TUNEL-positive cells and caspase-3 activation. Thus, the pivotal role of p66Shc in intestinal I/R-induced oxidative stress and apoptosis makes this adaptor protein a plausible target for disease treatment. Considering lack of inhibitors available at present, manipulating upstream regulators of the p66Shc pathway may provide a promising therapeutic option.

Pin1 has emerged as a novel regulator of biological processes that functions by modifying the target protein structure upon binding to a specific p-Ser/Thr-Pro motif and catalyzing cis-trans isomerization, thus adding another level of posttranslational regulation [37, 38]. It has been proposed that in murine embryonic fibroblasts, oxidative stress-activated PKCβ induces phosphorylation of p66Shc at Ser36, in turn triggering the interaction of Pin1 with p66Shc via recognition of the p-Ser36-Pro37 residue, resulting in the subsequent translocation of p66Shc to the mitochondria [28]. Recently, other PKC members and several stress kinases, such as c-Jun N-terminal kinase (JNK), apoptosis signal-regulating kinase 1 (ASK1) and p38 MAP kinase (p38 MAPK), have also been identified to be involved in p66Shc phosphorylation, depending on the cellular context or stress conditions [39-43]. Although multiple kinases may phosphorylate p66Shc at Ser36, Pin1 is the only known enzyme that can recognize the specific Ser36-Pro37 bonds after phosphorylation. Hence, due to Pin1's unique regulatory mechanism, we preferentially focus on this isomerase as a suitable target. In the present study, we observed that intestinal I/R stimulated binding of Pin1

to p66Shc. Moreover, the mitochondrial accumulation of p66Shc exhibited a similar time-dependent trend with the induction of Pin1 during intestinal I/R, implying that p66Shc translocation may be correlated with Pin1 expression. By using both pharmacological inhibition and genetic knockdown of Pin1 *in vivo* and *in vitro*, we demonstrated that binding of Pin1 is required for the consecutive translocation of p66Shc after intestinal I/R, as the mitochondrial level of p66Shc was significantly decreased under Pin1-deficient conditions. Taken together, we tested the above hypothesis that the Pin1/p66Shc pathway may be involved in intestinal I/R injury.

Pin1-mediated post-phosphorylation regulation can have profound effects on various cellular processes, including cell cycle progression, cell metabolism, DNA damage responses and oxidative stress [24, 25]. Previous studies have revealed diverse functions of Pin1 in oncogenesis and neurodegenerative and cardiovascular diseases. However, there is little information on the role of Pin1 in I/R injury. Lentsch's group was the first to critically examine the function of Pin1 during hepatic I/R injury and demonstrated that Pin1-/- mice exhibit significantly more liver injury after I/R than wild-type mice, indicating that Pin1 appears to confer protection against hepatic I/R injury [44]. In contrast, data from a model of ischemic stroke have suggested that Pin1 plays a pivotal role in promoting neuronal death and brain injury after cerebral I/R [45]. Here, our data showed that Pin1 is up-regulated in a time-dependent manner during intestinal I/R. Moreover, the isomerase activity of Pin1 was also increased. Acute inhibition of Pin1 by juglone administration in rats can significantly reduce intestinal I/R-induced local and systemic injuries, which was supported by decreased serum markers of tissue injury, inhibition of pro-inflammatory cytokine release, and improvement of histological damage. These results suggest that Pin1 activation may play a deleterious role in intestinal I/R injury. Thus, it seems that Pin1 has the opposite functions in the pathogenesis of I/R injury. We speculate that distinct animal models and cell lines and complex Pin1-signaling networks may collectively result in the divergent roles of this isomerase in different organ I/R injury. Other vital signaling molecules regulated by Pin1 during intestinal I/R need to be further investigated in future studies.

Pin1 blockade by the pharmacological inhibitor juglone blunted the I/R-induced translocation of p66Shc to the mitochondria, implying that the protective mechanism of juglone in intestinal I/R injury may be associated with p66Shc-mediated mitochondrial oxidative stress and apoptosis. Previous studies have demonstrated that in human aortic endothelial cells or HK-2 cells, Pin1 inhibition prevents hyperglycemia-induced p66Shc translocation to the mitochondria, hence blunting mitochondrial O_2^- generation [26, 46]. In line with these observations, we showed that inhibition of Pin1 by juglone markedly reduced the content of H_2O_2 in the I/R intestine. In *in vitro* studies, knockdown of Pin1 via siRNA prevented mitochondrial O_2^- overproduction under H/R conditions. Furthermore, oxidative stress has a close relationship with cell apoptosis during various pathophysiological processes. Aberrant intestinal epithelial cell apoptosis has been shown to play a determinant role in the pathogenesis of intestinal I/R injury [47]. P66Shc-induced ROS formation alters mitochondrial membrane permeability, facilitating the release of pro-apoptotic proteins, such as cytochrome *c*, which is responsible for the activation of the apoptosis execution enzyme caspase-3 [48]. In this study, we found that intestinal I/R caused a significant release of cytochrome *c* from the mitochondria to the cytosol, accompanied by caspase-3 activation and TUNEL-positive staining. In contrast, juglone treatment profoundly restored the mitochondrial localization of cytochrome *c,* attenuated caspase-3 activation and reduced apoptosis in the intestine after I/R. It is notable that intestinal I/R also involves extensive necrosis in the intestinal epithelium. Recent studies have shown that a programmed form of necrosis, termed necroptosis, has been demonstrated to be an essential contributor to intestinal I/R injury *in vivo* and *in vitro* [49, 50]. The parallel existence of necrotic and apoptotic pathways that induce epithelial cell death implies the complexity in the pathophysiology of intestinal I/R, so more experiments are required to further evaluate the relative contributions of necrosis and apoptosis to I/R-induced gut damage. Collectively, it is conceivable that the protective effect of juglone is partially attributed to the suppression of p66Shc translocation and the alleviation of p66Shc-mediated mitochondrial oxidative stress and subsequent apoptosis in intestinal I/R.

