Overexpression of IkBα in cardiomyocytes alleviates hydrogen peroxide-induced apoptosis and autophagy through inhibiting NF-κB activation

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Research

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Abstract

**Background:** Inflammation and oxidative stress play a predominant role in the initiation and progression of ischemia/reperfusion (I/R) injury, of which nuclear factor kappa B (NF-κB) is a crucial mediator. Overexpression of the inhibitor of κB alpha (IkBα) gene is hypothesized to have protective effects against apoptosis and autophagy in cardiomyocytes subjected to hydrogen peroxide (H₂O₂) through inhibiting the NF-κB pathway.

**Methods:** The IkBα^{S32A,S36A} gene was transfected via adeno-associated virus serotype 9 (AAV9) delivery into neonatal rat ventricular cardiomyocytes (NRVMs) prior to H₂O₂ treatment. NRVMs were divided into control, H₂O₂, GFP +H₂O₂, IkBα+H₂O₂, and pyrrolidine dithiocarbamate (PDTC)+H₂O₂ groups. Nuclear translocation of NF-κB p65 subunit was evaluated by immunofluorescence and Western blot. Cell viability was assessed by Cell Counting Kit-8 assay. Supernatant lactate dehydrogenase (LDH) and intracellular malondialdehyde (MDA) were measured to identify H₂O₂-stimulated cytotoxicity. Apoptosis was determined by Annexin V-PE/7-AAD staining, and the mitochondrial membrane potential (ΔΨm) was detected by JC-1 staining. Western blot was used to detect apoptosis- and autophagy-related proteins.

**Results:** IkBα transfection significantly increased cell viability and ΔΨm, but decreased the supernatant LDH and cellular MDA levels in cardiomyocytes exposed to H₂O₂. Meanwhile, IkBα overexpression decreased H₂O₂-induced apoptosis by upregulating the Bcl-2/Bax ratio and reduced autophagy by downregulating the expression of Beclin-1 and the LC3-2/LC3-1 ratio. These effects partly accounted for the ability of IkBα to inhibit the NF-κB signaling pathway, as evidenced by decreases in p65 phosphorylation and nuclear translocation. Indeed, the effects of inactivation of NF-κB signaling with the specific inhibitor, PDTC, resembled the cardioprotective effects of IkBα during H₂O₂ stimulation.

**Conclusion:** IkBα overexpression can ameliorate H₂O₂-induced apoptosis, autophagy, oxidative injury, and ΔΨm loss through inhibition of the NF-κB signaling pathway. These findings suggest that IkBα transfection can successfully resist oxidative stress-induced damage through inhibiting NF-κB activation, which may provide a potential therapeutic target for prevention of myocardial I/R injury.

**Introduction**

Acute myocardial infarction (AMI) is the leading cause of death worldwide, and reperfusion therapy is the most effective treatment for AMI [1]. Paradoxically, the process of myocardial reperfusion also induces a series of adverse cardiac events such as inflammation, necrosis, apoptosis and autophagy, finally leading to myocardial ischemia/reperfusion (I/R) injury [2]. Recent evidence has suggested that excessive inflammation and oxidative stress play a predominant role in the initiation and progression of I/R injury [3, 4].

Nuclear factor kappa B (NF-κB) is an inflammatory inducer and redox-sensitive transcription factor in most cell types [5]. The p65/50 heterodimer, the most common pattern of NF-κB dimer, normally exists as
a component of inactive cytoplasmic complexes bound to the inhibitor of κB alpha (IkBα). Upon stimulation, IkBα is phosphorylated, and undergoes ubiquitylation and proteasomal degradation, subsequently leading to phosphorylation and nuclear translocation of the NF-κB p65 subunit [6]. Activated NF-κB then initiates the expression of corresponding target genes, many of which may regulate apoptosis, inflammation and autophagy [7].

