

Radioprotective 105 kDa protein attenuates ischemia/reperfusion-induced myocardial apoptosis and autophagy by inhibiting the activation of the TLR4/NF- κ B signaling pathway in rats

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Abstract. Toll-like receptor 4 (TLR4) serves as an important inducer of apoptotic and autophagic responses in myocardial ischemia/reperfusion (I/R) injury (MIRI). Radioprotective 105 kDa protein (RP105) is a specific inhibitor of TLR4. However, the molecular mechanisms by which RP105 represses myocardial apoptosis and autophagy through TLR4-mediated signaling during I/R have not yet been fully elucidated. Therefore, in the present study, we aimed to examine whether adenovirus-mediated RP105 overexpression repressed myocardial apoptosis and autophagy by inhibiting the TLR4-driven mechanism in MIRI. Three days after the injection of virus or saline into the myocardium, Sprague-Dawley (SD) rats were subjected to 30 min of left anterior descending coronary artery occlusion and 6 h of reperfusion. Myocardial specimens were prepared for analysis. We performed immunohistochemical and histopathological analysis, the measurement of cardiac biomarkers, TUNEL assay, RT-qPCR and western blot analysis. The results indicated that the overexpression of RP105 contributed to an amelioration of

myocardial histological damage, decreased leakage of creatine kinase (CK) and lactate dehydrogenase (LDH), as well as a reduction in the number of TUNEL-positive cardiomyocytes. The levels of positively associated modulators of apoptosis and autophagy were also significantly downregulated by RP105, whereas Bcl-2, which plays an opposite role in inducing apoptosis and autophagy, was inversely upregulated. Furthermore, the overexpression of RP105 led to the repression of TLR4 activity and the phosphorylation of NF- κ B/p65, as well as the reduced production of the cytokines interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α). Taken together, these data suggest that RP105 protects the myocardium against apoptosis and autophagy, and plays a cardioprotective role during I/R injury. This is most likely due to the inactivation of TLR4/NF- κ B signaling pathway. Thus, RP105 may represent an innovative therapeutic target for attenuating MIRI.

Introduction

Acute myocardial infarction (AMI) is a leading cause of clinical morbidity and mortality (1-3). Currently, various reperfusion therapies, including pharmacological interventions (e.g., thrombolytics) and/or invasive manipulations (e.g., angioplasty) constitute the most effective strategies for the treatment of AMI (4). Paradoxically, the restoration of oxygen-rich blood to the ischemic tissue leads to additional myocardial impairment, a situation collectively referred to as myocardial ischemia/reperfusion (I/R) injury (MIRI) (5,6). Finding ways to limit MIRI and thereby achieve the maximal cardioprotective effects of clinical reperfusion strategies has always been a problem. Recent studies indicated that the apoptosis and autophagy of cardiomyocytes played an important pathogenic role in MIRI (5,7,8). Although the molecular mechanisms underlying MIRI have not been completely clarified, the mechanistic exploration of apoptosis and autophagy may unveil novel therapeutic approaches for the treatment of MIRI.

Apoptosis, named as type I programmed cell death, is genetically determined by the appropriate genes in response

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Abbreviations: TLR4, Toll-like receptor 4; RP105, radioprotective 105 kDa protein; MIRI, myocardial ischemia/reperfusion injury; AMI, acute myocardial infarction; LC3, light chain 3; TIR, Toll-IL-1 receptor; LAD, left anterior descending

Key words: radioprotective 105 kDa protein, ischemia/reperfusion, apoptosis, autophagy, Toll-like receptor 4, nuclear factor- κ B

to MIRI (9). Among these, the Bcl-2 family, mainly consisting of pro-apoptotic Bax and anti-apoptotic Bcl-2, determine the possibility of cardiomyocytes undergoing apoptosis or surviving under conditions of I/R (7,10,11). Autophagy, or type II programmed cell death, plays a critical role in the pathogenesis of MIRI (5,12,13). Beclin-1 and light chain 3 (LC3) are two pivotal regulatory genes involved in autophagy, and are both commonly recognized as indispensable markers of autophagy (3,14,15). The transfer of LC3-I to LC3-II leads to the formation of double-membrane vesicles named autophagosomes (3). Furthermore, close crosstalk occurs between these two forms of programmed cell death, which is modulated by intracellular signaling pathways (16,17). For instance, the cell surface receptor-mediated signaling pathways including pathways involving Toll-like receptor 4 (TLR4), simultaneously regulate apoptosis- and autophagy-associated pathogenesis in I/R injury (18). Furthermore, blocking TLR4-induced apoptosis and autophagy may confer a protective effect against MIRI (5,7). Thus, a better understanding of the mechanisms which initiate apoptotic and autophagic responses is critical for the study of MIRI and for the development of novel treatments.

TLR4, as an upstream common sensor of multiple intracellular pathways including TLR4/nuclear factor- κ B (NF- κ B) signaling, is commonly recognized as an important hallmark and potent stimulus of the apoptotic cascade as well as autophagic signaling (18–20). Previous findings have confirmed that TLR4 activity stimulated the downstream transcription factor NF- κ B; it induced the expression of NF- κ B target genes including interleukin (IL)-6 and tumor necrosis factor α (TNF- α), and led to the activation of the apoptotic cascade and autophagic responses (18). It has been demonstrated that IL-6 and TNF- α are the principal cytokines involved in the initiation of the apoptotic process (18). The precise mechanisms detailing how each of these signaling molecules participates in apoptosis and autophagy during MIRI remain elusive.

