Original Paper

RP105 Protects Against Apoptosis in Ischemia/Reperfusion-Induced Myocardial Damage in Rats by Suppressing TLR4-Mediated Signaling Pathways

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Key Words

RP105 • Ischemia/reperfusion injury • Myocardial apoptosis • TLR4

Abstract

Background: Myocardial apoptosis is heavily implicated in the myocardial damage caused by ischemia-reperfusion (I/R). Toll-like receptor 4 (TLR4) is a potent inducer of these apoptotic cascades. In contrast, the radioprotective 105 kDa protein (RP105) is a specific negative regulator of TLR4 signaling pathways. However, the precise mechanisms by which RP105 inhibits myocardium apoptosis via TLR4-associated pathways during I/R is not fully understood.

Methods: We utilized a rat model of myocardial ischemic reperfusion injury (MIRI). Animals were pre-treated with Ad-EGFP adenovirus, Ad-EGFP-RP105 adenovirus, saline, or nothing (sham). After three days, rats underwent a 30min left anterior descending coronary artery occlusion and a 4h reperfusion. Myocardial tissue was assessed by immunohistochemistry, TUNEL-staining, Western blot, quantitative RT-PCR, and a morphometric assay. **Results:** RP105 overexpression resulted in a reduction in infarct size, fewer TUNEL-positive cardiomyocytes, and a reduction in mitochondrial-associated apoptosis cascade activity. Further, RP105 overexpression repressed I/R-induced myocardial injury by attenuating myocardial apoptosis. This was mediated by inhibiting TLR4 activation and the phosphorylation of P38MAPK and the downstream transcription factor AP-1. **Conclusion:** RP105 overexpression leads to the de-activation of TLR4, P38MAPK, and AP-1 signaling pathways, and subsequently represses apoptotic cascades and ensuing damage of myocardial ischemic reperfusion. These findings may become the basis of a novel therapeutic approach for reducing of cardiac damage caused by MIRI.

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Introduction

Accumulating evidence indicates that the most viable therapeutic approach for the treatment of coronary artery disease is to re-establish the blood supply to ischemic myocardial tissue [1]. Paradoxically, coronary revascularization results in myocyte damage, accordingly called myocardial ischemic reperfusion (I/R) injury (MIRI) [2]. The mechanisms underlying MIRI have not been fully delineated. However, recent studies demonstrate that myocardial apoptosis is the most destructive action induced in MIRI [3]. Apoptosis is one of the main types of programmed cell death, and a number of apoptosis-related genes are expressed in response to MIRI [4]. Studies show that blocking the apoptotic pathway may exert a protective effect against MIRI [5, 6]. More recent studies have further characterized this relationship by identifying the receptor-mediated signaling and transduction pathways involved, including pathways involving toll-like receptor 4 (TLR4) [6, 7]. A better molecular understanding of these apoptotic properties is a key in understanding MIRI damage and future treatment.

We have previously shown that TLR4 mRNA and protein levels are both significantly up-regulated in MIRI [8], as have other groups [9]. Importantly, TLR4-dependent pathways are considered to be hallmarks of MIRI and potent inducers of apoptotic cascades [6]. TLR4 also modulates cell apoptosis by activating members of mitogen-activated protein kinase (MAPK) signaling pathways [10]. Further, the TLR4-dependent P38MAPK and transcription factor activator protein 1 (AP-1) signaling pathways have been identified to play a critical role in the pathogenesis of liver I/R injury [11]. *In vitro* studies show the activation of the TLR4, P38MAPK, and AP-1 lead to the activation of mitochondria-associated apoptotic cascades [12]. Precisely how each of these signaling pathways are involved in apoptosis during MIRI is currently unknown.

