Overexpression of BAG3 Attenuates Hypoxia-Induced Cardiomyocyte Apoptosis by Inducing Autophagy

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Key Words
Hypoxia • Bcl2-associated athanogene 3 (BAG3) • Autophagy • Cardiomyocyte

Abstract
Background: Hypoxia is a well-known factor in the promotion of apoptosis, which contributes to the development of numerous cardiac diseases, such as heart failure and myocardial infarction. Inhibiting apoptosis is an important therapeutic strategy for the treatment of related heart diseases caused by ischemia/hypoxic injury. Previous studies have demonstrated that BAG3 plays an important role in cardiomyocyte apoptosis and survival. However, the role of BAG3 in hypoxia-induced cardiomyocyte apoptosis remains to be clarified. Here, we demonstrate that BAG3 is induced by hypoxia stimuli in cultured cardiomyocytes. Methods: BAG3 expression level was measured in H9c2 cells treated with hypoxia for 48 h. Cell proliferation and apoptosis were tested using MTT assay and Annexin V FITC-PI staining assay, respectively. The mRNA or protein expression level of BAG3, LC3-I, LC3-II, Atg5, NF-κB p65 and phosphorylated NF-κB p65 were assessed by qRT-PCR and western blot assay, respectively. Results: Overexpression of BAG3 inhibited cell apoptosis and promoted proliferation in hypoxia-injured H9c2 cells. Furthermore, autophagy and NF-κB were activated by BAG3 overexpression, and the NF-κB inhibitor PDTC could inhibit the activation of autophagy induced by BAG3 overexpression. In addition, the autophagy inhibitor 3-MA partly impeded the inhibitory effect of BAG3 on hypoxia-induced cardiomyocyte apoptosis. Conclusion: these results suggested that overexpression of BAG3 promoted cell proliferation and inhibited apoptosis by activating autophagy though the NF-κB signaling pathway in hypoxia-injured cardiomyocytes.

Introduction
Cardiovascular disease, especially cardiac dysfunction caused by myocardial hypoxia, is one of the leading causes of death worldwide. Myocardial hypoxia is caused by the acute reduction of coronary blood flow, resulting in diminished nutrient and oxygen supply to...
the myocardium, and thus leading to myocardial infarction, ischemia reperfusion injury, and hypertrophy [1]. Hypoxia is a well-known factor in the promotion of apoptosis, which is responsible for the anatomic remodeling of the myocardium in pathological processes [2]. The dysregulation of myocardial cell apoptosis can lead to a variety of heart diseases, including heart failure and myocardial infarction with or without reperfusion injury [3]. Therefore, understanding the apoptotic mechanisms involved in ischemia/hypoxic stimuli is important for the development of therapeutic strategies aimed at the treatment of related heart diseases.

Recently, it has been reported that autophagy, which is a lysosome-mediated intracellular degradation mechanism, engages in a complex interplay with apoptosis [4]. Autophagy is activated under stress conditions to provide a survival advantage to cells during starvation, hypoxia or other stresses [5]. The process of autophagy is controlled by numerous autophagy-related proteins (ATGs). ATG complexes participate in stepwise reactions resulting in autophagosome formation, of which LC3-II is the most specific marker, being incorporated into the autophagosomal membrane during its elongation [6]. Autophagy is found to be involved in many cellular and physiological pathways, including development and differentiation [7], and is regarded as a source of energy production for cell functioning through the degradation of damaged or aged molecules and organelles [8]. It is reported that autophagy can function both as a pro-survival and a pro-death mechanism, depending on the context [9]. Whether autophagy enhances or inhibits cell death in response to cellular stress is controversial. Recent advances suggest that autophagy is activated and plays a defective role in neurodegenerative diseases, including Huntington’s, Parkinson’s and Alzheimer’s diseases [10, 11]. In addition, accumulating evidence has indicated that autophagy is implicated in many cardiovascular diseases, such as myocardial ischemia, heart hypertrophy, and cardiomyopathies [12, 13], and the activation of autophagy in oxidative stress, ischemia/reperfusion, or high-glucose injured cardiomyocytes was also proven [14-16]. However, it is still controversial whether activation of autophagy in cardiomyocyte injury may present a mechanism of cell death or may be a rescue mechanism activated after the injury.