The radioprotective 105 kDa protein (RP105) is a TLR4 homologue which lacks the intracytoplasmic Toll-IL-1 receptor (TIR) domain of TLR4 (21,22). RP105 is widely involved in the pathophysiological process of various cardiovascular diseases through directly inhibiting TLR4-driven pathogenesis during myocardial infarction, atherosclerosis and post-interventional vascular remodeling (22–24). Previously, we showed that RP105 protected the myocardium against I/R injury, and the underlying mechanism was ascribed to the inhibition of the apoptotic cascade response (25). There exists close crosstalk between apoptosis and autophagy, although the role of RP105 in reducing myocardial apoptosis and autophagy simultaneously during I/R injury is yet to be defined. Thus, we aimed to examine the molecular mechanisms through which RP105 protects cardiomyocytes exposed to I/R injury against apoptosis and autophagy. In this study, we demonstrated that the overexpression of RP105 ameliorates MIRI by limiting apoptosis and autophagy. RP105 may inhibit these two types of programmed cell death synergistically through inhibiting the activation of the TLR4/NF- κ B signaling pathways. Moreover, we hypothesized that the anti-autophagic potency of RP105 may be closely associated with the increase in binding between Bcl-2 and Beclin1.

Materials and methods

Preparation of adenoviral vector. An adenoviral vector encoding EGFP-RP105 (Ad-EGFP-RP105) or EGFP (Ad-EGFP) was generated under the control of AdMax adenovirus packing system (Microbix Biosystems Inc., Mississauga, ON, Canada) in accordance with the manufacturer's instructions. The resulting recombinant adenoviruses were picked and amplified in 293 cells (American Type Culture Collection, Manassas, VA, USA). The virus was then purified using an Adeno-XTM Virus Purification kit (BD Biosciences; Clontech, Mountain View, CA, USA). The viral titer was determined by a plaque assay, and finally concentrated to 1.5×10^{10} plaque-forming units (PFU).

Animals and experimental design. Adult male Sprague-Dawley (SD) rats weighing 220–250 g were used for all experiments in the present study. The experimental procedures were approved by the Institutional Animal Care and Use Committee of Wuhan University (Wuhan, China). Animal care conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. One week prior to surgery, SD rats were randomly divided into four groups ($n=10$), namely, the sham-operated group (SO group), the myocardial I/R group (I/R group), the myocardial I/R with Ad-EGFP group (I/R + Ad-E group), and the myocardial I/R with Ad-EGFP-RP105 group (I/R + Ad-E-R group). Three days after adenovirus delivery or saline injection, each rat underwent 30 min of left anterior descending (LAD) coronary artery occlusion and subsequently, 6 h of reperfusion.

Gene transfer in vivo and establishment of a rat model of MIRI. Ad-EGFP or Ad-EGFP-RP105 was transferred or saline was injected into the left ventricular wall of the rat heart. The animals were then subjected to a MIRI surgical operation three days post-transfection as reported previously (25). Briefly, the SD rats were anesthetized by an intraperitoneal injection of sodium pentobarbital (40 mg/kg), and were intubated orally with 100% oxygen using a rodent ventilator. After a left thoracotomy between the fourth and fifth intercostal space, Ad-EGFP (1.5×10^{10} PFU), Ad-EGFP-RP105 (1.5×10^{10} PFU) or saline in volume of 100 μ l was injected intramyocardially into five separate sites via a 30-gauge needle. All the five injection sites were situated in the left ventricular anterior wall around the LAD artery. The chest was closed and all rats received a single intramuscular injection of penicillin sodium (0.8 mg/g; North China Pharmaceutical Co., Ltd., Shijiazhuang, China).

Three days after virus delivery or saline injection, the MIRI model was established as previously described (7). Briefly, all rats were re-anesthetized and ventilated with 100% oxygen. A thoracotomy through the original incision site was re-performed. A 6-0 silk suture with a curved needle was placed under the origin of the LAD artery and medical latex tubing was located over the LAD artery. Myocardial ischemia was induced by tightening the suture for 30 min. Ischemia was confirmed by electrocardiogram changes (S-T segment elevation) and the immediate regional cyanosis in the anterior ventricular wall. The suture was then loosened to restore coronary circulation. At 6 h post-reperfusion, the rats were re-anesthetized and blood samples were collected through the jugular vein. The rats were then sacrificed by air embolism

methods and the myocardial tissue near the cardiac apex was obtained for further analysis. The SO group rats underwent the same operation with the exception of LAD artery ligation.

Immunohistochemistry analysis and fluorescence microscopy.

We detected the efficiency of adenoviral transfection by immunohistochemistry and fluorescence microscopy [immunofluorescence (IF) microscopy] as previously described (26). Briefly, the heart specimens were quickly fixed in 4.0% paraformaldehyde, embedded in paraffin, and then sectioned. After dewaxing and antigen retrieval using microwave processing, the slices were incubated in 1% goat serum albumin at room temperature (RT) for 30 min, and incubated with anti-EGFP antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) overnight at 4°C. The sections were then incubated for 60 min at 20-37°C in the dark with fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit IgG secondary antibody (Boster Biotech, Wuhan, China). Finally, the slices underwent 4',6'-diamidino-2-phenylindole (DAPI; Beyotime Biotech, Shanghai, China) staining to redye the nuclei of the cardiomyocytes. Images were captured using a fluorescence microscope (BX51; Olympus America, Inc., Center Valley, PA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from myocardial tissue using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA (4.0 µg) was reverse transcribed into cDNA according to the manufacturer's instructions (Fermentas, Glen Burnie, MD, USA). RT-qPCR was performed using a SYBR-Green/fluorescein qPCR Master Mix kit (Fermentas) with the ABI Prism 7500 system (Foster City, CA, USA). Data were normalized to β -actin gene expression, and calculated using the comparative quantification method ($2^{-\Delta\Delta C_t}$). The following sequence-specific primers were used to amplify gene products: RP105 forward, 5'-TGAGGGCCTCTGTGAAATGT-3' and reverse, 5'-GGAA GCACTGATTTGGCACA-3'; β -actin forward, 5'-CACGATG GAGGGGCCGACTCATC-3' and reverse, 5'-TAAAGACC TCTATGCCAACACAGT-3'.

