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

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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 considered to be a crucial mediator. Inactivation of NF-κB could benefit cardiomyocytes through inhibiting apoptosis. IκBα, an inhibitor of NF-κB, is hypothesized to protects cardiomyocytes from H_2O_2-induced apoptosis and autophagy through inhibiting the NF-κB pathway.

Methods: We designed an AAV9-delivered mutated IκBα$^{S32A, S36A}$ and investigated its effect on neonatal rat ventricular cardiomyocytes (NRVMs) in response to hydrogen peroxide (H_2O_2). NRVMs were divided into Normal (blank), Control (H_2O_2), GFP +H_2O_2, IκBα+H_2O_2, and Pyrrolidine dithiocarbamate (PDTC)+H_2O_2 groups. NF-κB p65 nuclear translocation was evaluated by immunofluorescence and western blot. Cell viability was assessed by a cell counting kit-8 kit. Supernatant lactate dehydrogenase (LDH) and intracellular malondialdehyde (MDA) were measured to identify H_2O_2-stimulated cytotoxicity. Apoptosis was determined by Annexin V-PE/7-AAD, and the mitochondrial membrane potential (△Ψm) was detected by JC-1. Western bolt was used to detect apoptosis and autophagy related proteins.

Results: Consequently, H_2O_2-treated NRVMs showed reductions in cell viability but increased IκBα degradation and NF-κB p65 nuclear translocation in a time-dependent manner. Furthermore, LDH and MDA content, LC3-Ⅱ/LC3-Ⅰ ratio, Bax and Beclin-1 expressions, and apoptotic cells were upregulated in NRVMs exposed to H_2O_2, whereas △Ψm and Bcl-2 expression were downregulated. Additionally, IκBα transduction or PDTC pretreatment both attenuated the nuclear translocation of the p65 subunit and reversed the H_2O_2-stimulated effects in NRVMs.

Conclusion: These findings suggest that IκBα could ameliorate H_2O_2-induced apoptosis and autophagy through targeted inhibition of NF-κB activation, which may guide strategies to prevent cardiac I/R jury.

Introduction

Acute myocardial infarction (AMI) is the leading cause of death in 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 necrosis, apoptosis, autophagy and inflammation, finally leading to myocardial ischemia/reperfusion (I/R) injury [2, 3]. Recent evidences have suggested that excessive inflammation and oxidative stress play a predominant role in the initiation and progression of I/R injury [4].

Nuclear factor kappa B (NF-κB) is an inflammatory inducer and redox-sensitive transcription factor existing in most cell types [5]. The p65/50 heterodimer, the most common pattern of NF-κB dimers, normally exist as components of inactive cytoplasmic complexes bounded with members of the inhibitor of κB (IκBα). Upon stimulation, IκBα is phosphorylated, and undergone ubiquitylation and proteasomal degradation, subsequently leading to the release and nuclear translocation of p65/p50 dimer [6].

Activated NF-κB then initiates expression of corresponding target genes, many of which may regulate apoptosis, inflammation and autophagy [7].

However, whether NF-κB is protective or detrimental for prevention cardiomyocyte apoptosis remains controversial [8]. Notably, our previous study indicated that p65 ribozyme could prevent cell apoptosis in H9C2 cardiomyocyte exposed to hydrogen peroxide (H_2O_2) [9]. Autophagy, an evolutionarily conserved “self-digestion”, plays dual roles in heart [10]. Recent researches on autophagy shows both protective [11] and deleterious [12] roles of autophagy in cardiomyocyte against oxidative stress. Evidences have elucidated a strong correlation exists between the modulation of NF-κB and autophagic response [13, 14]. In addition, it is noted that there exists a “cross-talk” between autophagy and apoptosis [15], and NF-κB is known as a mediator of the balance between them [16].

Therefore, we speculate that NF-κB activation is the key point of I/R injury, inhibiting NF-κB may be a targeting therapy. IκBα, as the key inhibitor of canonical NF-κB pathway, phosphorylation on Ser 32 and Ser 36 is necessary for its degradation and any mutation of these two serine will block IκBα degradation [6]. Recently, adeno-associated virus serotype 9 (AAV9) is defined as the best gene carrier due to their high efficiency in the heart [17]. H_2O_2, as one common reactive oxygen species (ROS), is generally utilized to mimic I/R injury in vitro [12]. Thus, we designed an AAV9-delivered mutated IκBα $^{S32A, S36A}$ gene to investigate the role of inhibition of NF-κB pathway in H_2O_2-induced apoptosis and autophagy in cardiomyocytes. Pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF-κB, was used as a positive control in this study.

