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Recombinant adeno-associated virus serotype 9 with p65 ribozyme protects H9c2 cells from oxidative stress through inhibiting NF-κB signaling pathway

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Abstract

Background Oxidative stress is a major mechanism underlying the pathogenesis of cardiovascular disease. It can trigger inflammatory cascades which are primarily mediated via nuclear factor-κB (NF-κB). The NF-κB transcription factor family includes several subunits (p50, p52, p65, c-Rel, and Rel B) that respond to myocardial ischemia. It has been proved that persistent myocyte NF-κB p65 activation in heart failure exacerbates cardiac remodeling. **Mechods** A recombinant adeno-associated virus serotype 9 carrying enhanced green fluorescent protein and anti-NF-κB p65 ribozyme (AAV9-R65-CMV-eGFP) was constructed. The cells were assessed by MTT assay, Annexin V–propidium iodide dual staining to study apoptosis. The expression of P65 and P50 were assessed by Western blot to investigate the underlying molecular mechanisms. **Results** After stimulation with H_2O_2 for 6 h, H9c2 cells viability decreased significantly, a large fraction of cells underwent apoptosis. We observed a rescue of H9c2 cells from H_2O_2-induced apoptosis in pretreatment with AAV9-R65-CMV- eGFP. Moreover, AAV9-R65-CMV-eGFP decreased H_2O_2-induced P65 expression. **Conclusions** AAV9-R65-CMV-eGFP protects H9c2 cells from oxidative stress induced apoptosis through down-regulation of P65 expression. These observations indicate that AAV9-R65-CMV-eGFP has the potential to exert cardioprotective effects against oxidative stress, which might be of great importance to clinical efficacy for cardiovascular disease.

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Keywords: Cardiomyocytes; Adenovirus; R65 ribozyme; Apoptosis; NF-κB pathway

1 Introduction

It has been proved that oxidative stress causes numerous biological effects ranging from alternation in signal transduction and gene expression to mutagenesis and promotes apoptosis.[1] It is well known that oxidative stress plays a significant role in the pathogenesis of various cardiovascular diseases including myocardial ischemia, arteriosclerosis, cardiomyopathy, transplant rejection, and heart failure.[2] Cardiomyocyte apoptosis is shown to be a highly regulated program of cell death causes loss of contractile tissue, compensatory hypertrophy and reparative fibrosis, all of which contribute to the development of cardiovascular diseases.[3] Thus, restraining the cardiomyocyte apoptosis induced by oxidative stress can result in improved prognosis of cardiovascular diseases. Oxidative stress can trigger inflammatory cascades which are primarily mediated via nuclear factor-κB (NF-κB).[4,5] NF-κB is a pivotal transcription factor that regulates the expression of numerous cellular genes, particularly those involved in the inflammatory response. Activation of NF-κB induces expression of a variety of gene products of which cytokines, chemokines, and adhesion molecules are implemented in IR injury.[6,7] NF-κB has been shown to play a key role in oxidative stress.

The NF-κB family has five subunits—p65, RelB, c-Rel, p50, and p52—that form homo- or hetero-dimers. Under resting conditions, inactive NF-κB dimers (classically p65/p50) are bound to inhibitor of κB (IκB) in the cytoplasm, whereas on stimulation, IκB kinase (IKK)-mediated IκB phosphorylation results in IκB ubiquitination and nuclear translocation of NF-κB.[8] Study suggested that targeted deletion of NF-κBp50 results in enhanced cardiac remodeling and functional deterioration following myocardial infarction by increasing matrix remodeling and in-

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flammation.[9] NF-κB activation in the murine failing heart is primarily the p65 subunit, with negligible p50.[10] Targeted blockade of p65 in the heart may be a useful therapeutic strategy to maintain homeostatic responses to endoplasmic reticulum stress and ameliorate myocyte loss in the remodeling heart.[10]