Western blot analysis. Western blot analysis was performed to assess the protein levels of RP105, TLR4, NF- κ B/p65, phosphorylated (p)-NF- κ B/p65, Bax, Bcl-2, Beclin-1, LC3, IL-6 and TNF- α according to the manufacturer's instructions. Briefly, the extracted proteins were separated by electrophoresis with SDS-polyacrylamide gel and then transferred onto nitrocellulose membranes. After blocking with 5% (w/v) nonfat dry milk for 2 h at RT, the membranes were incubated with primary antibodies overnight at 4°C. After washing 5 times, the membranes were then incubated with peroxidase-conjugated secondary antibodies for 2 h at RT. Bands were scanned and analyzed using an enhanced chemiluminescence (ECL) detection kit (Thermo Fisher Scientific, Waltham, MA, USA). GAPDH served as a loading control.

Histological analysis of myocardial injury. For histopathological observations, heart tissues were obtained at 6h post-reperfusion and immediately fixed in 4.0% paraformaldehyde solution overnight. After dehydration at RT by a graded alcohol series, specimen slices were embedded in

paraffin and sectioned at 4 µm. The sections were then stained with hematoxylin and eosin (H&E; Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) and examined under a light microscope (Leica Microsystems, Wetzlar, Germany).

Determination of serum activity levels of lactate dehydrogenase (LDH) and creatine kinase (CK). Blood samples were collected 6 h after reperfusion. LDH and CK assay commercial kits (Elabscience Biotechnology Co., Ltd., Wuhan, China) were used to analyze enzyme activity levels. All procedures were performed in accordance with the manufacturer's instructions. The results are expressed as international units per liter.

Determination of myocardial apoptosis. The terminal deoxynucleotidyl-transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay was performed in order to detect myocardial apoptosis. The TUNEL detection kit (Roche Applied Science, Basel, Switzerland) was used according to the manufacturer's instructions. In addition, the sections were co-stained with hematoxylin after TUNEL staining. Five microscopic fields of each section (x400 magnification; Leica Microsystems) were randomly chosen to count the number of TUNEL-positive cells and total cells. An apoptotic index (AI) score represented the ratio of apoptotic cardiomyocytes/total myocytes.

Statistical analysis. Statistical analysis was performed using SPSS software (version 14.0). Data are expressed as the means \pm standard deviation (SD). Differences between groups were assessed using one-way analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK)-q test. A p-value <0.05 was considered to indicate a statistically significant difference.

Results

Efficiency of adenoviral transfection into the rat myocardium. IF microscopy was performed to determine the efficiency of Ad-EGFP and Ad-EGFP-RP105 transfection into the myocardium at three days post-transfection. Successful adenovirus transfection was indicated by high EGFP (green) fluorescence in the Ad-EGFP- or Ad-EGFP-RP105-transferred myocardium (Fig. 1). However, the EGFP signal in the myocardium treated with saline or no injection was not detectable (SO or I/R groups, respectively) (images not shown).

Ad-EGFP-RP105 transfection in vivo increases RP105 expression. To determine the anti-apoptotic and anti-autophagic action of RP105 during I/R injury, we performed the MIRI surgical procedure after adenovirus transfection as described above. As shown in Fig. 2A, the mRNA expression of RP105 was markedly downregulated 2.10-fold in comparison with the SO group. However, the transduction of Ad-EGFP-RP105 into the myocardium prior to I/R markedly increased the mRNA expression of RP105 by 73.38% ($p < 0.05$ vs. I/R + Ad-E) (Fig. 2A). A similar pattern of RP105 protein expression was analyzed by western blot analysis. Compared with Ad-EGFP transduction, Ad-EGFP-RP105 transfection significantly increased the RP105 protein level after MIRI (0.74 ± 0.03 in the I/R + Ad-E-R group vs. 0.14 ± 0.01 in the I/R + Ad-E group, $p < 0.05$) (Fig. 2B). In addition, the mRNA and protein levels of RP105 showed no statistical difference between the I/R and I/R + Ad-E groups.