Materials And Methods

Reagents

The antibodies against p65 (#8242s), IκBα (#4812s), Bax (#2772), GFP (#2956), Beclin-1 (#3495), LC3 Ⅰ/Ⅱ (#12741) were from Cell Signaling Technology. Bcl-2 (ab196495), Histone H3 (ab1791), β-actin (ab8227) and horseradish peroxidase (HRP)-conjugated secondary antibody (ab205718) were from Abcam. RIPA buffer and Halt™ Protease and Phosphatase Inhibitor Cocktail were from Thermo Fisher Scientific. Enhanced chemiluminescence (ECL) reagent was from Millipore. Trypsin, bromodeoxyuridine (BrdU) and PDTC were from Sigma. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin solution were from Gibco. Collagenase II was from Worthington (USA). H_2O_2 was from Sangon.

Vectors design

Recombinant AAV-9 vectors were purchased from Virovek (Hayward, CA, USA) and generated by the recombinant baculovirus (rBac)-based system in SF9 cells as previously described [18]. Both recombinant AAV9 vectors were packaged as double-stranded DNA, which contains the enhanced green fluorescent

protein (GFP) gene (dsAAV9-eGFP) or mutant IκBα $^{S32A, S36A}$ gene (dsAAV9- IκBα) driven by the human cytomegalovirus (CMV) promoter.

Isolation, culture and treatment of rat cardiomyocytes

1-to 3-day-old Sprague Dawley rats (sex unlimited) were obtained from the Experimental Animal Center of Xinjiang Medical University. The protocol for the isolation and purification of neonatal rat ventricular cardiomyocytes (NRVMs) has been reported in our previous research [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 1.5 h differential adhesion, the suspensions of nonadherent cells were resuspended and cultivated in high-glucose DMEM containing 10% FBS, 1% penicillin-streptomycin, and 0.1 mM BrdU for 48 hours. The cardiomyocytes would be treated with H_2O_2 (100 μM) to establish a myocardial injury model according to our previous study [12]. Incubation of 100 μM PDTC for 60 min before H_2O_2 treatment was defined as the positive control group according to our preliminary experiments. The medium was replaced every 48 h.

AAV9 transfection of cardiomyocytes

After 48 h culture, NRVMs were transfected respectively with dsAAV9-GFP and dsAAV9- IκBα as previously described [12]. Briefly, cells were firstly transfected with dsAAV9 (multiplicity of infection, MOI = 5×10^6 vg/cell) in serum-free medium, then DMEM at equal volume with 20% FBS, 2% penicillin-streptomycin and 0.2 mM Brdu was added to every dish 3 h later. The images of GFP were captured using fluorescence inverted microscope (LEICA-DMI4000B, Germany) and green fluorescence intensities were analyzed using Image J software.

Measurement of cardiomyocyte vitality and cytotoxicity

A cell counting kit-8 (CCK-8; Dojindo, Japan) was used to assess the cell viability. In brief, 2×10^4 cells were seeded into a 96-well plate per well and transfected with GFP or IκBα for five days, respectively. After cells exposed to H_2O_2, 10 μl of CCK-8 stock solution was added to each well and incubated at 37 °C for 2 h. The absorbance was measured at 450 nm by GO microplate spectrophotometer (Thermo Fisher Scientific, USA). The extent of cell death was determined by quantifying LDH released into culture supernatant following the manufacturer's instructions of LDH Kit (Jiancheng Bioengineering Institute, China). Intracellular MDA, an indicator of oxidative injury, was also measured using the MDA assay kit (Jiancheng Bioengineering Institute, China).

Flow cytometry analysis

Cell apoptosis was measured using the PE Annexin V Kit I (BD Biosciences, USA). Briefly, cells were collected and resuspended in 1 × binding buffer. Thereafter, the solution (1×10^5 cells) supplemented with 5 μL PE Annexin V and 7-AAD were incubated in the dark for 15 min at room temperature. The apoptotic cells were identified by flow cytometry (Beckman Coulter, USA). All the experiments were performed in triplicate.

Western blot analysis

We extracted nuclear and cytoplasmic proteins following the instructions of Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific, USA) and applied the ratio of nuclear p65 to cytosolic p65 to reflect the degree of NF-κB translocation [20]. Total proteins were extracted with RIPA buffer containing Halt™ Protease and Phosphatase Inhibitor Cocktail. Equal amounts of protein were loaded and separated on SDS-PAGE precast gels (Invitrogen Life Technologies) and transferred to Millipore PVDF membrane. After blocking with 5% skim milk, the membranes were blotted overnight with specific primary antibodies (1:1000) against p65, IκBα, Bax, GFP, Beclin-1, LC3 Ⅰ/Ⅱ, Bcl-2, Histone H3, and β-actin at 4℃, followed by the incubation of anti-Rabbit HRP secondary antibody (1:5000) at room temperature for 2 h. ECL solution was added onto 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, USA).

