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Original Article

Hsp27 reduces cold ischemia-reperfusion injury in heart transplantation through regulation of NF-κB and PUMA signaling

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Abstract: Background and Objective: Global myocardial ischemia-reperfusion (I/R) injury after heart transplantation is believed to impair graft function and aggravate episodes of both acute and chronic rejection. The 27-kDa heat shock protein (Hsp27) has a potent ability to alleviate I/R after cardiac transplantation. The aim of this study was to investigate the anti-I/R injury effect of Hsp27 to elucidate the underling mechanisms. Methods: Heart grafts from BALB/c mice were preserved in University of Wisconsin (UW) solution (control) or UW solution containing pAAV-Hsp27 (Hsp27 solution) at 4°C for 48 h and subsequently transplanted into syngeneic recipients for 72 h. The heart grafts were then collected for histopathological and gene expression analyses. An *in vitro* I/R model (H9c2 cells or H9c2/Hsp27 cells) was constructed. Then, protein and mRNA expression of Hsp27, p65, p53 upregulated modulator of apoptosis (PUMA), interleukin (IL)-6, IL-1β, and tumor necrosis factor (TNF)-α in heart tissues and H9c2 cells were detected with western blot and reverse transcription polymerase chain reaction analyses. Caspase-3 activity was detected using a commercial assay, while protein levels of IL-6, IL-1β, and TNF-α were detected using specific enzyme-linked immunosorbent assays. NF-κB activity was detected with an electrophoretic mobility shift assay. Cell apoptosis was detected with the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay and flow cytometric analysis. Results: Cold I/R caused severe morphologic myocardial injury of heart grafts from wild type C57BL/c mice, whereas grafts from Hsp27 preservation showed less damage as demonstrated by decreased cell apoptosis/death and the preservation of the normal structure of the heart. Hsp27 inhibited I/R-induced injury as indicated by the reduction in cardiac troponin I activities and decreased cardiac tissue levels of the proinflammatory factors TNF-α, IL-1β, and IL-6. Hsp27 was further demonstrated to significantly inhibit nuclear translocation of p65 and p53 upregulated modulator of apoptosis (PUMA) expression. Conclusions: These results suggested that the cardioprotective effect of Hsp27 could be due to the suppression of the myocardial inflammatory response and apoptosis by blocking the NF-κB-dependent pro-inflammatory and NF-κB-dependent PUMA signaling pathways.

Keywords: Heart transplantation, ischemia-reperfusion (I/R) injury, inflammatory factor, apoptosis, heat shock protein (Hsp27), NF-κB, PUMA

Introduction

Cold preservation of donor organs, tissues, and cells with various specialized preservation solutions is a common strategy to minimize ischemic injury prior to transplantation [1]. The University of Wisconsin (UW) cold storage solutionhas been demonstrated to be both safe and effective for heart transplantation [2-4], and is associated with improved short-term survival and less acute ischemic necrosis in the early post-transplant period [5-7]. However, cold ischemic storage of the heart is still limited to 4 to 6 h because preservation solutions are unable to significantly inhibit ensuing ischemia-reperfusion (I/R) injury [8, 9]. Consequently, storage beyond the 4-6-h window leads to delayed organ function and eventual organ failure. The inability to preserve organs for longer periods contributes to the shortage of donor organs and the increased number of patients waiting for heart transplantations. Therefore,

the development of a novel organ preservation solution to reduce I/R injury and extend organ preservation time is critical for clinical organ transplantation.

I/R injury leads to organ damage in various ways. Increasing evidence indicates that inflammation is involved in the pathophysiology of myocardial I/R injury. Interleukin (IL)-1β is a prominent and early mediator of inflammation in I/R injury. I/R induces IL-1β expression in the heart, which prevents myocardial injury after I/R, suggesting that the deleterious effects of myocardial I/R are mediated, at least in part, by IL-1β [10, 11]. In addition, tumor necrosis factor (TNF-α) and IL-6 were also found to be secreted by activated neutrophils and macrophages during I/R injury of the heart and play important roles in inflammation-induced cardiac dysfunction [12, 13].

I/R-induced cell death was thought to occur by extrinsic factors, such as loss of energy supply, elaboration of inflammatory mediators and toxic molecules, and mechanical injury, a mode of cell death termed necrosis. However, it is now recognized that cell death can also be programmed by cellular signaling mechanisms via apoptosis [14]. P53 upregulated modulator of apoptosis (PUMA) is one of the most potent killers among the BCL-2 homology 3-only subgroup of BCL-2 family members that drives apoptosis induced by p53-independent or -dependent signals. PUMA was previously shown to be upregulated in neurons after transient global cerebral ischemia and inhibition of PUMA upregulation protected neurons from delayed ischemic death [15]. In addition, the absence of PUMA reduces infarct in isolated, perfused hearts subjected to I/R [16].