Adeno-associated virus (AAV) is a small, nonpathogenic, replication-defective parvovirus with a single-stranded DNA genome. The use of AAV vectors has emerged as a novel method for gene therapy targeting human diseases owing to the nonpathogenic capability of these vectors for transducing nondividing cells and long-term transgene expression.[11] Recombinant adeno-associated virus serotype 9 (rAAV9) is highly efficient in transducing the murine heart at lower doses.[12,13] Ribozymes are catalytic RNA molecules that can cleave other RNA molecules in a target-specific manner, thereby down-regulating the expression of any pathogenic gene product, thus making them potentially a broad new class of therapeutic agents.[14] Therefore, we sought to determine whether blocking of NF-κB pathway by rAAV9 mediated enhanced green fluorescent protein and anti-NF-κB p65 ribozyme could ameliorates the necrosis and apoptosis of myocardial cell after oxidative stress.

2 Methods

2.1 Vector design, construction, and production

Vectors of AAV9-CMV-eGFP (enhanced green fluorescent protein) AAV9-R65-CMV-eGFP were purchased from Virovek (USA).

2.2 Cell culture

Rat embryonic cardiomyoblast-derived H9c2 cardiomyocytes were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). H9c2 cardiomyocytes were cultured in high glucose Dulbecco's modified Eagle's Medium (DMEM; HyClone, USA) supplement with 10% (v/v) fetal bovine serum (FBS; Gibco, USA) and 1% (v/v) penicillin streptomycin (Gibco, USA) at 37°C in humidified atmosphere of 5% CO_2. For all experiments, cells were seeded at an appropriate density according to the experimental design and were grown to reach 70%–80% confluence before experiments were performed.

2.3 Cell viability assay

Cellular toxicities were analyzed in H9c2 cardiomyocytes using MTT methods. The H9c2 cells were seeded in 96-well plates. The cells were treated with 200 μmol/L H_2O_2 for 6 h. Subsequently, 20 μL MTT solution was added to each well. After 2 h incubation, the medium was carefully aspirated and the purple formazan crystals were solubilized with 100 μL DMSO. Optical density was measured at 570 nm and used to calculate the relative ratio of cell viability. Next, prior to H_2O_2 treatment, the cells were subjected to infection of rAAV9-eGFP and rAAV9-eGFP-R65. The multiplicities of infection (MOI) of rAAV9-eGFP and rAAV9-eGFP-R65 were selected from preliminary experiments (MOI = 6×10^5 vg/cell) demonstrating infection efficiency of more than 90% at the peak time (the peak time at the 5th day was selected from preliminary experiments).[15]

2.4 Flow cytometry analysis

H9c2 cardiomyocytes were cultured in 6-well plates for 24 h and then treated as described. Cells were harvested by trypsinization and rinsed with cold PBS twice. Cells were suspended by 200 mL binding buffer after centrifugation (4°C, 1000 r/min) for 5 min, and then treated with 10 mL Annexin V-FITC and 5 mL propidium iodide (PI) for 15 min at room temperature. Flow cytometric analysis of cells was performed with an Epics ALTRA Coulter flow cytometer. Cytometric analysis was repeated three times.

2.5 Western blot analysis

Nuclear extracts were obtained by NE-PER nuclear and cytoplasmic extraction reagents (pierce biotechnology). The protein content was determined using the DC protein assay kit (Bio-Rad). Equal amounts (10 μg) of protein fractions were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels (SDS-PAGE), in which the protein samples were evenly loaded. The proteins were then transferred onto nitrocellulose membranes in Trisglycine buffer at 100 V for 1 h. After blocking overnight in tris-buffered saline (TBS) containing 0.05% Tween (TBS-T) and 5% dry powdered milk, membranes were washed three times for 5 min each with TBS-T and then incubated overnight with appropriate primary antibodies (Abcam) at 4°C. Afterwards, they were washed thrice with TBS-T and incubated with secondary antibodies for 2 h at room temperature. Blots were developed with the enhanced chemiluminescence detection system according to manufacturer's instructions.

2.6 Statistical analysis

Results are presented as mean ± SD. For tests of significance between the groups, one-way analysis of variance (ANOVA) was performed. Comparisons between two groups were performed using unpaired Student's *t* test. Differences were considered significant at $P < 0.05$. All data were performed in at least three independent experiments.

