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Ambra1 modulates starvation-induced autophagy through AMPK signaling pathway in cardiomyocytes

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ABSTRACT

Recent research has revealed a role for Ambra1, an autophagy-related gene-related (ATG) protein, in the autophagic pro-survival response, and Ambra1 has been shown to regulate Beclin1 and Beclin1-dependent autophagy in embryonic stem cells and cancer cells. However, whether Ambra1 plays an important role in the autophagy pathway in cardiomyocytes is unknown. In this study, we hypothesized that Ambra1 is an important regulator of autophagy and apoptosis in cardiomyocytes. To test this hypothesis, we confirmed autophagic activity in serum-starved cardiomyocytes by assessing endogenous microtubule-associated protein 1 light chain 3 (LC3) localization, the presence of autophagosomes and LC3 protein levels. Cell apoptosis and viability were measured by annexin-V and PI staining and MTT assays. We determined that serum deprivation-induced autophagy was associated with Ambra1 upregulation in cardiomyocytes. When Ambra1 expression was reduced by siRNA, the cardiomyocytes were more sensitive to staurosporine-induced apoptosis. In addition, co-immunoprecipitation of Ambra1 and Beclin1 demonstrated that Ambra1 and Beclin1 interact in serum-starved or rapamycin-treated cardiomyocytes, suggesting that Ambra1 regulates autophagy in cardiomyocytes by interacting with Beclin1. Finally, we determined that starvation stress-induced activation of Ambra1 contributes to the attenuation of adaptive AMP-activated protein kinase (AMPK) signaling. In conclusion, Ambra1 is a crucial regulator of autophagy and apoptosis through AMPK signaling pathway in cardiomyocytes that maintains the balance between autophagy and apoptosis.

1. Introduction

In many developed countries, cardiovascular diseases are the major cause of disability and mortality [1]. Myocardial ischemia and reperfusion is a major source of cardiomyocyte injury within the context of a host of clinical pathologies [2]. Coronary disease is one of the most prevalent diseases afflicting humans [3]. The major consequences of coronary disease are myocardial ischemia (cell damage) and myocardial infarction (cell death and apoptosis), either of which can develop when blood supply to the myocardium decreases. Myocardial infarction is a complex process for which the regulatory mechanisms remain to be understood.

Autophagy, a dynamic catabolic process in which cellular components are delivered to the lysosome for degradation, has been implicated in a wide array of physiological processes and pathogenesis of diverse diseases [4–6]. Besides to its established roles in homeostasis maintenance and stress adaptation, autophagy also possesses functions in cell differentiation [7–11]. It is a closely regulated process that helps to maintain a balance among the synthesis, degradation, and subsequent recycling of cellular products. In addition, autophagy rescues colorectal cancer cells from death in response to starvation or anti-tumor drugs [12,13]. Autophagy is regulated by specific genes known as ATGs (autophagy-related genes). To date, more than 34 ATG genes have been identified in yeast.

Ambra1 is a newly discovered ATG gene, and the Ambra1 protein is a crucial regulator of autophagy. Ambra1 interacts with Beclin1 through the target lipid kinase Vps34/PI3KC3 to assemble a class III PI3K complex, which positively regulates the formation of autophagosomes [14]. A dynamic interaction between Ambra1 and BCL-2 exists in mitochondria and potentially regulates Beclin1-dependent autophagy and apoptosis [15]. The function of Ambra1 in autophagy and apoptosis has been explored in vitro in embryonic stem cells and human fibroblast 2FTGH cells [16], but

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the role of Ambra1 in cardiomyocytes has not been reported in the literature, and the role of this ATG protein in the autophagy and apoptosis pathways in cardiomyocytes is unknown.

In this study, we used cardiomyocytes to test the hypothesis that Ambra1 interacts with Beclin1 to promote autophagy and to inhibit apoptosis in cardiomyocytes. We sought to determine whether autophagy occurs in cardiomyocytes in response to apoptotic stimuli and whether Ambra1 regulates autophagy in cardiomyocytes by interacting with Beclin1. Our findings clearly suggest that Ambra1 functions at the intersection between autophagy and apoptosis. We found that Ambra1 interacts with Beclin1 to function as a pro-survival switch that inhibits apoptosis and induces autophagy, thereby preventing cardiomyocytes death in response to apoptotic agents.