Nuclear factor-kappa B (NF-κB) is a key transcription factor in the regulation of the acute inflammatory response, which plays a key role in I/R injury [17]. The dysregulation of NF-κB may lead to the excessive production of pro-inflammatory mediators, resulting in myocardium damage, heart failure, and even death [18]. Wang et al. found that PUMA is a direct target of NF-κB and mediates TNF-α-induced apoptosis both *in vitro* and *in vivo* [19]. Yan et al. recently reported that *Astragalus* saponins IV (AS-IV) provided protection against renal I/R injury by reducing apoptosis and inflammation through inhibition of NF-κB activity and PUMA expression [20]. Shi et al. found that targeting NF-κB activation using the NF-κB activation inhibitor dehydroxymethylepoxyquinomicin reduced cardiac I/R injury in a transplantation model [21].

Heat shock protein 27 (Hsp27) binds to and prevents the aggregation of denatured proteins in response to stress [22], and is constitutively expressed in human cardiomyocytes, while ex vivo studies using mouse hearts overexpressing Hsp27 have demonstrated that it protects the myocardium against I/R injury [23-25]. Seemampillai et al. recently found that overexpression of HSP-27 protects mouse hearts against acute rejection [26], although the underlying mechanisms of such protection remain unclear.

Hsp27 is a negative regulator of NF-κB in skeletal muscle [27], HeLa cells [28], and keratinocytes [29]. However, in U937 human leukemic cells, MEF cells, and rat colon carcinoma REG cells, Hsp27 appears to enhance NF-κB activation in response to either etoposide or TNF-α treatment [30], suggesting the outcome of NF-κB regulation by Hsp27 may vary with the cell type or stimuli. Several recent studies have reported that the heat stress response protects against tissue injury by increasing the expression of heat shock proteins (HSPs) and suppressing NF-κB activation [31]. In addition, heat shock treatment protects against angiotensin II-induced tissue inflammation by suppressing the inflammatory transcription factor NF-κB. This protection is related to high heat shock-induced expression of Hsp70 and Hsp27 [32].

However, the impact and underlying mechanisms of Hsp27 on cold I/R in heart transplantation remain unknown. The results of the present study showed that Hsp27 exerts a protective effect on cold I/R in heart transplantation through interaction with NF-κB signaling both *in vivo* and *in vitro*.

Materials and methods

Animals

C57B/6 wild type (WT) mice were purchased from Shanghai Laboratory Animal Research Center (Shanghai, China) and housed under conventional conditions at the Animal Care Facility in accordance with the guidelines established by the China Council on Animal Care. The

protocols of all animal experiments in this study were approved by the Animal Experiment Committee of the Affiliated Hospital of Qingdao Medical College (Guangdong Sheng, China). All experiments were conducted in strict adherence to relevant international guidelines with every effort to minimize animal suffering.

Plasmids and Hsp27 vector construction

The fragment of Hsp27 containing the open reading frame sequence was cleaved from the pUC19 cloning vector at the *Bam*HI and *Xho*I restriction sites and sub cloned at the corresponding sites in the pCDNA3.1 plasmid. The insert was cut at the *Eco*RI restriction sites and cloned into the corresponding sites of a recombinant adeno-associated virus (AAV) backbone (pAAV-Hsp27) containing the cytomegalovirus promoter. Packaging, propagation, and purification of AAV particles were conducted by Biogot Technology Co., Ltd. (Nanjing, China) using standard procedures [33].

Gene transfer in vivo

pAAV-Hsp27 was used to infect the grafted heart as previously described [34]. Heterotypic cardiac transplantation was performed in a syngeneic recipient as previously described [15]. The donor hearts were excised and immersed in pAAV-Hsp27 solution at 4°C for 48 h. Before anastomosis to the recipient was performed, the donor heart was flushed again with fresh pAAV-Hsp27 solution through the tube to wash out potentially harmful cellular metabolites that accumulate during the period of cold ischemia. Then, the tube was removed and the inferior vena cava was ligated permanently. The graft was revascularized via end-to-side anastomoses between the donor aorta and recipient abdominal aorta, and between the donor pulmonary artery and recipient inferior vena cava. Heart graft viability was monitored daily with direct abdominal palpation. The degree of pulsation was scored as follows: A, beating strongly; B, noticeable decrease in pulsation intensity; or C, complete cessation of pulsation.

Heterotopic heart transplantation with prolonged I/R

A syngeneic murine heterotopic heart transplantation was performed. Male C57BL/6 and Hsp27/C57BL/6 mice at 8 weeks of age were anesthetized with ketamine/xylazine solution and the hearts were excised and preserved in UW solution for 24 h at 4°C. After 24 h, the preserved hearts were then implanted into the same strain recipient mice as the donors. On day 3 post transplantation, the transplanted mice were sacrificed and the heart grafts were collected for histopathological examination and gene expression analysis.

