Int J Clin Exp Med 2020;13(9):6606-6615
www.ijcem.com /ISSN:1940-5901/IJCEM0111915

Original Article

Trehalose alleviates oxygen-glucose deprivation induced myocardial injury through the autophagy-lysosome pathway

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Received April 3, 2020; Accepted July 23, 2020; Epub September 15, 2020; Published September 30, 2020

Abstract: Objective: To investigate the role and mechanism of trehalose in oxygen-glucose deprivation (OGD) induced injury in H9c2 rat myocardial cells. Methods: H9c2 cells were treated with OGD in the presence of trehalose or not. Cell viability, LDH release, apoptosis and apoptotic related proteins (Bcl-2 and Bax) were measured by CCK-8, flow cytometric analysis, and Western blot (WB), respectively. The changes in autophagic flux, lysosomes, and endosomes were determined by detecting LC3, p62, Beclin 1, LAMP2, Rab7, and Rab9 using WB. Lysosomes were purified to determine the integrity by measuring luminal protease releasing. Inflammatory cytokines (TNF-α, IL-1β, IL-6, and IL-8) were measured by ELISA and the production of ROS, MDA, and SOD activity was detected by commercial kits. Results: Trehalose increased cell viability, reversed the ratio of Bax/Bcl-2, and blocked apoptosis induced by OGD. Trehalose decreased the accumulated LC3 and p62 induced by OGD and increased the protein levels of LAMP2, Rab7, and Rab9 damaged by OGD. Trehalose blocked the release of proteases from the lysosome induced by OGD. Trehalose also inhibited the production of inflammatory cytokines, decreased ROS and MDA levels, and increased SOD activity in OGD condition. Conclusion: Trehalose blocked OGD-induced myocardial apoptosis through protecting lysosome-mediated autophagic flux and inhibiting inflammatory and oxidative responses.

Keywords: Oxygen-glucose deprivation, trehalose, autophagy, lysosome, myocardial cells

Introduction

Myocardial infarction is one of the main injuries in cardiovascular diseases, which is the primary cause of death in many countries [1]. Cell death is a fundamental process in myocardial infarction and apoptosis plays a pivotal role in myocardial cell death [2]. Thus, the development of novel pharmacological therapies to ameliorate myocardial injury induced apoptosis is very important for clinical practice.

Myocardial infarction is known to trigger an intense inflammatory response that is important for cardiac repair [3]. Infarcted myocardium generates reactive oxygen species (ROS) and induces cytokines and chemokines. Excessive and prolonged inflammation may be involved in the development of heart failure following infarction [4]. Thus, inhibition of the inflammatory response is essential for the activation of myofibroblasts and effective healing [3]. The oxygen glucose deprivation (OGD) model has been used for studying ischemia injury in myocardial cells [5, 6]. OGD-induced myocardial injuries are accompanied by the production of ROS, mitochondrial permeability and apoptosis. Meanwhile, many inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8, are released during this process [7]. The inhibition of inflammation and apoptosis is regarded as an important way to attenuate OGD-induced damage in myocardial infarction.

Recently, autophagy has been recognized as a major regulator of cardiac homeostasis and function [8, 9]. Autophagy occurs through the lysosomal degradative pathway including the delivery of cytosolic components for degradation and recycling [10]. Previous study showed that deletion of the essential autophagy gene *ATG5* (autophagy-related gene 5) led to cardio-

myopathy [11]. Basal autophagy maintains cardiac structure and function and is activated under various stress conditions to limit damage [8]. Conversely, abnormal autophagic activity has been implicated in the development of cardiac diseases, including doxorubicin-induced cardiomyopathy [12] and proteinopathy in cardiac fibrosis [13]. In myocardial tissue, autophagy is believed to exert both beneficial and detrimental effects depends on different circumstances [8, 14, 15]. Modulation of autophagic activity maybe a promising therapeutic target in cardiac disease.