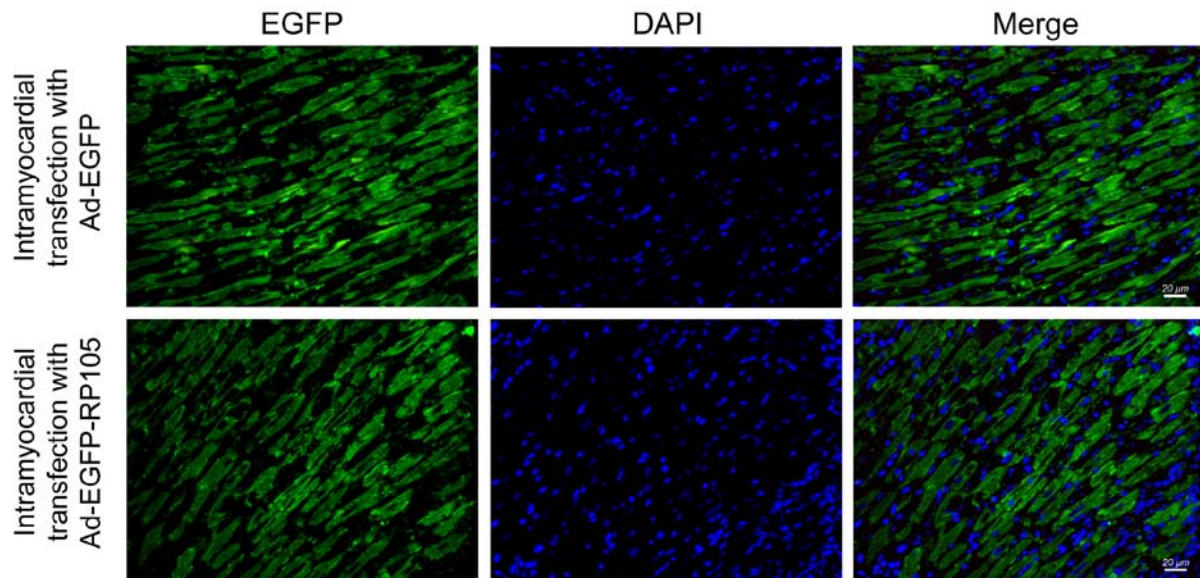


Figure 1. Efficiency of adenoviral transfection into the rat myocardium. Representative immunofluorescent images captured by immunofluorescence (IF) microscopy of the Ad-EGFP- and Ad-EGFP-RP105-transferred myocardium. Viral transfection was performed three days prior to IF microscopy. EGFP and the cardiomyocyte nuclei (DAPI) were labeled by green and blue fluorescence, respectively. Scale bar represents 20 μ m.

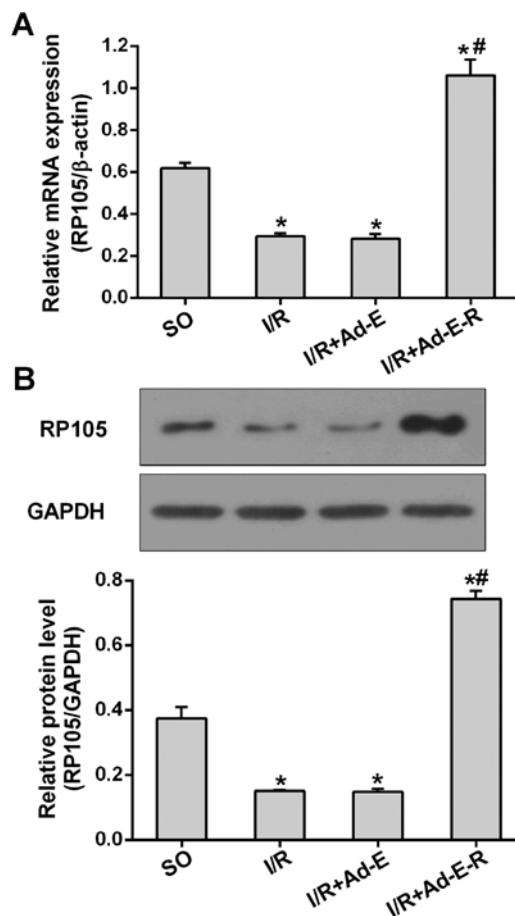


Figure 2. Ad-EGFP-RP105 transfection into the rat myocardium *in vivo* increases the expression of radioprotective 105 kDa protein (RP105). (A) mRNA expression of RP105 was detected by RT-qPCR. (B) Western blot analysis of RP105 expression. Upper panel, original representative western blots; lower panel, the corresponding densitometric analyses of RP105 protein expression. The results are presented as the means \pm SD (n=6 per group); * p <0.05 vs. sham operated (SO) group; # p <0.05 vs. the myocardial ischemia/reperfusion (I/R) group or the myocardial I/R with Ad-EGFP (I/R + Ad-E group) group. I/R + Ad-E-R group, the myocardial I/R with Ad-EGFP-RP105 group.

RP105 attenuates MIRI-induced pathological damage and elevations in myocardial enzyme activity. To determine whether antagonizing TLR4 with RP105 alleviates myocardial damage, myocardial histopathological analysis was performed as well as measurements of the activity of serum LDH and CK as the indices of injury in MIRI. H&E staining (x100 magnification) revealed that the structure of the myocardial tissues was completely preserved and remained organized in the sham-operated myocardium. However, the myocardial tissue from the rats in the I/R group demonstrated focal destruction of myocardial fibers with erythrocyte and neutrophil infiltration, enlarged intercellular spaces, extensive edema and myocyte necrosis. Pathological damage was markedly attenuated in the I/R myocardium transduced with Ad-EGFP-RP105 when compared with the I/R and I/R + Ad-E groups (Fig. 3A). With regard to the leakage of myocardial enzymes, only low activity levels of serum LDH and CK were detected in the SO group. The activity of LDH and CK in the other three I/R groups were all markedly increased relative to the SO group (Fig. 3B and C). However, the overexpression of RP105 significantly repressed the elevation of LDH and CK levels (I/R + Ad-E-R vs. I/R, p <0.05). In addition, the transduction of Ad-EGFP did not affect the myocardial leakage of LDH and CK in MIRI (I/R + Ad-E vs. I/R, p >0.05). These findings suggest that RP105 plays a cardioprotective role in MIRI.

RP105 represses the apoptosis of cardiomyocytes in MIRI. To determine the anti-apoptotic potency of RP105 in MIRI, we first detected the number of TUNEL-positive cardiomyocytes in order to evaluate the apoptosis of cardiomyocytes. The numbers of TUNEL-positive myocytes in all three I/R groups were significantly higher than that shown in the SO group (Fig. 4A). However, the overexpression of RP105 markedly reduced the AI compared with that in the Ad-EGFP-transduced tissues ($14.33 \pm 0.59\%$ in the I/R + Ad-E-R group vs. $24.32 \pm 1.13\%$ in the I/R + Ad-E group, p <0.05) (Fig. 4B). In agreement with