3 Results

3.1 Effect of rAAV9-eGFP and rAAV9-eGFP-R65 on cardiomyocytes viability

The viability of the myocytes was evaluated using the MTT test. No significant differences in cell viability were found between normal myocytes and myocytes treated with the indicated concentrations of rAAV9-eGFP and rAAV9-eGFP-R65 for 48 h (Figure 1), which indicates that the rAAV9-eGFP and rAAV9-eGFP-R65 inhibitory effect observed was not due to cytotoxicity.

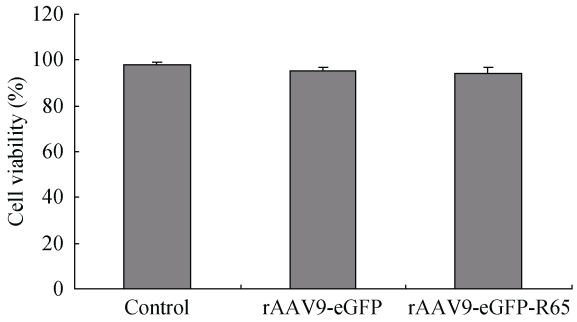

Figure 1. Effects of rAAV9-eGFP-R65 and rAAV9-eGFP on the viability of H9c2 cells. H9c2 cells were pretreated with rAAV9-eGFP and rAAV9-eGFP-R65 for five days. Cell viability were measured by the MTT assay. eGFP: enhanced green fluorescent protein; rAAV9: recombinant adeno-associated virus serotype 9.

3.2 AAV9-eGFP-R65 protects H9c2 cardiomyocytes from H_2O_2 induced cytotoxicity

In order to study whether rAAV9-eGFP-R65 was able to protect against cell injury induced by oxidative stress *in vitro*, we determined the H_2O_2-induced H9c2 cells toxity. H9c2 cardiomyocytes were pretreated with the indicated concentrations of rAAV9-eGFP and rAAV9-eGFP-R65 for five days then further treated with H_2O_2 (200 μmol/L), and cell viability measured by the MTT assay (Figure 2). The cells in control group were considered 100% viability. Viability of H_2O_2 treated cells significantly decreased to 58.32% in cell viability. The viability of cells after pretreatment with rAAV9-eGFP-R65 was around 82.28%. When the cells were pretreated with rAAV9-eGFP-R65, the cell viability increased significantly compared to H_2O_2 treated group and the rAAV9-eGFP treated group.

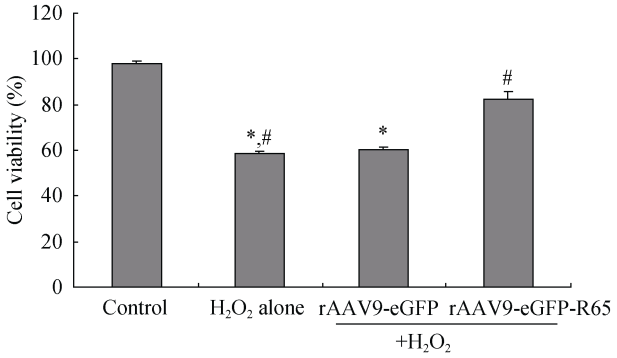

Figure 2. Protection of rAAV9-eGFP-R65 on H9c2 cells from H_2O_2-induced oxidative stress. Cell viability was measured by MTT assay. $^*P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with H_2O_2 treatment group. eGFP: enhanced green fluorescent protein; rAAV9: recombinant adeno-associated virus serotype 9.

3.3 rAAV9-eGFP-R65 protects H9c2 cardiomyocytes from H_2O_2 induced cell apoptosis

To confirm the effects of rAAV9-eGFP-R65 on H_2O_2-induced H9c2 cells apoptosis, the percentage of apoptotic cells was detected by Annexin V-FITC and PI double staining (Figure 3). As shown in Figure 3, the number of apoptotic cells in control group was 6.73%. After being treated with 200 μmol/L H_2O_2, about 43.21% of cells showed apoptosis characteristics. rAAV9-eGFP-R65 pretreatment decreased the percentage of the apoptotic cells compared with the groups that treated with H_2O_2 alone and rAAV9-eGFP. These results indicate that rAAV9-eGFP-R65 can protect H9c2 cardiomyocytes from H_2O_2 induced cell apoptosis.