Trehalose is a natural, non-reducing disaccharide that is detected in most organisms [16]. Trehalose has been shown to have neuroprotective effects in various diseases, including neurodegenerative diseases, at least through a mechanism involving autophagy induction [17]. It should be noted that the role of trehalose in autophagy induction is under debate, especially in OGD models in neuronal diseases [17]. For example, a recent study showed that trehalose inhibited transient global ischemia-induced neuronal death and OGD-induced death in neuroblastoma SH-SY5Y cells through the preservation of proteasome activity, not via induction of autophagy [18]. Interestingly, trehalose also plays a protective role in spinal cord ischemia in rabbits [19]. A recent study further showed that trehalose reduced myocardial infarction (MI)-induced cardiac remodeling and dysfunction through the activation of autophagy in an *in vitro* MI model [20]. However, the precise mechanism of trehalose in the MI model is still not clear.

In the present study, we provided evidence showing that trehalose protected rat myocardial cells from OGD-induced damage. Trehalose ameliorated the accumulation of autophagic vesicles induced by OGD through the preservation of lysosomal integrity and function. Furthermore, trehalose inhibited the OGD-induced inflammatory response and alleviated cell death under OGD condition. Our study provided the new insight into trehalose treatment in myocardial infarction.

Materials and methods

Cell culture and oxygen glucose deprivation

H9c2 rat myocardial cells were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured at 37°C in a humidified atmosphere of 95% air, 5% CO_2 (v/v) in DMEM medium (GIBCO, Gaithesburger, MD, USA) supplemented with 10% (v/v) fetal bovine serum (GIBCO), 100 U/ml penicillin G, 100 μg/ml streptomycin and 2 mM glutamine (GIBCO). Cells were exposed to OGD as described previously [21]. Briefly, cells were rinsed twice with serum-free DMEM and incubated in glucose-free DMEM and subsequently placed in an anaerobic chamber containing a mixture of 95% N_2 and 5% CO_2 at 37°C for 6 h. OGD was terminated by removing cells from the chamber and put back to the incubator with normal conditions. Trehalose (100 mM) treatment started at 6 h before OGD and continued up to the termination of reoxygenation. Cells in the control group were not treated with OGD and cells in normal were not treated with trehalose.

Antibodies and reagents

Primary antibodies included anti-LC3, anti-LAMP2, anti-Rab 7 and anti-Rab 9, anti-Bcl-2, anti-Bax, anti-Beclin 1 (Cell Signaling Technology, Danfoss, MA, USA), anti-p62, and anti-β-actin (Sigma, Saint Louis, MO, USA). Trehalose was obtained from Sigma (Saint Louis, MO, USA). Bafilomycin A1 was purchased from Enzo Life Sciences (Farmingdale, NY, USA).

Cell viability and apoptosis assay

H9c2 cells were plated at a density of 1 × 10^4 cells per well in 96-well plates for 24 h. After trehalose or OGD treatment, the proliferation of cells was evaluated using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) assay. Briefly, 10 μL CCK-8 was added to each well of 96-well plates for 1-2 h at 37°C and the absorbance was measured at 450 nm using a microplate reader (ELX 800, Bio-Tek, Winooski, VT, USA). For apoptosis assay, the cell samples were harvested and stained with Annexin V and propidium iodide (Beyotime Institute of Biotechnology, Nantong, China), then the cells were analyzed with flow cytometric analysis (Beckman Coulter Cytomics FC500, Brea, CA, USA).

LDH release cell death assay

H9c2 cells were plated at a density of 1 × 10^4 cells per well in 96-well plates for 24 hours, and then treated with OGD for the indicated time

course. The levels of lactate dehydrogenase (LDH) in the culture medium were measured at 490 nm using a microplate reader (ELX 800, Bio-Tek, Winooski, VT, USA) with a detection kit (Beyotime Biotech, Nanjing, China).

