Increased expression of RP105 decreases cardiomyocytes hypoxia/reoxygenation injury through the TLR4/NF-κB signaling pathway

Qinqin Yu², Jun Yang¹², Jian Yang¹², Wusong Dong¹², Xinxin Li¹², Xin Guo², Rui Yang³

¹Department of Cardiology, The First College of Clinical Medical Sciences, China Three Gorges University, Yichang 443000, Hubei Province, China; ²Institute of Cardiovascular Diseases, China Three Gorges University, Yichang 443000, Hubei Province, China; ³Department of Medicine, University of Vermont, Burlington, VT, USA

Received October 27, 2015; Accepted December 25, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: Radioprotective 105 (RP105) is a kind of TLR4 homologue lacking an intracellular signaling domain (TIR domain) and specifically inhibiting TLR4-signaling. This study was to investigate the role of RP105 in the cardiomyocytes undergoing the hypoxia/reoxygenation (H/R) treatment and to examine the underlying mechanism. Neonatal rat cardiomyocytes were infected with recombinant adenoviral vectors containing rat RP105 or GFP. GFP served as vector control. Untransduced cardiomyocytes were as H/R control. Two days after transduction, cardiomyocytes were subjected to 2 h anoxia followed by 4 h reoxygenation. Cardiomyocyte damage was evaluated by cell viability, lactate dehydrogenase (LDH), creatine kinase (CK) and creatine kinase-MB (CK-MB). The levels of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the culture medium were measured. The mRNA and protein expression of RP105, TLR4, MyD88 and NF-κB were detected by real-time PCR and western blot analysis. Here, we show that the levels of RP105 are significantly increased in cardiomyocytes transduced with Ad-RP105. Overexpression of RP105 enhances cell viability and reduces the release of LDH, CK and CK-MB. Furthermore, RP105 transduction suppresses TLR4, MyD88 and NF-κB expression and attenuates the release of TNF-α and IL-6 caused by H/R injury in the culture medium. We propose that RP105 protects cardiomyocytes against H/R injury, which is likely mediated by attenuation of inflammatory cytokine production in a TLR4/NF-κB signalling dependent manner.

Keywords: RP105, H/R injury, TLR4, NF-κB

Introduction

Toll-like receptor 4 (TLR4) participates in the innate immune and inflammatory responses, which is linked to myocardial ischemia/reperfusion (I/R) injury [1]. TLR4-mediated signalling predominately activates nuclear factor-κB (NF-κB), which is a crucial transcription factor determining innate immune and proinflammatory cytokines gene expression [2]. Several studies have shown a reduced myocardial I/R injury by antagonizing TLR4-mediated signaling pathway or utilizing TLR4 deficiency system [3-7]. Mice deficient in TLR4 have attenuated NF-κB binding activity under myocardial I/R injury [4, 6]. Specific blockade of TLR4 suppresses NF-κB activation, resulting in a reduction in the production of inflammatory cytokines [8]. NF-κB is a transcription factor that is not only involved in activating corresponding target genes, but in the myocardial I/R injury, it has also been linked to regulate inflammation [9].

Increasing number of accessory molecules has been shown to play an important role in TLR4 signaling, which opens the opportunities in manipulating TLR4 pathway with these proteins [10]. Among these proteins, radioprotective 105 (RP105), a homolog of TLR4 lacking the intracellular signaling domain (TIR domain), is expressed on human and mouse antigen-presenting cells and specifically inhibits TLR4-mediated signalling [11, 12]. RP105, along with its helper molecule myeloid differentiation factor 1 (MD1), bind to the TLR4 ectodomain and prevents ligand binding to the TLR4, thereby competitively inhibiting TLR4 signaling [12-14].

Here we show that infection of cardiomyocytes with adenovirus expressing RP105 significantly
Increased expression of RP105 decreases cardiomyocytes H/R injury

suppresses the inflammatory response during H/R conditions. Our results suggest that targeting TLR4 pathway using RP105 could be protective against H/R in cardiomyocytes and, consequently, of potential therapeutic value.

**Materials and methods**

**Preparation of adenoviruses**

Two recombinant adenoviral vectors based on the GV315-EGFP vector (purchased from Shanghai GeneChem Co.Ltd) were respectively constructed, expressing the rat RP105 gene and the green fluorescence protein (GFP) gene are under the control of the rat CMV promoter.