Immunofluorescence

Immunofluorescence was employed to identify the H_2O_2-induced nuclear translocation of the NF-κB p65 subunit in cardiomyocytes. Briefly, 2×10^5cells were seeded into confocal dishes. After H_2O_2 treatment, cardiomyocytes were fixed by 4% paraformaldehyde for 20 min and permeabilized with 0.25% Triton X-100 for 10 min, respectively. After blocking with 1% BSA for 1 h, cells were probed overnight with anti-p65 (1:200) at 4 °C, and incubated with FITC-conjugated secondary antibody (1:200) for 2 h at room temperature, followed by 10 minutes of DAPI staining for nuclei. Signals were detected using a confocal spectral microscope (Leica Microsystems, Germany).

Mitochondrial membrane potential measurement

JC-1 (Millipore, USA) is generally used as an ideal fluorescent probe to detect mitochondrial membrane potential (△Ψm) in cardiomyocytes. Briefly, we prepared 10 nmol/L JC-1 working solution prior to use, and then stained cardiomyocytes at 37 °C in the dark for 15 min. The double staining of cells by JC-1 was visible either as green or red fluorescence. Fluorescent images and intensities were obtained using a fluorescence microscope or Image J software. Generally, △Ψm was represented with the red-to-green fluorescence ratio, which dropped proportionally with the severity of cell injury.

Statistical analysis

All statistical analysis was performed with SPSS 22.0. Data were presented as mean ± SEM. One-way ANOVA was used for multiple group comparisons. A value of $p < 0.05$ was regarded as statistically significant.

Results

H_2O_2-induced activation of NF-κB in NRVMs

To confirm NF-κB activation induced by H_2O_2 in cardiomyocytes, the translocation of the NF-κB p65 subunit from the cytosol to the nucleus was assessed. NRVMs were incubated with 100 μM H_2O_2 for different durations (0, 15, 30, 60 min), respectively. The findings indicated that H_2O_2 elicited a dose-

dependent IκBα degradation and p65 translocation (Fig. 1A and B). The ratio of nuclear p65 to cytosolic p65 peaked at 60 min. Thus, 100 μM H_2O_2 treatment for 60 min was identified for the following researches.

Transduction efficiency of IκBα on NRVMs

To confirm transduction efficiency of dsAAV9- IκBα on NRVMs, cells were transfected with IκBα and GFP virus at a 5×10^6 MOI respectively. Compared with the normal group, the expressions of IκBα and GFP were significantly enhanced, and green fluorescence intensities of dsAAV9-GFP achieved more than 70% (Fig. 1C and D). Our findings verified the high-efficiency transduction of NRVMs using dsAAV9 vectors.

IκBα protected cardiomyocytes from H2O2-induced apoptosis

To assess the role of IκBα in H_2O_2-stimulated cardiomyocytes apoptosis, flow cytometry and western blot were both applied. The apoptosis rate (Fig. 2A) and Bax/Bcl-2 ratio (Fig. 2B) remarkedly increased in NRVMs exposed to H_2O_2, which were both attenuated by IκBα transduction or PDTC pretreatment. These results indicated that IκBα could prevent H_2O_2-induced cell apoptosis in NRVMs.

IκBα protected cardiomyocytes from H2O2-induced cell injury

△Ψm was assessed by JC-1 staining through calculating the red-to-green fluorescence intensity ratio. Compared to the normal group, △Ψm decreased significantly in the control or GFP group, but it was rescued by IκBα or PDTC treatment (Fig. 3A). Additionally, we determined the H_2O_2-stimulated injury using CCK8, LDH, and MDA assays. H_2O_2 treatment significantly decreased cell viability but elevated supernatant LDH and intracellular MDA levels, which were reversed by IκBα or PDTC treatment (Fig. 3B-D). Our findings demonstrated that IκBα played a protective role in H_2O_2-induced injury in NRVMs.

IκBα suppressed H2O2-induced NF-κB translocation and autophagy in NRVMs

To explore whether IκBα transduction could inhibit H_2O_2-induced NF-κB activation, we applied both western blotting and immunofluorescence staining. Compared to the normal group, H_2O_2 treatment in control or GFP group significantly elicited p65 translocation, which was successfully reversed by IκBα transduction and PDTC pretreatment (Fig. 4A and B). These results indicated that IκBα transduction effectively inhibited the NF-κB activation in H_2O_2-treated cardiomyocytes. Additionally, the autophagy-associated markers including Beclin-1 expression and LC3-Ⅱ/LC3-Ⅰ ratio were markedly upregulated in NRVMs exposed to H_2O_2, whereas these effects were inhibited by IκBα or PDTC treatment (Fig. 4C). Thus, we infer that IκBα could alleviate H_2O_2-induced autophagy via inhibiting the NF-κB signaling pathway.

Discussion

We found that IκBα degradation and NF-κB activation occurred in a time-dependent manner in NRVMs subjected to H_2O_2. Cells treated with H_2O_2 showed reductions in cell vitality and $\triangle\Psi m$, but elevations in LDH, MDA, apoptosis and autophagy. IκBα transduction or PDTC pretreatment ameliorated H_2O_2-induced cell injury via inhibiting the NF-κB translocation.