The radioprotective 105 kDa protein (RP105), a single-pass type I membrane protein belonging to the toll-like receptor family, is a specific inhibitor of the TLR4-triggered response [13-16]. The expression of RP105 is strongly correlated with the inflammatory response of MIRI [12]. Unpublished data from our lab show that overexpression of RP105 with a recombinant adenoviral vector protects myocardial damage from MIRI. These protective effects have been described to specifically repress the TLR4-dependent pro-inflammatory action in an animal model of MIRI. Several studies have demonstrated anti-apoptotic properties of RP105, though its ability to inhibit myocardial cell apoptosis during reperfusion after ischemic insults is just beginning to be investigated [17-19]. The aim of the present work was therefore to characterize the molecular mechanisms by which RP105 protects cardiomyocytes from cell apoptosis during MIRI. We show that overexpression of RP105 in the myocardium leads to the deactivation of the TLR4, P38MAPK, and AP-1 signaling pathways, and subsequently represses apoptotic cascades and ensuing MIRI.

Materials and Methods

Animals and experimental design

Forty adult male Sprague-Dawley rats weighing 220–250 g (SPF grade) were purchased from Vital River Laboratories (VRL), Beijing. Experimental procedures were approved by the Institutional Animal Care and Use Committee (Renmin Hospital, Wuhan University, Wuhan, China). All animal care was in accordance with the Guide for the Care and Use of laboratory animals published by the US National Institutes of Health (revised in 1996).

One week before surgery, rats were randomly allocated into four equal groups (n=10): (1) normal non-ischemic group (sham group); (2) myocardial I/R group (I/R group); (3) myocardial I/R with Ad-EGFP group (Ad-E group); and (4) myocardial I/R with Ad-EGFP-RP105 group (Ad-E-R group). On the fourth day after adenovirus or normal saline delivery, all of the rats underwent 30min of coronary occlusion followed by reperfusion for 4h with the exception of sham group. Details on surgery, the assessment of transductions, and transgene expression is described below.

Construction of adenoviral vectors

Construction of the adenovirus vector expressing EGFP-RP105 (Ad-EGFP-RP105) or EGFP (Ad-EGFP) was performed using the AdMax adenovirus system (Microbix Biosystems, Canada) according to the manufacturer's protocol. Recombinant adenoviruses were amplified in HEK293 cells, and were further purified using the Adeno-X™ Virus Purification Kit (BD Biosciences, Clontech) according to the manufacturer's recommendations. Viral titer was routinely concentrated to 1.5×10^{10} plaque-forming units (PFU), as determined by plaque assays.

Rat MIRI model and in vivo gene transfer

Adenovirus encoding EGFP or EGFP-RP105 was transduced, or saline was injected into the left ventricular wall of rat myocardium. Three days later, the *in vivo* MIRI model was performed as previously described [5]. Briefly, rats were anesthetized with sodium pentobarbital (40mg/kg; i.p.) and ventilated with oxygen using a small animal ventilator. After opening the chest gently between the fourth and fifth ribs and exposing the heart, rats received intramyocardial injections of 100µl solution containing Ad-EGFP (1.5×10^{10} PFU), Ad-EGFP-RP105 (1.5×10^{10} PFU), or saline, respectively, at five separate sites of the left ventricular anterior wall and the left anterior descending (LAD) coronary artery by using a 30-gauge needle. The chest was closed and all rats received a single intramuscular injection of penicillin sodium (0.8mg/g). Three days later, rats were re-anesthetized and the chest was re-opened to ventilate 100% oxygen via a small animal ventilator. As described above, the thorax was reopened and the LAD was identified. A 6-0 silk suture was loosely placed around the origin of the LAD and medical latex tubing (inner diameter, 2mm, socket) was placed over the LAD. Myocardial ischemia was induced by tightening the ligature around the tube for 30min. Successful surgical myocardial ischemia was confirmed by observing electrocardiogram S-T segment elevation and a blanched appearance of the ischemic region. After 30min, the suture was withdrawn to restore normal circulation for 4h of reperfusion. Rats were then sacrificed and myocardial tissue near the cardiac apex was harvested. Sham-operated rats underwent the same procedures without the occlusion of the LAD or reperfusion.

Assessment of transgene expression

We assessed the expression of EGFP or EGFP-RP105 in two ways three days after the intramyocardial injection with adenovirus. We performed immunohistochemistry (IHC) and fluorescence microscopy as well as quantitative RT-PCR.