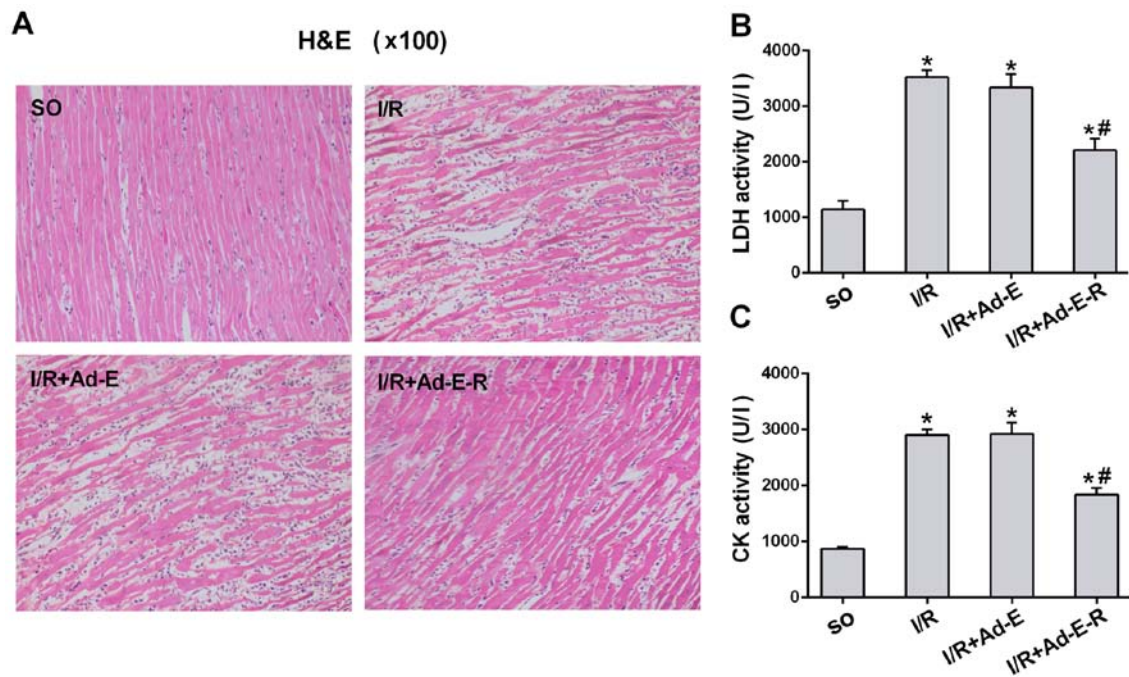


Figure 3. Radioprotective 105 kDa protein (RP105) ameliorates myocardial ischemia/reperfusion (I/R) injury (MIRI)-induced pathological damage and elevations in myocardial enzyme activity. (A) Representative images of the rat myocardium prepared for histological evaluation under a light microscope with H&E staining (original magnification, x100). (B) The activity of serum lactate dehydrogenase (LDH). (C) The activity of serum creatine kinase (CK). Data are expressed as the means \pm SD (n=6 per group); *p<0.05 vs. sham operated (SO) group, #p<0.05 vs. the myocardial I/R group or the myocardial I/R with Ad-EGFP (I/R + Ad-E group). I/R + Ad-E-R group, the myocardial I/R with Ad-EGFP-RP105 group.

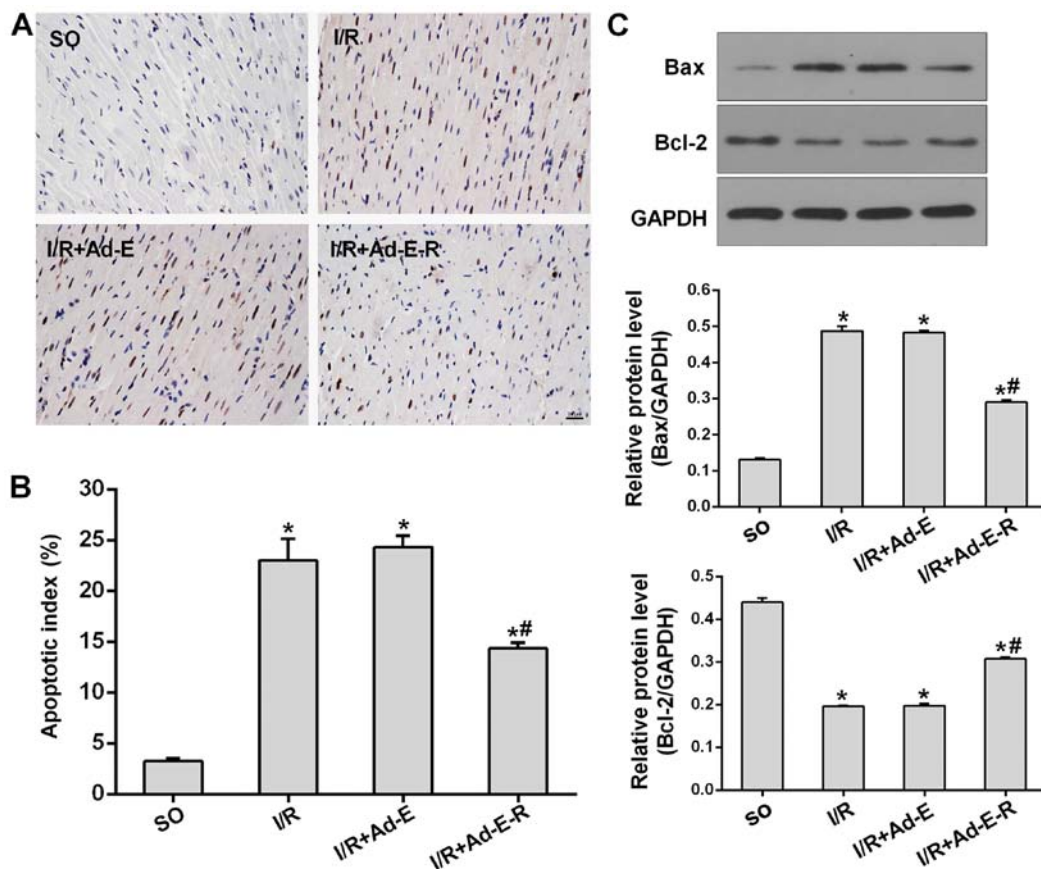


Figure 4. Radioprotective 105 kDa protein (RP105) represses the apoptosis of cardiomyocytes in myocardial ischemia/reperfusion (I/R) injury (MIRI). (A) Representative photomicrographs of TUNEL staining and (B) the quantification of the mean apoptotic index (AI) in each group. Scale bar represents 20 μ m. (C) Original representative western blots of Bax and Bcl-2 protein and quantitative analysis of the relative protein levels of Bax and Bcl-2. The results are expressed as the means \pm SD (n=6 per group); *p<0.05 vs. sham operated (SO) group, #p<0.05 vs. the myocardial I/R group or the myocardial I/R with Ad-EGFP (I/R + Ad-E group). I/R + Ad-E-R group, the myocardial I/R with Ad-EGFP-RP105 group.

