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NF-κB-mediated induction of autophagy in cardiac ischemia/reperfusion injury

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ABSTRACT

Ischemia/reperfusion (I/R) injury severely attenuates the benefit of revascularization after acute myocardial infarction, in which transcription factor NF-κB plays an important role. Recently, there is increasing evidence to suggest that autophagy is involved in this process. We sought to define the role of NF-κB in the induction of autophagy during cardiac I/R injury. The left circumflex coronary arteries of New Zealand white rabbits were ligated for 1.5 h, followed by reperfusion for 1 h to induce I/R injury. Production of reactive oxygen species (ROS) was detected in myocardial injury area following I/R injury. Furthermore, the results indicated that the cardiac area at risk (AAR) for ischemia has the most abundant expression of Beclin 1 in parallel to p65 expression after cardiac I/R injury. Inhibition of NF-κB significantly attenuated Beclin 1 expression and autophagy in the AAR, which was associated with a marked reduction in the extent of the AAR. Our data thus suggests that I/R injury promotes NF-κB activity, in response to ROS, to aggravate myocardial injury through the activation of Beclin 1-mediated autophagy.

1. Introduction

Autophagy is an evolutionarily conserved process that enables eukaryotic cells to capture cytoplasmic components for degradation within lysosomes. Environmental stressors such as hypoxia, oxidative stress and glucose deprivation rapidly stimulate autophagy. A basal level of autophagy is protective for cells through the degradation of damaged mitochondria and protein aggregates as well as recycling of catabolites to maintain ATP production [1]. On the other hand, excessive degradation of essential proteins and organelles may lead to bioenergetic failure and eventually organ dysfunction.

The development of percutaneous coronary intervention has brought marked benefit to patients with acute myocardial infarction. However, ischemia/reperfusion (I/R) injury during revascularization inevitably results in clinical complications such as arrhythmias and left ventricular dysfunction that lead to poor patient outcomes. Recently, increasing evidence suggests that autophagy is increased in the heart undergoing I/R injury [2]. Nevertheless, whether it is a causal or concomitant phenomenon of cell death remains controversial. Meanwhile, the molecular mechanism involved in the increase of I/R-associated autophagy is still unclear.

NF-κB is a redox-sensitive transcription factor existing in most cell types with the common pattern of p50/65 heterodimer. Generally, inactive NF-κB dimers bind with the inhibitor of NF-κB proteins (IkBs) and remain in the cytosol. Many stimuli, such as reactive oxygen species (ROS) and inflammatory factors, may lead to the activation of NF-κB. Activated NF-κB then translocates into the nucleus, where it activates corresponding target genes, many of which regulate apoptosis, inflammation and autophagy. The relationship between NF-κB and autophagy is a matter of debate. Some studies suggested that NF-κB upregulates autophagy by transactivating the autophagy-triggering protein Beclin 1 [3]. However, it has been reported that NF-κB inhibits autophagy in the context of TNFα-induced cell death in cancer cell lines and upon stimulation of macrophages using *Escherichia coli* [4]. Our preliminary data indicated that NF-κB mediated I/R injury-induced myocardial inflammation [5]. Whether NF-κB is involved in myocardial I/R injury-induced autophagy remains unknown. We speculated that I/R injury generates ROS to activate NF-κB p65, which upregulates the expression of Beclin 1, promoting the activity of autophagy, which eventually results in the aggravation of cardiac I/R injury. To this end, we utilized a specific NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC) in a rabbit myocardial I/R injury model and investigated the association between the activation of NF-κB, autophagy and the extent of myocardial I/R injury.

2. Materials and methods

2.1. Ethics statement

All animals used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals,

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NIH Publication, 1996 edition, and all the protocols in this study were approved by the Animal Subjects Committee of The People's Hospital of Hainan Province, Haikou, China.