A number of unique properties make Pin1 a particularly attractive candidate for disease diagnosis and treatment. Indeed, Pin1 is significantly overexpressed in ~60% of total human cancers, with high levels being positively correlated with poor clinical outcome [51-53]. Extensive research in the past years concentrating on Pin1 inhibitors has suggested that Pin l has the potential to be a novel therapeutic target for anticancer drugs [54]. Here, we propose that the p66Shc-mediated mitochondrial apoptosis signaling pathway is particularly dependent on Pin1 activation; hence, targeting Pin1 may also represent a promising strategy to prevent intestinal I/R injury in critically ill patients.

In conclusion, our study demonstrated that Pin1 was activated after intestinal I/R, which was responsible for the mitochondrial translocation of p66Shc and subsequent mitochondrial ROS generation. Inhibition of Pin1 activation by juglone protected rats from intestinal I/R injury, partially via the p66Shc-mediated mitochondrial apoptotic pathway. Our findings highlight that the pharmacological inhibition of Pin1 may represent a feasible prophylactic approach to counteract intestinal I/R injury.

Declarations of interest

The authors declare no conflict of interest.

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Author contribution statement

X.T., D.F. and J.Y. designed the study. D.F., G.W., Z.L. and Y.L. performed the experiments. D.F., G.W., Z.L., G.Z. and J.Y. analyzed and interpreted the data. F.L., S.N., W.Q. and Z.C. contributed materials and collected human intestinal samples. X.T. and D.F. wrote and revised the paper. Z.C. and X.T. provided fnancial support. All authors reviewed and approved the manuscript.

Clinical perspectives

- The adaptor protein p66Shc activation increases oxidative stress and apoptosis in intestinal I/R injury. However, the upstream regulators of the p66Shc pathway involved in intestinal I/R remain elusive. The aim of this study was to investigate the association between a prolyl-isomerase Pin1 and p66Shc during intestinal I/R and to evaluate the effect of inhibiting Pin1 *in vivo*.
- Increased Pin1 expression and activity in the intestine were required for the mitochondrial translocation of p66Shc during intestinal I/R. Pin1 inhibition could attenuate intestinal I/R-induced local gut damage and distant organ injury by suppressing p66Shc-mediated mitochondrial oxidative stress and apoptosis.
- The isomerase Pin1 may serve as a novel target for the treatment of intestinal I/R injury in clinic.

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Figure legends

Figure 1 p66Shc participates in the regulation of mitochondrial oxidative stress and apoptosis during intestinal I/R injury

(**A**) Representative immunoblot showing p66Shc protein expression in the intestines of rats subjected to 60 min of intestinal ischemia followed by 2-8 h of reperfusion or to sham operation (Sham) (n = 5 rats per group). (**B**) Immunoblot showing p66Shc protein expression in human intestinal samples from normal control intestines and ischemic intestines (n = 3 per group). (**C-I**) Caco-2 cells were transfected with a siRNA targeting p66Shc (si-p66Shc) or a negative control siRNA (si-NC) for 48 h. The cells were then exposed to hypoxic conditions for 12 h followed by normoxic conditions for 6 h to achieve H/R. Control cells were exposed to normal oxygen (Control) (n = 6 per group). (**C**) Representative immunoblot of p66Shc protein expression. (**D**) Cell viability was evaluated using CCK-8 assay. (**E**) Representative fluorescent images of Caco-2 cells stained with MitoSOX from Control, H/R, H/R+si-p66Shc and H/R+si-NC groups. Scale bar = 12.5 μm. (**F**) The bar graphs represent a summary of flow cytometry analyses of cells stained with MitoSOX dyes. (**G**) Left panels are representative fluorescent images of TUNEL and DAPI staining cells from Control, H/R, H/R+si-p66Shc and H/R+si-NC groups. Relative apoptotic rates are represented as TUNEL-positive cells/DAPI (right panels). Scale bar = 100 μm. (**H**) Caspase-3 activity. (**I**) Representative immunoblot showing both the cleaved (c-) and full length caspase-3 levels. All results are expressed as the means ± SD. *P < 0.05 compared with Normal; **P < 0.01 compared with Sham or Control; ##P < 0.01 compared with H/R.

Figure 2 Intestinal I/R-induced activation and enhanced interaction of Pin1 with p66Shc

Rats were subjected to sham operation (Sham) or 60 min ischemia followed by 2, 4, or 8 h of reperfusion (n = 5 rats per group). (**A**) Representative immunoblot showing p66Shc protein expression in mitochondrial fractions of the intestine. VDAC was

used as loading control for mitochondrial proteins. (**B**) Representative immunoblot showing Pin1 protein expression in the intestine. (**C**) Pin1 isomerase activity assays in the intestine. (**D**) Representative immunoblot showing the interaction of Pin1 with the adaptor p66Shc from sham and 2 h reperfusion intestine. The input represents the total protein extracts used in immunoprecipitation. IB, immunoblotting; IP, immunoprecipitation; IgG, negative control. All results are expressed as the means ± SD. **P* < 0.05, ***P* < 0.01 compared with Sham.

Figure 3 Inhibition of Pin1 blunts the mitochondrial translocation of p66Shc during intestinal I/R

(**A-C**) I/R rats were subjected to 60 min of intestinal ischemia followed by 2 h of reperfusion. Juglone or vehicle was administered intraperitoneally for 3 consecutive days at a dose of 1 mg/kg/d prior to surgery (*n* = 8 rats per group). (**A**) Representative immunoblot showing Pin1 protein expression in the intestine. (**B**) Representative immunoblot showing p66Shc protein expression in mitochondrial fractions of the intestine. (**C**) Representative immunoblot showing p-Ser36 p66Shc protein expression in cytosolic fractions of the intestine. β-actin and VDAC indicate loading controls for cytosolic and mitochondrial fractions, respectively. (**D-E**) Caco-2 cells were exposed to hypoxic conditions for 12 h followed by normoxic conditions for 6 h to achieve H/R in the presence or absence of Pin1 siRNA (si-Pin1). Si-NC was used as a negative control. (**D**) Representative immunoblot showing Pin1 protein expression (*n* = 3 per group). (**E**) An immunofluorescence microscopic analysis of p66Shc localization in Caco-2 cells is shown. The staining patterns for mitochondria (MitoTracker Red), p66Shc, and nuclei are indicated by red, green, and blue fluorescence, respectively. Scale bar = 12.5 μm. All results are expressed as the means ± SD. ***P* < 0.01 compared with Sham or Control; #*P* < 0.05, ##*P* < 0.01 compared with I/R. J, juglone.