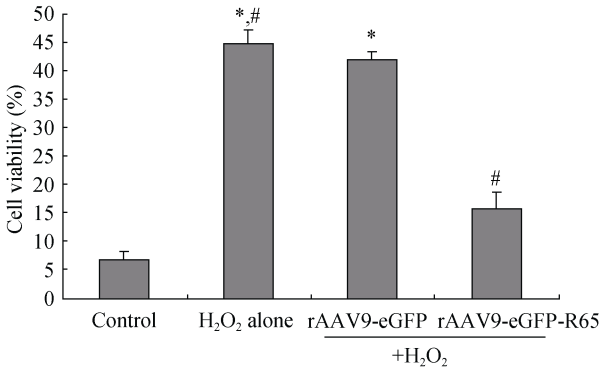

Figure 3. rAAV9-eGFP-R65 blocked apoptosis of H9c2 cells induced by H_2O_2. Apoptotic cells were detected by flow cytometry analysis. $^*P < 0.05$ compared with control, $^{\#}P < 0.05$ compared with H_2O_2 only treatment group. eGFP: enhanced green fluorescent protein; rAAV9: recombinant adeno-associated virus serotype 9.

3.4 Effect of rAAV9-eGFP-R65 treatment on H_2O_2 induced alterations of P65 and P50 proteins in H9c2 cardiomyocytes

To investigate whether rAAV9-eGFP-R65 could modulate the expression of P65 and P50 proteins, Western blot analysis was performed in H9c2 cardiomyocytes. The in-

tensity measurement for nucleus proteins were determined from the ratio of the integrated intensity of the P65 and P50 bands to the integrated intensity of the β-actin band in the same sample. As shown in Figure 4, H_2O_2 alone, pretreatment with rAAV9-eGFP and rAAV9-eGFP-R65 group resulted in an increase in P50 and P65 protein subunits in nucleus compared with the control cells. However, pretreatment with rAAV9-eGFP-R65, the expression of P65 decrease compared with the H_2O_2 alone cells, $P < 0.05$.

4 Discussion

Oxidative stress has been increasingly recognized as a contributing factor in promoting cell death in response to a variety of signals and pathophysiological condition.[16] It results from a disturbance in the balance between the production of reactive oxygen species (free radicals) and antioxidant defenses is discussed in relation to the pathophysiology of atherosclerosis and the main clinical manifestations of cardiovascular disease.[17] Many chemical and physiological inducers of oxidative stress cause apoptosis.[18] The main oxygen species responsible for oxidative stress include H_2O_2, the free radical superoxide anion (O^{2-}) and the hydroxyl radical (OH^-). In this study, H_2O_2 was used to induce apoptosis in H9c2 cells, as it is a well-established model to study oxidative stress-induced cardiomyocyte apoptosis. The MTT assay revealed that the cell viability induced by H_2O_2 increased with AAV9-R65 pretreatment. Similarly, flow cytometry analysis illustrated that AAV9-R65 pretreatment blocks H9c2 apoptosis induced by H_2O_2.