2.2. Animal heart ischemia/reperfusion studies

New Zealand white male rabbits weighing 2.0–3.0 kg (aged 3–5 months) were anesthetized using sodium pentobarbital (30 mg/kg iv.) and surgery was performed as previously described [5]. Briefly, we performed 1.5 h of ligation at the site of the 1/5 proximal left circumflex (LCX) coronary artery followed by 1 h of reperfusion in order to introduce myocardial I/R injury (10 rabbits per group). Rabbits were pre-treated with saline or PDTC (20 mg/kg diluted in saline) intravenously 0.5 h before the onset of I/R injury and therefore were divided into I/R and I/R + PDTC groups accordingly. Control animals underwent the same surgical procedure except that the sutures passing around the LCX were not tied (sham group).

2.3. Myocardial damage measurement

Myocardial damage was measured using Evan's blue and Thioflavin S staining methods as previous described [6]. 6% thioflavin S (1 ml/kg, Sigma) was injected into the left atrium precisely 1 h after reperfusion. Then, the LCX coronary artery was retied at the site of the previous occlusion and 4% Evan's blue (2 ml, Alfa Aesar) was injected into the left atrium to measure the ischemic risk area. The rabbits were subsequently euthanized using sodium pentobarbital. The heart was quickly removed, sliced into five transverse sections, weighed and photographed. The left ventricular area (LV), area at risk for ischemia (AAR, defined as the area not stained by the blue dye), non-ischemic area (N, defined as the area stained by the blue dye) and the area of no-reflow (NR, defined as the non-fluorescent area) for all slices were calculated by computer-assisted planimetry using Imagepro-plus (IPP) software. The AAR was expressed as a percentage of LV. Samples from all three portions of LV cardiac tissue were processed accordingly for subsequent analysis.

2.4. Reagents

The antibodies used in this study were purchased from the following vendors: p-NF-κB p65 (Ser536) and Beclin 1 were from Cell Signaling Technology (Beverly, MA); NF-κB p65, β-Actin were from Santa Cruz Biotechnology(Santa Cruz, CA). PDTC was purchased from Sigma.

2.5. Lipid peroxidation determination

Cardiac malondialdehyde (MDA) was determined quantitatively in order to estimate the extent of lipid peroxidation in the damaged tissue and cells. Left ventricular tissue from sham-operated animals served as controls. Frozen tissues from the N, AAR, and NR zones of the I/R injury group were homogenized in lysis buffer. After centrifugation (1600g × 10 min), 100 μl of supernatants were analyzed for MDA using a lipid peroxidation assay kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions.

2.6. Western blot

Myocardial tissue was homogenized in a chilled lysis buffer with protease inhibitor cocktail (Roche). Protein concentrations were determined using the BCA assay (Pierce) with bovine serum albumin serving as a standard, and clarified lysates were boiled in SDS sample buffer. Samples were separated by 10% or 15% SDS–PAGE and transferred onto a polyvinylidene difluoride (PDVF) membrane (Millipore). Membranes were blocked in 5% non-fat milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, incubated overnight with relevant antibodies at 4°C, washed, probed with species-specific secondary antibodies coupled to horseradish peroxidase, and visualized using ECL chemiluminescence.

2.7. Electron microscopy

Electron microscopy analysis was performed as described previously. Samples were taken from the central part of the AAR zones of myocardium in the sham, I/R and I/R + PDTC groups ($n = 3$ for each group). Ultra-thin sections (1 mm) were fixed for 30 min with ice-cold 2.5% glutaraldehyde in 0.1 M cacodylate buffer, embedded in Epon, and processed for transmission electron microscopy by standard procedures in a blinded fashion at ×6,000 or ×12,000 magnification.

2.8. Immunohistochemistry

Heart samples from each group were isolated and fixed in 4% paraformaldehyde solution. The samples were cut into 5 μm serial sections. After microwave antigen retrieval, sections were incubated with antibodies NF-κB p65 (1:50, Santa Cruz) and Beclin 1 (1:50, Cell Signaling) for 60 min at room temperature, followed by incubation for 30 min with the secondary antibody. The sections were developed in 3,3′-diaminobenzidine solution under microscopic observation and counterstained with hematoxylin. Negative control slides in which the primary antibodies were supplied as IgG were included in all assays. Images were viewed and captured by use of a Nikon Labophot 2 microscope equipped with a Sony CCD-I/Ris/RGB colour video camera attached to a computerized imaging system.