Bcl2-associated athanogene 3 (BAG3) protein belongs to the BAG family, which interacts with the ATPase domain of the heat shock protein (Hsp) 70 through the BAG domain (110-124 amino acids). It has been reported that BAG3 affects a number of biological processes, including cell proliferation, apoptosis, migration, cytoskeletal organization, and autophagy [17, 18]. BAG3 is induced by cellular stresses, such as oxidative and heat stress, nutrient deprivation, and transient forebrain ischemia [19-22]. The involvement of BAG3 in autophagy has been suggested and BAG3 was described as a mediator of a novel autophagy pathway. Recently, it was found that the expression of BAG3 is upregulated under oxidative stress in human cardiomyocytes and promotes myocardocyte proliferation and a survival response to stressful stimuli [23]. However, the role of BAG3 in hypoxia-induced cardiomyocyte apoptosis remains to be elucidated.

In this report, we used H9c2 cell lines to investigate the expression of BAG3 and to assess the biological function of BAG3 protein under hypoxic stimulus. In addition, the relationship between BAG3 and autophagy in the process of hypoxia-induced cardiomyocyte apoptosis was explored.

**Materials and Methods**

*Cell culture and hypoxia treatment*

H9c2 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in high-glucose Dulbecco’s modified Eagle’s medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml of penicillin, 100 mg/ml streptomycin (Sigma, St. Louis, MO, USA), and 5% CO₂ at 37 °C in a humidified atmosphere for 48 h.

For hypoxia treatment, cells were serum-starved overnight, and then cultured in normal medium in an incubator containing a gaseous mixture of 94% N₂, 5% CO₂ and 1% O₂ at 37 °C for 48 h. As a normoxia control, cells were incubated under the same conditions except the O₂ concentration was 21%.
Plasmids and siRNA transfection
To knock down BAG3 expression, the BAG3 siRNA (5′-AAG GUU CAG ACC UAC UUG GAA-3′) and scrambled RNA (5′-CAG UCG CGU UUG GCA GUG G-3′) were transfected into H9c2 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. After incubation for 48 h, the cells were harvested and BAG3 expression was determined.

To overexpress BAG3, H9c2 cells were transfected with the expression vector pcDNA3-BAG3 encoding full-length BAG3 or empty pcDNA3 vector (Vehicle) using Lipofectamine 2000 regent (Invitrogen) and incubated for 48 h, after which the cell lysates were used for analysis by RT-qPCR and western blotting.

Quantitative real-time PCR (qRT-PCR)
Total RNA was extracted from cultured H9c2 cells using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. After evaluating the integrity and purity of the extracted RNA, 0.1 µg of RNA was reverse transcribed to cDNA using the MMLV Reverse Transcriptase kit (Takara Biotechnology, Dalian, China) according to the manufacturer’s instructions. qRT-PCR was performed using 20 µl reaction volumes with SYBR Premix Ex Taq Takara (TaKaRa) using the LightCycler 480 SYBR green I Master (Roche Diagnostics, GmbH) with a Roche 480 LightCycler. The PCR parameters were as follows: 95 °C for 30 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. All reactions were performed in triplicate. The relative expression levels of the target genes tested were calculated using the 2-ΔΔCt method and normalized to GAPDH.

Western blot
Total proteins were extracted from cultured H9c2 cells using RIPA (Beyotime, Nantong, China), and their concentrations were determined by the BCA (bicinchoninic acid) Protein Assay Kit (Pierce, Rockford, IL, USA). Protein samples (100 µg) were separated in 10% SDS-PAGE and transferred to PVDF membranes. The membranes were blocked using 1x PBS containing 0.1% Tween-20 and 5% w/v nonfat dry milk and then immunoblotted with polyclonal rabbit anti-ATG5 polyclonal antibody, mouse monoclonal anti-p-NF-kB p65 and anti-NF-κB p65 (1:2,000 dilution, Cell Signaling Technology, Danvers, MA, USA), rabbit anti-BAG3 antibody (1:1000 dilution), rabbit anti-LC3 polyclonal antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and rabbit anti-GAPDH (1:1000 dilution; Santa Cruz Biotechnology) overnight at 4 °C. Membranes were then incubated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:3000; Santa Cruz Biotechnology) for 1 h at room temperature. The protein bands were developed using enhanced chemiluminescence (ECL) Plus Western Blotting Detection System (GE Healthcare) and exposed to Amersham Hyperfilm™ ECL (GE Healthcare). For quantification of protein expression, the X-ray films were scanned and analyzed using Image J 1.41o software (National Institutes of Health (NIH), Bethesda, MD, USA).