However, whether NF-κB is protective or detrimental for cardiomyocyte apoptosis remains controversial [8]. Notably, our previous study indicated that p65 ribozyme could prevent cell apoptosis in H9C2 cardiomyocytes exposed to hydrogen peroxide (H₂O₂) [9]. Autophagy, an evolutionarily conserved form of “self-digestion”, plays dual roles in the heart [10]. Recent studies on autophagy have shown both the protective [11] and deleterious [12] roles of autophagy in cardiomyocytes against oxidative stress. Evidence has revealed a strong correlation between the modulation of NF-κB and the autophagic response [13, 14]. In addition, cross-talk between autophagy and apoptosis has been noted [15], and NF-κB is known to mediate the balance between autophagy and apoptosis [16].

Therefore, it is thought that NF-κB activation is the key point of I/R injury; thus, inhibiting NF-κB may be a targeted therapy for I/R injury. Phosphorylation of IkBα, the key inhibitor of the canonical NF-κB pathway, at Ser 32 and Ser 36 is necessary for its degradation, and any mutation of these two serine residues blocks IkBα degradation [6]. Recently, adeno-associated virus serotype 9 (AAV9) was demonstrated to be the best gene carrier due to its high efficiency in the heart [17]. H₂O₂, a common reactive oxygen species (ROS), is generally utilized to mimic I/R injury in vitro [12]. Thus, the IkBαS32A, S36A gene was transfected into cardiomyocytes via AAV9-mediated delivery to investigate the role of inhibition of the NF-κB pathway in H₂O₂-induced apoptosis and autophagy. Pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF-κB, was used as a positive control in this study.

**Materials And Methods**

**Ethics statement**

The experimental protocol was approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (No. IACUC-20180223-69). One-to three-day-old neonatal Sprague-Dawley (SD) rats were purchased from the Experimental Animal Center of Xinjiang Medical University, and handled in accordance with the recommendations in the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health.

**Reagents**

Briefly, rabbit anti-Bax polyclonal antibody (#2772) and rabbit anti-p65 (#8242), anti-p-p65 (#3033), anti-IkBα (#4812), anti-GFP (#2956), anti-Beclin-1 (#3495), and anti-LC3 #12741 (Abcam, Cambridge, UK) monoclonal antibodies were all obtained from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-Bcl-2 (ab196495), anti-Histone H3 (ab1791) and anti-β-actin (ab8227) polyclonal antibodies and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (ab205718) were obtained from Abcam (Cambridge, UK). RIPA
buffer and Halt™ Protease and Phosphatase Inhibitor Cocktail were obtained from Thermo Fisher Scientific (Waltham, MA, USA). Enhanced chemiluminescence (ECL) reagent and JC-1 were obtained from Millipore (Bedford, MA, USA). Trypsin, PDTC and bromodeoxyuridine (BrdU) were obtained from Sigma (St. Louis, MO, USA). Dulbecco’s modified Eagle’s medium (DMEM), Fetal bovine serum (FBS), and penicillin-streptomycin solution were obtained from Gibco (Grand Island, NY, USA). Collagenase II was obtained from Worthington (Minnesota, USA). H$_2$O$_2$ was obtained from Sangon (Shanghai, China).

Vector design

Recombinant AAV-9 vectors generated by a recombinant baculovirus (rBac)-based system in SF9 cells as previously described were purchased from Virovek (Hayward, CA, USA) [18]. Both recombinant AAV9 vectors were packaged as double-stranded DNA, and contained the enhanced green fluorescent protein (eGFP) gene (dsAAV9-GFP) or the IKBa$^{S32A, S36A}$ gene (dsAAV9-IkBα) driven by the human cytomegalovirus (CMV) promoter.

Isolation and culture of rat cardiomyocytes

The protocol for the isolation and purification of neonatal rat ventricular cardiomyocytes (NRVMs) was reported in our previous study [19]. Briefly, the hearts of 1- to 3-day-old SD neonatal rats were dissected and digested with 0.1% trypsin and 0.08% collagenase II. Following differential adhesion twice for 50 min each time, nonadherent cells were resuspended and cultivated in high-glucose DMEM containing 10% FBS, 1% penicillin-streptomycin, and 0.1 mM BrdU for 48 h. The medium was replaced every 48 h.