2.9. Statistical analysis

Data were expressed as the mean ± SD. Comparisons of quantitative data were analyzed using Student's *t*-test between two groups or by one-way ANOVA followed by the Newman–Keuls test for multiple groups. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Discrimination of myocardial damage area in rabbit I/R injury heart model

P65 is a main subunit of the transcription factor NF-κB complex. To explore the pivot role of p65 in cardiac I/R injury, New Zealand white male rabbits either underwent a myocardial I/R injury procedure of 1.5 h of ischemia followed by 1 h of reperfusion (I/R group) or received PDTC 20 mg/kg intravenously for 0.5 h prior to I/R injury (I/R + PDTC group) (Fig. 1A). Sham-operated rabbits served as control. Evan's blue and Thioflavine S staining techniques were utilized to determine different pathologic zones of the heart undergoing I/R injury. The area at risk (AAR), the distal myocardium supplied by obstructed coronary artery, was not stained by Evan's blue. The remaining blue area was recognized as the non-ischemic area (N) (Fig. 1B, top). Thioflavin S is a specific endothelial fluorescent dye. Non-fluorescent myocardium was defined as no-reflow area (NR) (Fig. 1B, bottom).

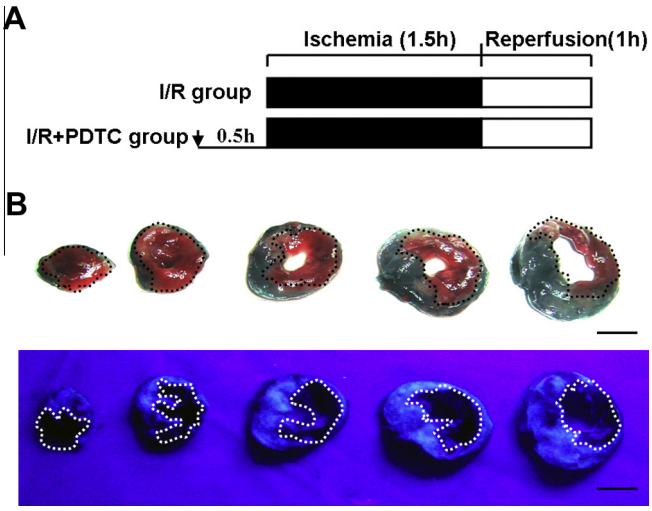

Fig. 1. The construction of the I/R injury model and exploration of the AAR and NR zones in rabbit hearts. Male New Zealand white rabbits underwent 1.5 h of left circumflex coronary artery ligation followed by reperfusion for 1 h in the presence or absence of PDTC treatment (20 mg/kg, intravenously) 0.5 h prior to ischemia/reperfusion (I/R). (A) Details of I/R protocol were shown. (B) Non-ischemia area (N) (blue area), area at risk (AAR) (non-blue area, indicated by black broken lines) and no-reperfusion area (NR) (non-fluorescence area, indicated by white broken lines) zones of the heart were determined by Evan's blue (top) and Thioflavin S (bottom) staining, respectively. Scale bar, 0.5 cm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Cardiac I/R injury triggers ROS release and induces activation of NF-κB p65 and Beclin 1 in AAR area of rabbit heart

One of the most well documented types of mediators of I/R injury are reactive oxygen species (ROS), which are implicated as a stimulator of p65 activity. To determine the production of ROS in each zone of the heart with I/R injury, we evaluated levels of malondialdehyde (MDA), a reliable parameter of lipid peroxidation, in samples from the N, AAR and NR zones of the heart. MDA levels of the AAR and NR zones were 3.59 and 4.45 times those of the sham group, respectively ($P < 0.05$) (Fig. 2A). PDTC, also known as a potent antioxidant, suppressed MDA levels in the AAR and NR zones by 58.73% and 67.55%, respectively ($P < 0.05$). To explore the expression of NF-κB p65 protein in each area, Western Blot analysis was performed with samples from the N, AAR and NR zone of rabbit hearts undergoing I/R injury (Fig. 2B). P65 protein level was significantly elevated in the AAR zone, which was 4.01 times and 2 times that of the N and NR zones respectively ($P < 0.05$) (Fig. 2C), as measured by the quantification the Western blot bands from Fig. 2B. Phosphorylation of p65 at Ser536 in the AAR zone was significantly elevated as compared to the N zone. Interestingly, Beclin 1had 2.78 and 2.67 times expression in AAR compared to N and NR, respectively ($P < 0.05$) (Fig. 2C). This finding is particularly interesting because Beclin 1 plays an essential role in mediating the localization of other autophagic proteins to pre-autophagosomal structures as part of a class III phosphatidylinositol 3-kinase complex.