2. Materials and methods

2.1. Cell isolation and culture

Primary rat cardiomyocyte culture isolation of cardiacmyocytes was performed as previously described [32]. Briefly, the hearts were harvested and placed in PBS. Then, the hearts were minced and dissociated with 1 mg/mL type I collagenase. Cardiomyocytes were enriched by preplating for 90 min to eliminate fibroblasts. More than 90% of the cells were identified as cardiacmyocytes by immunocytochemistry with anti-sarcomeric β-actin (Sigma). The cells were then cultured in DMEM with 10% FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin and maintained in 5% CO_2 at 37 °C. The cells were seeded at a density of 4×10^4 cells/mL in 96-well microplates for MTT assays or at a density of 3×10^5 cells/mL in 6-well microplates for LDH, CK, MDA activity assays and antioxidant enzyme assays, and for the measurement of other biochemical indicators. The culture medium was changed every second day. After 48 h, cultured cardiomyocytes were used in subsequent experiments.

2.2. RNA interference

The following siRNA oligonucleotides corresponding to human Ambra1 cDNA were purchased from Genepharma (Shanghai, China): 5′-AGAACTGCAAGATCTA-3′. The cells were transfected with 100 pmol Ambra1 siRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 6-well plates. The negative control siRNA was transfected under the same conditions. The transfection was repeated for two days to achieve a high efficiency. Approximately 24 h after the second transfection, 2×10^5 cells/well were plated in 6-well plates and cultured with the indicated agents. Protein expression was measured by Western blot 48 h after transfection.

2.3. Reagents and antibodies

The Beclin 1 and other polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, California). Rapamycin and polyclonal antibodies for GAPDH and LC3-II were purchased from Sigma–Aldrich. The coding sequence of MAP1-LC3 fusion with GFP was synthesized and cloned into the pcDNA3.1(+) to construct the LC3-GFP-expressing plasmid.

2.4. Quantitative GFP-LC3 analysis and electron microscopy

Quantitative GFP-LC3 light microscopy autophagy assays were performed in cardiomyocytes with various treatments. The cardiomyocytes grown to 80% confluency were transfected with a GFP-LC3-expressing plasmid, using Lipofectamine 2000 (Invitrogen). After 24 h transfection, cells were subjected to rapamycin (200 nM) for another 24 h and analyzed by fluorescence microscopy. In another two groups, cells were pretreated with starvation, 2 h later, cells were subject to 3MA (100 μM) for another 24 h and analyzed by fluorescence microscopy. Electron microscopy was performed to determine the autophagic vacuoles in cardiomyocytes with or without starvation treatment. Images of thin sections were observed under a transmission electron microscope (JEM1230, Japan).

2.5. RNA isolation and real-time PCR

Total cellular RNA from 2 to 5×10^5 cells was prepared with Trizol, and reverse transcription (RT) was performed with M-MLV Reverse Transcriptase (Promega). Primer sequences and conditions are available upon request. For quantitative analysis of mRNA expression of Ambra1, Beclin 1, Atg5 or Atg 7, real-time PCR was conducted by LightCycler 480 (Roche applied), 1 μg RNA per sample was converted to cDNA and used for real-time PCR. Data were normalized based on β-actin.

2.6. Western blot

Cell extracts were prepared by a standard protocol, and proteins were detected by Western blot using polyclonal (human) anti-Beclin 1 antibody, monoclonal (human) anti-Atg5 antibody, monoclonal (human) anti-Atg7 antibody, or polyclonal (rabbit) anti-LC3 or GAPDH antibody (Sigma–Aldrich). Goat anti-mouse IgG or goat anti-rabbit IgG (Pierce) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (Super Signal West Femto, Pierce) were used for detection.

2.7. Cell viability assay

Cell viability was determined by MTT assay. The cardiomyocytes were seeded in 96-well plates, and after 24 h, the medium was replaced with alpha-MEM medium containing 1% FBS. At 0, 12 h, 24 h and 48 h post-treatment, the incubation medium in the test wells was replaced with 50 μl 1× MTT solution, and the cells were incubated for 2 h at 37 °C. After incubation, the MTT solution was discarded, and 150 μl DMSO was added to dissolve the precipitate completely at room temperature. The optical density was then measured at 570 nm using a spectrophotometer. The cell viability was expressed as relative viable cells (%) to control cardiomyocytes.

2.8. Measurement of apoptosis

Percentage of cells actively undergoing apoptosis was determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to manufacturer's instructions. Briefly, after incubation under starvation for 48 h, the cardiomyocytes were harvested and resuspended in binding buffer. Cells were mixed with annexin VFITC and PI. After incubating for 15 min in the dark, analysis was performed by flow cytometry.