For IHC, four hours after myocardial I/R, tissue was immediately fixed in 4.0% paraformaldehyde, embedded in paraffin, and sectioned. Sections were dewaxed and microwave antigen retrieval was carried out. Slices were incubated in 1% goat serum albumin at room temperature (RT) for 30min, incubated with anti-RP105 antibody (Santa Cruz, CA, USA) overnight at 4°C, and then incubated with Fluor Cy3 (red)-conjugated rabbit anti-goat IgG (Boster Biotech, China) in a humidified chamber for 60min at 20–37°C in the dark. Sections were washed three times in PBS. They were then incubated with 1% goat serum albumin at RT for 30min, incubated with anti-EGFP antibody (Santa Cruz, USA) overnight in a humidified chamber at 4°C, incubated with fluorescein isothiocyanate (FITC)-labeled secondary goat anti-rabbit IgG (Boster Biotech, China) in a humidified chamber for 60min at 20–37°C in the dark, and lastly stained with 4',6'-diamidino-2-phenylindole (DAPI). Fluorescence microscopy (Olympus America, BX51) was then carried out.

We then performed quantitative RT-PCR. Total RNA was isolated from myocardial samples with Trizol Reagent (Invitrogen) following the manufacturer's instructions. For mRNA detection, 4.0µg RNA was reverse transcribed with a commercial cDNA synthesis kit (Fermentas). qRT-PCR was performed with SYBR green/fluorescein qPCR Master Mix kit (Fermentas) with the ABI Prism 7,500 system. mRNA levels were normalized to β -actin and data were analyzed using the comparative quantification method ($2^{-\Delta\Delta C_t}$). Reverse transcription was performed at 42°C for 1h. PCR was run as follows: 50°C for 2min, 95°C for 10min, and 40 cycles of 95°C for 30s and 60°C for 30s. The following primers were used to amplify gene products:

RP105, F: 5'-TGAGGGCCTCTGTGAAATGT-3'

RP105, R: 5'-GGAAGCACTGATTTGGCACA-3'

EGFP, F: 5'-TTTATGGTGAGCAAGGGCGAG-3'

EGFP, R: 5'-TTTTGGTGAGATGAACTTCAG-3'

β -actin, F: 5'-CACGATGGAGGGCCGACTCATC-3'

β -actin, R: 5'-TAAAGACCTCTATGCCAACACAGT-3'

Measurement of myocardial infarct size

An Evans Blue/triphenyltetrazolium chloride (TTC) double-staining was performed to determinate the myocardial infarct area as previously described [5]. Briefly, after surgery and 4h of reperfusion treatment, the LAD was re-occluded. 1ml of 2.0% Evans blue was then injected intravenously. Stained hearts were quickly removed, frozen, and sliced into 5 sections (2mm thick). Sections were incubated in 1.5% TTC for 15min at 37°C. Viable myocardium at ischemic risk stained red with TTC, whereas unstained, damaged tissue appeared white. Staining was quantitated using image analyzer software (Image-Pro Plus 5.0). The infarct size was presented as a percentage of myocardium area at risk (AAR).

Apoptosis assessment using TUNEL assay

The *in vivo* terminal deoxynucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay was used to detect apoptotic cardiomyocytes induced by MIRI. Myocardial tissue was fixed with 10% formaldehyde for 24h, washed, dehydrated, and embedded in paraffin. TUNEL staining was then performed according to the manufacturer's instructions in the TUNEL detection kit (Roche Applied Science, Basle, Switzerland). In addition, sections were counterstained with hematoxylin after TUNEL staining. Five microscopic fields of each section (400× magnification) were randomly chosen to count TUNEL-positive cells and the total cells. An apoptosis index (AI) score was calculated with the ratio of apoptotic cardiomyocytes vs. total myocytes.

Western blot analysis

Western blot analysis was carried out to measure the expression level of multiple proteins in myocardial tissue. The protocol was performed as previous described [5]. Briefly, extracted protein was separated on a 10% SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes. Membranes were blocked with 5% (w/v) nonfat dry milk in Tris-buffered saline for 2h at RT, and incubated with the respective primary antibody including rabbit anti-RP105 (Santa Cruz, SC-27841), anti-TLR4 (Santa Cruz, sc-10741), anti-P38MAPK (Santa Cruz, sc-535), anti-p-P38MAPK (Santa Cruz, sc-7973), anti-c-Jun (Santa Cruz, sc-44), anti-p-c-Jun (Santa Cruz, sc-101721), anti-Bax (Santa Cruz, sc-526), anti-Bcl-2 (Santa Cruz, sc-492), anti-cytochrome c (Bioworld, BS1089), anti-caspase-9 (Santa Cruz, sc-56076), anti-caspase-3 (Santa Cruz, sc-1225), and mouse anti-GAPDH at the recommended dilution. After three washes, peroxidase-conjugated secondary antibodies were incubated for 2h at RT. GAPDH was used as a loading control. Bands were detected with an ECL detection kit (Thermo).