Ischemia/reperfusion (I/R) injury severely attenuates the benefit of revascularization after acute myocardial infarction (AMI) and hence has become an important focus of cardiovascular research [2]. It is currently believed that the inflammatory response induced by AMI is essential for heart repair, but the excessive generation of ROS and inflammation following reperfusion therapy will exacerbate heart damage [21].

NF-κB signaling pathway plays a key role in the inflammatory response, oxidative stress, apoptosis, and autophagy in heart [8]. As is known, the nuclear translocation of p65 subunit is a sign for NF-κB activation [20]. Previous studies [22–24] identified that H_2O_2 treatment for different durations (30 min-24 h) elicited a significant p65 nuclear translocation in NRVMs. In line with these studies, we found that p65 was time-dependently translocated from cytoplasm to nucleus with IκBα degradation in NRVMs subjected to H_2O_2.

However, whether NF-κB activation could protect or exacerbate cardiomyocyte is still a matter of debate. The early study demonstrated that activation of NF-kB reduced cell apoptosis in hypoxic cardiomyocyte [25], whereas most of recent studies have shown that NF-κB is a pro-apoptotic transcription factor correlated with myocardial injury [26] and blocking NF-κB activity will prevent myocardial apoptosis [27]. Gray et al [22] recently report that ROS generated by ischemia-reperfusion can rapidly activate calmodulin kinase II (CaMKII), which deteriorates cell injury through inducing IκBα degradation and nuclear p65 accumulation in NRVMs exposed to H_2O_2. Importantly, knock-out CaMKIIδ gene significantly attenuates area of myocardial infarction by inhibiting IκBα degradation and NF-κB activation. All these findings reveal that NF-κB activation deteriorates the prognosis of heart in I/R injury.

Herein, we hypothesized that directly overexpress IκBα to prevent NF-κB activation may play a better role in protecting cardiomyocyte. Then, we designed a dsAAV9-IκBα $^{ser\ 32A,36A}$ mutant to prevent IκBα degradation from phosphorylation at serine 32 and 36 sites, which could be successfully transfected into cardiomyocytes. Western blot and immunofluorescence demonstrated that IκBα transfection could successfully maintained cytoplasmic IκBα level and suppressed the p65 translocation in NRVMs exposed to H_2O_2. Consistent with our speculation, IκBα elevated cell viability, decreased LDH and MDA level and attenuated apoptosis, implying a protective role of IκBα in H_2O_2-induced cell injury in NRVMs. The mechanisms may be account for the role of NF-κB in mediating the expression of various proteins that promote or inhibit apoptosis. It is noted that NF-κB regulates the expression of certain anti-apoptotic genes, for example Bcl-2 [28], and increasing the ratio of Bcl-2/Bax will decrease cell apoptosis. In this study, treatment with IκBα mutant or PDTC significantly reduced Bax/Bcl-2 ratio in NRVMs subjecting to

H_2O_2. These data indicate that IκBα protects NRVMs against H_2O_2-induced apoptosis by decreasing the ratio of Bax/Bcl-2.

It is reported that opening of the mitochondrial permeability transition pore (MPTP) in the first few minutes of reperfusion lead to △Ψm loss and is responsible for the necrotic and apoptotic cell death processes exhibiting differential contributions to infarct size [29]. Thus, △Ψm loss reflects the dysfunction of mitochondrial, represents a sign of early stage apoptosis and is a critical determinant of I/R injury [30]. Our previous study demonstrates that oxidative stress induces a significant decrease of △Ψm [12]. In this study, we observe that H_2O_2 treatment results in attenuated △Ψm and enhanced Bax expression in NRVMs, and the effect was reversed by pretreatment with IκBα or PDTC. NF-κB [31] and Bax [32] are reported to be involved in regulation of △Ψm. Herein, we suggest that IκBα decreases cell injury and apoptosis via inhibiting NF-κB activation and Bax expression, finally elevating △Ψm after H_2O_2 stimulation.

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

However, the communication between autophagy and NF-κB is bidirectional. It is reported that autophagy is required for the activation of NF-κB [13], in turn, NF-κB further increases autophagosome maturation via upregulating Beclin-1 and LC3 expression in I/R injury [35]. Importantly, PDTC attenuates Beclin-1 expression and formation of autophagosomes by suppressing I/R injury-induced NF-κB activation [16]. In accordance with these findings, our results unveil that NRVMs treated with H_2O_2 could induce p65 translocation, enhance Beclin-1, increase LC3-Ⅱ/LC3-I ratio and decrease P62 expression, which are rescued by IκBα transfection or PDTC treatment. These results imply that IκBα could protect cardiomyocytes by inhibiting H_2O_2-induced autophagy. In addition, it is noticed that Bcl-2 can bind to Beclin-1 to inhibit autophagy [36]. Our study also demonstrates that IκBα transfection elevates the expression of Bcl-2, which may disturb the function of Beclin-1 and thus further inhibit H_2O_2-induced autophagy, implying a “cross-talk” between apoptosis and autophagy.