Figure 4 Inhibition of Pin1 activation by juglone ameliorates intestinal I/R injury

Rats were treated with juglone or vehicle and subsequently subjected to sham

operation or 60 min of intestinal ischemia followed by 2 h of reperfusion (n = 8 rats per group). (**A**) H&E staining of representative intestinal sections from the sham, I/R, sham juglone pretreatment, and I/R juglone pretreatment groups. Scale bar = 100 μm. (**B**) Histopathological injury scores of the intestinal mucosa were quantified as described in the Materials and Methods. (**C-G**) Serum levels of (C) ALT, (D) AST, (E) LDH, (F) TNF-α and (G) IL-6. (H) The survival rate of the rats (n = 15 rats per group). All results are expressed as the means ± SD. *P < 0.05 compared with vehicle; **P < 0.01 compared with Sham; #P < 0.05, ##P < 0.01 compared with I/R.

Figure 5 Pin1 inhibition decreases gut mitochondrial oxidative stress after intestinal I/R

(**A**) Rats were subjected to sham operation or 60 min of intestinal ischemia followed by 2 h of reperfusion with or without pretreatment with juglone or vehicle. Intestinal H_2O_2 levels were measured in isolated mitochondria (n = 8 rats per group). (**B-C**) Caco-2 cells were transfected with Pin1 siRNA or negative control as indicated. The cells were then exposed to hypoxic conditions for 12 h followed by normoxic conditions for 6 h to achieve H/R. Control cells underwent normal oxygen (n = 6 per group). (**B**) Representative fluorescent images of MitoSOX staining cells from Control, H/R, H/R+si-Pin1 and H/R+si-NC groups. Scale bar = 12.5 μm. (**C**) The bar graphs represent a summary of flow cytometry analyses of cells stained with MitoSOX dyes. All results are expressed as the means ± SD. **P < 0.01 compared with Sham or Control; ##P < 0.01 compared with I/R or H/R.

Figure 6 Inhibition of Pin1 activation by juglone prevents cytochrome *c* release and ameliorates gut apoptosis after intestinal I/R

Rats were subjected to sham operation or 60 min of intestinal ischemia followed by 2 h of reperfusion with or without pretreatment with juglone or vehicle (n = 8 rats per group). (**A**) Representative immunoblot showing cytochrome c release in the intestine. VDAC and β-actin indicate loading controls for mitochondrial and cytosolic fractions, respectively. Bar graphs represent percentage change of cytochrome c in the cytosol

versus mitochondria. (**B**) Representative immunoblot showing both the cleaved (c-) and full length caspase-3 levels in the intestine. (**C**) TUNEL staining (green) of paraffin-embedded intestinal tissue sections. Representative images for sham, I/R, sham juglone pretreatment, and I/R juglone pretreatment groups. Nuclei are stained with DAPI (blue). Scale bar = 50 μm. All results are expressed as the means ± SD. $^{**}P < 0.01$ compared with Sham; $^{\#\#}P < 0.01$ compared with I/R.

Figure 7 Pin1 inhibition by juglone alleviates the distant lung injury induced by intestinal I/R

Rats were subjected to sham operation or 60 min of intestinal ischemia followed by 2 h of reperfusion with or without pretreatment with juglone or vehicle (n = 8 rats per group). (**A**) H&E staining of representative lung sections from the sham, I/R, sham juglone pretreatment, and I/R juglone pretreatment groups. Scale bar = 100 μm. (**B**) The lung injury scores were calculated as described in the Materials and Methods. (**C**) Lung edema was estimated by comparing wet-to-dry weight ratios. (**D**) Lung MPO activity. All results are expressed as the means ± SD. $^{**}P < 0.01$ compared with Sham; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with I/R.