Nuclear transfer is one of the characteristics of p65 activation. To visualize the location and activity of p65, we performed immunohistochemical staining with polyclonal anti-p65 antibodies on tissue sections of the AAR zone from all three groups. I/R injury increased nuclear p65 expression in the AAR zone as compared to sham group, whereas expression was reduced in the I/R + PDTC group (Fig. 2D). Quantification of positive nuclear staining for p65 in sections from each group revealed that I/R injury induced p65 expression by 5.05 times compared to levels in the sham group ($P < 0.01$). PDTC treatment vigorously inhibited p65 expression by 90.5% in contrast to the I/R injury group ($P < 0.01$) (Fig. 2E).

3.3. I/R injury induces Beclin 1-mediated autophagy in the AAR zone in a p65-dependent manner

PDTC treatment suppressed I/R injury-enhanced p65 protein levels and Beclin 1 protein levels as well as the level of p65 phosphorylation at Ser536 in the N and AAR zones of the heart (Fig. 3A). Quantification of immunoblotting bands indicated that p65 protein expression in the AAR zone of myocardium of the I/R + PDTC group was suppressed by 82.5% in contrast to that of the I/R injury group ($P < 0.05$). Accordingly, PDTC treatment inhibited I/R injury-induced Beclin 1 protein expression in the AAR zone by 89.2% ($P < 0.05$) (Fig. 3B). Immunohistochemical staining revealed substantial cytoplasmic expression of Beclin 1 in the AAR zone myocardium of the I/R injury group. The opposite was true in the AAR zone of the I/R + PDTC group (Fig. 3C). Knowing that Beclin 1 is required for the initiation of the formation of the autophagasome in autophagy, we therefore sought to explore the relationship between p65 and autophagy in I/R injury. Electron microscopy (EM) experiments were performed to visualize autophagosomes in the AAR zone of myocardium from each group. EM showed numerous autophagic vacuoles scattered in the disordered myofibers of the AAR zone of the I/R injury group (Fig. 3D, b). With higher magnification, autophagsomes containing degraded organelles ranging from mitochondria to digested cytoplasmic materials were identified (Fig. 3D, d–f). In contrast, the AAR zone of the I/R + PDTC group hardly exhibited any autophagic vaculoles under EM (Fig. 3D, c).

3.4. PDTC inhibits the extent of the AAR zone in rabbit myocardial I/R injury model

We measured the extent of ischemia, i.e., the AAR zone, through pathologic staining of Evan's blue in the rabbit heart tissue of the I/R group and I/R + PDTC group to explore the role of p65 in cardiac I/R injury. Representative transverse sections of cardiac papillary muscles treated with PDTC exhibited a decrease in the extent of the I/R injury-associated AAR zone (Fig. 4A). PDTC treatment lead to a 52.3% reduction of AAR/LV ($P < 0.05$) as calculated with IPP software (Fig. 4B).

4. Discussion

Ischemia/reperfusion (I/R) injury severely attenuates the benefit of revascularization after acute myocardial infarction and hence has become an important focus of cardiovascular research. Transcription factor NF-κB and autophagy may be involved in myocardial I/R injury [7]. Production of excessive oxygen free radicals by reperfusion is one of the most important factors contributing to I/R injury. With the release of reactive oxygen species (ROS), downstream redox-sensitive signaling pathways including the NF-κB pathway are activated [8]. Activity of p65, a subunit of NF-κB, during reperfusion exceeds activity during ischemia, presumably due to the overwhelming release of ROS during reperfusion. We identified different pathologic zones according to gross pathologic tissue staining in our study. Our results indicate that the p65 protein and phorsphorylation at Ser536 are expressed most plentifully in the area at risk (AAR). Furthermore, p65 immunohistochemical staining confirmed the activation of p65 in the AAR zone through the detection of p65 nuclear transfer. It has been reported that the antioxidant NAC suppresses I/R injury-induced NF-κB expression and attenuates a patient's cardiac injury [9]. Accordingly, our results reveal that PDTC suppresses the abundant release of ROS as well as the activity of p65 in the AAR zone.