Conclusions

Our reports showed that pretreatment with dsAAV9- IκBα or PDTC could protect NRVMs from H_2O_2-induced apoptosis, autophagy, and oxidative injury via restraining the NF-κB signaling pathway. The research suggests that targeting the NF-kB pathway may be a good strategy for developing interventions in myocardial I/R injury. However, the present findings have been obtained on neonatal cardiomyocytes in vitro, which may have a discrepancy from integral animal experiment due to the complicated features of

the in vivo environment, further in vivo studies will be conducted to confirm the cardioprotective effects of IκBα on therapy of ischemic heart disease.

Declarations

Ethics approval and consent to participate

All animal experimental designs and protocols were approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University

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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

IκBα: inhibitor of kappa B alpha；NF-κB: nuclear factor kappa B; I/R: ischemia/reperfusion; NRVMS: neonatal rat ventricular cardiomyocytes; H_2O_2: hydrogen peroxide; GFP: green fluorescent protein; PDTC: pyrrolidine dithiocarbamate; CCK-8: cell counting kit-8; LDH: lactate dehydrogenase; MDA: malondialdehyde; △Ψm: mitochondrial membrane potential; ROS: reactive oxygen species.

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Figures

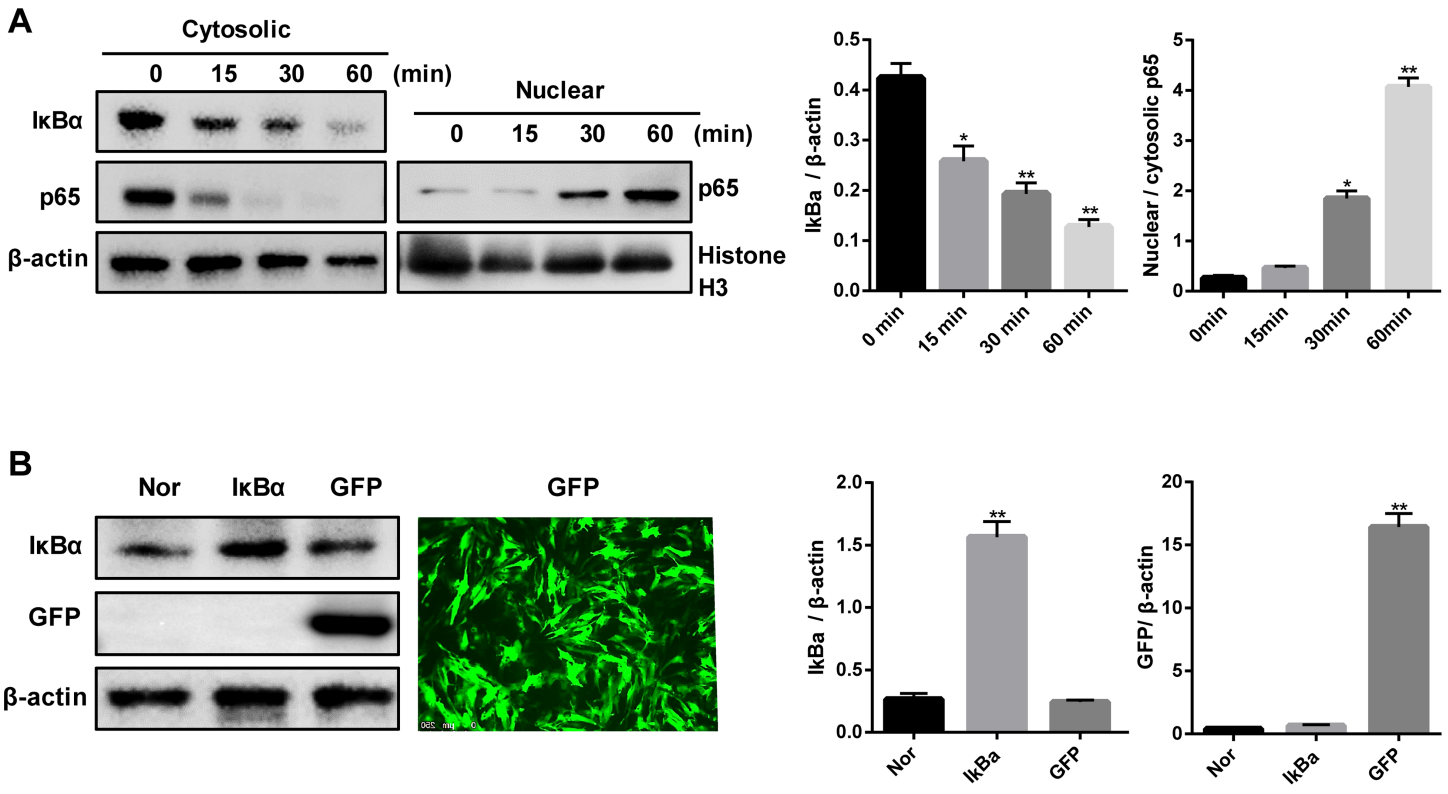

Figure 1

Effects of the H2O2 and dsAAV9-IκBα vectors on NRVMs. (A) Western blotting and quantified cytosolic or nuclear protein levels of IκBα 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). (B) Representative images of GFP were captured using a fluorescence inverted microscope in NRVMs (scale bar: 250 μm) and western blotting to detect the protein expression of IκBα and GFP. (n = 3, **p <0.01 vs. Normal (Nor)).

