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# Alpha-lipoic acid protects cardiomyocytes against hypoxia/reoxygenation injury by inhibiting autophagy

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ABSTRACT

Hypoxia/reoxygenation (H/R) is an important in vitro model for exploring the molecular mechanisms and functions of autophagy during myocardial ischemia/reperfusion (I/R). Alpha-lipoic acid (LA) plays an important role in the etiology of cardiovascular disease. Autophagy is widely implicated in myocardial I/R injury. We assessed the degree of autophagy by pretreatment with LA exposed to H/R in H9c2 cell based on the expression levels of Beclin-1, LC3II/LC3I, and green fluorescent protein-labeled LC3 fusion proteins. Autophagic vacuoles were confirmed in H9c2 cells exposed to H/R using transmission electron microscopy. Our findings indicated that pretreatment with LA inhibited the degree of autophagy in parallel to the enhanced cell survival and decreased total cell death in H9c2 cells exposed to H/R. We conclude that LA protects cardiomyocytes against H/R injury by inhibiting autophagy.



## 1. Introduction

Coronary heart disease, including acute coronary syndrome, is one of the main causes of morbidity and mortality worldwide [1]. Successful reperfusion therapy for acute coronary syndrome can attenuate myocardial infarction and prevent heart failure, leading to reduced mortality [2,3]. However, a side effect of this therapy is myocardial ischemia/reperfusion (I/R), which can lead to tissue damage and pathological remodeling [4]. Thus, it is necessary to protect against myocardial I/R injury as an adjunct therapy for the treatment of post-myocardial infarction [5].

Autophagy is the primary catabolic process of the lysosome-dependent turnover of damaged proteins and organelles and plays an important role in the removal of dysfunctional cytosolic components [6]. Autophagy is widely implicated in heart diseases, including cardiomyopathies, ischemic heart disease, heart failure, and I/R injury [7]. The degree of autophagy is mediated by ischemia but is increased during myocardial I/R [10]. However, the role of autophagy in contributing to the cell survival and death in the heart remains controversial [11].

Hypoxia/reoxygenation (H/R) simulates I/R in a cell culture model [8]. H/R is known to injure the cells through oxidant production and mitochondrial damage, which leads to the production of inflammatory cytokines and the activation of the inflammatory cell signaling pathways [9]. Recently, several studies have assessed autophagic roles during myocardial I/R. Ma et al. showed that autophagosome clearance was impaired, which contributed to cell death [12]. During H/R injury, autophagy primarily acts as a protective mechanism that may prevent cell death [13]. However, another study reported that enhanced autophagy contributes to cell death in the heart [14]. Thus, it is still unclear whether the autophagy plays a beneficial or detrimental role in the heart.

Alpha-lipoic acid (LA), or 1, 2-dithiolane-3-pentanoic acid, is known to possess antioxidative and anti-inflammatory properties and plays an important role in the etiology of cardiovascular disease [15]. Recently, increasing attention has focused on the antioxidant functions of LA [16]. LA is effective in both the prevention and treatment of oxidative stress in a number of models or clinical conditions, including diabetes [17] and I/R injury [18]. Therefore, LA may serve as a protective agent against the risk factors of cardiovascular disease [19]. Interestingly, it has been reported that LA suppressed the degree of autophagy [20]. We posited that LA exerts a protective effect on cardiomyocytes from H/R injury via the inhibition of autophagy.

Thus, the main purposes of this study were (1) to investigate the protective effects of LA subjected to H/R in cardiomyocytes and (2) to determine the relationship between LA and autophagy during H/R in cardiomyocytes.

## 2. Materials and methods

### 2.1. Reagents

LA was obtained from Sigma–Aldrich (St. Louis, MO, USA) and diluted in 10% dimethyl sulfoxide (DMSO) (Cell Signaling

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Technology, Inc., Beverly, MA). The final concentration of DMSO never exceeded 0.1% in either control or treated cells. Beclin-1 and LC3B antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Gibco Laboratories (Carlsbad, CA, USA).