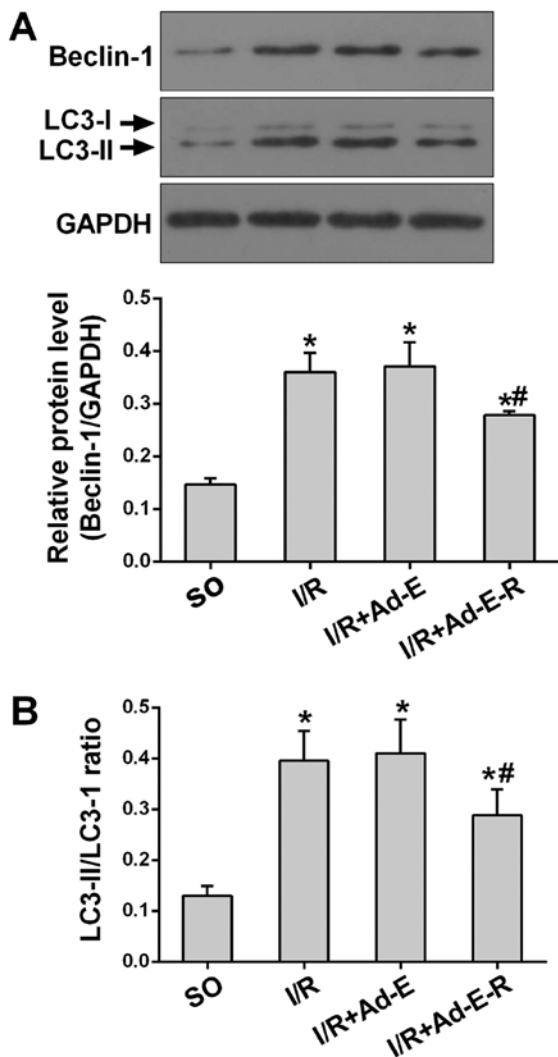


Figure 5. Radioprotective 105 kDa protein (RP105) suppresses autophagic signaling in myocardial ischemia/reperfusion (I/R) injury (MIRI). (A) Original representative immunoblots of Beclin-1, light chain 3 (LC3)-I and LC3-II are presented as well as quantitative analysis of Beclin-1 protein levels. (B) The ratio of LC3-II/ LC3-I. Data are presented as the means \pm SD ($n=6$ per group). * $p<0.05$ vs. sham operated (SO) group, # $p<0.05$ vs. the myocardial I/R group or the myocardial I/R with Ad-EGFP (I/R + Ad-E group). I/R + Ad-E-R group, the myocardial I/R with Ad-EGFP-RP105 group.

the results of the TUNEL assay, RP105 overexpression also led to the limitation of apoptotic activation in MIRI. This was evidenced by a 39.92% reduction in the levels of the pro-apoptotic Bax protein, as well as inversely a 1.55-fold upregulation of the levels of the anti-apoptotic Bcl-2 protein relative to that in the Ad-EGFP-transduced tissues (Fig. 4 C; $p<0.05$). Additionally, Ad-EGFP transduction had no significant effect on the percentage of TUNEL-positive cells and apoptotic activation (I/R + Ad-E group vs. I/R group, $p>0.05$).

RP105 suppresses autophagic signaling during MIRI. Beclin-1 and LC3 act as two indispensable mediators which regulate autophagic signaling. To determine whether antagonizing the TLR4 pathway with RP105 attenuates the autophagic process in I/R injury, we detected alternations in Beclin-1 and LC3 (LC3-II and LC3-I) protein expression by western blot analysis (Fig. 5A and B). As shown in Fig. 5, only low levels of Beclin-1 protein

and a low LC3-II /LC3-I ratio were measured in the SO group. Myocardium samples from all three I/R groups demonstrated elevated levels of Beclin-1 protein and the LC3-II/LC3-I ratio relative to the SO group. However, overexpression of RP105, markedly reduced the levels of Beclin-1 and the LC3-II/LC3-I ratio by 24.98 and 29.73%, respectively (I/R + Ad-E-R group vs. I/R + Ad-E group, $p<0.05$) (Fig. 5A and B). Ad-EGFP transduction had no significant effect on the levels of autophagic signaling (I/R + Ad-E group vs. I/R group, $p>0.05$).

RP105 reduces the expression of TLR4, NF- κ B, IL-6 and TNF- α in MIRI. To determine whether RP105 modulates the activity of the transcription factor NF- κ B and the production of pro-apoptotic cytokines (IL-6 and TNF- α) through antagonizing TLR4, we detected the levels of TLR4, NF- κ B/p65, p-NF- κ B/p65 (p-p65), IL-6 and TNF- α by western blot analysis (Fig. 6 A-C). Compared with the SO group, the rat myocardium from all three other I/R groups demonstrated increased protein levels of TLR4, p-p65, IL-6 and TNF- α . However, RP105 overexpression markedly suppressed the elevated levels of TLR4, p-p65, IL-6 and TNF- α by 44.27, 34.77, 51.51 and 45.63%, respectively (I/R + Ad-E-R group vs. I/R + Ad-E group, $p<0.05$), and exerted no effect on the total p65 protein level. Ad-EGFP transduction had no significant effect on the expression levels of those proteins (I/R + Ad-E group vs. I/R group, $p>0.05$).