Figure 1

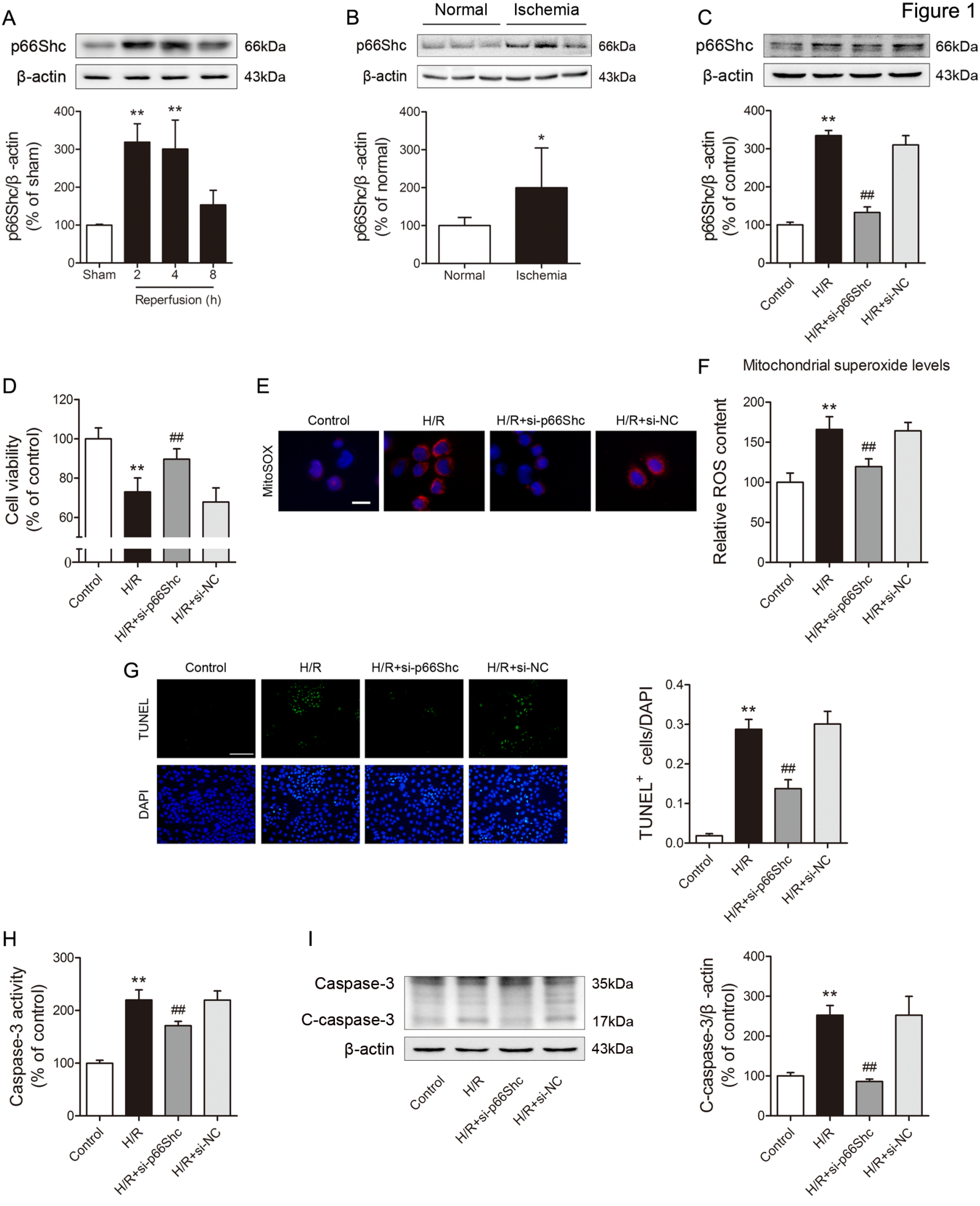

A
p66Shc
β-actin
66kDa
43kDa
p66Shc/β -actin (% of sham)
Sham
2
4
8
Reperfusion (h)
B
Normal
Ischemia
p66Shc/β -actin (% of normal)
C
p66Shc/β -actin (% of control)
Control
H/R
H/R+si-p66Shc
H/R+si-NC
D
Cell viability (% of control)
E
MitoSOX
F
Mitochondrial superoxide levels
Relative ROS content
G
TUNEL
DAPI
TUNEL+ cells/DAPI
H
Caspase-3 activity (% of control)
I
Caspase-3
C-caspase-3
35kDa
17kDa
C-caspase-3/β -actin (% of control)