### 2.2. Cell culture and experimental design

H9c2 cardiomyocytes derived from rat myocardium were obtained from the American Type Culture Collection (Manassas, VA, USA). H9c2 cells were cultured in DMEM with 10% fetal bovine serum (FBS) without penicillin/streptomycin in a humidified atmosphere of 5% $CO_2$ at 37 °C. The medium was changed every 2 days. LA was added 12 h prior to H/R [21]. H9c2 cells were assigned to 4 groups: (1) Control: H9c2 cells were maintained in normoxic condition without LA treatment; (2) LA: H9c2 cells were treated with LA and incubated in normoxic condition; (3) H/R: H9c2 cells were subjected to 4 h of Hypoxia followed by 3 h of reoxygenation; (4) LA + H/R: H9c2 cells pretreated with LA were subjected to 4 h of Hypoxia followed by 3 h of reoxygenation. H9c2 cells were treated with LA (300 μM) for the indicated times as described previously [21].

### 2.3. Hypoxia/reoxygenation

Hypoxia/reoxygenation (H/R) was performed as previously described [22]. H/R conditions were simulated by culturing cells in a hypoxia chamber (Billups-Rothenberg, Inc., San Diego, CA, USA). H9c2 cells were cultured in nutrient-rich medium for 3 days and washed with phosphate-buffered saline (PBS). Hypoxia was achieved by placing the cells in a hypoxia chamber filled with 5% $CO_2$ and 95% $N_2$ at 37 °C for 4 h. After hypoxia exposure, the cells were reoxygenated with 5% $CO_2$ and 95% $O_2$ for 3 h in DMEM with 10% FBS.

### 2.4. MTT assay

The viability of H9c2 cardiomyocytes was measured using the MTT Cell Proliferation Assay kit according to the manufacturer's instructions [23]. H9c2 cells were seeded in flat-bottomed 96-well microtiter plates at a density of $1 \times 10^4$ cells/well. After the treatment, MTT was added to achieve a final concentration of 0.5 g/L. The cells were incubated in 5% CO2 at 37 °C for 4 h. DMSO was perfused into each well to dissolve the formazan crystals after the medium was removed. The plate was shaken for 10 min at a low speed. Finally, sample absorbance measurements were determined at 570 nm by a microplate reader.

$$\text{Viability}(\%) = (\text{OD}_{570,\text{sample}} - \text{OD}_{570,\text{blank}})/(\text{OD}_{570,\text{control}} - \text{OD}_{570,\text{blank}}) \times 100$$

### 2.5. Flow cytometry

The assay was performed according to the manufacturer's instructions [24]. Briefly, both attached and floating cells were harvested, washed with PBS, suspended in Annexin V binding buffer (10 mmol/L HEPES, pH 7.4; 2.5 mmol/L $CaCl_2$, 140 mmol/L NaCl), stained with Annexin V-FITC, and analyzed by flow cytometry. For each experiment, 10,000 cells were analyzed using the ELITE Flow Cytometer (BD Biosciences, San Jose, CA) and Cell Quest software (BD Biosciences).

### 2.6. Western blotting

The proteins isolated from cultured H9c2 cardiomyocytes were determined with a western blot analysis [25]. The samples were denatured by boiling for 5 min in loading buffer, subjected to 10% SDS–PAGE, and then electroblotted onto nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ, USA). Next, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h. The membranes were washed with Tris-buffered saline-Tween 20 (TBST) buffer for 5 min and then incubated with horseradish peroxidase-conjugated antibodies at room temperature for 1 h. After being washed again with TBST buffer for 5 min, immunolabeled bands were detected using an enhanced chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA). The primary antibodies were used at a dilution of 1:1000, and secondary antibodies were used at a dilution of 1:2000. GADPH antibody (1:5000 dilution; Cell Signaling Technology, Inc., Beverly, MA) was used as the loading control. The blots were scanned using an Epson Perfection PhotoScanner. Finally, data analysis was performed with Image J by measuring the densities of immunoreactive bands.