Statistical analysis

All data were expressed as mean ± SD. Statistical comparisons between mean values of groups were performed using one-way analysis of variance (ANOVA) in SPSS software (version 14.0). Student-Newman-Keuls (SNK)-q tests were performed to analyze multiple comparisons. A p-value of <0.05 was considered to be statistically significant.

Results

In vivo myocardial expression of EGFP or EGFP-RP105 after intramyocardial adenovirus gene delivery

To examine whether targeting TLR4 pathway using RP105 could protect myocardium from apoptotic cell death in rat models of MIRI, we injected adenoviral vectors expressing either Ad-EGFP-RP105 or Ad-EGFP into the myocardium. Immunofluorescence microscopy was used to visualize cardiomyocytes (Fig. 1). Adenoviral transduction using both Ad-EGFP-RP105 and Ad-EGFP successfully allowed for visualization of myocardial cells, as seen by the high EGFP signal (Fig. 1A–B). Only low levels of endogenous RP105 (red) were detected in the myocardium transduced with Ad-EGFP control vector (Fig. 1C). Viral transduction using Ad-EGFP-RP105 drastically increased the expression of RP105 (red) in myocardium (Fig. 1D). In addition, most Ad-EGFP-RP105-transduced cardiomyocytes expressed high levels of RP105, as indicated by high levels of colocalization (Fig. 1F and J).

To further analyze expression levels of the transgenes, EGFP mRNA was measured by quantitative RT-PCR. EGFP mRNA from myocardium treated with saline injections (sham

Fig. 1. Expression and colocalization of RP105 and EGFP in the adenovirus gene injection. Respective representations of five IHC photomicrographs for EGFP (green), RP105 (red), and DAPI-labeled nuclei of cardiomyocytes (blue). Either Ad-EGFP (left, A,C,E,G, and I) or Ad-EGFP-RP105 (right, B,D,F,H, and J) were injected three days prior to IHC. Areas in yellow indicate colocalization (E and F). Scale bar=20 μ m.

control) was not detectable (Fig. 2A). Adenoviral transduction of Ad-EGFP (Ad-E) or Ad-EGFP-RP105 (Ad-E-R) indeed expressed EGFP (Fig. 2A–B). No significant differences in expression levels were seen between Ad-E and Ad-EGFP-RP105 groups.

RP105 expression is reduced after MIRI

To investigate the involvement of RP105 in the anti-apoptotic action during ischemia and reperfusion conditions, we performed the surgical MIRI procedure described above. The expression of RP105 was measured by quantitative RT-PCR assay and Western blot analysis. Relative to non-ischemic sham control, the levels of RP105 mRNA from I/R myocardium were markedly reduced (Fig. 2D) and RP105 protein levels were significantly decreased (Fig. 2C and E). To determine whether intramyocardial transduction of Ad-EGFP-RP105 would induce RP105 overexpression after MIRI, we transduced rat myocardium with adenoviral vector Ad-EGFP control or Ad-EGFP-RP105. RP105 expression was not altered by the transduction of Ad-EGFP. RP105 expression, however, was increased in I/R myocardium transduced with Ad-EGFP-RP105, as shown by both quantitative RT-PCR (Fig. 2D) and Western blot analysis (Fig. 2C and E).