Decker et al. [10] reported that autophagy was activated by cardiac I/R injury early in 1980. Rabbit cardiomyocytes exhibited autophagosomes after reperfusion for 20–40 min. Oxidative

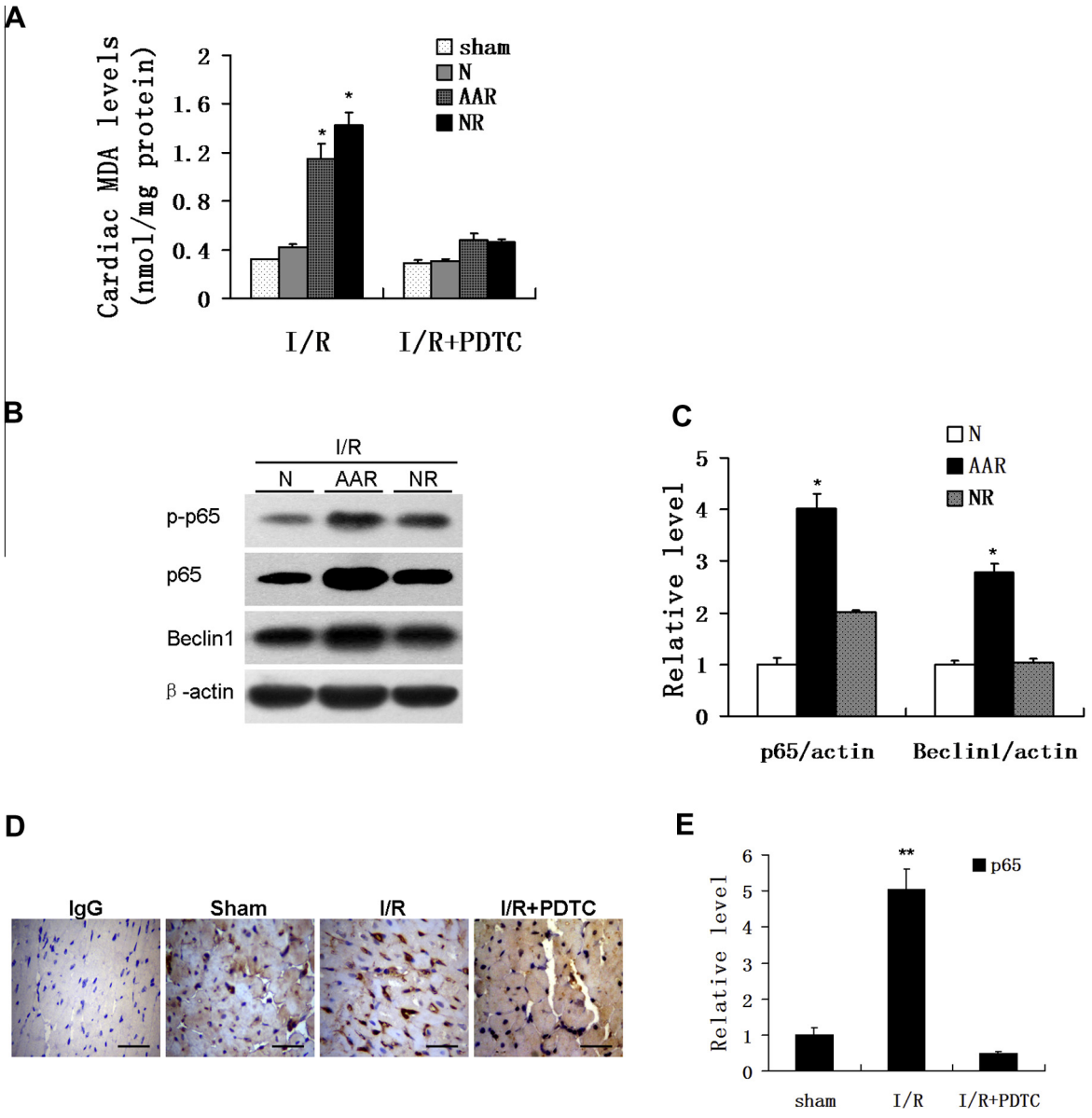

Fig. 2. Myocardial I/R injury produces ROS and activates NF-κB p65 and Beclin 1 in the AAR zone of rabbit hearts. Male New Zealand white rabbits underwent 1.5 h of left circumflex coronary artery ligation followed by reperfusion for 1 h. (A) Free radical generation in the N, AAR, NR zones of the heart from the I/R group and I/R + PDTC group were analyzed by measuring the lipid peroxidation products via MDA levels. Sham-operated animals served as controls. $^{*}P < 0.05$ ($n = 4$/group). (B) Western blots for p-p65, p65 and Beclin 1 were performed with the protein extracts from the N, AAR, NR zones of myocardium after I/R injury. (C) Bar graph showing the quantification of the immunoreactive bands obtained as (B). $^{*}P < 0.05$ (versus N and NR; $n = 4$/group). (D) Immunohistochemistry staining for p65 was performed in the AAR zone of heart sections of the sham, I/R and I/R + PDTC groups. IgG staining served as a negative control. Scale bar, 50 μm. Figures were representative images of at least three different heart samples and images were viewed and captured blindly by two observers. (E) Positive nuclear staining for p65 in sections from each group was quantified. $^{*}P < 0.05$.