### 2.7. GFP-LC3 assay

The method used to evaluate fluorescent LC3 puncta *in vivo* has been described previously [26]. Briefly, H9c2 cells were plated into 24 wells and transiently transfected with GFP-LC3 plasmid for 24 h. The cells were then fixed in 3.7% paraformaldehyde. The number of GFP-LC3 punctate dots per cell in GFP-LC3-positive cells was counted under a Nikon D-Eclipse C1 confocal laser scanning microscope. The nuclear numbers were also evaluated by counting the DAPI-stained nuclei in the same fields at the same magnifications. The number of GFP-LC3 puncta/cell was evaluated as the total number of dots divided by the number of nuclei in each microscopic field.

### 2.8. Transmission electron microscopy (TEM)

TEM was performed as previously described [27]. H9c2 cells were collected by centrifugation, washed with PBS, and perfused with 4% paraformaldehyde and 1.5% glutaraldehyde in 0.1 M cacodylate buffer. The cells were then fixed in Karnofsky's fixative, post-fixed in 1% osmium tetraoxide, dehydrated in a graded series of acetone concentrations, and embedded in EMbed 812/Araldite (Electron Microscopy Sciences, Fort Washington, PA, USA). Sections of 98 nm thickness were mounted on copper slot grids with Parlodion and stained with uranyl acetate. The discs were examined with a Philips CM100 electron microscope (FEI, Hillsboro, OR, USA).

### 2.9. Statistical analysis

All data are presented as the means ± standard error of the means and were statistically analyzed using SPSS software, version 13.0. The data were analyzed using one-way ANOVAs to determine statistical significance and were further evaluated using the Bonferroni post hoc tests. A value of $P < 0.05$ was considered to be statistically significant.

## 3. Results

### 3.1. LA protects against H/R-induced the cell death in H9c2 cells

Fig. 1 shows the cellular morphologies of H9c2 cells in every groups under an inverted phase contrast microscope (×100). The cells grown under control or LA group had normal fusiform

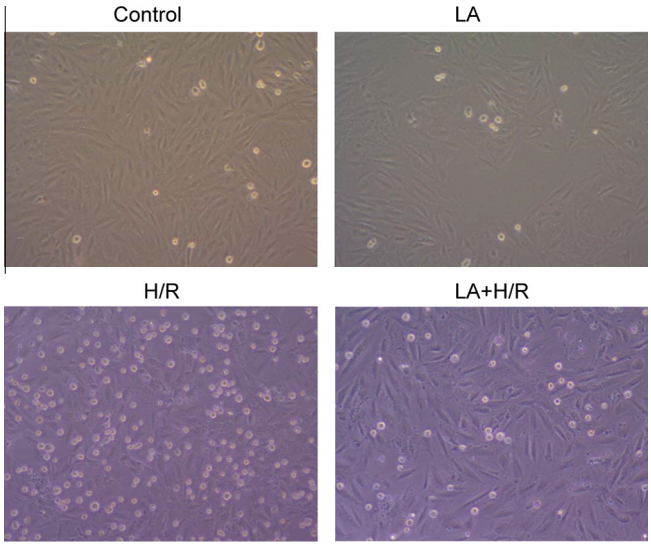


**Fig. 1.** LA protects against H/R-induced the cell death in H9c2 cells. Under the inverted phase contrast microscope (×100), control and LA groups displayed the normal fusiform morphology. H/R group showed the extensive blebbing morphology and decreased cell survival, while pretreatment with LA prevented against the injury.

morphology, whereas H/R group showed the extensive blebbing morphology and decreased cell survival. Compared to the H/R group, LA + H/R group increased cell survival with slighter blebbing.