Western blot analysis

Cells were washed twice with PBS and lysed with ice-cold lysis buffer containing 40 mM Tris-HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP40 and EDTA-free complete protease inhibitor and Phospho-Stop inhibitor (Roche, Basel, Switzerland). Cell lysates were centrifuged at 11,000 rcf for 10 min at 4°C. The supernatants were collected for protein concentrations using the BCA protein assay kit (Pierce, Rockford, IL, USA). Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes (Millipore, Bellerica MA, USA). The membranes were blocked with 5% non-fat milk in TBST buffer for 1 h at room temperature and then incubated with primary antibodies overnight at 4°C as follows: anti-LC3 (1:1000), anti-p62 (1:2000), anti-LAMP2 (1:1000), anti-Bcl-2 (1: 1000), anti-Bax (1:1000), anti-Beclin 1 (1: 1000), anti-Rab 7 (1:1000), anti-Rab 9 (1: 1000), or β-actin (1:10000). Then, the membranes were visualized using a SuperSignal West Pico substrate chemiluminescence detection kit (Pierce, Rockford, IL, USA). The signal intensities of protein bands were quantitatively analyzed with Image J software and normalized to the loading control β-actin.

Lysosome fractionation

Lysosomes were isolated with the Lysosome Enrichment Kit (Thermo-Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The activities of lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) and acid phosphatase were detected with NAG quantitative analysis kit and acid phosphatase activity quantitative analysis kit (GENMED, Shanghai, China) according to the manufacturer's instruction.

Enzyme-linked immunosorbent assay (ELISA)

After trehalose or OGD treatment, the levels of cytokines in the supernatant of the H9c2 cells were measured using TNF-α, IL-1β, IL-6, and IL-8 kits (R&D systems, Minneapolis, MN, USA) according to the manufacturers' instruction.

Measurement of ROS, MDA, and SOD

The levels of ROS were measured with the superoxide sensitive fluorescent dye dihydroethidium (DHE, Beyotime Institute of Biotechnology, Nantong, China). The levels of MDA and SOD activities were measured by respective kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the instructions of the manufactures.

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Statistical analysis was carried out using GraphPad Prism (GraphPad Software, San Diego, CA), either by two-tailed unpaired t-test or by one-way ANOVA followed by Dunnett's t-test or by two-way ANOVA followed by Bonferroni's test. Differences were considered significant when $P < 0.05$.

Results

Trehalose protected myocardial cells from OGD-induced cell injury

To investigate whether trehalose has a protective effect in the OGD model of myocardial cells, H9c2 cells were subject to OGD treatment and the cell viability and LDH release were measured. As shown in **Figure 1**, OGD treatment significantly decreased cell viability and increased LDH release (**Figure 1A**), while pretreatment of cells with trehalose partially rescued the decreased cell viability and inhibited the release of LDH (**Figure 1B**). Trehalose treatment at different time courses didn't affect the cell viability or dramatically induce LDH release (**Figure 1C**). Furthermore, trehalose inhibited OGD-induced apoptosis (**Figure 1D**). Finally, the downregulation of Bcl-2 and the upregulation of Bax induced by OGD were significantly reversed in the present of trehalose (**Figure 1E**). These data indicated that trehalose could protect the myocardial cells from OGD-induced apoptosis.

Trehalose improved the autophagic flux damage by OGD

To determine the autophagic activity in the OGD model, the autophagy marker LC3 and autoph-