stress, endoplasmic reticulum stress, and protein degradation induce autophagy [11]. Beclin 1 is the first mammalian protein described to mediate autophagy by forming a complex with class III PI3 K and upregulating autophagy via formation of autophagosomes and the initiation of autophagy. It has been suggested that Beclin 1 is upregulated during myocardial reperfusion rather than during ischemia [12]. In Beclin 1 knockout cardiomyocytes, formation of autophagosomes during reperfusion was severely suppressed. *In vitro* research on cultured cardiomyocytes indicated that urocortin downregulated the expression of Beclin 1 as well as autophagy and reduced cell death [13]. I/R injury stimulated Beclin 1-associated autophagic flux in adenovirus and cardiac-specific mRFP-GFP-LC3 transgenic mice [14]. Overexpression of Beclin 1 promoted autophagy in I/R injury, whereas silencing of Beclin 1 by siRNA decreased autophagy. Indeed, Beclin 1 plays an essential role in cardiac I/R injury-associated autophagy. However, to date, both the location in which Beclin 1 expression occurs during I/R injury and the mechanism by which it plays a role remain unkown. Our research is the first study to observe that AAR zone of rabbit heart expresses abundant Beclin 1 in parallel to p65 expression after I/R injury. Some studies indicate that p65 upregulates Beclin 1 mRNA and protein levels which is coupled to an increase in autophagy [15]. Nevertheless, Djavaheri-Mergny et al. proposed that NF-κB activation downregulates tumor necrosis factor-alpha-induced autophagy in cancer cell lines and this process was not relevant with Beclin 1 [16]. The relationship between p65 and Beclin 1 in I/R injury is poorly defined. PDTC, a well-defined specific NF-κB inhibitor, has proven to be a useful tool to investigate the NF-κB signaling pathway. The inhibitory effect of PDTC on p65 activity was confirmed by Wes-