### 3.2. LA attenuates H/R-induced total cell death in H9c2 cells

Annexin-V FITC/PI staining was used to quantify the anti-apoptotic effects of LA. H9c2 cells in control and LA groups showed normal apoptosis (Fig. 2A and B). The total cell death rates in the H/R group were significantly increased ($P < 0.05$ vs. control, Fig. 2A and B). However, Pretreatment with LA significantly attenuated the elevated H/R-induced total cell death ($P < 0.05$ vs. H/R, Fig. 2A and B). These results strongly demonstrated that LA could attenuate H/R-induced total cell death in H9c2 cells.

### 3.3. LA improves cell viability during H/R in H9c2 cells

We assessed cell viability with MTT assays in every groups. Control and LA groups showed similar cell viability ($P > 0.05$, Fig. 2C). Compared with the control, the cell viability was markedly inhibited after H/R treatment ($P < 0.05$, Fig. 2C). Interestingly, Pretreatment with LA significantly improved cell viability ($P < 0.05$ vs. H/R, Fig. 2C). These findings indicated that LA could improve the cell viability in H9c2 cells subjected to H/R.

### 3.4. LA inhibits the expression levels of autophagy markers in H9c2 cells subjected to H/R

It is known that autophagy is regulated by autophagy-related proteins, such as Beclin-1, LC3-II/LC3-I, and LC3II [28]. Our findings indicated that there was no apparent change of autophagy-related proteins between control and LA groups during H/R in H9c2 cells (Fig. 3A, C and D). The expression levels of autophagy were