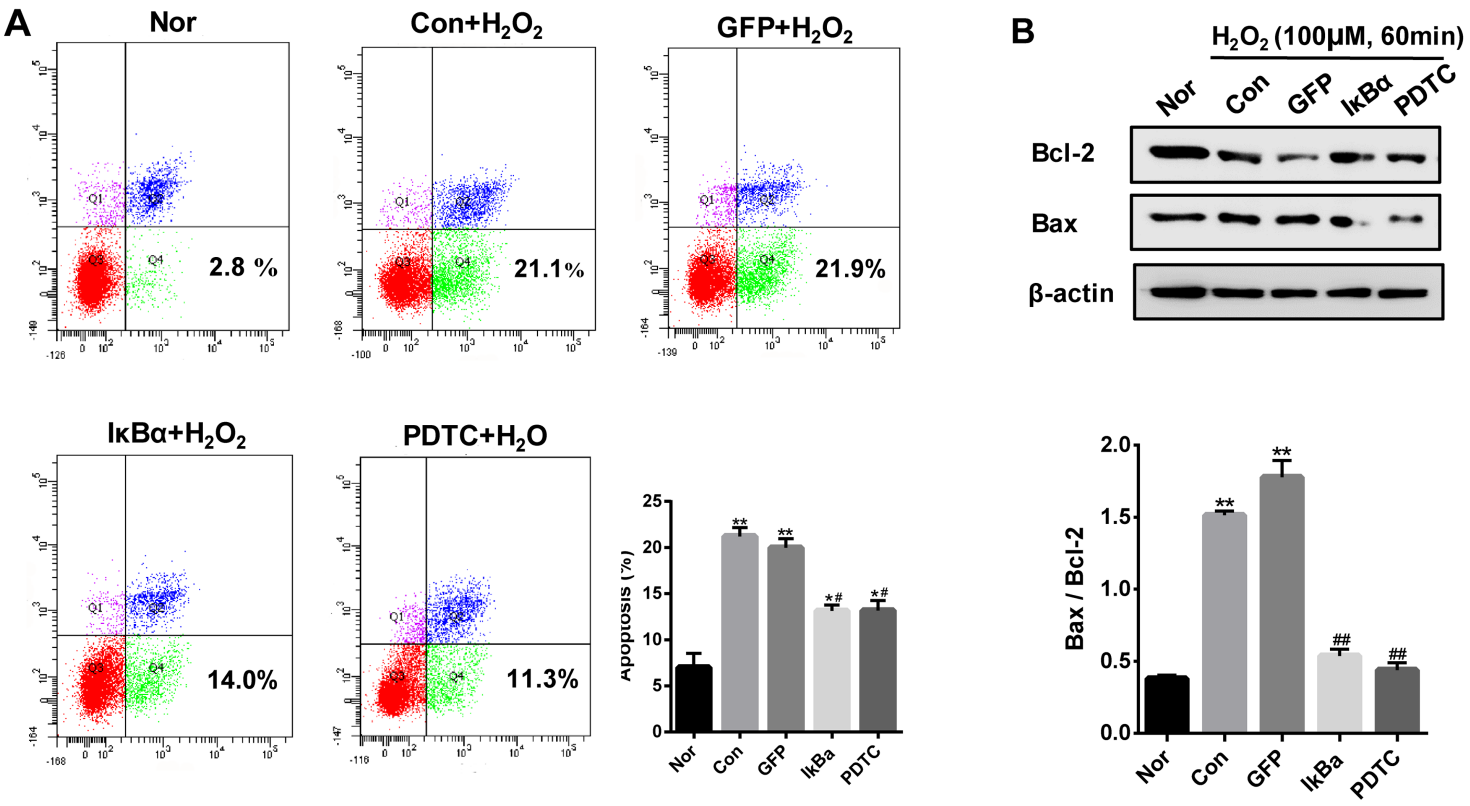

Figure 2

Protective effects of IκBα or PDTC on H2O2-induced apoptosis in NRVMs. (A) Flow Cytometry indicated that IκBα or PDTC attenuated H2O2-induced apoptosis in NRVMs (n =3, *p <0.05 and **p <0.01 vs. Nor, # p <0.05 vs. Control (Con). (B) Western blot results showed that IκBα or PDTC decreased the ratio of Bax/Bcl-2 in NRVMs exposed H2O2 (n = 3, **p <0.01 vs. Nor, ## p <0.01 vs. Con).