Up-regulation of RP105 decreased infarct size after MIRI

To determine whether antagonizing the TLR4 pathway with RP105 could attenuate the myocardial damage in I/R injury, heart tissue was harvested and analyzed after surgical

Fig. 2. RP105 expression was reduced after MIRI. A–B: Quantitative RT-PCR gel and quantitation from the four groups for β -actin and EGFP. C and E: Protein expression levels of RP105, as examined by Western blot. D: mRNA expression of RP105 was detected by quantitative RT-PCR. RP105 expression was reduced in the experimental model of MIRI in rats, while delivery of Ad-EGFP-RP105 into the myocardium increased the expression of RP105. Values are expressed as mean $\pm$ SD, $n=6$ /group, * $p<0.05$ vs. sham group; # $p<0.05$ vs. Ad-E or Ad-E-R group. NS indicates no significant difference at the 0.05 level.

induction of MIRI. Interestingly, although infarct myocardium was found in all groups, the myocardium transduced with Ad-EGFP-RP105 had less infarct tissue, as indicated by the white tissue (Fig. 3A–B). Although transduction of Ad-EGFP had no significant effect on the damage and prognosis after myocardial I/R in comparison with saline injection (I/R), overexpression of RP105 significantly reduced the mean infarct size of the AAR relative to the Ad-E group (Fig. 3A, B), indicating that RP105 played a cardioprotective role in MIRI.

Up-regulation of RP105 alleviated apoptosis of cardiac myocytes during reperfusion injury

To investigate whether RP105 is involved in anti-apoptotic actions during MIRI, we performed a TUNEL assay with cardiac myocyte cells. Significantly more apoptotic cells were detected in all three MIRI groups compared to sham animals. Transduction of Ad-EGFP had no significant effect on the percentage of TUNEL-positive cells compared to saline injection. However, overexpression of RP105 partially rescued the myocardium apoptosis caused by I/R relative to Ad-EGFP transduction (Fig. 4A, B).

Up-regulation of RP105 suppressed the expression of TLR4/P38MAPK/AP-1 signal pathways

To determine whether RP105, a specific inhibitor of TLR4 signaling, modulates the activity of P38MAPK and AP-1 through the regulation of TLR4, we measured the levels of TLR4, P38MAPK, phospho-P38MAPK (p-P38MAPK), c-Jun, phospho-c-Jun (p-c-Jun) and GAPDH as an endogenous control using Western blot and densitometric analysis (Fig. 5).

Fig. 3. RP105 overexpression decreased infarct size after MIRI. A: Representative pictures of Evans and TTC staining for the areas of myocardial infarction. Dark blue area, normal tissue; red-stained area, ischemic tissue; pale white area, infarcted tissue. B: The infarct size is presented as a percentage of the area at risk. Values are means $\pm$ SD, n=6/group, *p<0.05 vs. sham group, #p<0.05 vs. Ad-E or Ad-E-R group.

Fig. 4. RP105 overexpression reduced apoptosis of cardiac myocytes during reperfusion injury. A: Representative photomicrographs of TUNEL staining of myocardium from each of the four groups (400 $\times$ magnification). Scale bar=20 μ m. B: Quantification of the mean apoptotic index in each group. Values are means $\pm$ SD, n=6/group, *p<0.05 vs. sham group, **p<0.05 vs. Ad-E or Ad-E-R group.

Only low levels of TLR4, P38MAPK, p-P38MAPK, c-Jun, and p-c-Jun were found in the non-ischemic group (sham control). Myocardium from I/R injury shared elevated levels of TLR4 and downstream molecules including P38MAPK, p-P38MAPK, c-Jun and p-c-Jun (Fig. 5A-E). I/R injury plus adenoviral transduction of Ad-EGFP had no significant effect on the levels of TLR4, P38MAPK, p-P38MAPK, c-Jun, or p-c-Jun proteins (Fig. 5A, B, D, E). Interestingly, overexpression of RP105 significantly reduced the levels of TLR4, p-P38MAPK, and p-c-Jun by 35.09%, 33.90%, and 30.50%, respectively in I/R myocardium (Fig. 5A, B, D, E). Adenoviral transduction of RP105 had no significant effect on total cellular levels of P38MAPK or c-Jun (Fig. 5A, B, D, E).