In vitro I/R model

H9c2 myoblasts were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA). H9c2 cells were plated in a six-well plate at a concentration of 80,000 cells/well overnight. DMEM was replaced by deoxygenized phosphate-buffered saline (PBS) and then placed in an In vivo 2 hypoxia workstation (The Baker Company, Sanford, ME, USA) with 0% O_2 at 10°C for 16 h. After hypoxia treatment for 24 h, the PBS was replaced with new complete culture medium and the cells were incubated in a normoxic culture environment with 5% CO_2 and 28% O_2 at 37°C for 24 h.

Adenovirus infection

H9c2 cells were infected with pAAV-Hsp27 as previously described [35]. Briefly, to overexpress Hsp27, H9c2 cells (80,000/well) were infected with human pAAV-Hsp27 at a multiplicity of infection of 100 in 600 μL of FBS-free DMEM for 6 h. Then, 600 μL of culture medium containing 20% FBS was added to the infected cells and the cells were cultured overnight.

Enzyme-linked immunosorbent assay (ELISA)

Quantitative detection of human IL-1β, IL-6, and TNF-α in heart tissues was determined using designated ELISA kits, respectively (Immunological Science, Shanghai, China). Each assay was performed in duplicate, following the manufacturer's instructions. The absorbance of each well of 96- and 384-well plates (American Instrument Exchange, Inc., Haverhill, MA, USA) was read with a Multiskan Ascent 96/384 Plate Reader (MTX Lab Systems, LLC Bradenton, FL, USA).

Western blot analysis

Protein (40 μg) was loaded into the wells of sodium dodecyl sulfate polyacrylamide gels

and then separated by electrophoresis and transferred to nitrocellulose filters, which were blocked with 5% non-fat milk and incubated with anti-Hsp27, anti-p65, anti-PUMA, anti-IL-1β1, anti-IL-6, and anti-TNF-α antibodies diluted to 1:500 in 0.5% milk-Tris-buffered saline-Tween solution overnight. After incubation with a secondary anti-rabbit antibody (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:2000, the amount of protein was detected with an Amersham Electrochemiluminescence Pluskit (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA).

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). The RNA concentration was measured witha NanoDrop spectrophotometer (NanoDrop Technologies, LLC, Wilmington, DE, USA) and quality-checked on a 1% agarose gel. Then, 2 μg of RNA were reverse-transcribed into complementary DNA (cDNA) using the Maxima First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA).

PCR was performed using Dream Taq Green Polymerase (Thermo Fisher Scientific) with the following primer pairs: Hsp27 (sense: 5'-GCG TGT CCC TGG ATG TCA AC-3', antisense: 5'-TGT ATT TCC GCG TGA AGC AC-3'); PUMA, (sense: 5'-*GC*G GAT CCA TGA AAT TTG GCA TGG GGT C-3', antisense: 5'-*CCG* CTC GAG CTA CAT GGT GCA GAG AAA GTC-3'), IL-1β (sense: 5'-GTC TTC CGC CTC TCG GTA AT-3', antisense: 5'-AGA GAT ACG GAT CGC ACA GG-3'), TNF-*α* (sense: 5'-CTT TTG GAG TTT GAG GTA GTA TAC CTA-3', antisense: 5'-GCT GCG CAG AAT GAG ATG AGT TGT C-3'); IL-6 (sense: 5'-AAA TGC CAG CCT GCT GAC GAA G-3', antisense: 5'-AAC AAC AAT CTG AGG TGC CCA TGC TAC-3'), and GAPDH (sense: 5'-CAG CGA CAC CCA CTC CTC CAC CTT-3', antisense: 5'-CAT GAG GTC CAC CAC CCT GTT GCT-3'). The PCR was performed with the following cycling conditions: initial denaturation at 95°C for 2 min followed by 40 cycles ofdenaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, and a final extension step at 72°C for 10 min. The PCR products were analyzed with agarose gel electrophoresis on a 2% agarose gel.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as described by Schreiber et al. [36]. For the EMSA, equal amounts of nuclear proteins (5 μg) were added to 12 μL of H_2O in a reactionmixture containing poly (deoxyinosinic-deoxycytidylic) acid (1 μg/μL; Roche, Basel, Switzerland), bovine serum albumin (10 μg/mL), Buffer D (20 Mm HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.5 mM ethylenediaminetetraacetic acid, 0.25% NP-40, 2 mM dithiothreitol, 0.1% phenylmethane sulfonyl fluoride), Buffer F (20% Ficoll 400, 100 mM HEPES, pH 7.9, 300 mM KCl, 10 mM dithiothreitol, 0.1% phenylmethane sulfonyl fluoride), and 25 ng of a [^{33}P]-labeled NF-κB binding oligonucleotide. Then, the reaction mixture was incubated for 30 min at room temperature. Subsequently, the extracts were separated using a non-denaturing 6% polyacrylamide gel, which was dried on a vacuum gel dryer for 60 min at 80°C, then exposed to Imaging Plate (model BAS-MS 2340; Fujifilm, Tokyo, Japan) overnight and finally analyzed with a fluorescent image analyzer (model FLA-3000; Fujifilm).