MTT assay
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was employed to investigate cell survival. In brief, H9c2 cells transfected with BAG3 siRNA and pcDNA3 BAG3 were cultured in a 96-well plate. After incubation under hypoxic conditions for 48 h, 20 µl MTT (5 mg/ml, Sigma) were added into each well. The plates were cultured for an additional 4 h before 150 µl dimethyl sulfoxide/well (DMSO, Sigma) were added. Absorbance was measured at 490 nm with a microplate spectrophotometer.

CCK-8 assay
H9c2 cells were seeded in a 96-well plate with hypoxia treatment for 48 h. Then CCK-8 solution (Dojin Chemical, Kumamoto, Japan) was added to each well and the plate was incubated at 37 °C for 4 h. Absorbance was measured at 450 nm using a microplate reader (Bio-Rad).

Assessment of apoptosis
The Annexin V FITC-PI staining assay was used to detect apoptotic H9c2 cells. In brief, after hypoxia treatments, cells were harvested and washed with PBS. Apoptosis staining was performed using an Annexin V FITC-PI apoptosis detection kit (BD Biosciences, Piscataway, NJ, USA) according to the manufacturer’s instructions. Stained cells were analyzed using FACSCalibur™ Flow cytometry (BD Biosciences) with CellQuest software.
Statistical analysis

All data are reported as the mean ± SD. Statistical analyses between groups were performed using One-way analyses of variance (ANOVA). \( P < 0.05 \) was considered to indicate a significant result.

Results

**BAG3 is upregulated in hypoxia-injured cardiomyocytes**

To confirm that our experimental protocol successfully induced hypoxic stress in H9c2 cells, the vascular endothelial growth factor (VEGF) protein expression level was monitored. The results showed that VEGF protein was significantly increased by hypoxia treatment (Fig. 1A). Besides, MTT and Annexin V FITC-PI staining assay showed that cell proliferation was remarkably inhibited (Fig. 1B) and apoptosis (Fig. 1C) was induced by hypoxia. Those results indicating that hypoxic responses were successfully induced in our assay.

Next, we assessed the expression of the BAG3 mRNA and protein using RT-qPCR and western blot assay. The results showed that BAG3 mRNA expression was significantly upregulated in the hypoxia group relative to the control (Fig. 1D). Western blot analysis of protein lysates from the hypoxia-injured H9c2 cells revealed that BAG3 protein was detectable at low levels in the control group, while levels were remarkably elevated in the hypoxia group (Fig. 1E).

**Overexpression of BAG3 promoted cell proliferation and inhibited cell apoptosis in hypoxia-injured cardiomyocyte**

To figure out the role of upregulated BAG3 in hypoxia-injured cardiomyocytes, pcDNA3-BAG3 and BAG3 siRNA were used to overexpress or silence BAG3 expression, respectively.

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**Fig. 1.** BAG3 is highly expressed in hypoxia-injured H9c2 cells. Cells were cultured and treated with hypoxia for 48 h. (A) VEGF protein expression level was assessed by western blot. (B) Cell proliferation was measured using MTT assay. (C) Cell apoptosis was evaluated using the Annexin V FITC-PI staining assay. (D) qRT-PCR analysis was used to evaluate the mRNA expression of BAG3. (E) Western blot analysis was used to determine the protein expression level of BAG3. The data are expressed as the mean ± SD (n=6). Statistical comparisons were performed using one-way ANOVA. \( *P < 0.05 \) vs. control group.
(Fig. 2A), and then cell proliferation and apoptosis were tested in hypoxia-injured H9c2 cells. The results showed that overexpression of BAG3 significantly promoted cell proliferation and inhibited apoptosis in hypoxia-injured cells, while BAG siRNA produced the opposite effect (Fig. 2B and C).