2.9. Co-immunoprecipitation assays

The supernatants from cardiomyocytes cell lysates were incubated with anti-sera bound to protein A-sepharose beads (Amersham Bioscience) for 2 h at 4 °C and washed extensively. To elute the bound proteins, 100 mM glycine–HCl (pH 2.5, precipitated with 5% trichloroacetic acid) was added, and the samples were washed with ice-cold acetone and resuspended in SDS sample buffer.

2.10. Statistical evaluations

For GFP-LC3 dot number analysis, relative mRNA expression of Ambra1 or beclin 1 to β-actin, MTT measurements, statistical evaluations are presented as mean ± SE. Data were analyzed using the Student's *t* test.

3. Results

3.1. Starvation induces apoptosis and autophagy and increases Ambra1 activity in cardiomyocytes

In this study, the autophagy was induced by starvation. The cardiomyocytes were cultured in serum-free medium for various time periods and were transfected with GFP-LC3, a biomarker for autophagy. LC3, a microtubule-associated protein light chain 3 (MAP-LC3), typically exhibits diffuse cytosolic distribution [17,18]. The C-terminal fragment of LC3 is cleaved immediately after synthesis to yield a cytosolic form termed LC3-I. A subpopulation of LC3-I is further converted to the LC3-II autophagosome-associated form. When autophagy is activated, the LC3-I protein localized in the cytoplasm is cleaved, lipidated, and inserted as LC3-II into autophagosome membranes.

To confirm that the autophagosomes were induced by starvation, we examined serum-starved cardiomyocytes via fluorescence microscopy and electron microscopy (EM). Representative fluorescence images, shown in the Fig. 1A, indicated that incubating cardiomyocytes in serum-free media in the presence of chloroquine, a lysosome inhibitor, led to the redistribution of LC3 to punctate structures and increased the number of LC3-GFP-positive vesicles in cells. The ultra-structures of cardiomyocytes were observed by EM microphotography, shown in the Fig. 1B. The EM images confirmed the induction of autophagy at various time points as indicated by autophagosome visualization.