Apoptosis in vitro by flow cytometric analysis

Cell apoptosis was determined with a fluorescein isothiocyanate (FITC)-Annexin Vapoptosis kit (BD Pharmingen, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. In brief, cells were washed with ice-cold PBS and resuspended in binding buffer (10 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$) at a concentration of 1×10^6 cells/mL and then stained with annexin V-FITC and propidiumiodide for 15 min in the dark before analysis with aflow cytometer (Beckman Coulter, Inc., Miami, FL, USA).

Measurement of caspase-3 activity

Caspase 3 activity was determined using a commercial colorimetric assay according to the manufacturer's instructions. Briefly, heart tissues and H9c2 cells were lysed with lysis buffer on ice. The lysates were centrifuged at 14,000 g at 4°C for 15 min. Cytosolic protein was mixed with 10 μL of caspase 3-specific substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (2 mM) for 2 h at 37°C. The absorbance of each sample was monitored at 405 nm using a Spectramax M5 Microtiter Plate Luminometer (Molecular Devices, Sunnyvale, CA, USA).

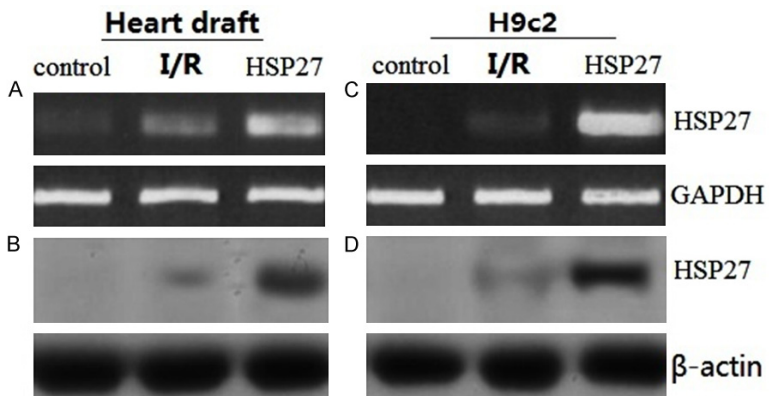

Figure 1. rAAV-mediated Hsp27 gene transfer. The donor hearts were preserved in UW solution or/andpAAV-Hsp27solution at 4°C for 24 h. Hsp27 mRNA and protein expression levels were detected with RT-PCR (A) and western blot (B) analyses, respectively. H9c2 cells were transfected with pAAV-Hsp27 prior to exposure to cold hypoxia at 10°C for 16 h followed by reperfusion at 37°C for 24 h. Hsp27 mRNA and protein expression levels were detected with RT-PCR (C) and western blot (D) analyses, respectively.

Detection of cell apoptosis in I/R-injured heart tissues using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay

To detect DNA strand breaks, the TUNEL reaction assay was performed using a commercial kit in accordance with the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany). In brief, heart tissue sections were incubated with 50 μL of terminal deoxynucleotidyl transferase enzyme and TUNEL reaction mixture for 1 h at 37°C in the dark. The sections were then washed with PBS (1×) for 3-5 min. The slides were mounted using 4',6-diamidino-2-phenylindole (DAPI)-Fluoromount-G (Southern Biotech, Birmingham, AL, USA), covered with a glass cover slip, and analyzed under a fluorescence microscope. The number of TUNEL-positive cells was expressed as the ratio of DAPI-TUNEL double-labeled nuclei to the total number of DAPI-stained nuclei. For each specimen, the cells were counted in four fields and an average value was calculated for each experimental group by an investigator blinded to the experimental groups.

Blood sampling for measurement of plasma cardiac troponin I

At the end of the experiment, about 2 mL of blood was collected from the heart into a tube containing disodium ethylenediaminetetraacetic acid (22 mg/mL) as an anticoagulant, then mixed thoroughly and centrifuged at 3000 rpm for 15 min for determination of plasma cardiac troponinI concentration using an ELISA kit, in accordance with the manufacturer's instructions (Life Diagnostics, Inc., West Chester, PA, USA).

Histopathological processing

Hearts from each experimental group were fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Then, 4 μm-thick sections were placed on adhesive slides and stained with hematoxylin and eosin (H&E) for morphological assessment of cardiac injury using a semi-quantitative scale of 0 to 4 (with 4 being the most severe) [17]. Hearts that failed to function immediately were assigned a score of 5. In functioning cardiac grafts, the myocardium was assessed for myocytolysis (dissolution of myocytes), myocardial necrosis, and inflammatory infiltrate. The degree of injury was measured by the extent of myocardium involved in the biventricular section as 1 < 10%, 2 ≥ 10% and ≤ 30%, 3 ≥ 30% and < 60%, and 4 ≥ 60%.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism V software (GraphPad Software, Inc., La Jolla, CA, USA). Statistical significance was calculated using the Student's *t*-test. Data are presented as the mean ± standard deviation. A probability (*P*) value of < 0.05 was considered statistically significant.