Cardiomyocytes were infected with Ad-GFP adenovirus at various multiplicities of infection (MOI) for 48 h in Dulbecco’s modified Eagle’s medium (DMEM). GFP expression was assessed with a fluorescence microscope.

**Cell culture**

The investigation conformed to the NIH Guide for the Care and Use of Laboratory Animals (NIH publication no. 85-23, revised 1996), and all study procedures were approved by the Animal Care Committee of Wuhan University. Neonatal rat cardiomyocytes were obtained from the ventricles of 1- to 3-day-old Sprague-Dawley rats and cultured as described elsewhere. Briefly, the ventricles were harvested and minced into pieces approximately 1 mm³. The tissue fragments were isolated by digestion with 0.125% trypsin and 0.08% collagenase I for 5 times at 37°C. To reduce fibroblast contamination, cells were preplated for 1.5 h in DMEM containing 10% fetal bovine serum (FBS), 100 U/ml penicillin and 0.1 µg/ml streptomycin. Then non-adherent cells were seeded in 6-well plates at a density of 1 × 10⁶ cells/ml, incubating for 72 h before the experiment.

**Hypoxia/reoxygenation**

According to a previously described method, 20 hypoxia was achieved by placing the confluent beating cardiomyocytes in a hypoxia chamber filled with 95% N₂-5% CO₂ at 37°C for 2 h, and then the cells were reoxygenated with a gas mixture of 95% O₂-5% CO₂ for 4 h in DMEM with 10% FBS.

**Cell viability assay**

The Dojindo cell counting kit 8 (CCK8) assay (Tokyo, Japan) was used to assess the cell viability, and the experimental procedure was based on the manufacturer’s instructions. The cardiomyocytes were seeded into a 96-plate at 1 × 10⁶ cells per well and incubated for 72 h before being treated as described above. The absorbance of each well was read at a 450 nm with a reference at 630 nm on a microplate reader (Bio-Rad Laboratories, Hercules, CA). The percentage of cell viability was calculated according to the following equation: % cell viability = (mean absorbance in test wells)/(mean absorbance in control well) × 100.

**Measurement of LDH, CK and CK-MB**

The extent of cell injury was monitored by measuring the concentration of LDH, CK and CK-MB contained in culture medium. The activities of LDH and CK were measured according to manufacturers’ manual (JianCheng Bioengineering Institute, Nanjing, China). The supernatant was collected to measure the CK-MB level, through using commercially available kits (Elabscience Biotechnology Co., Wuhan, China).

**Western blot analysis**

Total proteins were obtained from cardiomyocytes by using RIPA lyses buffer. Fifty micrograms of denatured proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane. To detect the proteins of interest, an anti-RP105 antibody (1:300, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), an anti-TLR4 antibody (1:800, Bioworld Technology Co., Minnesota, USA), an anti-MyD88 antibody (1:700, Bioworld Technology), an anti-NF-κB antibody (1:5,000, Hangzhou GoodHere Biotechnology Co., Ltd., Hangzhou, China) were used to blot the membrane. Blots were then incubated with horseradish peroxidase-conjugated IgG secondary antibody, and the signal was detected with an ECL kit.

**Quantitative RT-PCR analysis**

Total RNA was extracted from cells by the Trizol reagent (Invitrogen Corp.) according to the manufacturer’s protocol, and then it was reversely
Increased expression of RP105 decreases cardiomyocytes H/R injury

transcribed into cDNA from total-RNA with oligo (dt). RT-PCR analysis was performed on the ABI ViiA7 Real Time PCR System by using SYBR green as the detection fluorophore. The primer sequences used were as follows: RP105, forward primer 5'-TGGGGACATTTGAGGACATT-3' and reverse primer 5'-GCTGTTAGGTCCAGCTCCTG-3'; TLR4, forward primer 5'-TGCTCAGACATACTGGCAGTTTC-3' and reverse primer 5'-CTGGACTATGCTTTTCCAACACC-3'; MyD88, forward primer 5'-GAGATCCGCGAGTTTGAGAC-3' and reverse primer 5'-CTTGAGTTTCAGGTGGCTTGA-3'; NF-κB, forward primer 5'-GAGGTGTCGTCCCATCGTAG-3' and reverse primer 5'-GAGGTGTCGTCCCATCGTAG-3'; β-actin, forward primer 5'-CACGATGGGAGGGCGGACTCATC-3' and reverse primer 5'-TAAAGACCTCTATGCCAACACAGT-3'. β-actin was used as internal control. The fold change of mRNA was calculated based on the method of the 2^△△Ct.