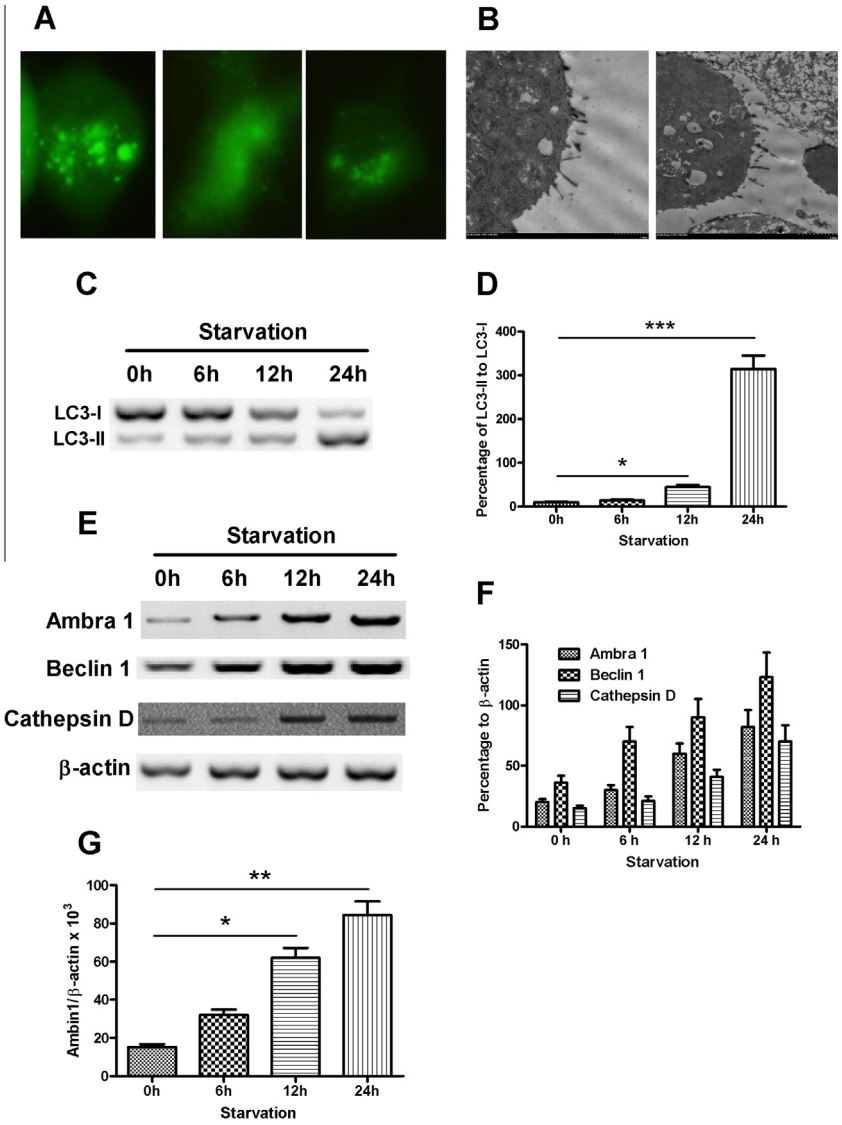

Fig. 1. Autophagy is induced by starvation in cardiomyocytes. (A) Representative images of fluorescent LC3 puncta after GFP-LC3 transduction. The cardiomyocytes were transfected with a plasmid that expresses a GFP-LC3 fusion protein. After 24 h, the cardiomyocytes were cultured with growth medium (10% serum) (left), serum-free medium (middle), or serum-free medium +10 μM chloroquine (right) for another 24 h. (B) Representative electron micrographs of starvation-induced autophagy in cardiomyocytes. Control group (left) or starvation for 24 h (right). (C) Western blot analysis of LC3 expression in cardiomyocytes. (D) Quantitation of the optical density of LC3-II/LC3-I at various time points. (E) Western blot of Ambra1, Beclin1 and cathepsin D expression after serum starvation at various time points. (F) Quantitation of the optical density of Ambra1, cathepsin D and Beclin1. (G) The relative Ambra1 expression after serum starvation for the indicated times as determined by real-time PCR. Experiments in this figure were repeated three times. $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

Next, immunoblot analysis was used to measure the levels of LC3 and other ATG-related proteins in cardiomyocytes starvation-induced autophagy model. As shown in Fig. 1C and D, incubating cardiomyocytes in serum-free media in the presence of chloroquine resulted in LC3-II accumulation. The LC3-II/LC3-I ratio increased after incubation with chloroquine in serum-free media. Ambra1 levels increased during autophagy as well as Beclin1 levels (Fig. 1E and F).

Since autophagy is a constitutive process involving the activation of lysosomal enzymes and the subsequent degradation of substrates, the alteration of cathepsin D expression was measured [19]. Cathepsin D expression was upregulated by serum starvation (Fig. 1E). The quantification of the optical densities of Ambra1, Beclin1 and cathepsin D in serum-starved cardiomyocytes is presented in Fig. 1F. We further performed real-time PCR to determine that Ambra1 mRNA expression was increased (Fig. 1G), which distinguish Ambra1 up-regulation was due to transcriptional, not post-translational regulation. These findings demonstrated that autophagy was triggered by starvation and that Ambra1 was associated with autophagy.

3.2. Inhibition of Ambra1 impairs autophagy and promotes serum starvation-induced apoptosis in cardiomyocytes

Ambra1 is an anti-apoptotic ATG protein that promotes autophagy [20]. Therefore, we hypothesized that Ambra1 was a negative regulator of autophagy and apoptosis in cardiomyocytes. To verify this hypothesis, we firstly used siRNA to knock down Ambra1 expression in cardiomyocytes (Fig. 2A and B).

We dynamically monitored autophagosome formation by immunofluorescence in Ambra1 siRNA and negative control cells cultured in serum-free media. Inhibition of Ambra1 significantly resulted in a decrease in the number of LC3-GFP-positive vesicles (Fig. 2C and D). The results revealed a significant reduction in visible dots in the Ambra1 siRNA groups (siAmbra1) compared with the negative control groups. The negative and siAmbra1 cardiomyocytes were cultured in serum-free medium in the presence of chloroquine, and LC3-II expression was measured by Western blot. We observed LC3-II accumulation in the negative control and the Ambra1 siRNA groups (Fig. 2E and F). Nevertheless, there was a greater decrease in the LC3-II/LC3-I ratio in the Ambra1 siRNA group than in the negative control group. These data suggested that Ambra1 was pro-autophagic in cardiomyocytes.