Results

hsp27 expression in heart grafts and H9c2 cells in vitro in response to I/R injury

The excised donor hearts were placed in UW solution or/and pAAV-Hsp27 solution at 4°C for 24 h. The results showed that 24 h cold I/R decreased expression of Hsp27 mRNA and protein as compared to the control grafts without I/R injury or I/R injury alone, while perfusion of the heart with pAAV-Hsp27 resulted in a significant increase in Hsp27 levels (**Figure 1A**, **1B**).

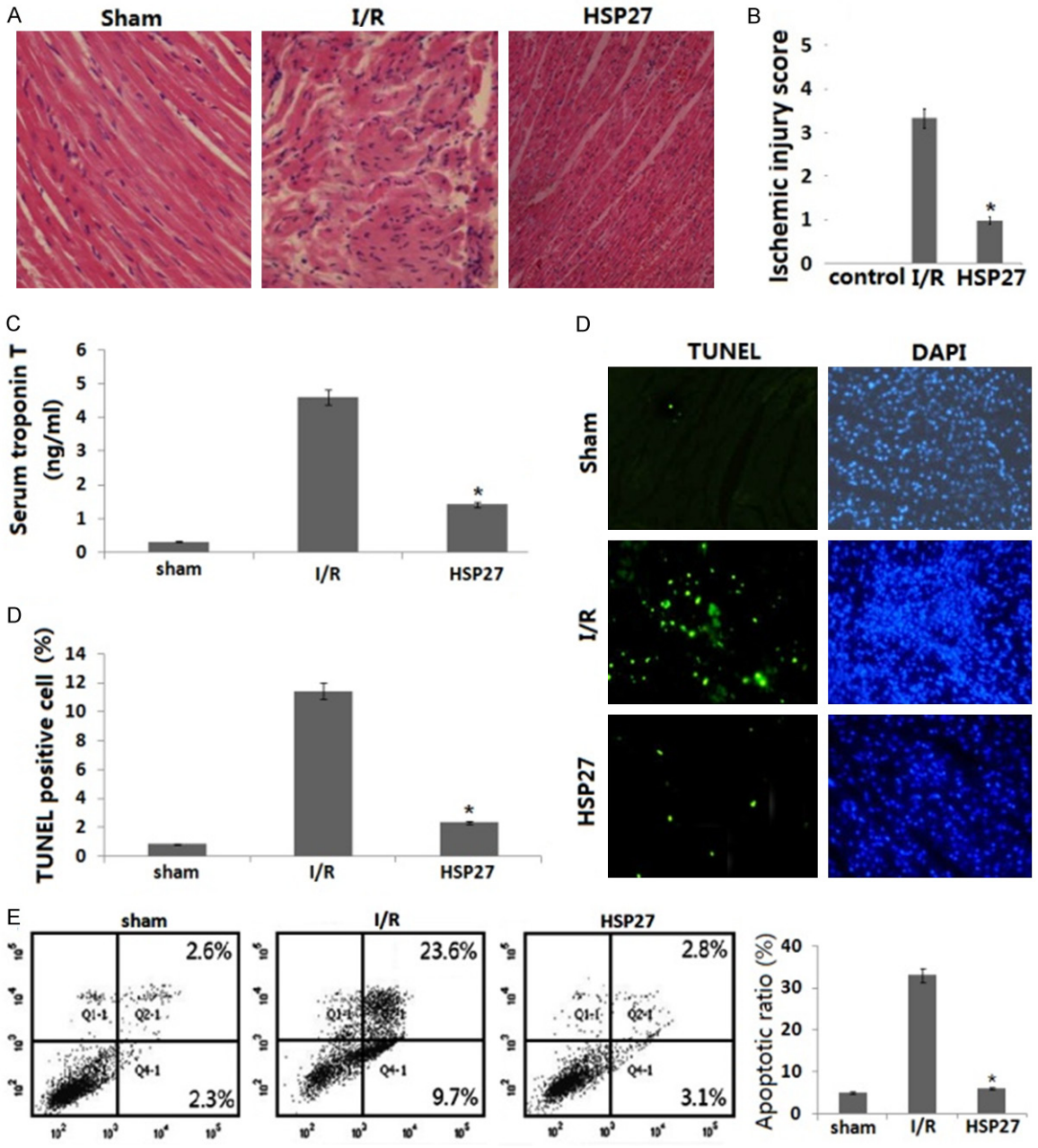

Figure 2. Hsp27 prevents cell apoptosis induced by I/R both *in vitro* and *in vivo*. A. Three days after transplantation, the heart grafts were harvested for H&E staining. B. The ischemic scores of both groups. C. Serum TnT levels. D. Cell apoptosis was detected with TUNEL assay in grafted hearts. E. H9c2 cells apoptosis was detected by double staining with FITC-labeled annexin-V and PI and subjected to flow cytometry (*$P < 0.01$ vs. control).