Discussion

Accumulating evidence indicates that apoptosis- and autophagy-associated myocyte death play key roles in the induction of MIRI (9,19,27). Our present study demonstrated that the overexpression of RP105 via a recombinant adenoviral vector is a promising cardioprotective strategy to induce anti-apoptotic and anti-autophagic effects in MIRI *in vivo*. Firstly, we showed that intramyocardial injection with adenoviral vectors prior to the I/R procedure successfully led to the overexpression of RP105 in rat hearts. Secondly, we proved that the overexpression of RP105 significantly contributed to fewer numbers of TUNEL-positive cardiomyocytes and inhibited apoptotic and autophagic signaling in comparison with rats subjected to saline or Ad-EGFP injection in MIRI. These outcomes were accompanied by the attenuation of I/R injury-induced myocardial pathological damage and reduced leakage of LDH and CK. Finally, mechanistic studies demonstrated that the overexpression of RP105 markedly inhibited the activation of the TLR4/NF- κ B pathway, and reduced the expression of pro-apoptotic cytokines IL-6 and TNF- α . Taken together, these findings strongly confirm a cardioprotective effect of RP105, as it protects the myocardium against apoptosis and autophagy in MIRI.

Previous studies have shown that TLR4 plays a critical role as a pivotal sensor in inducing cell apoptosis during MIRI (19,20). By contrast, TLR4 deficiency markedly attenuates I/R-induced myocardial apoptosis (7). However, limited information is available regarding the molecular mechanism underlying TLR4-mediated intracellular signaling in I/R-induced myocardial apoptosis *in vivo*. The transcription factor NF- κ B acts as the principal downstream signaling target of TLR4 (28). The phosphorylation of p65, an impor-

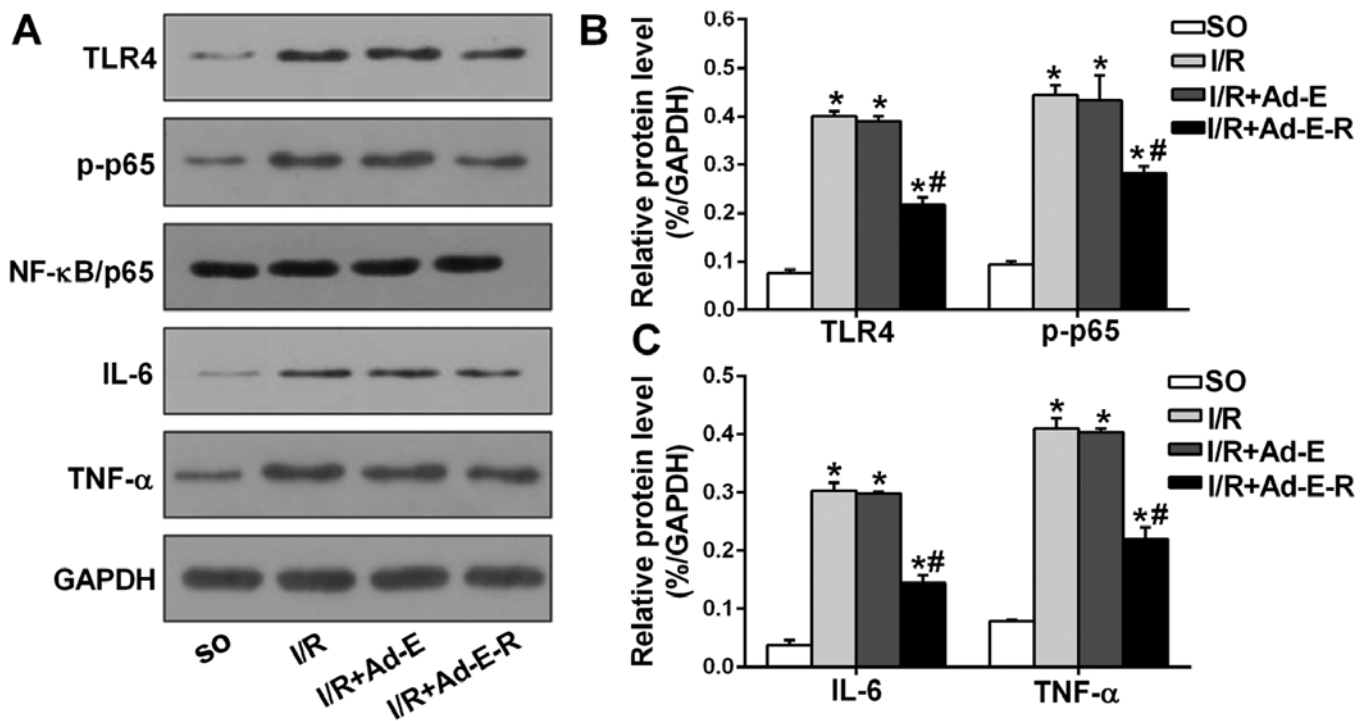


Figure 6. Radioprotective 105 kDa protein (RP105) reduces the expression levels of Toll-like receptor 4 (TLR4), nuclear factor- κ B (NF- κ B), interleukin 6 (IL-6) and tumor necrosis factor α (TNF- α) in myocardial ischemia/reperfusion (I/R) injury (MIRI). (A) Representative western blots of TLR4, NF- κ B/p65, p-p65, IL-6 and TNF- α . (B) Relative protein levels of TLR4 and p-p65. (C) Relative protein levels of IL-6 and TNF- α . Values are presented as the means \pm SD (n=6 per group), *p<0.05 vs. sham operated (SO) group, #p<0.05 vs. the myocardial I/R group or the myocardial I/R with Ad-EGFP (I/R + Ad-E group). I/R + Ad-E-R group, the myocardial I/R with Ad-EGFP-RP105 group.