We measured the cell viability after Ambra1 knockdown by using an MTT assay. The cell viability was decreased with serum deprivation (12 and 24 h) in all treatment groups. Cell viability was markedly lower in the serum-starved Ambra1 siRNA group compared with the serum-starved negative control siRNA group (Fig. 2G). Next, we investigated whether Ambra1 downregulation would result in increased cell death under stress by using the Ambra1 knockdown cardiomyocytes. In cardiomyocytes, the negative group were more resistant to stress-induced apoptosis than the siAmbra1 groups after 24 h of serum starvation (Fig. 2H). Ambra1 expression was knocked down by siRNA to determine whether the cells were more sensitive to apoptosis. The cardiomyocytes were stained with annexin-V and PI and analyzed by flow cytometry. Staurosporine treatment resulted in minimal cell death, whereas significant cell death was detected in the Ambra1 siRNA groups (Fig. 2I). These results indicated that Ambra1 may be an anti-apoptotic factor in cardiomyocytes.

3.3. Ambra1 triggers autophagy by binding to Beclin1

To understand how Ambra1 triggers autophagy, we investigated the mechanism by which Ambra1 affected autophagocyte formation. The autophagocyte formation is initiated by Beclin1-Class III PI3K complex; however, it has not been determined whether Ambra1 interacts with this complex in cardiomyocytes. We determined whether endogenous Ambra1 interacted with endogenous Beclin1 upon the induction of autophagy in cardiomyocytes. The interaction between endogenous Ambra1 and endogenous Beclin1 was confirmed by co-immunoprecipitation in cardiomyocytes. We used anti-Ambra1 or anti-Beclin1 antibodies to pull down the protein complexes, and found that the Ambra1-Beclin1 interaction was more significant in autophagy-stimulated cells than in non-stimulated cells (Fig. 3A and B). Similar results were observed in rapamycin-treated cardiomyocytes (Fig. 3C and D). These findings confirmed that Ambra1 bound to Beclin1 during autophagy.

3.4. Activation of AMPK mediates Ambra1-induced autophagy in cardiomyocytes

Next we focused on the underlying mechanism of autophagy induction by Ambra1. Previous studies show that activation of AMPK is important for autophagy induction [21,22]. First, we examined AMPK activation in Ambra1-treated cardiomyocytes. AMPK activation was reflected by AMPKα1 phosphorylation at Thr 172 and ACC phosphorylation at Ser 79. As demonstrated, a profound AMPK activation was observed in cardiomyocytes after Ambra1 treatment (Fig. 4A), as AMPKα1 and ACC phosphorylation were both significantly increased (Fig. 4A). Co-IP results confirmed that Ambra1 induced AMPK/Ulk1 association in cardiomyocytes, which appeared to cause Ulk1 phosphorylation in (Fig. 4B). AICAR, the AMPK agonist [23], promoted Ulk1 phosphorylation and LC3B-II/beclin-1 expression in cardiomyocytes (Fig. 4C). On the other hand, AMPKα1/2 RNAi stable-knockdown significantly inhibited Ambra1-induced autophagy in cardiomyocytes (Fig. 4D), correspondingly, cardiomyocytes cell viability loss and apoptosis were increased (Fig. 4E and F). These results indicated that activation of AMPK by Ambra1 mediates autophagy and apoptosis-resistance in cardiomyocytes.

4. Discussion

Ambra1 promotes autophagy in numerous cell types [24,25], but the role of Ambra1 in cardiomyocytes remains unknown. In this study, we examined the role of Ambra1 in autophagy and apoptosis in cardiomyocytes. Our novel findings suggested that Ambra1 not only promoted autophagy in this cardiomyocytes but also had the ability to control the switch between autophagy and apoptosis in these cells, thereby altering cellular fate. In addition, our results indicated that Ambra1 induced autophagy in cardiomyocytes by interacting with Beclin1.

Ischemia/reperfusion (I/R) injury includes a series of events that may occur together or separately: reperfusion arrhythmias, myocardial stunning in ‘‘reversible mechanical dysfunction’’, microvascular damage and cell death [26–28]. Clinical studies have also shown that autophagy is an intracellular lysosomal degradation process that is characterized by the formation of double-membrane vesicles in cytoplasm. Therefore autophagy plays key physiological cellular functions such as degradation of long-lived proteins, organelle turnover, adaptation to nutrient depletion, extension of lifespan, cellular development, differentiation, and anti-aging [29]. Consequently, autophagy can preserve viability or be a self-destructive process that leads to cell death [30]. Our data indicated that autophagy is activated in cardiovascular in vitro and that autophagy contributes to cardiovascular cell survival in the cellular microenvironment. In our study, immunofluorescent staining of the autophagosome marker LC3 revealed that autophagy was induced by serum-starvation in cardiovascular cells.