Figure 2

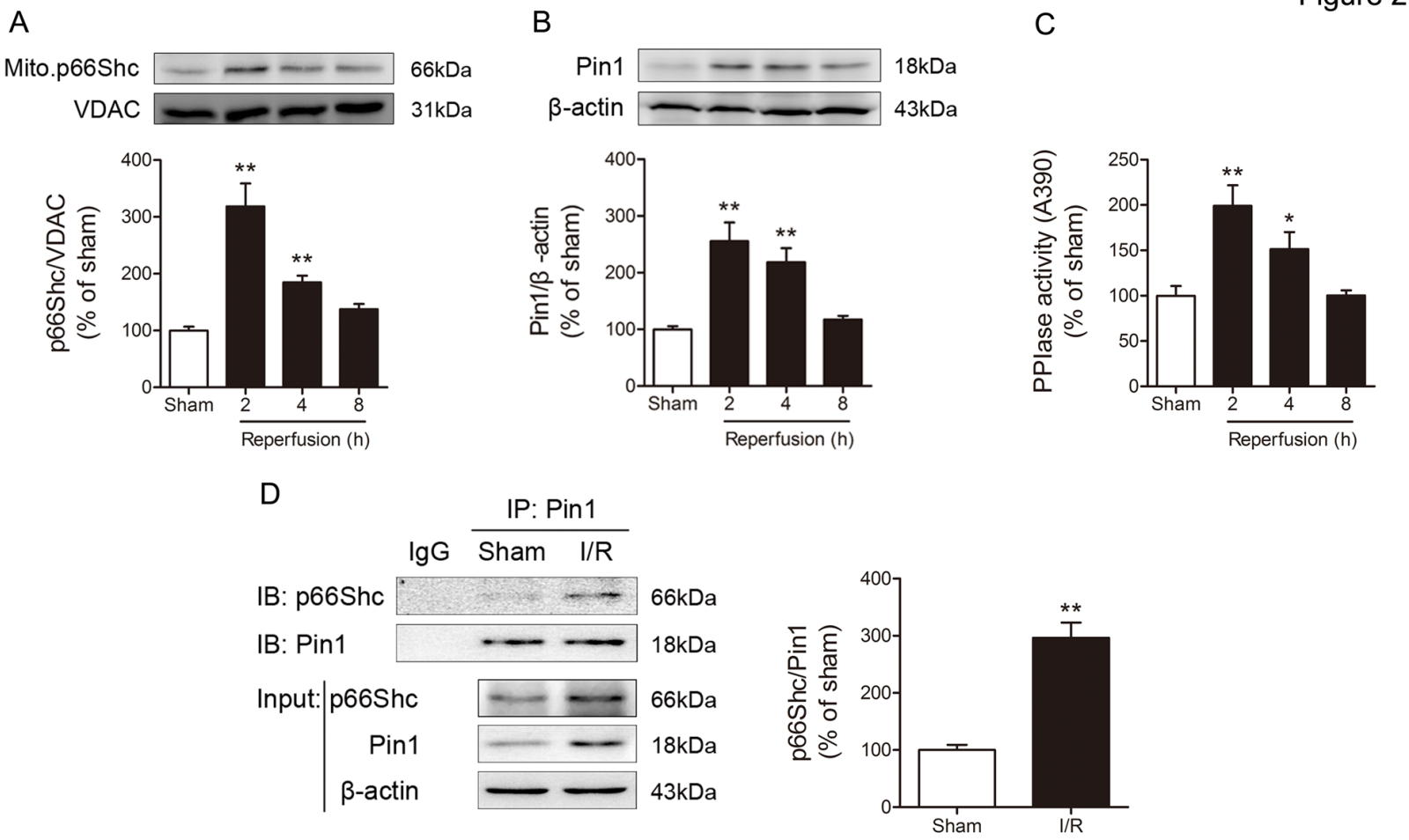

A
Mito.p66Shc
66kDa
VDAC
31kDa
p66Shc/VDAC (% of sham)
400
300
200
100
0
**
**
Sham
2
4
8
Reperfusion (h)
B
Pin1
18kDa
β-actin
43kDa
Pin1/β -actin (% of sham)
400
300
200
100
0
**
**
Sham
2
4
8
Reperfusion (h)
C
PPlase activity (A390) (% of sham)
250
200
150
100
50
0
**
*
Sham
2
4
8
Reperfusion (h)
D
IP: Pin1
IgG
Sham
I/R
IB: p66Shc
66kDa
IB: Pin1
18kDa
Input:
p66Shc
66kDa
Pin1
18kDa
β-actin
43kDa
p66Shc/Pin1 (% of sham)
400
300
200
100
0
**
Sham
I/R

Figure 3

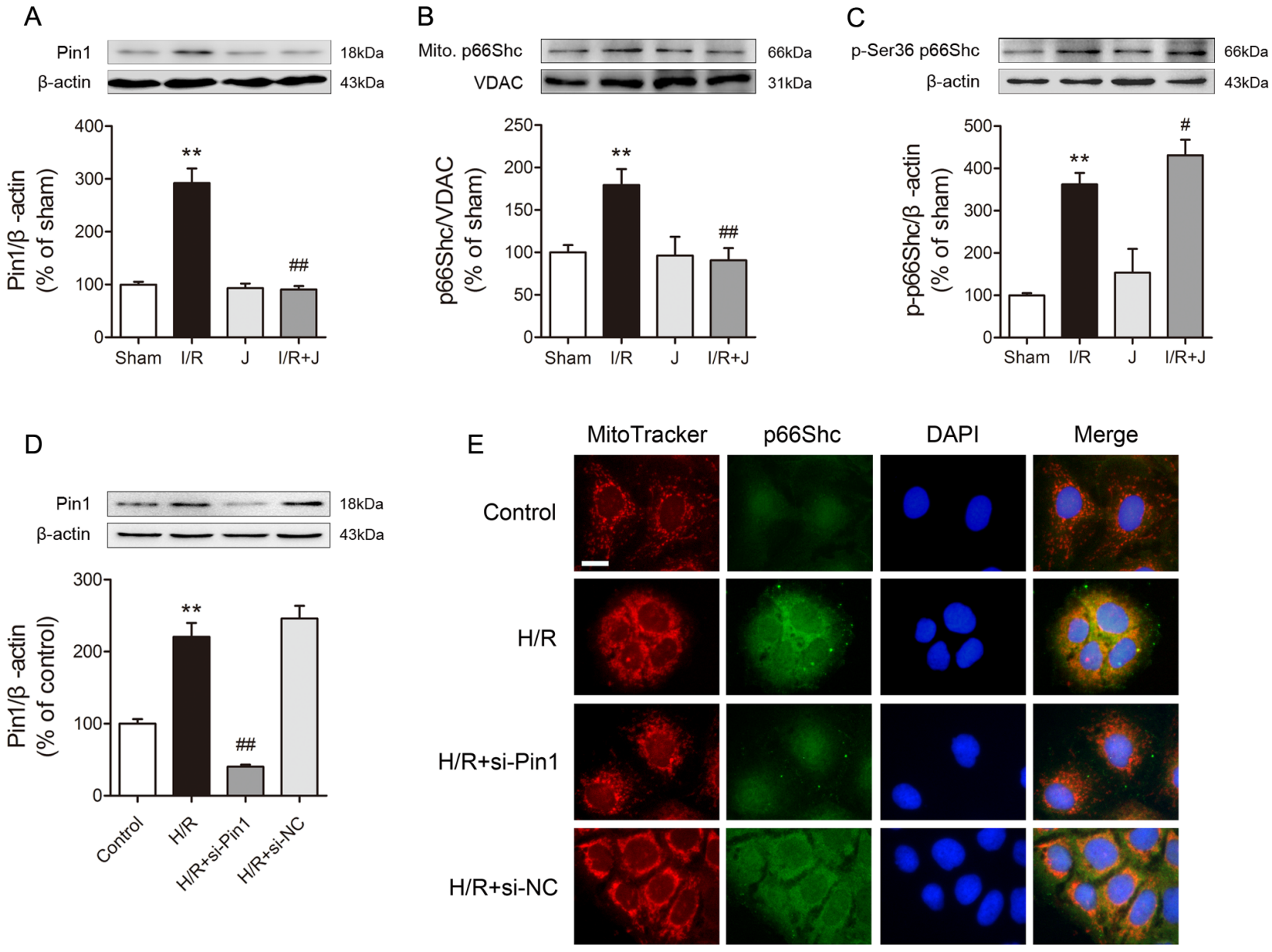

A
Pin1
β-actin
18kDa
43kDa
Pin1/β -actin (% of sham)
400
300
200
100
0
**
##
Sham
I/R
J
I/R+J
B
Mito. p66Shc
VDAC
66kDa
31kDa
p66Shc/VDAC (% of sham)
250
200
150
100
50
0
**
##
Sham
I/R
J
I/R+J
C
p-Ser36 p66Shc
β-actin
66kDa
43kDa
p-p66Shc/β -actin (% of sham)
500
400
300
200
100
0
**
#
Sham
I/R
J
I/R+J
D
Pin1
β-actin
18kDa
43kDa
Pin1/β -actin (% of control)
300
200
100
0
**
##
Control
H/R
H/R+si-Pin1
H/R+si-NC
E
MitoTracker
p66Shc
DAPI
Merge
Control
H/R
H/R+si-Pin1
H/R+si-NC