Hsp27 mRNA and protein expression levels were also increased in H9c2 cells transfected with pAAV-Hsp27 prior to exposure to cold hypoxia at 10°C for 16 h followed by reperfusion at 37°C for 24 h *in vitro* as compared to the control grafts without I/R injury or I/R injury alone (**Figure 1C**, **1D**).

Hsp27 protects H9c2 cells in vitro in response to I/R and grafted hearts from I/R injury in heart transplantation

The transplanted hearts from WT mice did not start to beat after implantation, while those from pAAV-Hsp27 mice strongly beat at steady

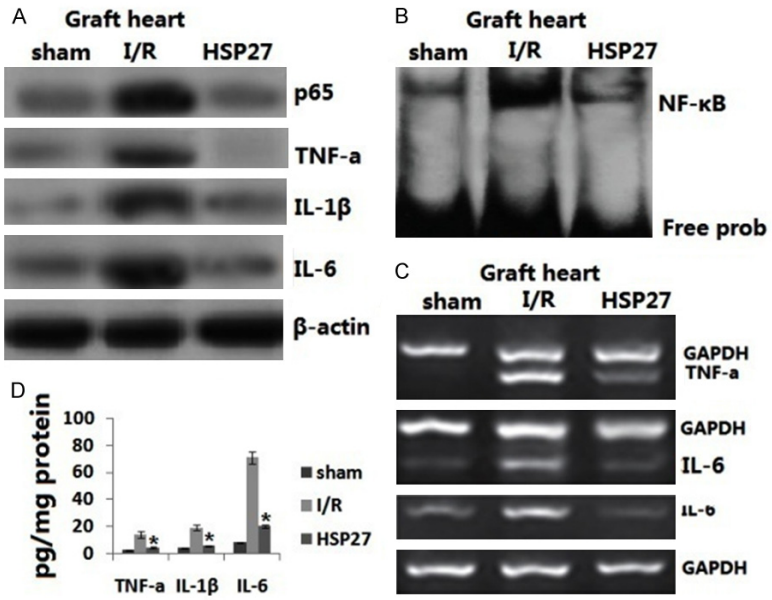

Figure 3. Hsp27 reduces inflammatory cytokine expression in heart grafts. A. Protein expression of p65, TNF-α, IL-6, and IL-1β in I/R grafts was detected by western blot analysis. B. NF-κB activity was detected with EMSA in I/R grafts. C. mRNA expression of TNF-α, IL-6 and IL-1β in I/R grafts was detected with western blot. D. Protein levels of TNF-α, IL-6, and IL-1β in grafts, as detected with ELISA (*$P < 0.01$ vs. the I/R group).

rates immediately upon revascularization. Three days after transplantation, the heart grafts were harvested to assess I/R injury by examining histopathological changes via H&E staining. The ischemia score was significantly higher in the cold I/R groups than in the pAAV-Hsp27-treated groups, indicating that pAAV-Hsp27 protected the heart from I/R injury (**Figure 2A**, **2B**). Detection of cardiactroponin T (TnT) production showed that at 72 h post-transplantation, the production of TnT was reduced in the pAAV-Hsp27-treated group, as compared to the cold I/R alone group (**Figure 2C**). In addition, TUNEL-positive cardiomyocytes were more frequently observed in the WT heart grafts group as compared to the pAAV-Hsp27 groups (**Figure 2D**). Apoptosis of H9c2 cells was assessed by annexin V-propidium iodide double staining (**Figure 2E**), which confirmed that I/R resulted in cell apoptosis and was significantly inhibited by pAAV-Hsp27 transfection.

Hsp27 reduces proinflammatory cytokine production in heart grafts in vivo and H9c2 cells in vitro

NF-κB plays a key role in the inflammatory response during I/R injury. Many cytokines, such as TNF-α, IL-6, IL-1, and ICAM-1, have been reported to be involved in the process of I/R injury, and their expression can be regulated by NF-κB. Also, Hsp27 is reported to dramatically inhibit NF-κB translocation and reduce the release of inflammatory cytokines following I/R injury. In the present study, I/R increased NF-κB p65 (p65) translocation, as determined by western blot analysis (**Figure 3A**), and increased NF-κB activity, as determined with the EMSA (**Figure 3B**), whereas the expression of p65 in the Hsp27 groups was significantly decreased, as compared with the I/R grafts and the grafts without I/R injury. These data indicate that Hsp27 inhibits nuclear translocation of p65.