Detection of TNF-α and IL-6 by ELISA

The levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in the culture medium were detected by using the commercial enzyme-linked immunosorbent assay (ELISA) kits, as indicated by the manufacturer (Elabscience Biotechnology Co., Wuhan, China).

Figure 1. Adenoviral infection of Ad-RP105 induces overexpression of RP105 in normoxia cultured rat cardiomyocytes. (A) Cardiomyocytes were infected with Ad-GFP for 48 h at the indicated MOI and observed by fluorescence microscopy (magnification: × 100). (a) No infection (blank). (b-f) Cardiomyocytes were infected with the virus at (b) MOI 5, (c) MOI 20, (d) MOI 50, (e) MOI 100 and (f) 200. (B) Extracted mRNA from control cardiomyocytes, cardiomyocytes infected with Ad-GFP control and cells infected with Ad-RP105 were analyzed for RP105 expression by qPCR. Error bars represent the mean ± SD (n = 3). #P < 0.05, compared with the control group and the Ad-GFP group.
Increased expression of RP105 decreases cardiomyocytes H/R injury

Data analysis

All data are presented as mean ± SD. Statistical significance between groups was determined by using one-way ANOVA. A value of \( P < 0.05 \) was considered statistically significant.

Results

Adenoviral infection of Ad-RP105 induces overexpression of RP105 in normoxia cultured rat cardiomyocytes

Neonatal rat cardiomyocytes were infected with rat RP105 (Ad-RP105) or an adenovirus through encoding a GFP (Ad-GFP) for forty-eight hours. Figure 1Aa-Af shows neonatal rat cardiomyocytes were infected with a different MOI of Ad-GFP (Figure 1A). As expected, cardiomyocytes infected with Ad-RP105 had significantly increased levels of RP105 expression, as measured by Quantitative real-time PCR (qPCR) (Figure 1B). Therefore, Ad-RP105 vector infection induces RP105 overexpression in cardiomyocytes, and a MOI of 20 was used for the subsequent experiments.

Infection of Ad-RP105 in H/R cardiomyocytes induces overexpression of RP105

To determine whether infection of RP105 in H/R cardiomyocytes would induce overexpression of RP105, we infected cardiomyocytes with Ad-RP105. GFP served as infection control. Forty-eight hours after infection, we examined RP105 protein and mRNA levels. As expected, levels of RP105 were increased in the H/R + Ad-RP105 group compared with the H/R group and the H/R + Ad-GFP group. Interestingly, the levels of RP105 were significantly reduced in H/R cardiomyocytes compared with normoxia cells. In addition, compared with the control group, RP105 protein and mRNA levels markedly attenuated in the H/R group and the Ad-GFP + H/R group (Figure 2).

Overexpression of RP105 attenuates H/R-induced injury in cultured rat cardiomyocytes

To evaluate the effect of overexpression of RP105 on H/R-induced cellular injury, we assessed cell viability with CCK-8 assays and LDH, CK and CK-MB activity in control cardiomyocytes or cardiomyocytes infected with Ad-GFP or Ad-RP105 under normoxia or H/R conditions. Compared with the normoxic control group, the cell viability was significantly inhibited after H/R treatment (\( P < 0.05 \)) (Figure 3). Interestingly, under H/R conditions, the cell viability were significantly improved in cardiomyocytes infected with Ad-RP105 compared with control cardiomyocytes or cells infected with Ad-GFP (\( P < 0.05 \)) (Figure 3). Therefore, overexpression of RP105 attenuates H/R induced cell injury in neonatal rat cardiomyocytes.