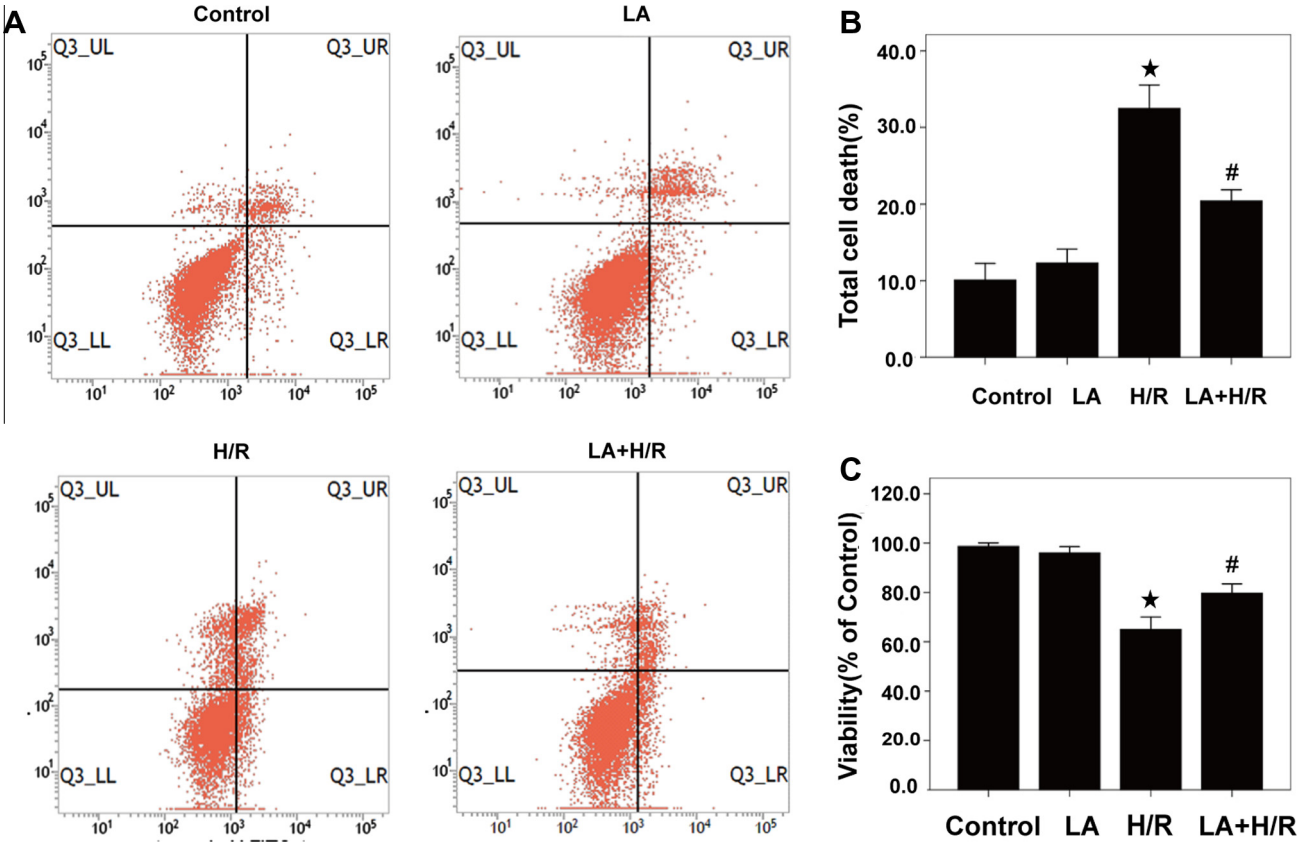


**Fig. 2.** LA improves the cell viability and attenuates the total cell death in H9c2 cells subjected to H/R. (A) Quantitative assessment of total cell death by annexin V-FITC/PI staining. Intact cells were V−/PI−, early apoptotic cells were V+/PI−, late apoptotic cells were V+/PI+ and necrotic cells were V−/PI+. (B) Flow cytometry results were displayed as quantitative bar graphs. (C) Cell viability was measured with an MTT assay. The figures are representative images of three different samples. Data are expressed as means ± SEM. $n = 3$, $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. H/R.