In addition, low p-P38MAPK/P38MAPK ratios and p-c-Jun/c-Jun ratios were found in the sham group, while myocardium from I/R injury showed increased p-P38MAPK/P38MAPK ratios and p-c-Jun/c-Jun ratios (Fig. 5F). I/R injury followed by adenoviral transduction using Ad-EGFP had no significant effect on the levels of these ratios. Overexpression of RP105 significantly lessened the p-P38MAPK/P38MAPK ratio and p-c-Jun/c-Jun ratio in I/R myocardium by 39.06% and 28.29%, respectively (Fig. 5F). Thus, overexpression of RP105 can specifically de-activate TLR4 and attenuate the phosphorylation of P38MAPK and c-Jun/AP-1.

Up-regulation of RP105 altered the expression of apoptosis-associated molecules

To further analyze the mechanisms by which RP105 inhibits myocardium apoptosis during MIRI through the regulation of TLR4 signaling pathway, apoptotic proteins were measured by Western blot. Only low levels of pro-apoptotic molecules were measured in the sham control, including Bax, cytochrome *c*, caspase-9 (procaspase-9), cleaved caspase-3, and anti-apoptotic protein Bcl-2. Myocardium from I/R injury showed elevated levels of pro-apoptotic molecules and reduced levels of anti-apoptotic proteins (Fig. 6). Additionally, I/R injury followed by adenoviral transduction using Ad-EGFP had no significant effect on the levels of mitochondria-associated apoptosis molecules. Interestingly, overexpression of RP105 significantly inhibited the levels of Bax, caspase-9, cleaved caspase-3, and cytochrome *c*. The anti-apoptotic protein Bcl-2 was significantly down-regulated in all three I/R groups.

Discussion

A number of recent studies show that apoptotic pathways play a central role in the pathophysiology of MIRI [20, 21]. The work presented here demonstrates that selective overexpression of RP105 via adenovirus vectors is a promising cardioprotective method to elicit anti-apoptotic effects, as it ameliorates MIRI *in vivo*. We first confirm that intramyocardial injection of adenoviral vectors followed by ischemia and reperfusion treatment contributes to the overexpression of RP105 in myocardial tissue. Next, we show that overexpression of RP105 with Ad-EGFP-RP105 prior to MIRI markedly suppressed infarct size per AAR in comparison with rats subjected to saline or Ad-EGFP vectors. This is consistent with the drastic reduction in the number of TUNEL-positive myocytes compared to the Ad-EGFP control and the non-transduced I/R control. In addition, overexpression of RP105 significantly reduces the expression of TLR4, and simultaneously attenuates the phosphorylation of P38MAPK and transcriptional factor c-Jun/AP-1. Together these findings strongly suggest a cardioprotective and anti-apoptotic role of RP105 in MIRI.

In the next set of experiments, we focused on TLR4-mediated apoptosis. Earlier studies have described a role for TLR4 in cardiomyocyte apoptosis during I/R injury [22]. Ding et al. provided evidence that TLR4 deficiency protects against reperfusion-induced apoptosis [6]. However, more precise mechanisms of TLR4-mediated intracellular signaling in MIRI have not been clarified. P38MAPK has also been reported to play a crucial role in cardiac cardiomyocyte apoptosis during MIRI [23]. In its phosphorylated state, P38MAPK augments myocardial apoptosis [24]. TLR4 also seems to be involved in mediating cell apoptosis by activating MAPK signaling pathways [10]. P38MAPK expression may require the activation of the transcription factor, AP-1 [11]. AP-1 subunits belong to c-Jun and c-Fos protein families, and their activities are mainly mediated by MAPKs [25]. c-Jun is considered to be a dominant component of the AP-1 transcription factor complex. It binds to the AP-1 sequence as c-Jun homodimers or c-Jun and c-Fos heterodimers [26]. Phosphorylation of c-Jun is its most important regulatory mechanism, and enhances c-Jun/AP-1 transcriptional activity [27]. Recent studies reveal that AP-1 may induce apoptosis of cardiac cells and are involved in the pathologic conditions of MIRI [28]. TLR4, P38MAPK, and AP-1 signaling pathways have all been shown to be up-regulated in the liver following I/R injury [11]. However, little is known about the regulation of TLR4, P38MAPK, and AP-1 in the heart following I/R. We show here for the first time that TLR4, P38MAPK, and c-Jun/AP-1 signaling pathways are up-regulated, and causally aggravate I/R-induced myocardium apoptosis. In addition, specifically attenuating the expression of TLR4, P38MAPK, and c-Jun/AP-1 can robustly reduce myocardium apoptosis. These results suggest that selective inhibition of TLR4 and downstream signaling molecules may be regarded as candidates for potential therapeutic targets for MIRI.