Overexpression of RP105 reduces the expression of TLR4, MyD88 and NF-κB on protein and mRNA levels

To determine the involvement and potential causal role of RP105 in myocardial I/R injury, we examined the effect of overexpression of RP105 by transduction of RP105 on TLR4, MyD88 and NF-κB expression in control cardiomyocytes or cardiomyocytes infected with Ad-GFP or Ad-RP105 under normoxia or H/R conditions. QPCR and western blot showed that the expression of TLR4, MyD88 and NF-κB were significantly upregulated after H/R treatment compared with the normoxic control group (\( P < 0.05 \)). Interestingly, under H/R conditions, the
Increased expression of RP105 decreases cardiomyocytes H/R injury

expression of TLR4, MyD88 and NF-κB were significantly down-regulated in cardiomyocytes infected with Ad-RP105 compared with control cardiomyocytes or cells infected with Ad-GFP (P < 0.05). These results strongly demonstrate that overexpression of RP105 suppresses the expression of TLR4, MyD88 and NF-κB. The data also indicate that RP105 infection efficiency is sufficient to down-regulate TLR4, MyD88 and NF-κB expression in the cardiomyocytes (Figure 4).

Overexpression of RP105 inhibits the production of TNF-α and IL-6 induced by H/R in cardiomyocytes

Cardiomyocytes subjected to H/R had a significantly increase of TNF-α and IL-6 production in the culture medium compared with the normoxic control group (P < 0.05). Interestingly, under H/R conditions, TNF-α and IL-6 production was significantly attenuated in cardiomyocytes infected with Ad-RP105 compared with control cardiomyocytes or cells infected with Ad-GFP (P < 0.05). No significant difference in TNF-α and IL-6 production was observed between control cardiomyocytes and cells infected with Ad-GFP under H/R conditions. These results indicate that RP105 could significantly attenuate the levels of proinflammatory cytokine production (Figure 5).

Discussion

The novel findings in the present study are as follows. First, the adenoviral vector-mediated infection system could efficiently deliver RP105 into cardiomyocytes. Second, infection cells with RP105 significantly prevented the loss of cell viability resulting from H/R, and suppressed the release of LDH, CK, and CK-MB from injured cardiomyocytes. Third, the level of proinflammatory cytokines (TNF-α and IL-6) induced by H/R in cardiomyocytes were atten-
Increased expression of RP105 decreases cardiomyocytes H/R injury

Myocardial I/R injury is characterized by innate immune and inflammatory response mediated by TLRs [4-6]. It has been documented that the TLR4-mediated Myd88-dependent signalling pathway plays a key role in myocardial I/R injury [4, 6]. The TLR4 signaling pathway has been investigated thoroughly, mainly signaling through Myd88-mediated pathway to transcription factors NF-κB [15-16]. Activation of NF-κB translocates into the nucleus, where it stimulates corresponding target genes expression, many of which regulate inflammation [9]. Studies from our group have shown that TLR4 is up-regulated in the myocardial I/R injury pathological process [17]. In addition, other investigators have found that TLR4, Myd88 and NF-κB expression are increased in myocardial I/R models [5, 18]. Interestingly, several studies have shown that inhibition of TLR4 significantly reduces myocardial infarction size after myocardial I/R injury [5, 7]. This opens the question for us that whether RP105, an endogenous inhibitor of TLR4, can block TLR4 signaling during cardiomyocytes H/R injury.

Formal phylogenetic analysis demonstrates that RP105 specifically belongs to the TLR4 subfamily of TLRs [12, 13]. In contrast to TLR4, RP105 lacks a TIR domain which containing only 6-11 intracytoplasmic amino acids [12-14]. RP105, along with its helper molecule MD1, bind to the TLR ectodomain and prevents ligand binding to the TLR4 [12-14]. Thus, it is suggested that RP105 is actually an inhibitor of TLR4 signaling [12, 13]. Divancovic et al. have reported that RP105 expression directly mirrors that

Figure 4. Overexpression of RP105 reduces the expression of TLR4, MyD88 and NF-κB on protein and mRNA levels. (A) Control cardiomyocytes or cardiomyocytes infected with control Ad-GFP or Ad-RP105 for 48 hours under normoxia or H/R conditions were analyzed for TLR4, Myd88, NF-κB and GAPDH as loading control by immunoblotting. TLR4, MyD88 and NF-κB mRNA expression indicated in (B-D) was measured by qPCR. Error bars represent the mean ± SD (n = 3). #P < 0.05, compared with the control group; *P < 0.05, compared with the H/R group and the H/R + Ad-GFP group.
Increased expression of RP105 decreases cardiomyocytes H/R injury