AAV9 transfection of cardiomyocytes

After 48 h of culture, NRVMs were transfected with dsAAV9-GFP or dsAAV9-IkBα as previously described [12]. Briefly, cells were first transfected with dsAAV9 (multiplicity of infection, MOI = 5×10$^6$ vg/cell) in serum-free medium, after which DMEM at an equal volume containing 20% FBS, 2% penicillin-streptomycin and 0.2 mM BrdU was added to every dish 3 h later. Images showing GFP were captured using a fluorescence inverted microscope (Leica DMI4000B, Wetzlar, Germany), and the green fluorescence intensities were analyzed using ImageJ software.

Experimental design and cell grouping

The experiment was designed is to explore whether AAV9-delivered IKBα$^{S32A, S36A}$ gene transfection could protect cardiomyocytes against H$_2$O$_2$-induced apoptosis and autophagy via inhibition of NF-κB activation. Cardiomyocytes were starved with serum-free DMEM for 12 h to ensure cell synchronization before H$_2$O$_2$ stimulation. The experimental cardiomyocytes were randomly divided into 5 groups as follows: the (1) control group, the primary cardiomyocytes in which were cultivated under normal conditions; (2) H$_2$O$_2$ control group (H$_2$O$_2$): the model control group, the primary cardiomyocytes in which were subjected to 100 μM H$_2$O$_2$ alone [12]; (3) GFP control group (GFP): the vector control group, the primary cardiomyocytes in which were transfected with dsAAV9-GFP virus for 5 days before being
subjected to 100 μM H₂O₂; (4) IκBα treatment group (IκBα): the treatment group, the primary cardiomyocytes in which were transfected with dsAAV9-IκBα virus for 5 days before being subjected to 100 μM H₂O₂; and the (5) PDTC treatment group (PDTC): the positive control group, the primary cardiomyocytes in which were pretreated with 100 μM PDTC for 60 min before being subjected to 100 μM H₂O₂.

**Measurement of cardiomyocyte vitality and cytotoxicity**

The Cell Counting Kit-8 (CCK-8; Dojindo, Japan) assay was used to assess cell viability. In brief, 2×10⁴ cells were seeded into each well of a 96-well plate and transfected with GFP or IκBα for five days. After the cells were exposed to H₂O₂, 10 μL of CCK-8 stock solution was added to each well and incubated at 37 °C for 2 h. The absorbance at 450 nm was measured with a GO microplate spectrophotometer (Thermo Fisher Scientific, USA). The extent of cell death was determined by quantifying lactate dehydrogenase (LDH) released into the culture supernatant with an LDH Kit (Jiancheng Bioengineering Institute, Nanjing, China). Intracellular malondialdehyde (MDA), an indicator of oxidative injury, was also measured using an MDA assay kit (Jiancheng Bioengineering Institute).

**Flow cytometry analysis**

Cell apoptosis was measured using PE Annexin V Kit I (BD Biosciences, NJ, USA). Briefly, cells were collected and resuspended in 1× binding buffer. Thereafter, the solution (1×10⁵ cells) supplemented with 5 μL of PE Annexin V and 7-AAD was incubated in the dark for 15 min at room temperature. The apoptotic cells were identified by flow cytometry (Beckman Coulter, CA, USA). All the experiments were performed in triplicate.

**Western blot analysis**

Nuclear and cytoplasmic proteins were extracted following the instructions of a Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, USA). Total proteins were extracted with RIPA buffer containing Halt™ Protease and Phosphatase Inhibitor Cocktail. The detection of phosphorylated p65 in the total lysate and the nuclear p65 to cytosolic p65 ratio were both applied to identify activation of the NF-κB signaling pathway [20]. Equal amounts of protein were loaded and separated on precast SDS-PAGE gels (Invitrogen, Grand Island, NY, USA) and transferred to Millipore PVDF membranes. After blocking with 5% skim milk, the membranes were blotted overnight with specific primary antibodies against p65 (1:1000), p-p65 (1:500), IκBα (1:1000), Bax (1:1000), GFP (1:1000), Beclin-1 (1:1000), LC3Ⅱ/Ⅰ (1:1000), Bcl-2 (1:1000), Histone H3 (1:1000), and β-actin (1:1000) at 4 °C, followed by incubation with anti-rabbit HRP secondary antibody (1:5000) at room temperature for 2 h. ECL solution was added to the membranes to visualize signals. β-actin and Histone H3 were regarded as loading controls. Images were captured and analyzed by Image Lab 4.0 software (Bio-Rad Laboratories, Hercules, CA, USA).