**Overexpression of BAG3 upregulated the autophagy related genes**

Next, to evaluate the effect of BAG3 on cardiomyocyte autophagy, we analyzed the expression of the conversion of LC3-I to LC3-II, a hallmark of autophagy induction and autophagy-related gene Atg5. The results revealed that the ratio of LC3II/I was increased with pcDNA3 BAG3 treatment and decreased with BAG3 siRNA treatment compared with hypoxia-injured cells (Fig. 3A). The mRNA abundances of Beclin1 and Atg5 increased with pcDNA3-BAG3 treatment, whereas the mRNA abundances of these genes were decreased by BAG3 siRNA treatment (Fig. 3B).
Fig. 3. Overexpression of BAG3 enhanced autophagy in hypoxia-injured H9c2 cells. Cells were transfected with BAG siRNA and pcDNA-BAG3, and the induction of autophagy by BAG3 was determined by measuring the LC3 and ATG5 protein levels using western blot assay. (A) The ratio of LC3II/I was evaluated and data were expressed as fold increases or decreases relative to the control. (B) The protein level of ATG5 was also determined by western blot assay. The optical densities of the respective protein bands were analyzed using Image J 1.41o software and normalized to the loading control (GAPDH). The data are expressed as the mean ± SD (n = 6). Statistical comparisons were performed using one-way ANOVA. *P < 0.05 vs. Hypoxia group.

Fig. 4. Autophagy is activated by BAG3 through NF-κB signaling pathway. (A) Cells transfected with BAG siRNA and pcDNA-BAG3 were cultured and treated under hypoxic conditions for 48 h. The protein expression level of phosphorylated NF-κB p65 and NF-κB p65 were evaluated using western blot analysis. (B) H9c2 cells were treated with pcDNA3-BAG3 or NF-κB inhibitor PDTC. The ratio of LC3II/I was evaluated and data were expressed as fold increases or decreases relative to control. (C) H9c2 cells were treated with pcDNA3-BAG3 or NF-κB inhibitor PDTC. The protein level of ATG5 was determined by western blot assay. Data are expressed as the mean ± SD (n = 6). Statistical comparisons were performed using one-way ANOVA. *P < 0.05 vs. Hypoxia group. #P < 0.05 vs. pcDNA3-BAG3.
Activation of NF-κB by BAG3 resulted in activation of autophagy

NF-κB-mediated induction of autophagy in cardiac ischemia/reperfusion injury has been reported [24], thus we measured the effect of BAG3 on the activity of NF-κB. The results showed that overexpression of BAG3 significantly upregulated the protein expression of phosphorylated NF-κB p65 compared with hypoxia-injured cells, and knockdown of BAG3 had the opposite effect (Fig. 4A). Furthermore, the NF-κB inhibitor PDTC significantly suppressed the elevations in ATG5 and the ratio of LC3II/I (Fig. 4B). This finding demonstrated that BAG3 activates autophagy through the NF-κB signaling pathway.

Inhibition of autophagy by 3-MA partly promoted proliferation and inhibited apoptosis induced by overexpression of BAG3 in hypoxia-injured cardiomyocytes

To assess the role of autophagy in BAG3-regulated proliferation, hypoxia-induced apoptosis was inhibited by autophagy inhibitor 3-MA, and the role of autophagy was determined using MTT and CCK-8 assays, and apoptotic cells were tested by Annexin V-FITC assay. The results implied that BAG3 promoted cell proliferation and cell viability inhibited by hypoxia, and this effect was significantly inhibited by NF-κB inhibitor PDTC and autophagy inhibitor 3-MA (Fig. 5A and B). The results of the Annexin V-FITC assay showed that BAG3 significantly inhibited hypoxia-induced apoptosis. Moreover, PDTC and 3-MA both impeded the inhibitory effect of BAG3 on hypoxia-induced cardiomyocyte apoptosis (Fig. 5C).