The inflammatory cytokines TNF-α, IL-1, and IL-1β in I/R grafts were detected by western blotting (**Figure 3A**), RT-PCR (**Figure 3C**), and ELISA (**Figure 3D**). The levels of each of these inflammatory cytokines were significantly decreased in the Hsp27 groups, ascompared to the grafts with and without I/R injury.

Hsp27 protects heart cells from I/R injury through the PUMA signaling pathway

PUMA, a pro-apoptotic factor belonging to the Bcl-2 family, functions as an inducer of apoptosis in several cancer cells. PUMA was previously shown to be upregulated in cardiomyocytes after transient ischemia and inhibition of PUMA upregulation protected the heart from delayed ischemic death. The results of the present study showed that I/R increased PUMA protein and mRNA levels in the WT grafts, while those of the Hsp27+ I/R-injured grafts were significantly decreased as compared with the WT I/R injured grafts (**Figure 4A**, **4B**).

Caspase-3 activity appears to contribute to apoptosis andactivation of caspase-3 was found with the induction of PUMA expression in many cells. In the present study, caspase-3 activity was significantly reduced in the Hsp27

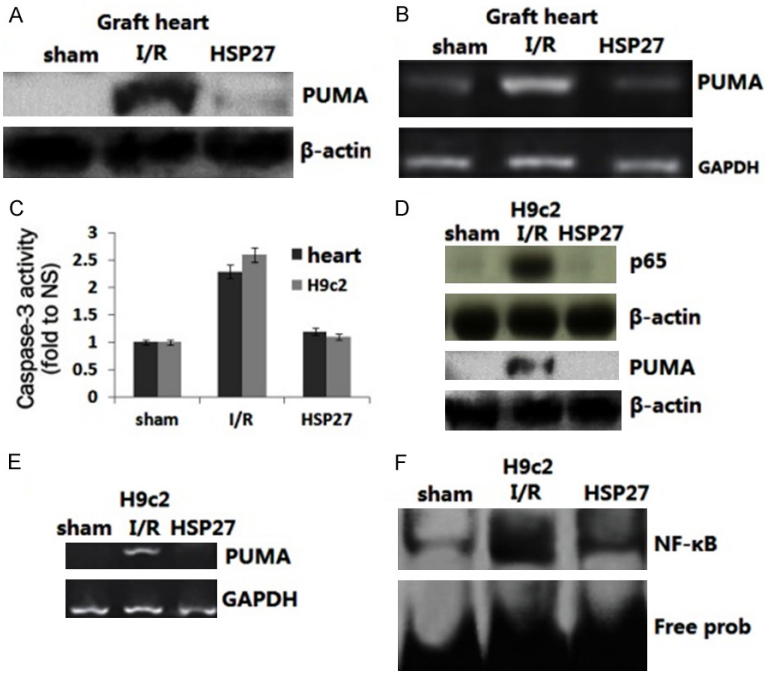

Figure 4. Hsp27 decreased the expression of PUMA in H9c2 cells and heart grafts. (A) Expression of PUMA protein (A) and mRNA (B) levels in heart graft tissues were detected with western blot and RT-PCR analyses, respectively. (C) Caspase-3 activity in I/R grafts and H9c2 cells. Protein (D) and mRNA (E) expression levels of P65 and PUMA in H9c2 cells were detected with western blot and RT-PCR analyses, respectively. (F) NF-κB activity was detected by EMSA in H9c2 cells (*$P < 0.01$ vs. the I/R group).

I/R injured grafts group, as compared to the WT heart grafts group (**Figure 4C**). Also, PUMA protein and mRNA levels as well as caspase-3 activity were significantly decreased in the H9c2/Hsp27 cells as compared to the I/R H9c2 cells (**Figure 4C-F**).

It has been reported that activation of NF-κB signaling could lead to the upregulation of PUMA during I/R injury. To determine whether the protective effect of Hsp27 in response to I/R injury occurs through inhibition of the NF-κB signaling pathway, phosphorylation of Rel A p65 was detected with western blotting and NF-kB activity by EMSA. The result showed that over-expression of Hsp27 reduced the phosphorylation of Rel A p65 and NF-κB activity in heart grafts (**Figure 3A**, **3B**) and I/R Hsp27/H9c2 cells (**Figure 4D-F**), suggesting that Hsp27 prevents activation of the NF-κB/PUMA signaling pathway.

Discussion

The results of this study demonstrated that overexpression of Hsp27 by pAAV-Hsp27 transfection protected heart grafts from cold I/R injury in heart transplantation and that upregulation of Hsp27 can protect donor hearts from cold I/R injury through inhibition of inflammation and apoptosis. Furthermore, the underlying mechanism of Hsp27-induced cardio-protection is the inhibition of the NF-κB-dependent pro-inflammatory and PUMA signaling pathways.