Figure 4

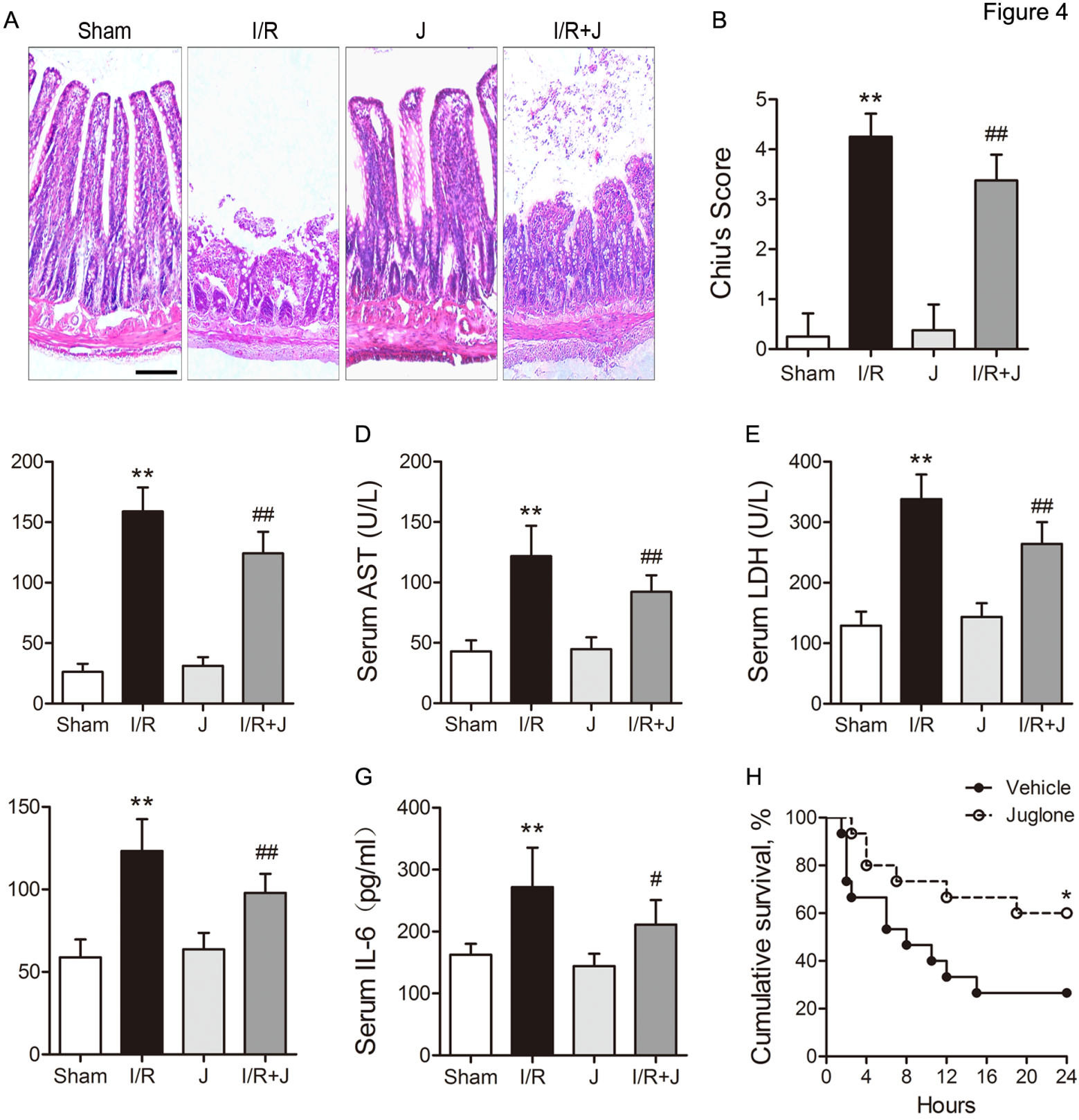

A
Sham
I/R
J
I/R+J
B
Chiu's Score
**
##
Sham
I/R
J
I/R+J
C
Serum ALT (U/L)
D
Serum AST (U/L)
E
Serum LDH (U/L)
F
Serum TNF-α (pg/ml)
G
Serum IL-6 (pg/ml)
#
H
Vehicle
Juglone
Cumulative survival, %
*
Hours