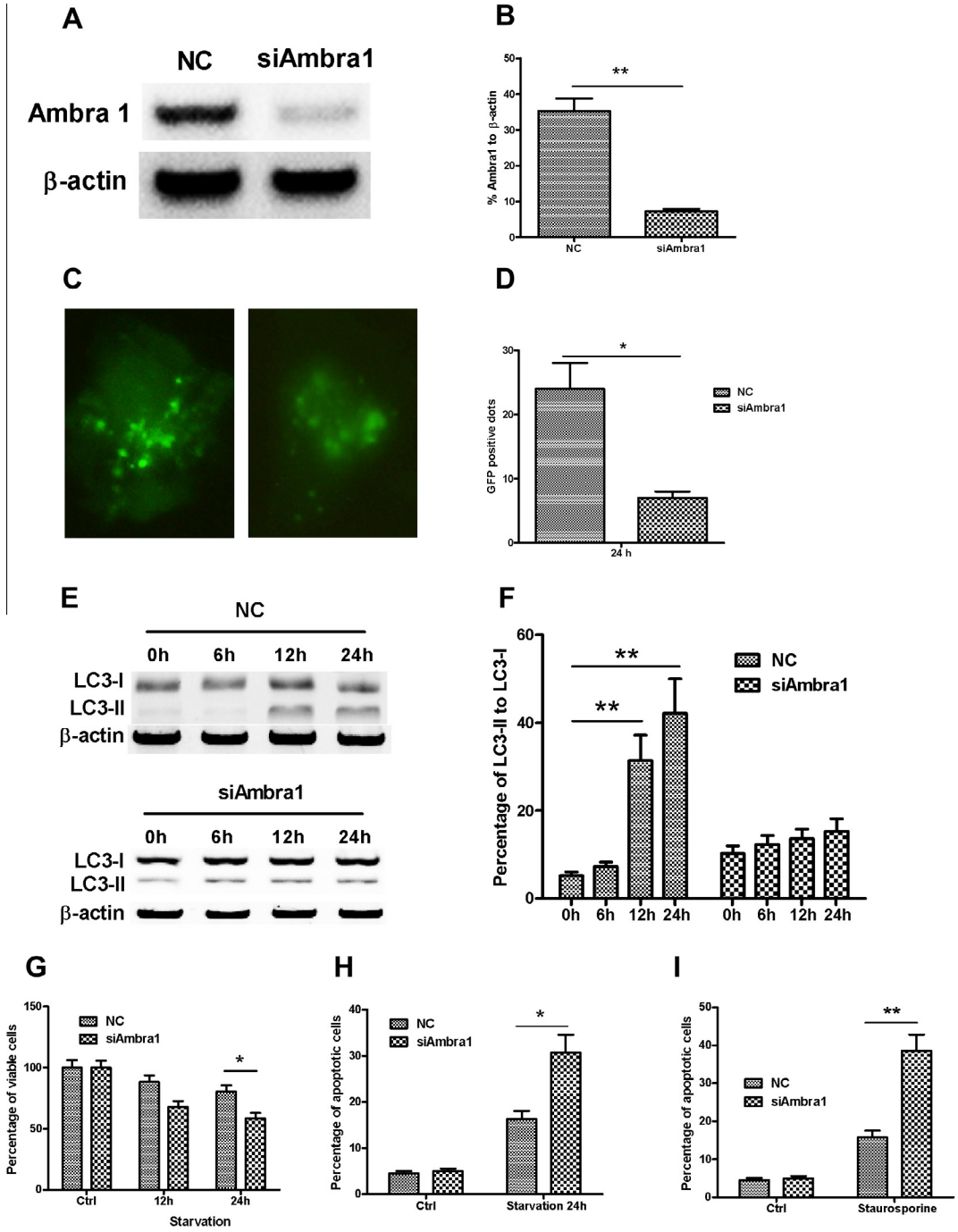

Fig. 2. Inhibition of Ambra1 impairs autophagy and promotes serum starvation-induced apoptosis in cardiomyocytes. (A) Western blot analysis of Ambra1 expression in cardiomyocytes after transient transfection with negative control (NC) or Ambra1-siRNA oligonucleotides (siAmbra1). (B) Quantitation of the optical density of the Ambra1 bands after transfection. (C) Representative images of fluorescent LC3 puncta after GFP-LC3 transduction. The cardiomyocytes were transfected with a GFP-LC3 plasmid and negative control siRNA (left) or Ambra1-siRNA oligonucleotides (right). After 24 h, the cardiomyocytes were treated with serum starvation for another 24 h. (D) The quantification of positive dots in the negative control and si-Ambra1 groups. (E) LC3 levels in negative control and si-Ambra1 serum-starved cardiomyocytes. (F) The quantification of the optical density in the negative control and si-Ambra1 groups of different time points. (G) The cardiomyocytes cultured in serum-free medium for 12 or 24 h. Cell viability was determined by MTT assay. D: The cardiomyocytes serum starved for 24 h. Cell death was measured by flow cytometry. F: The cardiomyocytes treated with staurosporine (2 mM for 6 h). Cell death was measured by flow cytometry. Experiments in this figure were repeated three times. $^{*}p < 0.05$, $^{**}p < 0.01$, or $^{***}p < 0.001$.