Discussion

The human BAG family of proteins comprises six family members (BAG 1–6) that function as molecular chaperone regulators. BAG3 is found at high levels in several disease models and is especially high in skeletal and cardiac muscle in vivo. Marco et al. [23] investigated the role of BAG3 in cardiomyocyte proliferation, survival and response to stressful stimuli, and announced that there may be an important role for BAG3 in the process of damaged cardiomyocyte regeneration through its anti-apoptotic activity and promotion of cell survival. The present study indicates that BAG3 mRNA and protein are expressed most plentifully in the hypoxia-treated H9c2 cells. As a common pathophysiological feature of heart diseases, including cardiac hypertrophy, diabetes and myocardial infarction, which are the leading causes of morbidity and mortality worldwide, hypoxia induces inflammation, cardiomyocyte apoptosis, and cardiac fibrosis that can lead to left ventricular dilatation and
heart failure [25]. Cardiomyocyte apoptosis induced by hypoxia is an important pathological phenomenon in the heart [26]. This study showed that overexpression of BAG3 promotes cardiomyocyte survival and inhibits apoptosis, which suggested a protective effect of BAG3 in the hypoxia-injured cardiomyocyte.

Autophagy, which is a predominantly cytoprotective process through de novo-formed membrane-enclosed vesicles that engulf and consume cellular components, has been linked to cell survival and apoptosis [27]. BAG3 could participate, along with HSP70 or HSPB8, in the degradation of misfolded proteins through mediation of a novel autophagocytic pathway [17]. Moreover, BAG3 is found to antagonize BAG1 and shift from the proteasome to the autophagosome [28]. Thus, we speculated that there might be a relationship between BAG3 and autophagy in the inhibitory effect of BAG3 on hypoxia-induced H9c2 cell apoptosis. The results of this study imply that BAG3 overexpression stimulates autophagy. Furthermore, our results suggest that BAG3 inhibits hypoxia-induced H9c2 cell apoptosis by promoting autophagy. Both autophagy and apoptosis are well-controlled biological processes that play essential roles in development, tissue homeostasis, and disease; however, the interplay between autophagy and apoptosis is quite complex. There are three different types of interplay with autophagy and apoptosis: the partner, the enabler, and the antagonist [27]. Researches have reported that inducing autophagy could inhibit oxidative stress and high-fat diet-induced apoptosis [29, 30]. However, Zhang et al. [31] indicated that autophagy in hypoxia/reoxygenation injury promoted cardiomyocyte apoptosis. In the present study, autophagy did not lead to cell death, but instead acted to attenuate apoptosis, which is consistent with Xu et al. [32] and Li et al. [33], who reported that enhanced autophagy increased cell viability and inhibited apoptosis in cardiomyocytes following ischemia/reperfusion. Thus, the role of autophagy in cardiomyocyte injury is inconsistent and may have a connection with the duration of hypoxia.

NF-κB is known to control multiple cellular processes, and several molecules involved in autophagy are dependent upon NF-κB [34, 35]. BAG3 is known to sustain NF-κB activation by inhibiting the delivery of kappa B kinase γ (IKKγ) inhibitor to the proteasome [36, 37]. Yunoki et al. [38] reported that the phosphorylation of p65 and IkBγ were decreased by BAG3 knockdown. Our results showed that overexpression of BAG3 upregulated the phosphorylation of p65, indicating that BAG3 activated NF-κB signaling pathway. Furthermore, the inhibitor of NF-κB blocked the effect of BAG3 on autophagy, which suggested that overexpression of BAG3 activated autophagy though NF-κB signaling. In conclusion, our data demonstrate that BAG3 is upregulated in hypoxia-injured H9c2 cells, and overexpression of BAG3 protects H9c2 cells from hypoxia-induced apoptosis. Furthermore, autophagy is involved in inhibiting hypoxia-induced apoptosis and BAG3 activates autophagy through NF-κB signaling pathway. All in all, this work suggests that overexpression of BAG3 attenuates hypoxia-induced cardiomyocyte apoptosis by inducing autophagy via NF-κB signaling pathway.

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Disclosure Statement

All authors declared no conflicts of interest.
Reference