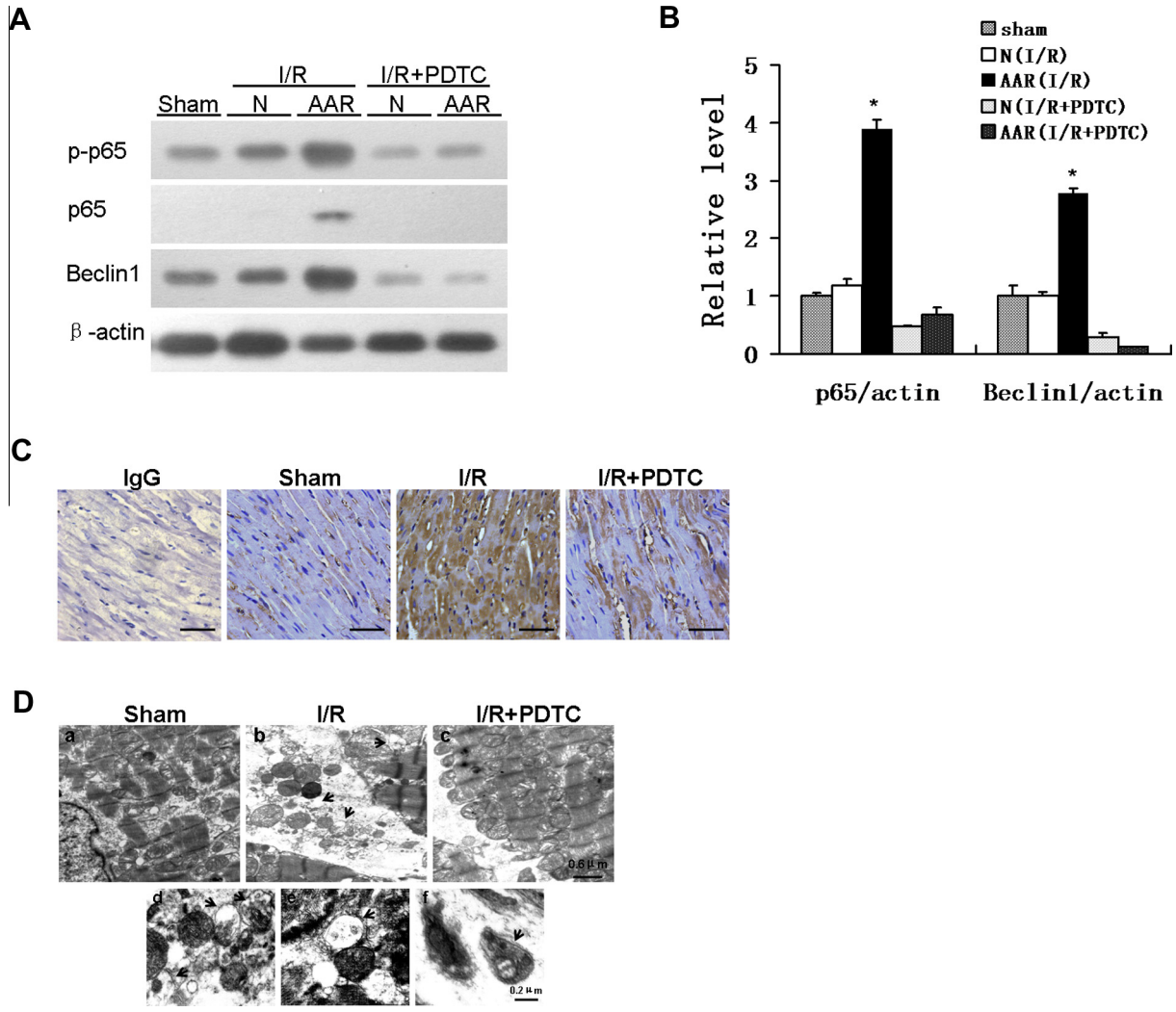

Fig. 3. PDTC inhibited I/R injury-induced p65, Beclin 1 expression as well as autophagy in the AAR zone of the heart. (A) Western blots for p-p65, p65 and Beclin 1 were performed with the protein extracts from the Sham group andthe N and AAR zones of myocardium from the I/R group and the I/R + PDTC group. (B) Bar graph showing the quantification of the immunoreactive bands obtained as (A). Results are expressed as mean ± SD. $^{*}P < 0.05$. (C) Immunohistochemical staining for Beclin 1 was performed in the AAR zone of heart sections of the sham, I/R and I/R + PDTC groups. IgG staining served for negative control. Scale bar, 50 μm. Figures were representative images of at least three different heart samples. Images were viewed and captured blindly by two observers. (D) a–c, Representative electron micrographs of left ventricular tissue sections of sham (a), I/R (b) and I/R + PDTC (c) groups were shown. Bar, 0.6 μm. d–f, higher magnifications of I/R-induced autophagosomes containing digested cytoplasmic materials (d and e) and mitochondria (f). Bar, 0.2 μm. The arrowheads denote the presence of autophagic vacuoles.