tant subunit of NF- κ B, plays a central role in promoting target gene expression, including the cytokines IL-6 and TNF- α , which are the main cytokines involved in the initiation of apoptosis (18,29). Shen *et al* have confirmed that the activity of TLR4/NF- κ B promoted the production of IL-6 and TNF- α , and subsequently led to the enhancement of the apoptotic cascade in the setting of hepatic I/R injury (18). Herein, our data showed, for the first time to the best of our knowledge, that the TLR4, p-NF- κ B/p65, and IL-6/TNF- α signaling pathways were upregulated in MIRI, and therefore promoted apoptosis. These results, taken together with other findings (19,29), regarding the TLR4/NF- κ B-IL-6 (TNF- α)-mediated apoptotic mechanism broaden our understanding of the multiple biological functions of TLR4, beyond being a canonical inflammatory sensor in MIRI.

Another such response that is modulated through TLR4-mediated signaling which may play significant role in the pathogenesis of MIRI is autophagy. Autophagy is an evolutionarily conserved process of cellular catabolism, mainly modulated by two indispensable regulatory proteins, Beclin-1 and LC3. Previous findings have shown that autophagy is regulated by a TLR4-mediated mechanism, and further aggravates hepatic I/R injury (18). It has been observed that NF- κ B is mainly activated by TLR4. Notably, independent of its pro-inflammatory and pro-apoptotic effects, NF- κ B also acts as an important mediator in regulating autophagy. Zeng *et al* demonstrated that the phosphorylation of NF- κ B/p65 aggravates myocardial damage through promoting Beclin-1-modulated autophagy in I/R injury (27). The specific repression of p-NF- κ B/p65 expression was shown to reduce

Beclin-1 expression, and subsequently limit autophagy in MIRI (27). In the present study, we showed that the activity of the TLR4/p-NF- κ B/p65 signaling pathway strongly elevated autophagic signaling in MIRI. The new contributing factor of TLR4-mediated autophagy further widens its pathogenic action, and especially confers a potent therapeutic approach for reducing MIRI.

As autophagy and apoptosis are both induced by the activity of TLR4 signaling pathway, we hypothesized that selective inhibition of TLR4 and the intercellular signaling pathway may be a valuable approach for the treatment of MIRI. RP105 is a key inhibitor of TLR4-mediated signaling, through which it attenuates pathophysiological processes in various cardiovascular disease settings such as myocardial infarction, atherosclerosis and post-interventional vascular remodeling (22-24,30). Previously, we showed that RP105 protected the myocardium against I/R injury, and the cardioprotective effect was attributed to inhibition of apoptosis (25). However, the molecular mechanism underlying the inhibitory effect of RP105 on the apoptosis of myocytes during I/R injury remains largely unknown. The novel contribution of RP105 in regulating autophagy also remains unrecognized. In the present study, we validated that RP105 overexpression alleviated myocardial apoptosis induced by I/R injury. The effect of RP105 on the suppression of autophagic process was also expounded for the first time, to the best of our knowledge. We demonstrated that the potential mechanisms of protection closely correlated with the inactivation of the TLR4/NF- κ B pathway. Our findings therefore demonstrated the anti-apoptotic and anti-autophagic effects of RP105

and suggested a possible mechanism underlying these effects in MIRI. Notably, previous findings manifested the dichotomous role of RP105 in mediating TLR4 (31), although recently it has been validated that RP105 deficiency significantly aggravated cardiac dysfunction by amplifying the TLR4 pathway in AMI (23). In light of the above-mentioned observations and the evidence obtained from our previous study, we proposed that RP105 may be a therapeutic target of value in the treatment of MIRI by negatively mediating the TLR4 pathway. Further studies examining the crosstalk between autophagic and apoptotic functions of RP105 (in terms of how one affects the other or to determine whether they are independently linked) are warranted in order to determine how TLR4 signaling exerts potential therapeutic effects in the setting of MIRI.

Certain common factors and components coordinate and modulate the apoptotic and autophagic pathways (3,16). The activity of the apoptotic cascade may induce autophagy, whereas signals that reduce apoptosis also limit autophagy (32). For instance, anti-apoptotic Bcl-2 was shown to interact with the BH3 domain of Beclin-1 in order to modulate apoptosis and autophagy simultaneously and also maintained the inactivation of autophagy (33,34). A recent study demonstrated that the upregulation of Bcl-2 increased the interaction between Bcl-2 and Beclin-1, thereby leading to the downregulation of autophagy in the setting of hepatic I/R injury (18). Herein, we confirmed that RP105 attenuated myocardial autophagy through inhibiting the TLR4/NF- κ B pathway and therefore downregulated Beclin-1 in MIRI. On the other hand, we also speculated that the overexpression of RP105 followed by the increased expression of Bcl-2 may play an important role in inhibition of autophagy due to the enhanced binding between Beclin1 and Bcl-2.

These results have revealed the role of the TLR4/NF- κ B signaling pathway in inducing apoptosis and autophagy and therefore shown that modulating RP105 may be an effective strategy in ameliorating MIRI. However, further investigations into the precise mechanism that links the inactivation of the TLR4-mediated signaling pathway with inhibition of the apoptotic cascade and autophagic signaling are warranted. Moreover, with regard to the viewpoint that the contribution of autophagy in MIRI is subject to controversy (14), further research is still required in order to validate whether RP105 is a potential anti-autophagic target, and more specifically, for the attenuation of MIRI. In conclusion, our findings may have identified a novel method for limiting I/R-induced myocardial apoptosis and autophagy, provided innovative cardioprotective insights as well as a potential therapeutic target for the treatment of MIRI.

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