Figure 5

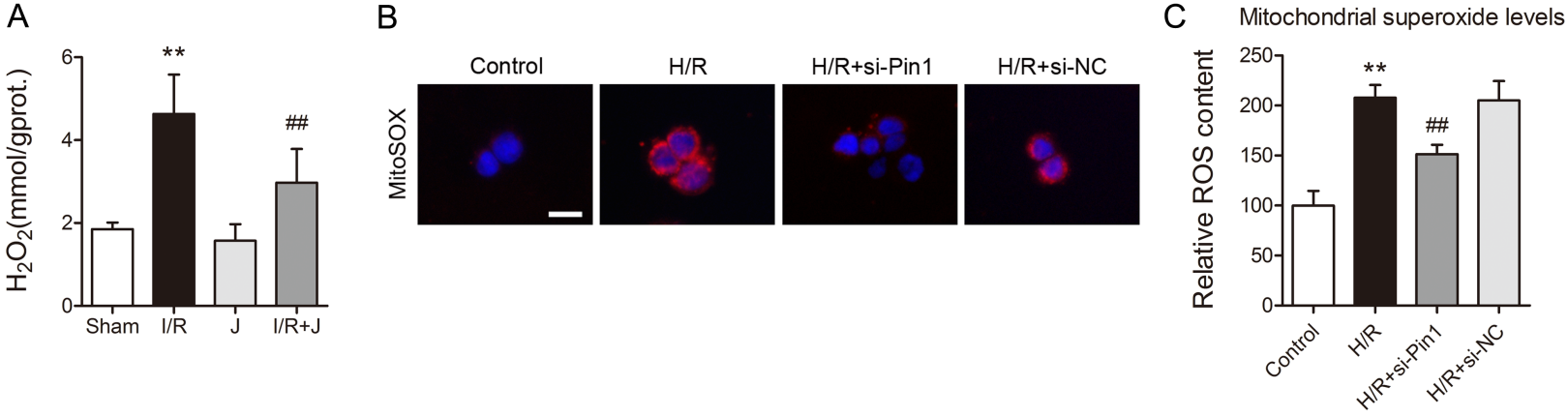

A
H_2O_2(mmol/gprot.)
6
4
2
0
**
##
Sham
I/R
J
I/R+J
B
Control
H/R
H/R+si-Pin1
H/R+si-NC
MitoSOX
C
Mitochondrial superoxide levels
Relative ROS content
250
200
150
100
50
0
**
##
Control
H/R
H/R+si-Pin1
H/R+si-NC