tern blot and immunohistochemical staining in our study. Intriguingly, our results demonstrated PDTC suppressed I/R injury-induced activation of Beclin 1 and formation of autophagosomes. This finding supports the hypothesis that p65 is involved in I/R injury-induced autophagy by mediating Beclin 1, which is in agreement with recent studies. Ryu et al. [17] suggested that p65-Ser529 NF-κB subunit phosphorylation induces TNF-α-mediated autophagic astroglial death following status epilepticus. Criollo et al. [18]reported that IKK promotes the autophagic pathway in a manner independent of NF-κB. NF-κB was also shown to activate autophagy during heat shock response, which increased survival of heat-treated Hela cells, without the involvement of the Beclin 1/class III PI3 K complex in the signaling pathway [19]. On the contrary, Fabre et al. suggested that in CD34(+) bone marrow blasts from myelodysplastic syndrome patients as well as those of patient-derived cell lines (P39 and MOLM13), inhibition of NF-κB activity precipitated a bioenergetic crisis that led to an autophagic stress response [20]. These contradictory phenomena might result from different molecular mechanisms involved in different diseases.

Whether I/R injury-induced autophagy is beneficial or detrimental to cell survival is controversial [21]. An *in vitro* experiment indicated that 3-methyladenine downregulation of autophagy threatens cardiomyocyte survival [22]. Heart I/R injury-induced autophagy aided in removal of Bnip3-assiociated dysfunctional mitochondria [23]. Both studies suggest that autophagy performs a protective function in cardiac I/R injury. Conversely, Urocortin reduced I/R injury-associated death of cardiomyocytes by inhibition of Beclin 1. Moreover, suppression of Cathepsin, an autophagy degradation lysosomal enzyme, reduced the reperfusion injury of cardiomyocytes [13]. Our *in vivo* data suggests that PDTC treatment reduces cardiac injury as indicated by the decrease of AAR/LV ratio probably through suppression of p65-dependent autophagy.

In conclusion, our data suggests that NF-κB, probably in response to ROS release, promotes Beclin 1-associated autophagy and contributes to cardiac injury in the setting of myocardial ischemia–reperfusion injury. Therefore, inhibition NF-κB activity may protect cardiomyocytes from detrimental autophagy in patients with acute myocardial infarction undergoing reperfusion therapy.

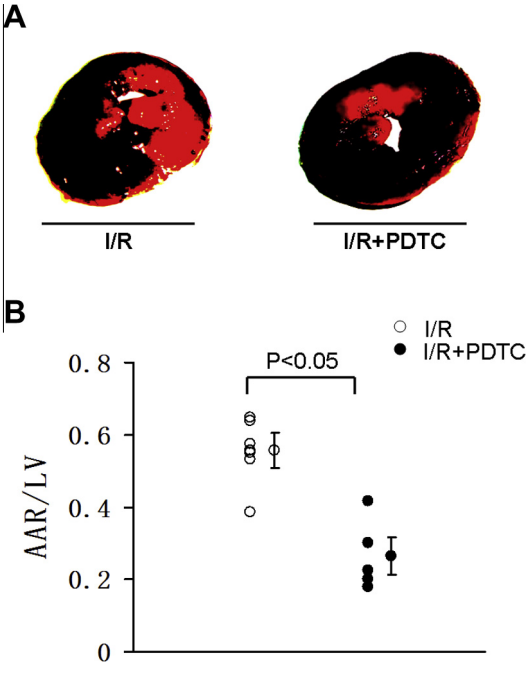

Fig. 4. PDTC attenuates the extent of the I/R injury-induced AAR zone. (A) Representative gross appearances of LV tissue sections after Evan's blue staining in rabbits of the I/R group and I/R + PDTC group. (B) Statistical analysis of AAR/LV area ratio in the I/R group and I/R + PDTC group. (I/R, $n = 7$; I/R + PDTC, $n = 5$).

5. Disclosures

None.

Acknowledgments

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