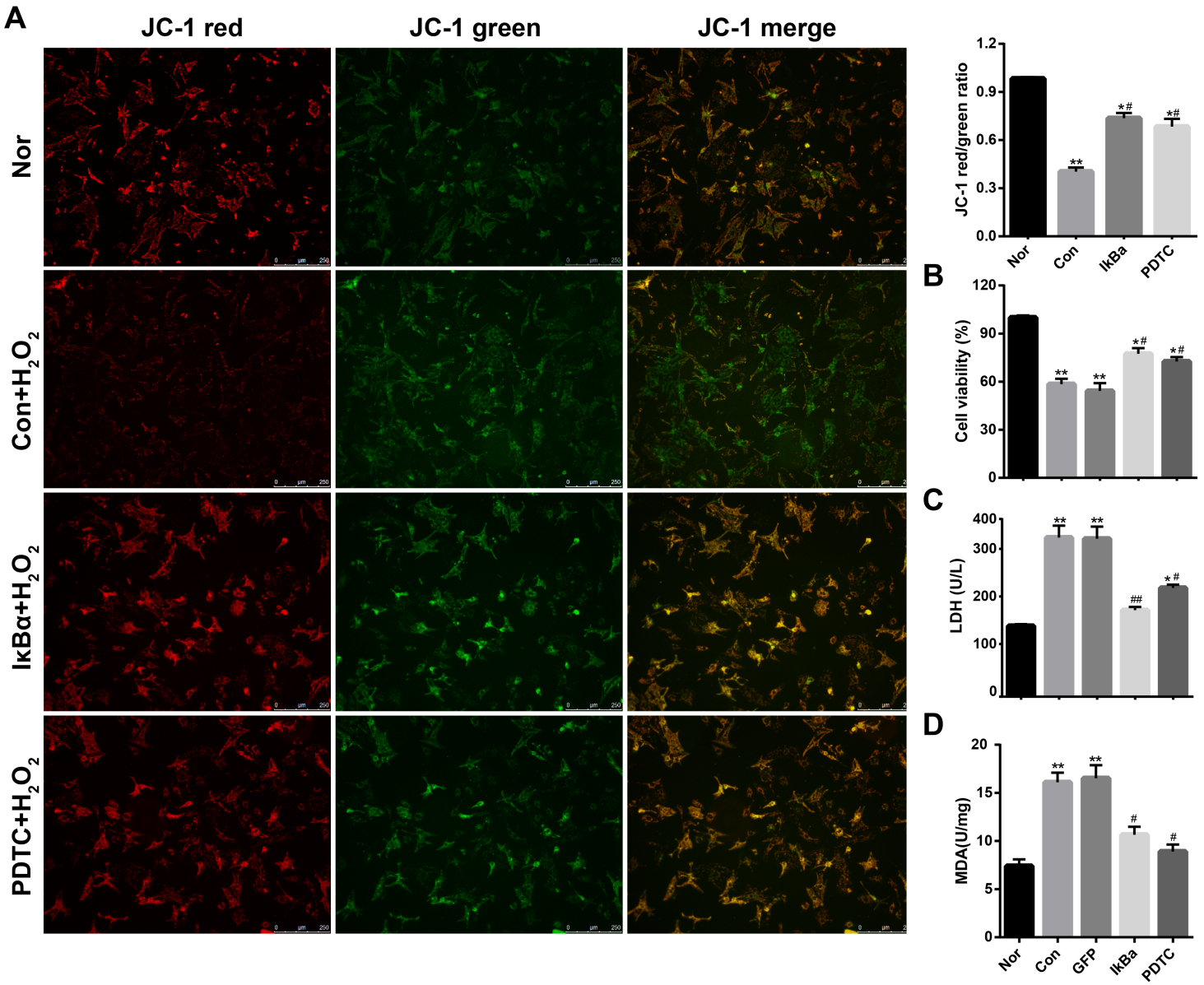

Figure 3

Protective effects of IκBα or PDTC on H2O2-induced injury in NRVMs. (A) Representative images of JC-1 were captured by a fluorescence inverted microscope in NRVMs exposed to H2O2 (scale bar: 250 μm). The results showed that IκBα or PDTC elevated the ratio of red to green fluorescence intensity (n = 3, *p <0.05 and **p <0.01 vs. Nor, #p <0.05 vs. Con). (B) CCK-8 results demonstrated that IκBα or PDTC increased the cell viability in NRVMs. (n = 3, *p <0.05 and **p <0.01 vs. Nor, #p <0.05 vs. Con). (C) IκBα or PDTC reduced the levels of supernatant LDH in NRVMs subjected to H2O2 (n= 3, *p <0.05 and **p <0.01 vs. Nor, #p <0.05 and ##p <0.01 vs. Con). (D) IκBα or PDTC decreased the content of intracellular MDA in H2O2-treated NRVMs. (n = 3, **p <0.01 vs. Nor, #p <0.05 vs. Con).