**Immunofluorescence**
Immunofluorescence was employed to identify H$_2$O$_2$-induced nuclear translocation of the NF-κB p65 subunit in cardiomyocytes. Briefly, 2×10$^5$ cells were seeded into confocal dishes. After H$_2$O$_2$ treatment, cardiomyocytes were fixed with 4% paraformaldehyde for 20 min and permeabilized with 0.25% Triton X-100 for 10 min. After blocking with 1% BSA for 1 h, cells were probed overnight with anti-p65 antibody (1:200) at 4 °C, and incubated with Alexa Fluor 594-labelled secondary antibody (Invitrogen, 1:200, labelled with red fluorescence) for 2 h at room temperature, followed by 10 min of DAPI staining of nuclei (labelled with blue fluorescence). Signals were detected using a confocal spectral microscope (Leica SP8, Germany).

Measurement of the mitochondrial membrane potential

JC-1 is an ideal fluorescent probe used to detect the mitochondrial membrane potential (ΔΨm) in cardiomyocytes. Briefly, a 10 nmol/L JC-1 working solution was prepared prior to use, and cardiomyocytes were stained at 37 °C in the dark for 15 min. Cells doubly stained with JC-1 were visible as either green or red fluorescence. Fluorescent images and intensities were obtained using a fluorescence microscope and ImageJ software. Generally, ΔΨm is represented by the red to green fluorescence ratio, which decreases in proportion with the severity of cell injury.

Statistical analysis

All statistical analyses were performed with SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as the mean ± SEM. Multiple comparisons were carried out using one-way analysis of variance (ANOVA) followed by Bonferroni’s post-hoc test. A value of $P < 0.05$ indicated statistical significance.

Results

H$_2$O$_2$-induced activation of NF-κB in NRVMs

The results indicated that H$_2$O$_2$ elicited time-dependent IκBα degradation and p65 translocation after the NRVMs were incubated with 100 μM H$_2$O$_2$ for different durations (0, 15, 30, 60min), respectively. (Fig. 1A-C). The ratio of nuclear p65 to cytosolic p65 peaked at 60 min. Consistent with the nuclear translocation of p65, the level of p-p65/p65 increased following H$_2$O$_2$ stimulation with the incubation time (Fig. 1D and E), and was highest at 60 min. Thus, treatment with 100 μM H$_2$O$_2$ for 60 min was identified for use in the following experiments.

Efficiency of IκBα transfection in NRVMs

As shown in Fig. 1F, the green fluorescence signal was robust and the dsAAV9-GFP transfection efficiency in NRVMs reached more than 70%. Western blot analysis showed that the GFP protein was more highly expressed in the GFP group than in the other groups, while the IκBα protein level was significantly elevated in the IκBα group compared with the control and GFP groups (Fig. 1G-I).
**IκBα protected cardiomyocytes from H$_2$O$_2$-induced apoptosis**

The proportion of apoptotic cells in control group was 7.0 ± 1.5%. After treatment with 100 μM H$_2$O$_2$, the apoptotic rate of cardiomyocytes in the H$_2$O$_2$ group and GFP group increased to 21.20 ± 0.95% and 19.97 ± 0.97%, respectively, which were decreased by IκBα or PDTC pretreatment (Fig. 2A). Indeed, compared with the levels in the control group, the anti-apoptotic protein Bcl-2 was downregulated, but the pro-apoptotic protein Bax was upregulated in NRVMs exposed to H$_2$O$_2$, leading to a higher Bax/Bcl-2 ratio, but this effect was completely abolished by pretreatment with IκBα or PDTC (Fig. 2B).

**IκBα protected cardiomyocytes from H$_2$O$_2$-induced cell injury**

Compared to that in the control group, ΔΨm was significantly decreased in the H$_2$O$_2$ and GFP groups, but this decrease was rescued by IκBα or PDTC treatment (Fig. 3A). Additionally, H$_2$O$_2$ treatment significantly decreased cell viability but elevated supernatant LDH and intracellular MDA levels; however, these changes were reversed by IκBα or PDTC treatment (Fig. 3B-D).