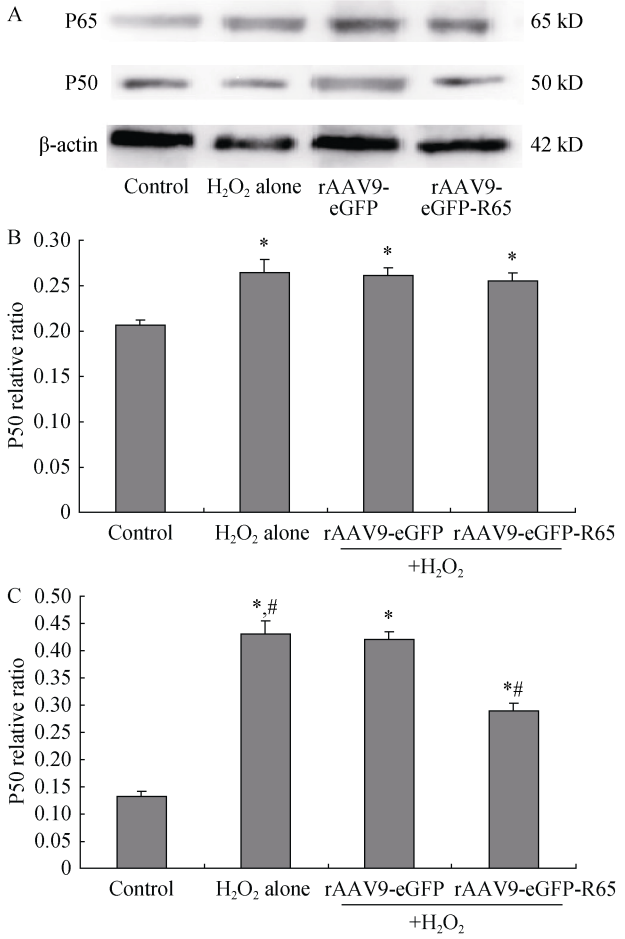

Figure 4. Effect of rAAV9-eGFP-R65 on the expression of proteins in H_2O_2-treated H9c2 cells. (A): The expression of P65, P50 in treated H9c2 cells was detected by Western blot analysis. (B&C): The ratio between the band of interest and the β-actin band. *$P < 0.05$ compared with control, #$P < 0.05$ compared with H_2O_2 only treatment group. eGFP: enhanced green fluorescent protein; rAAV9: recombinant adeno-associated virus serotype 9.

There are many reasons mediated oxidative stress that trigger inflammatory cascades. These events are primarily mediated via NF-κB.[4,5] NF-κB is a key transcription factor that regulates inflammatory processes.[19] Several recent studies reported that NF-κB is involved in the pathogenesis of heart failure.[20,21] Activation of NF-κB induces activation of genetic programs that lead to transactivation of cytokines, chemokines, and matrix metalloproteinases (MMPs), promoting inflammatory and fibrotic responses that participate in the progression of myocardial remodeling. Recent studies have suggested that inhibition of NF-κB activation may reduce the proinflammatory reactions and modulate the extracellular matrix and provide an effective approach to prevent adverse cardiac remodeling after myocardial infarction.[22,23]

AAV vector-mediated gene transfer is a novel method for the treatment of human disease because of its effective and stable transduction in target organs. Among various serotypes, rAAV9 is highly efficient in transducing the murine heart at lower doses.[12] We have proved rAAV9-eGFP-R65 can be stably and efficiently expressed in H9c2 cells without causing cell growth inhibition.[24] Ribozymes are catalytic RNA molecules that can cleave other RNA molecules in a target-specific manner, thereby down-regulating the expression of any pathogenic gene product, thus making them potentially a broad new class of therapeutic agents.[25] In our previous study, we have used rAAV9 vector to deliver the constitutive activation of the mitogen-activated protein kinase 1 gene into cardiomyocyte and it has been proved that AAV9 vector-mediated can efficiency transfect the aim gene, and the aim gene expression increased rapidly over the entire experimental period.[26] Our study found that rAAV9-eGFP-R65 down-regulated H_2O_2-induced expression of the protein P65. These results demonstrated that the antiapoptotic effect of rAAV9-eGFP-R65 was probably due to blocking the NF-κB signal.

In conclusion, our study demonstrates that rAAV9-eGFP-R65 rescues H9c2 cells from H_2O_2-induced cell death. Notably, the mechanism whereby rAAV9-eGFP-R65 prevents apoptosis appears to involve down-regulated H_2O_2-induced P65 expression. It is found that rAAV9-eGFP-R65 inhibited H_2O_2-induced NF-κB activation. Our current *in vitro* study suggests that rAAV9-eGFP-R65 exerts a protective effect against H_2O_2-induced apoptosis in H9c2 rat cardiomyocytes by preventing the transcription factor NF-κB. Future investigations will be necessary to determine the upstream molecular mechanisms as well as their *in vivo* relevance of our findings. Our data suggest that rAAV9-eGFP-R65 may have an important role in preventing oxidant-induced heart disease.

Acknowledgements

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