Previous studies have indicated that Ambra1 regulates autophagy in multiple cell types, including neuronal and 2F cells [13,31]. However, the role of Ambra1 in cardiomyocytes has not been reported. Our study demonstrated that starvation induced Ambra1 expression and that Ambra1 knockdown suppressed autophagy in response to starvation in cardiomyocytes. However, the link between Ambra1, autophagy and apoptosis remains obscure. Our present study suggests for the first time that apoptosis is induced at least partially by the retardation of autophagy caused by Ambra1 impairment. Consistent with other studies [32,33], we demonstrated that AMPK/mTOR-dependent autophagy is a mechanism that protects against starvation stress in cardiomyocytes.

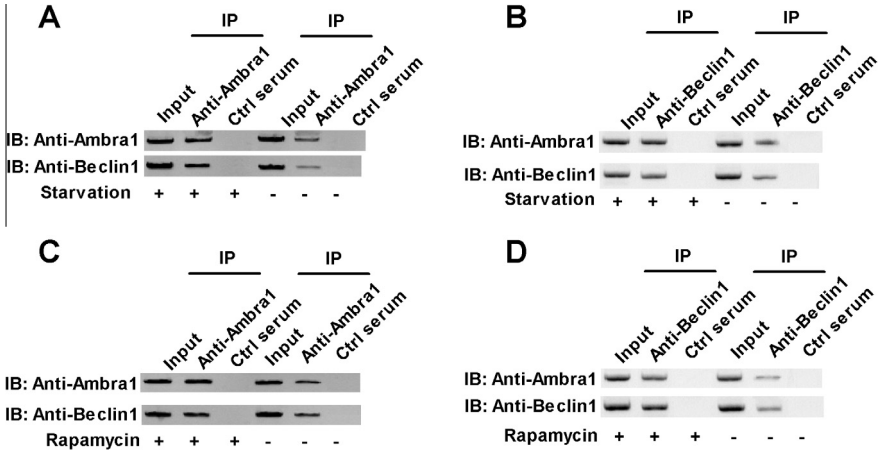

Fig. 3. Ambra1 interacts with Beclin1 in cardiomyocytes. (A and B) Co-immunoprecipitation assays with cardiomyocytes lysates. The cardiomyocytes were serum-starved or not (control) for 24 h. Next, the cells were incubated with anti-Ambra1 antisera or control sera (A) or anti-Beclin1 antisera or control sera (B) covalently bound to protein A-sepharose beads. After extensive washes, the eluted proteins were separated and subjected to Beclin1 and Ambra1 immunoblotting. The procedure for (C and D) is similar to that for (A and B) with the exception that the cells were treated or not with rapamycin (100 nM for 24 h) instead of being serum-starved.