Figure 6

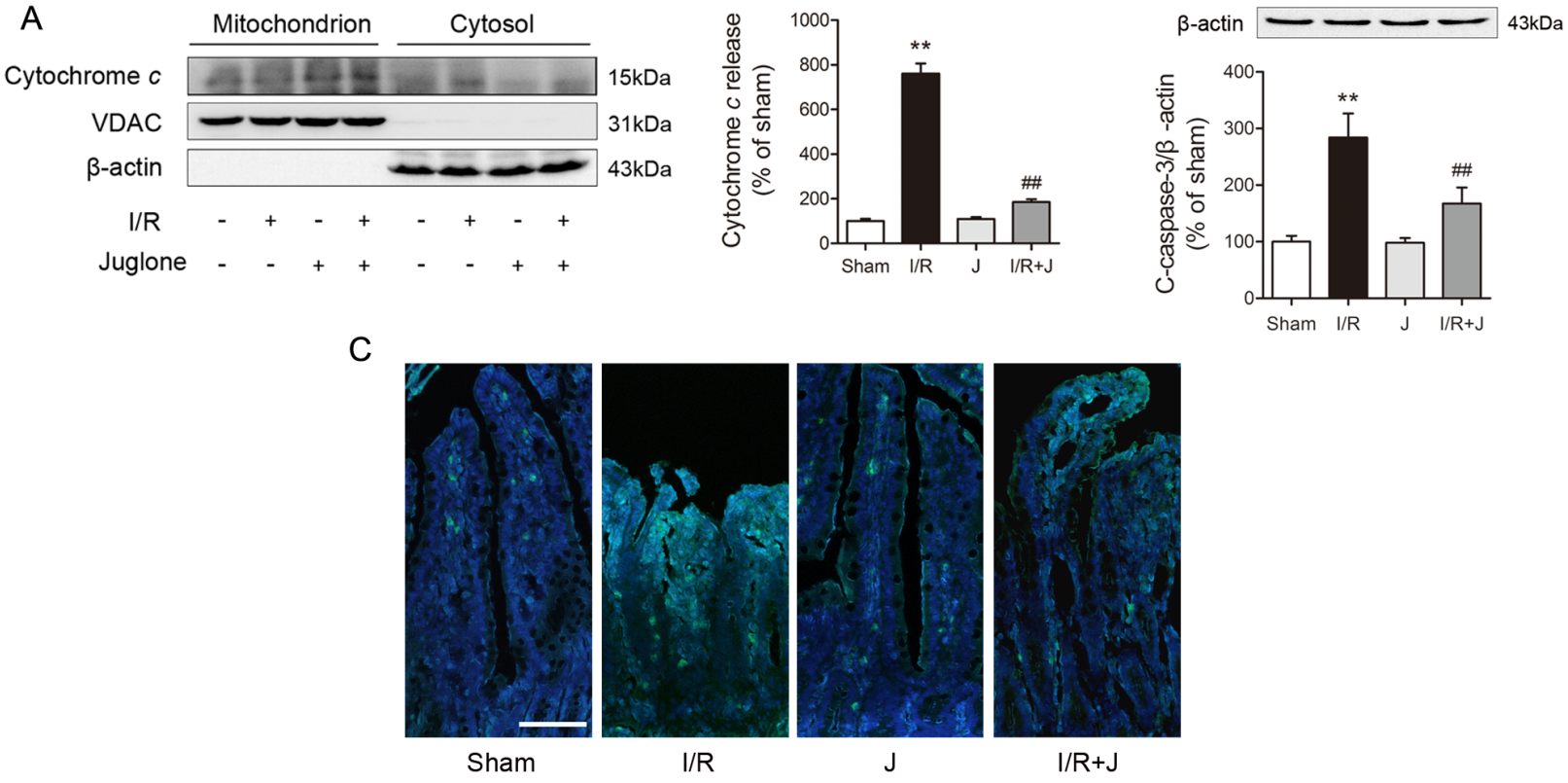
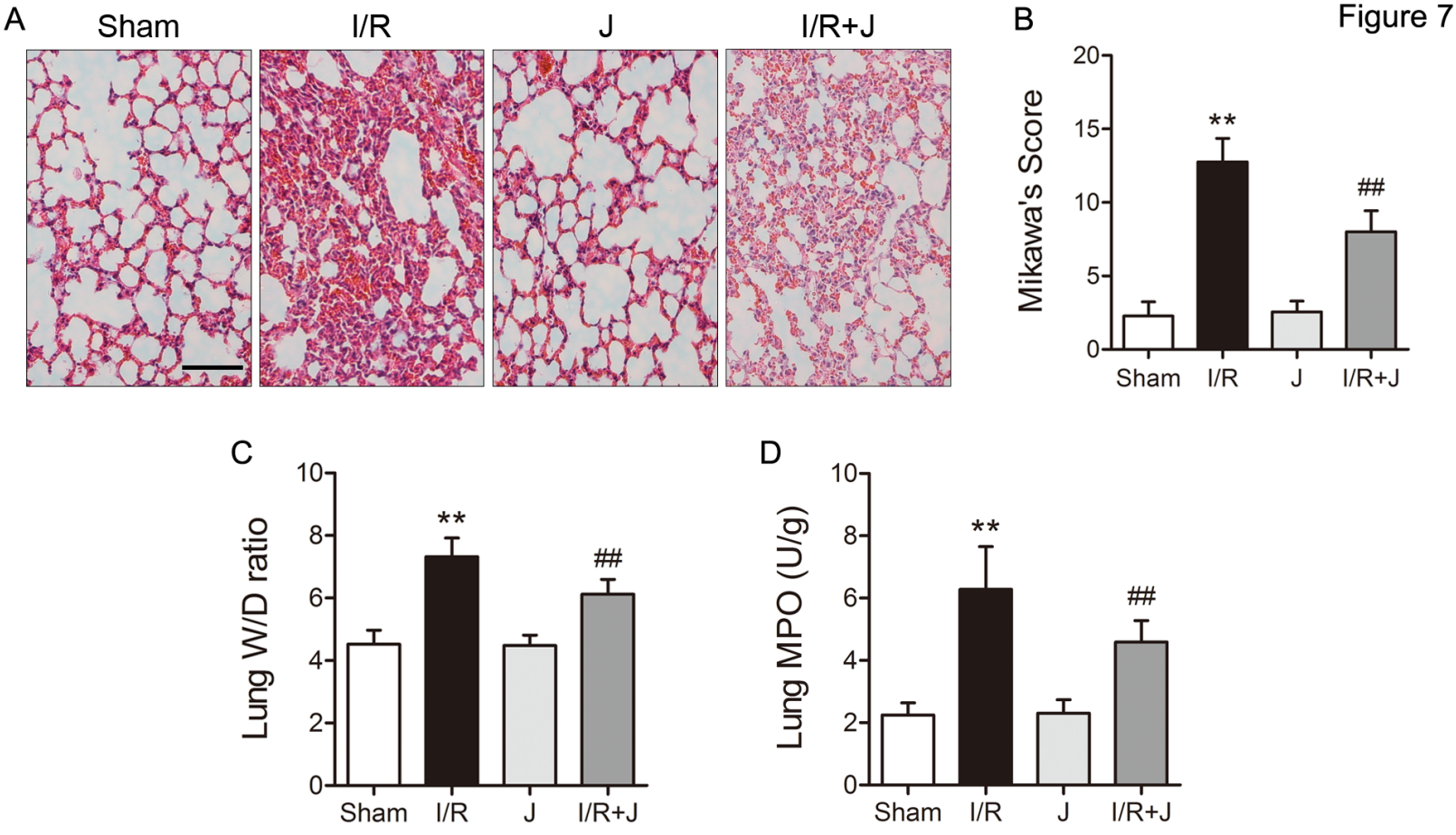
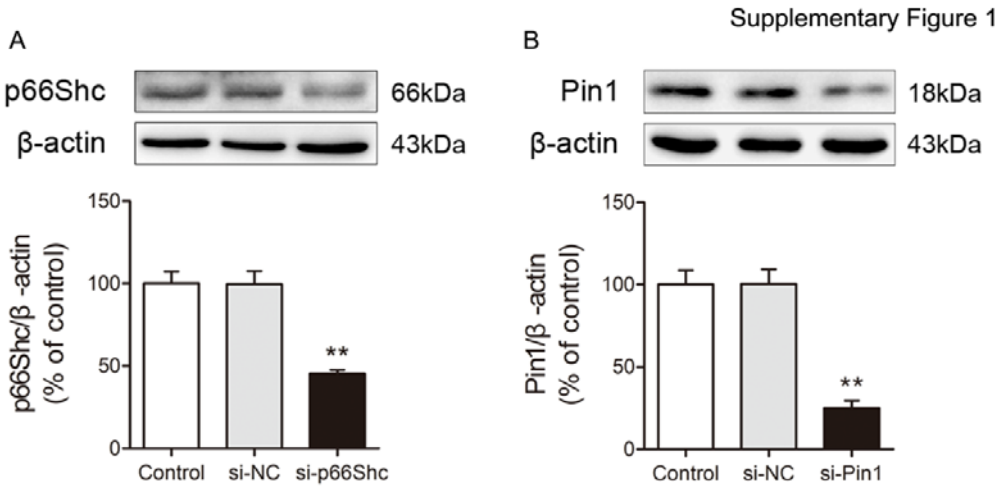

A
Mitochondrion
Cytosol
Cytochrome c
VDAC
β-actin
15kDa
31kDa
43kDa
I/R
Juglone
Cytochrome c release (% of sham)
Sham
I/R
J
I/R+J
B
Caspase-3
C-caspase-3
β-actin
35kDa
17kDa
43kDa
C-caspase-3/β -actin (% of sham)
C
Sham
I/R
J
I/R+J

Figure 7


A
Sham
I/R
J
I/R+J
B
Mikawa's Score
20
15
10
5
0
**
##
Sham
I/R
J
I/R+J
C
Lung W/D ratio
10
8
6
4
2
0
**
##
Sham
I/R
J
I/R+J
D
Lung MPO (U/g)
10
8
6
4
2
0
**
##
Sham
I/R
J
I/R+J



Supplementary Figure S1 siRNA-mediated knockdown of p66Shc and Pin1

Representative immunoblot showing p66Shc and Pin1 protein expression in Caco-2 cells after transfection with a negative control, p66Shc and Pin1 specific siRNA ($n = 3$ per group). Results are expressed as the means ± SD. $^{**}P < 0.01$ compared with Control.