I/R triggers a vigorous inflammatory response, augmented by the generation and release of various cytokines that ultimately exacerbates tissue injury, although the precise mechanism of I/R injury has not been fully revealed [37]. Increasing evidence indicates the importance of inflammation in the pathophysiology of myocardial I/R injury. For instance, interventions targeting inflammatory mediators were found to substantially reduce myocardial I/R injury [38]. In particular, the neutralization of IL-1β reduces I/R injury, suggesting that IL-1β is a key mediator in the pathophysiology of myocardial I/R injury [39]. It has also been observed that the neutralization of IL-6 and TNF-α in mice resulted in a significant aggravation of I/R injury in renal and lung tissues [40-42]. Studies have reported that exercise triggers the simultaneous increase of various antagonistic mediators, such as IL-1β, TNFα, IL-6, and IL-10, and that HSPs can inhibit the activities of inflammatory mediators and protect against stress [43-46].

In this study, expression levels of the pro-inflammatory cytokines IL-6, IL-1β, and TNF-α were increased in a cold I/R model, while overexpression of Hsp27 was impeded. Heat shock treatment was found to protect against angiotensinII-induced hypertension and inflammation in the rataorta, which may be related to the interactions of HSPs with the NF-KB pathway [47]. Moreover, overexpression of Hsp27 inhibited the nuclear translocation of NF-κB p65 and activation of NF-κB. These data suggest that

attenuation of inflammation by Hsp27 is mediated by inhibition of the NF-κB signaling pathway. This finding is in agreement with a report on inflammation in which overexpression of Hsp27 decreased the release of pro-inflammatory mediators by keratinocytes via inhibition of NF-κB signaling [48], and targeting Hsp27 increased the expression levels of NF-κB and NF-κB-mediated pro-inflammatory mediators [49]. Overall, these findings highlight a novel circumstance in which Hsp27 protects against inflammation and further supports Hsp27 as a cardio-protective agent against inflammation under ischemic conditions.

Cell apoptosis is one of the manifestations of I/R injury in organ transplantation [50-52]. Here, the TUNEL assay results showed that Hsp27 decreased the number of apoptotic cells in heart grafts. Additionally, the *in vitro* study with H9c2 cells showed that overexpression of Hsp27 reduced apoptosis of H9c2 cells under cold hypoxia/reperfusion stress. Hsp27 is an ATP-independent chaperone that can interfere with the transduction of apoptotic signaling at several steps, which has been implicated in the preservation of mitochondrial integrity and reduced release of cytochrome c. Hsp27 can also bind directly to cytochrome c, there by preventing activation of procaspase-9, and can bind to and inhibit the activation of procaspase-3 [53]. However, it remains unclear as to which of these activities of Hsp27 are essential for protection against heat shock-induced cell death.

PUMA functions through other Bcl-2 family members, such as Bax, Bcl-2, and Bcl-xL, to induce mitochondrial dysfunction and caspase activation [54, 55]. A study reported the induction of *PUMA* by I/R through oxidative stress in a p53-independent manner, while targeted deletion of *PUMA* attenuated I/R-induced apoptosis and tissue injury in the small intestine [56], suggesting that *PUMA* mediates I/R-induced intestinal apoptosis through the mitochondrial pathway. The results of the present study showed that cold I/R increased PUMA expression and activated caspase-3 in the WT grafts, as compared to the control heart grafts without 24-h cold I/R. Also, the increase in PUMA expression and caspase-3 activity can be reversed by over-expression of Hsp27, indicating a significant effect of Hsp27 on PUMA expression and a possible mechanism of the protective function of Hsp27 against apoptosis. Our *in vitro* study with H9c2 cells also showed that overexpression of Hsp27 by infecting these cells with a human Hsp27 decreased PUMA expression under cold I/R conditions.

Parcellier et al. reported that the pro-apoptotic activities of Hsp27 are controlled through the enhancement of NF-κB activity [57]. In contrast, Liu et al. reported that the anti-apoptotic properties of Hsp27 occur through inhibition of NF-κB activity [58]. Therefore, it appears that NF-κB can paradoxically suppress or promote apoptosis depending on the cell or tissue specificity. A recent study found that NF-κB activation induced apoptosis of breast cancer cells through the NF-κB-dependent upregulation of PUMA [59]. The results of the present study revealed that overexpression of Hsp27 inhibited NF-κB activation and PUMA expression, followed by decreased apoptosis in heart grafts and H9c2 cells. Hence, the protection of Hsp27 might occur through the regulation of NF-κB signaling and subsequent inhibition of PUMA signaling.

In conclusion, this study is the first to demonstrate that Hsp27is a promising target to prevent cold I/R injury in heart transplantation and to show an association of Hsp27 with NF-κB and PUMA. It was also demonstrated that the protective effect of Hsp27 is mediated by the NF-κB and PUMA signaling pathways.

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Disclosure of conflict of interest

None.

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