**IκBα suppressed H$_2$O$_2$-induced NF-κB activation and autophagy in NRVMs**

Compared with control group, H$_2$O$_2$ treatment significantly elicited p65 translocation, and increased p-p65/p65 ratio, and these changes were successfully reversed by IκBα or PDTC pretreatment (Fig. 4A and B). Consistently, H$_2$O$_2$ increased the expression of p-p65 in NRVMs, but IκBα or PDTC dramatically downregulated the H$_2$O$_2$-induced expression of p-p65. Meanwhile, Beclin-1 and the LC3-II/LC3-I ratio, the autophagy-associated markers, were markedly upregulated in NRVMs exposed to H$_2$O$_2$, whereas these effects were inhibited by IκBα or PDTC treatment (Fig. 4C).

**Discussion**

This study shows that IκBα degradation and NF-κB activation occurred in a time-dependent manner in NRVMs subjected to H$_2$O$_2$. Cells treated with H$_2$O$_2$ showed reductions in cell vitality and ΔΨm but elevations in LDH and MDA levels, apoptosis and autophagy. IκBα transfection or PDTC pretreatment ameliorated H$_2$O$_2$-induced cell injury through inhibiting NF-κB activation.

I/R injury severely attenuates the benefit of revascularization after AMI and hence has become an important focus of cardiovascular research [2]. The inflammatory response induced by AMI is essential for heart repair, but excessive generation of ROS and inflammation following reperfusion therapy exacerbate heart damage [21].

The NF-κB signaling pathway plays a key role in the inflammatory response, oxidative stress, apoptosis, and autophagy in the heart [8]. Phosphorylation and nuclear translocation of the p65 subunit are signs of NF-κB activation [20]. Previous studies [22-24] identified that H$_2$O$_2$ treatment for different durations (30 min-24 h) elicited significant p65 phosphorylation and nuclear translocation in NRVMs. In line with these
studies, p65 was time-dependently phosphorylated and translocated from the cytoplasm to the nucleus with IκBα degradation in NRVMs subjected to H$_2$O$_2$.

However, whether NF-κB activation protects or damages cardiomyocytes remains a matter of debate. An early study demonstrated that activation of NF-κB reduced cell apoptosis in hypoxic cardiomyocytes [25], whereas most recent studies [26, 27] have shown that NF-κB is a pro-apoptotic transcription factor correlated with myocardial injury, and blockade of NF-κB activity prevents myocardial apoptosis. Gray et al [22] recently reported that ROS generated by ischemia-reperfusion could rapidly activate calmodulin kinase II (CaMKII), which decreased cell injury through inducing IκBα degradation and nuclear p65 accumulation in NRVMs exposed to H$_2$O$_2$. Importantly, knockout of the CaMKIIδ gene significantly attenuated myocardial infarct size by inhibiting IκBα degradation and NF-κB activation. All these findings show that NF-κB activation deteriorates the heart in I/R injury.

Herein, we hypothesized that direct overexpression of IκBα to prevent NF-κB activation may have a good effect in protecting cardiomyocytes. Then, dsAAV9-IκBα$_{\text{Ser}^{32A,36A}}$ was designed to prevent IκBα degradation due to its phosphorylation at the Ser 32 and Ser 36 sites, and successfully transfected into cardiomyocytes. Western blot and immunofluorescence demonstrated that IκBα transfection successfully maintained cytoplasmic IκBα levels and suppressed p65 phosphorylation and translocation in NRVMs exposed to H$_2$O$_2$. Additionally, IκBα elevated cell viability, decreased LDH and MDA levels, and attenuated apoptosis, implying the protective role of IκBα in H$_2$O$_2$-induced cell injury in NRVMs. The mechanisms may account for the role of NF-κB in mediating the expression of various proteins that promote or inhibit apoptosis. Notably, NF-κB regulates the expression of certain anti-apoptotic genes, such as Bcl-2 [28], and an increased ratio of Bcl-2/Bax decreases cell apoptosis. In this study, treatment with IκBα or PDTC significantly elevated the Bcl-2/Bax ratio in NRVMs subjected to H$_2$O$_2$. These data indicate that IκBα protects NRVMs against H$_2$O$_2$-induced apoptosis by decreasing the ratio of Bax/Bcl-2.