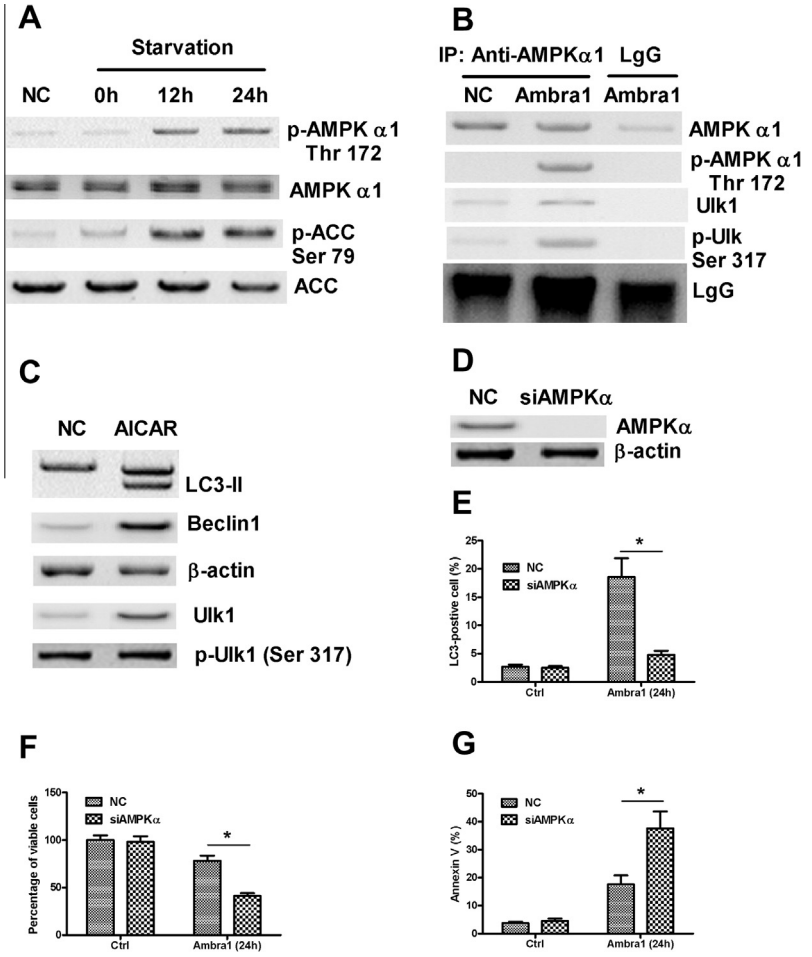

Fig. 4. Activation of AMPK mediates Ambra1-induced autophagy in cardiomyocytes. (A) The cardiomyocytes cells were treated with vehicle, or 10 μM of Ambra1, phospho (p-) and total AMPKα1 (Thr 172) and ACC (Ser 79) were examined by Western blots. P-AMPKα and ACC were quantified. (B) The association between AMPKα (total and p-) and Ulk1 (total and p-) in vehicle-, or Ambra1 10 μM, 24 h)-treated cardiomyocytes were examined by co-IP. (C) The cardiomyocytes were treated with vehicle ("NC"), or 1 mM of AICAR for 24 h, p- and total Ulk1, LC3-II, beclin-1 and β-actin were examined by Western blots. (D–F) Control or AMPKα stable knockdown cardiomyocytes were treated with 10 μM of Ambra1 for 24 h, LC3 puncta (D), cell viability (E) and Annexin V percentage (F) were detected. Experiments in this figure were repeated three times. $^{*}p < 0.05$.

Although we identified AMPK as a main mediator of autophagy that is controlled by Ambra1, we did not exclude other mechanisms, which could be carried on in future.

Our study confirmed that Ambra1 is an important factor in the pathways that regulate tumor cell survival in cardiomyocytes. In addition, we discovered one Ambra1-related autophagy pathway, which may exist in cardiomyocytes. Reports have suggested that Ambra1 and beclin1 dynamically interact to regulate apoptosis [25]. Moreover, apoptosis can regulate autophagy, and autophagy can inhibit apoptosis [34,35]. However, the detailed mechanisms by which apoptosis and autophagy are regulated in cardiomyocytes have not been fully elucidated. Future studies in animal models with knockdown or colon-specific overexpression of Ambra1 are necessary to confirm that Ambra1 plays a key role in regulating autophagy and apoptosis in cardiomyocytes in vivo.

In conclusion, this study ascertained the role of Ambra1 as a regulator of autophagy and apoptosis in cardiomyocytes. It has been proved that Ambra1 is a key pro-survival factor in cardiomyocytes that promotes autophagy by binding to Beclin1 and inducing Beclin1-mediated autophagy through the AMPK/mTOR axis. Also, we determined that Ambra1 knockdown rendered cardiomyocytes more susceptible to apoptosis, suggesting that Ambra1 may be an important negative regulator of apoptosis in these cells. Future studies are necessary to explore the detailed mechanisms that link the regulation of autophagy and apoptosis in other autophagy-related pathway.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.017.

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