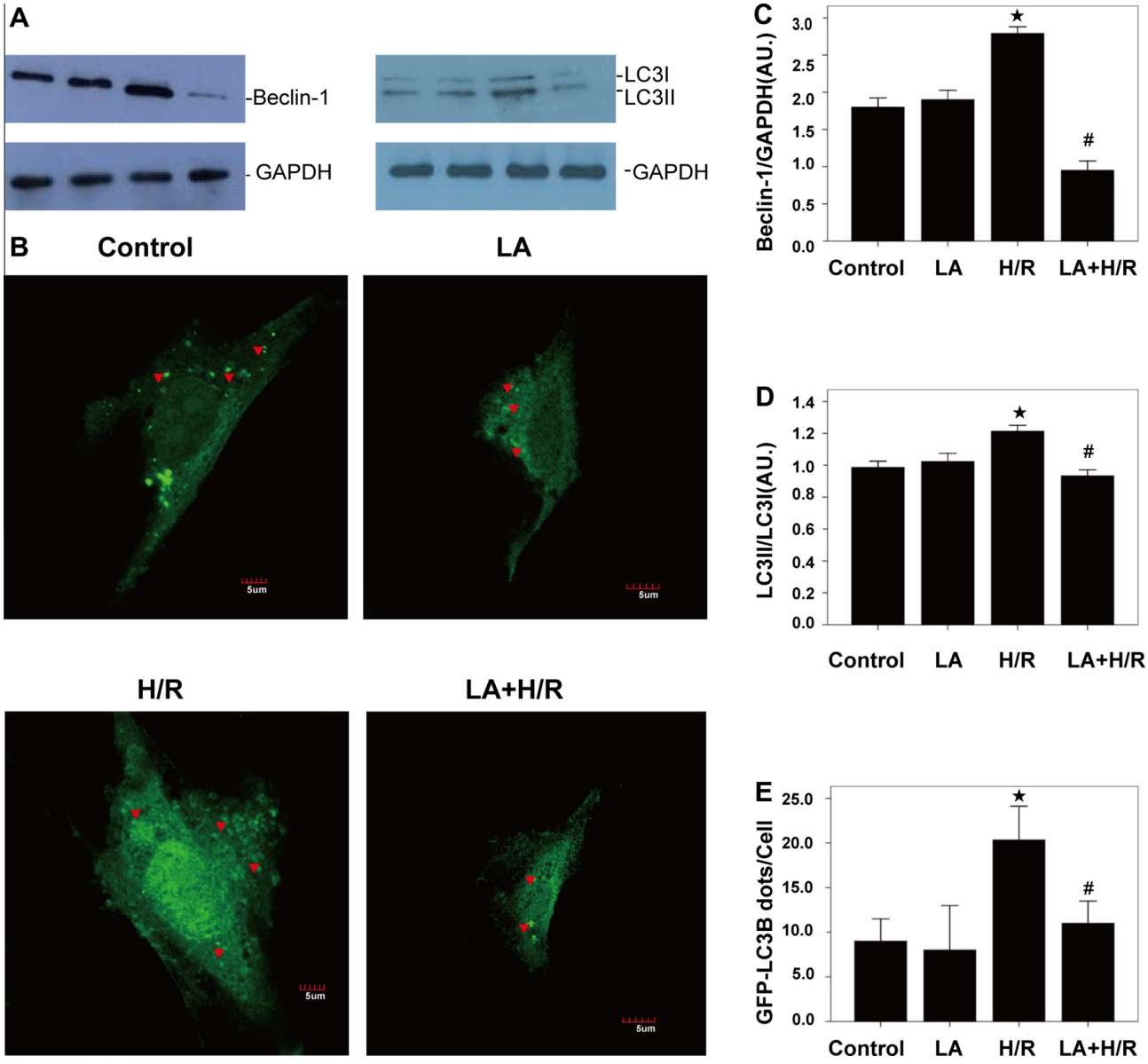


**Fig. 3.** LA inhibited the degree of autophagy in H9c2 cells subjected to H/R. (A) Western blotting was performed with H9c2 cells for the expression of autophagic marker proteins including Beclin-1, LC3II and LC3I. (B) Confocal fluorescent microscopic images of H9c2 cells showed the staining of LC3B (green, Alexa Fluor 488). (C and D) The quantification of Beclin-1 and LC3-II/LC3-I was performed using gene tools from Syngene. (E) The quantification of the staining of LC3B particles was measured with Adobe Photoshop software. The figures are representative images of three different samples. Data are expressed as means ± SEM. $n = 3$, $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. H/R. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

upregulated in H9c2 cells exposed to H/R ($P < 0.05$ vs. control, Fig. 3A, C and D). At the same time, the expression levels of Beclin-1 and LC3-II/LC3-I in the LA + H/R group were significantly reduced ($P < 0.05$ vs. H/R, Fig. 3A, C and D). It can be concluded that the degree of autophagy was enhanced in H9c2 cells exposed to H/R and inhibited by LA prior to H/R.

### 3.5. LA prohibits GFP-LC3 fusion proteins in H9c2 cells subjected to H/R

Furthermore, we transfected H9c2 cells with GFP-LC3 and examined the abundance of GFP-LC3 dots in every groups (Fig. 3B). Positive fluorescence dots were expressed as AVs where the GFP-LC3B fusion proteins were located. To quantify the GFP-LC3 dots, the percentage of cells showing numerous punctate GFP-LC3B structures was determined. Our results demonstrated that the GFP-LC3 dots were no differential in control and LA groups ($P > 0.05$ Fig. 3E).however, the GFP-LC3 dots were increased in H9c2 cells subjected to H/R ($P < 0.05$ vs. control, Fig. 3E). After pre-treatment with LA, the GFP-LC3 dots significantly were decreased in H9c2 cells exposed to H/R ($P < 0.05$ vs. H/R, Fig. 3E). The data indicated that LA prohibited GFP-LC3 fusion proteins in H9c2 cells subjected to H/R.