Opening of the mitochondrial permeability transition pore (MPTP) in the first few minutes of reperfusion leads to ΔΨm loss and is responsible for necrotic and apoptotic cell death processes, contributing differentially to myocardial infarct size [29]. Importantly, inhibition of the opening of the MPTP attenuated I/R injury. Thus, ΔΨm loss reflects mitochondrial dysfunction, indicates early-stage apoptosis and is a critical determinant of I/R injury [30]. A previous study demonstrated that H$_2$O$_2$ induced a significant decrease in ΔΨm [12]. In this study, H$_2$O$_2$ treatment attenuated ΔΨm and enhanced Bax expression in NRVMs, and these effects were reversed by pretreatment with IκBα or PDTC. NF-κB is involved in the regulation of mitochondrial dysfunction [31], and Bax antagonizes the anti-apoptotic ability of Bcl-2 and simultaneously promotes permeability of the mitochondrial outer membrane and reduces the level of ΔΨm [32]. The results herein suggest that IκBα decreases cell injury and apoptosis by inhibiting NF-κB activation and Bax expression, ultimately elevating ΔΨm after H$_2$O$_2$ stimulation.

Autophagy, a cellular process of lysosome-mediated degradation of cytoplasmic
components or damaged organelles, is thought to be an adaptive response and protective for cell survival [10]. However, autophagy causes a redox effect in cardiomyocytes upon different stimuli. Evidence supports the benefit of autophagy to cardiomyocytes during myocardial ischemia through its improvement of myocardial energy metabolism and organelle recycling [33], but excessive autophagy causes lethal damage in cells during cardiac I/R injury [21], which is mediated in part by the upregulation of Beclin-1 expression [34].

However, the communication between autophagy and NF-κB is bidirectional. Autophagy is required for the activation of NF-κB [13]; in turn, NF-κB further increases autophagosome maturation by upregulating Beclin-1 and LC3-Ⅰ expression in I/R injury [35]. Importantly, PDTC attenuates Beclin-1 expression and the formation of autophagosomes by suppressing I/R injury-induced NF-κB activation [16]. In accordance with these findings, the treatment of NRVMs with H₂O₂ induced p65 phosphorylation and translocation, enhanced Beclin-1 expression, and increased the LC3-Ⅰ/LC3-I ratio, these effects were rescued by IκBα transfection or PDTC treatment. These results imply that IκBα can protect cardiomyocytes by inhibiting H₂O₂-induced autophagy. In addition, Bcl-2 can bind Beclin-1 to inhibit autophagy [36]. This study also demonstrates that IκBα transfection elevated the expression of Bcl-2, which may disturb the function of Beclin-1 and thus further inhibit H₂O₂-induced autophagy, implying cross-talk between apoptosis and autophagy.

This study has some limitations. The present findings were obtained from neonatal cardiomyocytes in vitro, and may differ from findings in animal experiments due to the complicated features of the in vivo environment. Further animal studies should be conducted to confirm the cardioprotective effects of IκBα against I/R injury.

Conclusions

The findings of this study show that pretreatment with dsAAV9-κBα or PDTC protected NRVMs from H₂O₂-induced apoptosis, autophagy, mitochondrial dysfunction, and oxidative damage by restraining the NF-κB signaling pathway, suggesting that IκBα transfection can protect cardiomyocytes against cardiac oxidative damage. Thus, AAV9 vectors, as the high-efficiency gene carrier to heart, may be used to carry IκBα gene to protect the heart by targeted inhibition of myocardial NF-κB in future preclinical or clinical studies, which may provide a promising gene therapy for preventing cardiac I/R injury.

Declarations

Ethics approval and consent to participate

All animal experimental protocols were approved by the Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University (No. IACUC-20180223-69).

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Authors' contributions

MH, CXC, MHS, MTG performing the research. YNY, XMG, XM provided guidance on the whole study. MH and XCC analyzed the data and drafted the paper. Design and final approval of the version (YTM, BDC).

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Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this article.