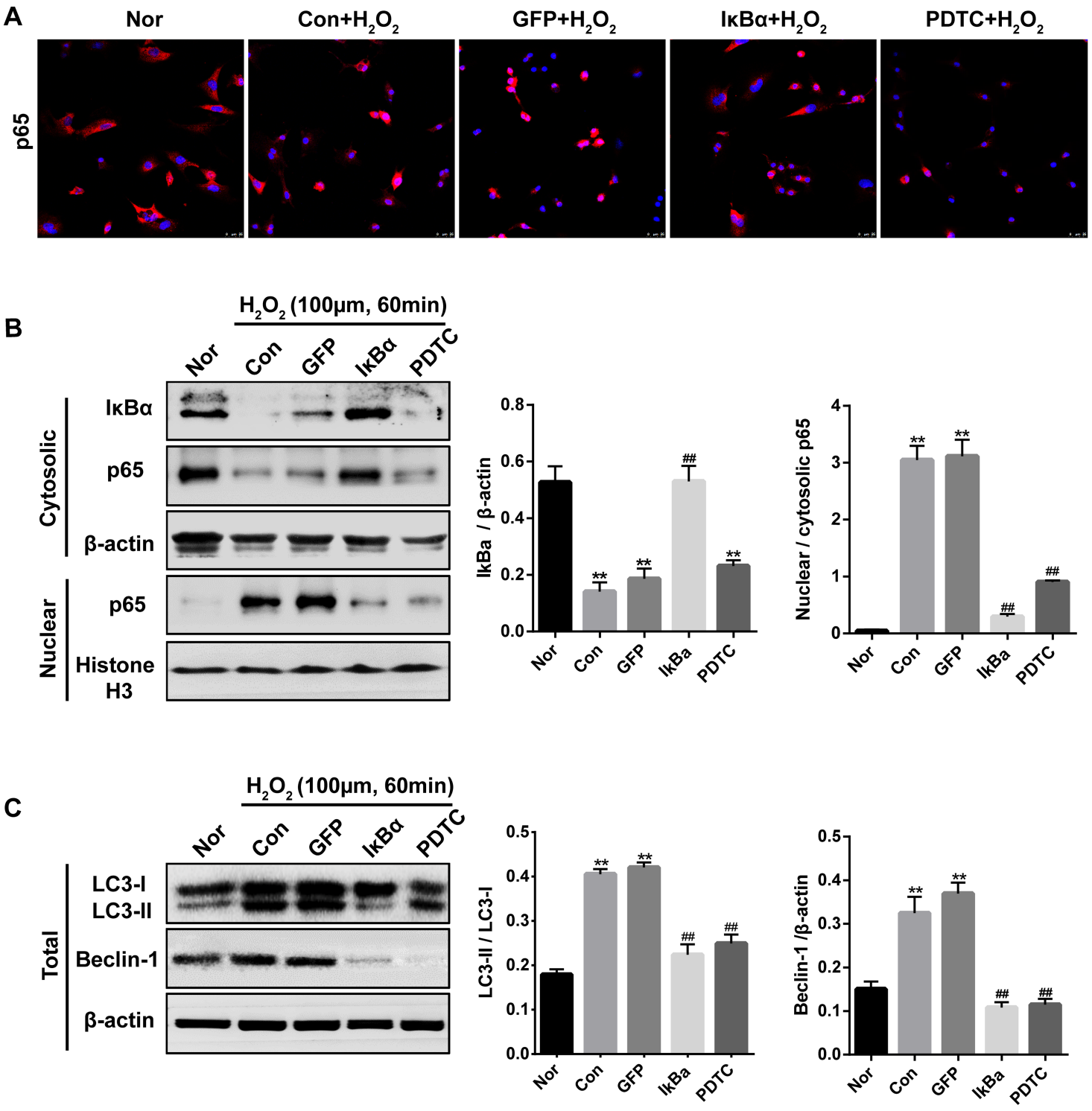

Figure 4

IκBα attenuated autophagy in H2O2-treated NRVMs via inhibition NF-κB translocation. (A) Distribution of NF-κB p65 was detected using immunofluorescence: red fluorescence represented p65 and the blue fluorescence indicated DAPI nuclei staining (scale bar: 25 μm). The results showed that IκBα or PDTC inhibited H2O2-induced p65 nuclear translocation. (B) The results showed that IκBα transduction maintained the cytosolic IκBα level and suppressed p65 nuclear translocation (n = 3, **p <0.01 vs. Nor,

##p <0.01 vs. Con). (C) Total lysates western bolt demonstrated IκBα or PDTC reversed the upregulation of Beclin-1 expression and LC3-Ⅱ/LC3-Ⅰ ratio induced by H2O2 stimulation (n = 3, **p <0.01 vs. Nor, ##p <0.01 vs. Con).