### 3.6. LA attenuates the formation of AVs during H/R in H9c2 cells

To confirm the effects of LA on the formation of AVs, we imaged the ultrastructural morphology by TEM in every groups. TEM showed that most AVs had double or multiple membrane boundaries, surrounding mitochondria or other cellular organelles (Fig. 4A). The number of AVs in control and LA groups was consistent ($P > 0.05$ Fig. 4B). The AVs were greater during H/R ($P < 0.05$ vs.

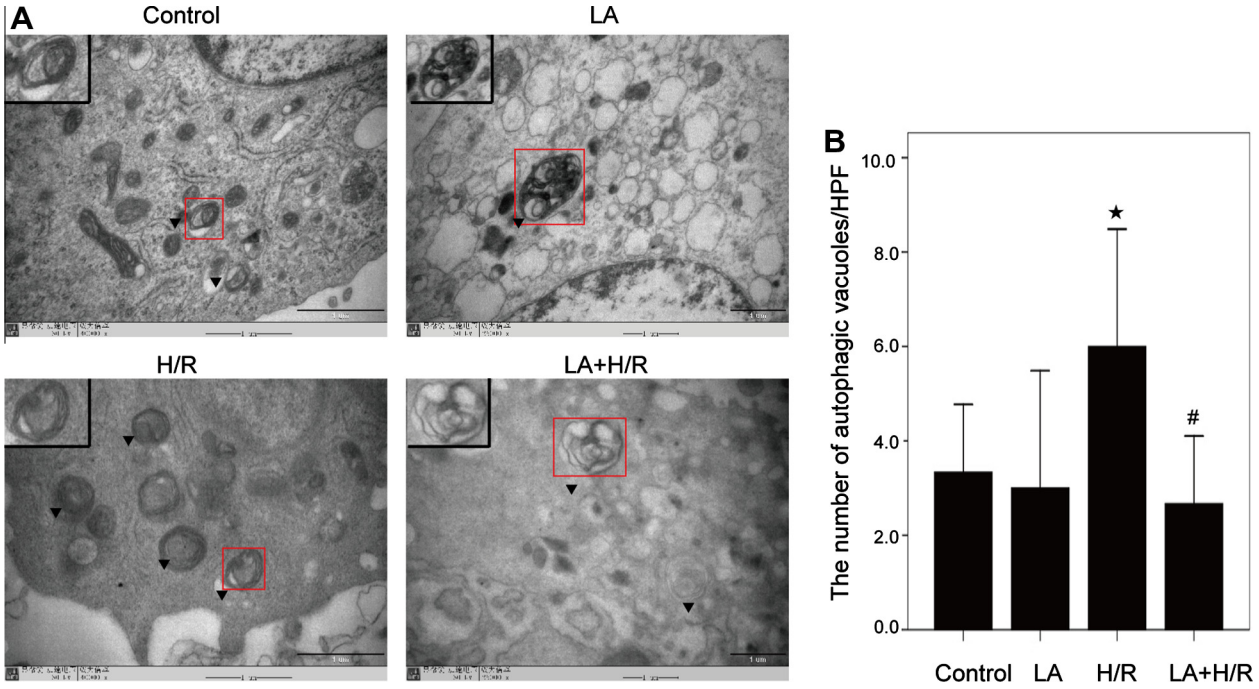


**Fig. 4.** Autophagic vacuoles (AVs) were detected by TEM in H9c2 cells subjected to H/R. (A) AVs (arrows) by TEM were measured in H9c2 cells. Scale bar = 1.0 μm (insets). Inset images were represented as the boxes in red. (B) AVs in different groups were quantified and presented as bar graphs. Data are expressed as means ± SEM. $n = 3$, $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. H/R. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

control, Fig. 4B). After pretreatment with LA, the AVs were less evident in H9c2 cells exposed to H/R ($P < 0.05$ vs. H/R, Fig. 4B). The data again suggest that LA attenuated the formation of AVs in H9c2 cells during H/R.