Competing interests

The authors declare no competing interests.

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Abbreviations

NF-κB: nuclear factor kappa B; IκBα: inhibitor of kappa B alpha; NRVMS: neonatal rat ventricular cardiomyocytes; I/R: ischemia/reperfusion; AMI: Acute myocardial infarction; H2O2: hydrogen peroxide; GFP: green fluorescent protein; PDTC: pyrrolidine dithiocarbamate; CCK-8: Cell Counting Kit-8; LDH: lactate dehydrogenase; MDA: malondialdehyde; MPTP: mitochondrial permeability transition pore; ΔΨm: mitochondrial membrane potential; ROS: reactive oxygen species.

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Figures
Figure 1

Effects of H2O2 and AAV9 vectors on NRVMs. (A-C) Western blot and quantified cytosolic or nuclear protein levels of IkBα and p65 in NRVMs exposed to 100 µM H2O2 for 0, 15, 30, 60 min, respectively (n = 3, *P <0.05 and **P <0.01 vs. H2O2 (0 min). (D-E) Western blot and quantified total protein levels of p-p65 and p65 in NRVMs subjected to 100 µM H2O2 for 0, 15, 30, 60 min, respectively (n = 3, *P <0.05 and **P <0.01 vs. H2O2 (0 min). (F) The green fluorescence intensity of GFP in NRVMs (scale bar: 250 µm). (G-I) Western blot to detect the protein expression of IkBα and GFP. (n = 3, ** P <0.01 vs. Control).
IκBα reduced cell apoptosis in NRVMs exposed to H2O2. (A) Flow cytometry indicated that IκBα or PDTC attenuated cell apoptosis in NRVMs exposed to H2O2 (n = 3, * P < 0.05 and ** P < 0.01 vs. Control, # P < 0.05 vs. H2O2 group. (B) Western blot findings indicated that IκBα or PDTC reduced the ratio of Bax/Bcl-2 in NRVMs subjected to H2O2 (n = 3, ** P < 0.01 vs. Control, ## P < 0.01 vs. H2O2 group).
Figure 3

IkBα reduced cell injury in NRVMs exposed to H2O2. (A) Representative images of JC-1 staining in different groups (scale bar: 250 µm). The results indicated that IkBα or PDTC elevated the ratio of red to green fluorescence intensity (n = 3, * P < 0.05 and ** P < 0.01 vs. Control, # P < 0.05 vs. H2O2 group). (B) CCK-8 results demonstrated that IkBα or PDTC increased the cell viability in NRVMs stimulated by H2O2 (n = 3, * P < 0.05 and ** P < 0.01 vs. Control, # P < 0.05 vs. H2O2 group). (C) IkBα or PDTC reduced the levels of supernatant LDH in NRVMs subjected to H2O2 (n= 3, * P < 0.05 and ** P < 0.01 vs. Control, # P < 0.05 and ## P < 0.01 vs. H2O2 group). (D) IkBα or PDTC decreased the content of intracellular MDA in H2O2-treated NRVMs (n = 3, ** P < 0.01 vs. Control, # P < 0.05 vs. H2O2 group).
**Figure 4**

IkBα suppressed H2O2-induced NF-κB activation and autophagy in NRVMs. (A) Distribution of NF-κB p65 (red fluorescence) was detected using immunofluorescence (scale bar: 25 µm). The results showed that IkBα or PDTC inhibited H2O2-induced p65 nuclear translocation. (B) Western blot and quantified cytosolic or nuclear protein levels of IkBα and p65 in NRVMs exposed to 100 µM H2O2. The results showed that IkBα transduction maintained the cytosolic IkBα level and suppressed p65 nuclear translocation (n = 3, ** P <0.01 vs. Control, ## P <0.01 vs. H2O2 group). (C) Western blot and quantified total protein levels of p-p65, p65, Beclin-1 and LC3-I/LC3-II in NRVMs exposed to 100 µM H2O2. The findings showed that IkBα or PDTC decreased p-p65/p65 ratio, Beclin-1 expression and LC3-I/LC3-II ratio after H2O2 stimulation. (n = 3, ** P <0.01 vs. Control, ## P <0.01 vs. H2O2 group).