RP105 is a member of the leucine-rich repeat family and has homology to TLR4. It is a key negative regulator of TLR4 signaling pathways, and may do so as RP105/myeloid differentiation protein-1(MD-1) or TLR4/MD-2 complexes [13, 14]. The RP105/MD-1

complex directly binds to the TLR4/MD-2 complex, and subsequently reduces binding capacity of TLR4 to its ligands. In this way, RP105 serves as a specific, endogenous inhibitor of TLR4-mediated response during multiple pathological conditions, including cardiovascular system disease [13, 14]. Previous reports show that B cells treated with anti-RP105 antibody can rescue B cells from apoptosis [17]. Another study demonstrated that RP105 protects B cells against apoptosis, and that RP105-negative B cells are more susceptible to corticosteroid-induced apoptosis *in vitro* [18]. The molecular mechanisms of RP105 in myocardium apoptosis in MIRI are unclear. In the current study, we found that over-expression of RP105 via intramyocardial delivery reduces cardiac damage following ischemic reperfusion by attenuating cardiomyocyte apoptosis. Further, we show that the underlying mechanisms are closely linked to the de-activation of TLR4, P38MAPK, and c-Jun/AP-1 signaling pathways. Thus, our data suggest that RP105 may be a promising target against I/R-induced myocardium apoptosis by inhibiting TLR4-mediated signaling pathways.

We also provide evidence that the underlying mechanism of RP105's action in MIRI involves the de-activation of TLR4, P38MAPK, and c-Jun/AP-1 signaling pathways. Other work shows that cardiomyocyte apoptosis is modulated by Bcl-2 family proteins, including pro-apoptotic factor Bax and anti-apoptotic protein Bcl-2 [29]. Bax directly modulates the opening of mitochondrial permeability transition pore, thereby contributing to the release of cytochrome *c* into the cytosol and activates apoptotic signaling pathways [30]. Apoptotic cascade events are initiated via complex caspase signaling cascades including cell surface death receptor-induced activation of caspase-8 and the mitochondrial apoptotic pathway, which then activate caspase-3 and -9 [31, 32]. *In vitro* experiments indicate that the activation of the TLR4 and P38MAPK signaling pathways and AP-1 phosphorylation downregulate uncoupling protein 2 (UCP-2), which causally leads to the mitochondria-dependent apoptotic pathways [12]. UCP2 is a carrier protein located on the inner mitochondrial membrane [33]. Activation of the TLR4, P38MAPK, and AP-1 signaling pathways results in the localization of Bax to the mitochondria, which increases mitochondria-dependent apoptosis, as identified by detecting increased release of cytochrome *c* into the cytosol and cleavage of caspase-9 and caspase-3 [34]. Our work indicates that increased expression of RP105 attenuates the pro-apoptotic proteins Bax, caspase-9, and cleaved caspase-3, as well as the release of cytochrome *c* into cytosol. Further, the Bcl-2 expression is reduced. This may be due to the inhibition of the TLR4, P38MAPK, and AP-1 signaling pathways. These results further support our conclusion that RP105 contributes to anti-apoptotic activity on myocardium damage induced by I/R. We find that RP105 ameliorates the mitochondrial apoptosis pathway, and is characterized by decreased release of cytochrome *c* into the cytosol, the down-regulation of caspase-9 and cleaved caspase-3, the repression of Bax, and finally, the enhancement of Bcl-2. Although RP105 may serve as a critical mediator in reducing MIRI, the precise molecular mechanisms that link the de-activation of TLR4, P38MAPK, and AP-1 signaling pathways with the attenuation of mitochondrial apoptosis pathways is warranted.

Taken together, our study suggests that RP105 possesses the ability to reduce MIRI by modulating apoptotic cascades. These findings may lead to novel ways to reduce reperfusion-induced myocardial apoptosis and facilitate a cardio-protective mechanism.

Disclosure Statement

The authors declare no conflict of interest.

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