Figure 5. Overexpression of RP105 inhibits the production of TNF-α and IL-6 induced by H/R in cardiomyocytes. Levels of IL-6 and TNF-α were measured by ELISA in culture supernatant from control cardiomyocytes or cardiomyocytes infected with control Ad-GFP or Ad-RP105 for 48 hours under normoxia or H/R conditions. Error bars represent the mean ± SD (n = 6). #P < 0.05, compared with the control group; *P < 0.05, compared with the H/R group and the H/R + Ad-GFP group.

of TLR4 on antigen-presenting cells [12]. Others have shown RP105 presence on vascular smooth muscle cells (VSMC) and smooth muscle cells of the airway [19]. Our study shows that RP105 is also expressed by cardiomyocytes. Recently, RP105 has been reported as a critical regulator of vascular remodeling via its TLR4 inhibitory function [19]. However, the role of RP105 in myocardial I/R injury remains unclear at this point. To evaluate the role of overexpression of RP105 in the TLR4/MyD88-mediated NF-κB activation pathway, we infected neonatal rat ventricular myocytes with either RP105 or GFP.

The measures of cell viability and level of LDH, CK, and CK-MB are usually used as indicators of cardiomyocyte injury. LDH, CK and CK-MB are three typical myocardium injury biomarkers, which are often released from ischemic cardiomyocytes during myocardial infarction and other ischemic injury [20]. Several studies have reported that cardiomyocytes H/R injury can also significantly increase the activity of LDH, CK and CK-MB [21, 22]. We found that pretreatment with Ad-RP105 markedly improved cell viability and reduced the levels of LDH, CK, and CK-MB in cardiomyocytes subjected to H/R, implying that RP105 could have a beneficial effect on cardiomyocytes exposed to H/R.

To determine the involvement and potential causal role of RP105 in myocardial I/R injury, we examined the effect of overexpression of RP105 by infection of RP105 on TLR4, MyD88 and NF-κB expression in control cardiomyocytes or cardiomyocytes infected with Ad-GFP or Ad-RP105 under normoxia or H/R conditions. We analyzed the levels of TLR4, MyD88 and NF-κB via western blotting as well as qPCR. Interestingly, the levels of TLR4, MyD88 and NF-κB are suppressed during RP105 overexpression, implicating that infection of RP105 increases the negative regulator of TLR4-mediated MyD88-dependent signaling pathway, and decreases expression of TLR4, which appears to be one of the mechanisms responsible for cardioprotective against H/R injury.

It is widely accepted that activation of the TLR4/NF-κB signaling directly and indirectly correlates to the releasing of proinflammation factor TNF-α and IL-6 in myocardial I/R injury. Other investigators and we have reported that myocardial I/R significantly increases the level of TNF-α and IL-6 [7, 17, 23]. These proinflammatory cytokines appear to be directly involved in the progression of myocardial I/R injury [24]. Overexpression of RP105 blocks TLR4 downstream signal pathways after cardiomyocytes H/R injury. The activation of NF-κB is interrupted, and the production of inflammatory cytokines are reduced. The present results showed that RP105 attenuated TLR4, strongly inhibited the expression of TNF-α and IL-6 after cardiomyocytes undergoing H/R by inhibiting NF-κB. Similarly, it has been reported that TLR4 mediates NF-κB signaling and initiates inflammatory responses [8, 9].

Therefore, our data suggest a novel cardioprotection mechanism in I/R injury, in which overexpression of RP105 specifically inhibits TLR4/MyD88-dependent NF-κB signaling pathway and subsequently suppresses inflammatory responses.
Increased expression of RP105 decreases cardiomyocytes H/R injury. Thus, we suggest a potential therapeutic approach against H/R injury by utilizing RP105 as a tool to inhibit TLR4 signaling pathway. So, we conclude that RP105 protects cardiomyocytes against H/R injury by inhibiting inflammation.

Acknowledgements

We thank Cardiovascular Research Institute of Wuhan University for providing technical assistance. We would like to thank Rui Yang for assistance with preparation of manuscript. This work was supported by the National Natural Science Foundation of China (Grant No. 81170133, 81200088) and Hubei Province’s Outstanding Medical Academic Leader program.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jun Yang, Department of Cardiology, The First College of Clinical Medical Sciences, China Three Gorges University, 183 Yiling Road, Yichang 443003, Hubei Province, China. Tel: +86-15971627440; Fax: +86-717-6482302; E-mail: yangjun@ctgu.edu.cn

References


Increased expression of RP105 decreases cardiomyocytes H/R injury