## 4. Discussion

This study was the first to explore the cardioprotective effects of LA against H/R injury in H9c2 cells. The underlying mechanisms may be largely attributed to the inhibition of autophagy. The primary findings in this study include the following: (1) LA improved cell viability and reduced total cell death during H/R in H9c2 cells, (2) LA decreased the expression levels of autophagy markers and GFP-LC3B fusion proteins in H9c2 cells subjected to H/R and (3) LA attenuated the formation of AVs during H/R in H9c2 cells.

In a previous study, treatment solely with LA did not change cell viability [29]. In this study, we also observed that the cell viability were no differential in control and LA groups. However, pretreatment with LA markedly improved cell viability and reduced total cell death in H9c2 cells exposed to H/R. Consistent with our findings, He et al. demonstrated that LA protected the heart against myocardial ischemia–reperfusion injury [30]. Other researchers have studied that LA protects against renal ischemia–reperfusion injury in rats [31]. Collectively, these data indicate that LA may have a beneficial effect on cardiomyocytes subjected to H/R.

To investigate how LA mediated H/R-induced autophagy, we examined the expression levels of Beclin-1 and LC3II/LCI, which have been regarded as autophagy markers [32]. We found that the expression levels of autophagy were upregulated in H9c2 cells exposed to H/R ($P < 0.05$ vs. control, Fig. 3C and D). Interestingly, pretreatment with LA caused a decline in the expression levels of autophagy in H9c2 cells subjected to H/R ($P < 0.05$ vs. H/R, Fig. 3C and D). At the same time, pretreatment with LA markedly improved cell viability ($P < 0.05$ vs. H/R, Fig. 2C). Therefore, our results demonstrated that LA-induced protection against H/R injury was mediated through the mechanism of inhibiting autophagy. Similarly, it has been reported that LA protected against arsenic trioxide-induced autophagy in U118 human glioma cells [33].

To further elucidate the relationship between the protective effect of LA and autophagy, we transfected H9c2 cells with GFP-LC3B fusion proteins. We observed the abundance of GFP-LC3B dots in H9c2 cells subjected to H/R (Fig. 3B). Interestingly, after pre-treatment with LA, the GFP-LC3B dots significantly decreased in H9c2 cells exposed to H/R ($P < 0.05$ vs. H/R, Fig. 3E). Thus, the protective effect of LA via the inhibition of autophagy was further validated by the observed decrease of GFP-LC3B dots in H9c2 cells exposed to H/R. In addition, we also detected autophagic vacuoles (AVs) by TEM in H9c2 cells. The number of AVs in LA + H/R group was fewer in H9c2 cells exposed to H/R ($P < 0.05$ vs. H/R, Fig. 4B). These results suggested that the protective effect of LA was mainly attributed to the inhibition of autophagic activity.

In summary, our findings indicated that pretreatment with LA inhibited the degree of autophagy in parallel to the enhanced cell survival and decreased total cell death in H9c2 cells exposed to H/R. Thus, we conclude that LA protects cardiomyocytes against H/R injury by inhibiting autophagy.

Overall, this study demonstrates for the first time that LA protects cardiomyocytes against H/R injury by inhibiting autophagy. However, the underlying mechanisms are still unclear. It will be worthwhile for future studies to investigate other regulating signal pathways. There are a few limitations of the present study. First, H9c2 cell lines may not be an ideal model for primary neonatal rat cardiomyocytes. Second, the MTT assay is a primary screening test that does not discriminate between cells undergoing necrosis and apoptosis. Third, our experiment was limited to in vitro conditions. Therefore, the results of our study should be interpreted with caution, especially in any application of these findings.

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