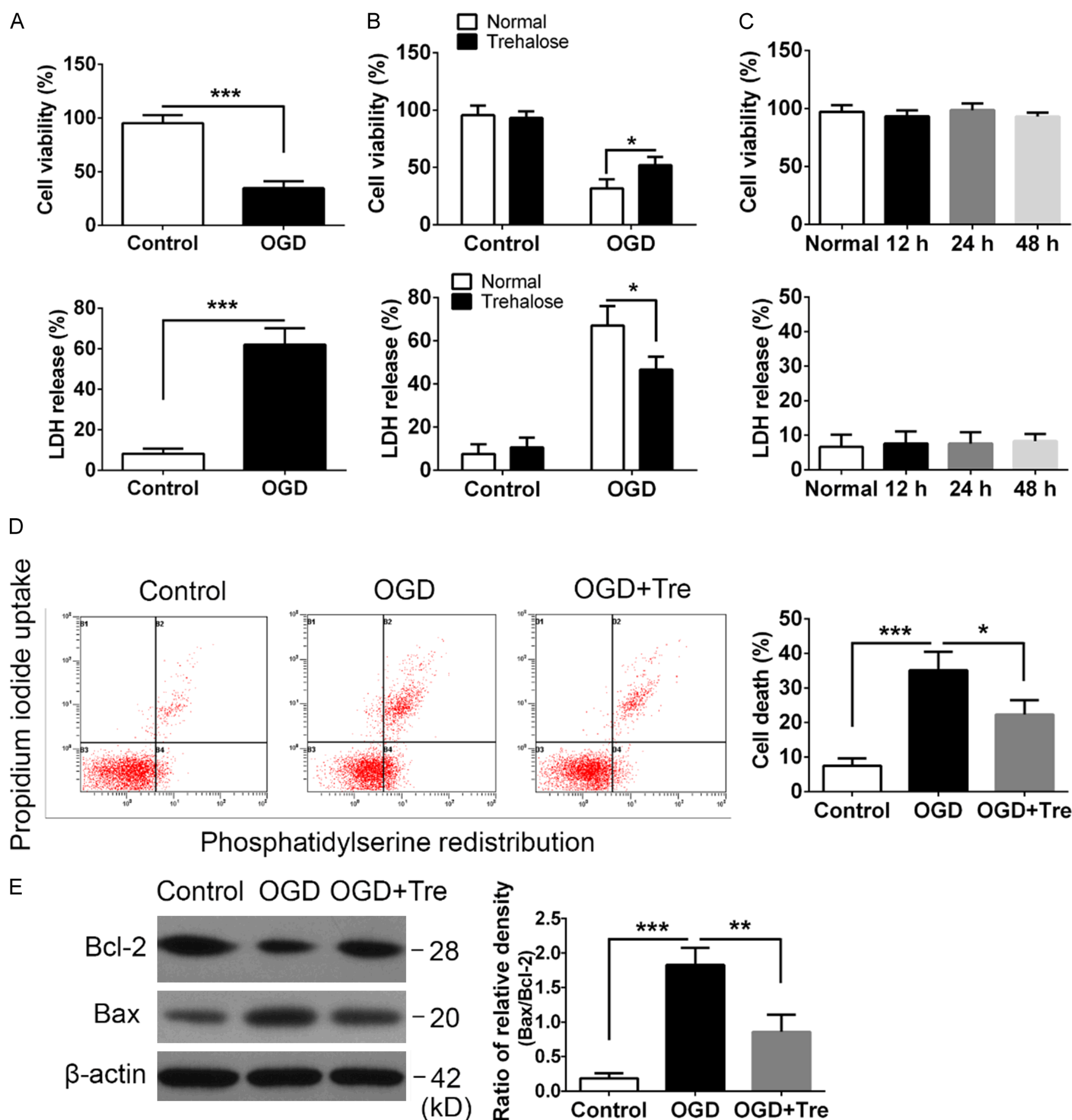

Figure 1. Trehalose blocked oxygen glucose deprivation (OGD)-induced damage in myocardial cells. (A) H9c2 cells were exposed to OGD for 6 h and the cell viability and lactic dehydrogenase (LDH) release were determined. (B) H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD and the cell viability and LDH release were determined. (C) H9c2 cells were treated with trehalose (100 mM) for the indicated time courses and the cell viability and LDH release were determined. (D) H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD. Annexin V/Propidium iodide (PI) staining was performed to determine apoptosis and the percentage of apoptotic cells was quantified. (E) H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD and the protein levels of Bcl-2 and Bax were examined by Western blot and quantified. Statistical analysis was performed by the two-tailed unpaired t-test (A), or by two-way Analysis of Variance (ANOVA) followed by Bonferroni's test (B), or by one-way ANOVA followed by Dunnett's t-test (in C-E). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs the indicated groups. Data represent mean ± SD for combined data from three independent experiments. OGD for oxygen glucose deprivation; LDH for lactic dehydrogenase; SD for standard deviation.

agic substrate p62 were detected. As shown in **Figure 2**, OGD induced the upregulation of LC3 and p62 (**Figure 2A**). To determine the autophagic flux change under OGD conditions, cells were treated with Bafilomycin A1 (Baf A1) to block the final stage fusion of the autophagosome with the lysosome. As expected, Baf A1 induced the accumulation of LC3-II in the control group. The upregulation of LC3-II and p62 induced by OGD induction didn't further in-

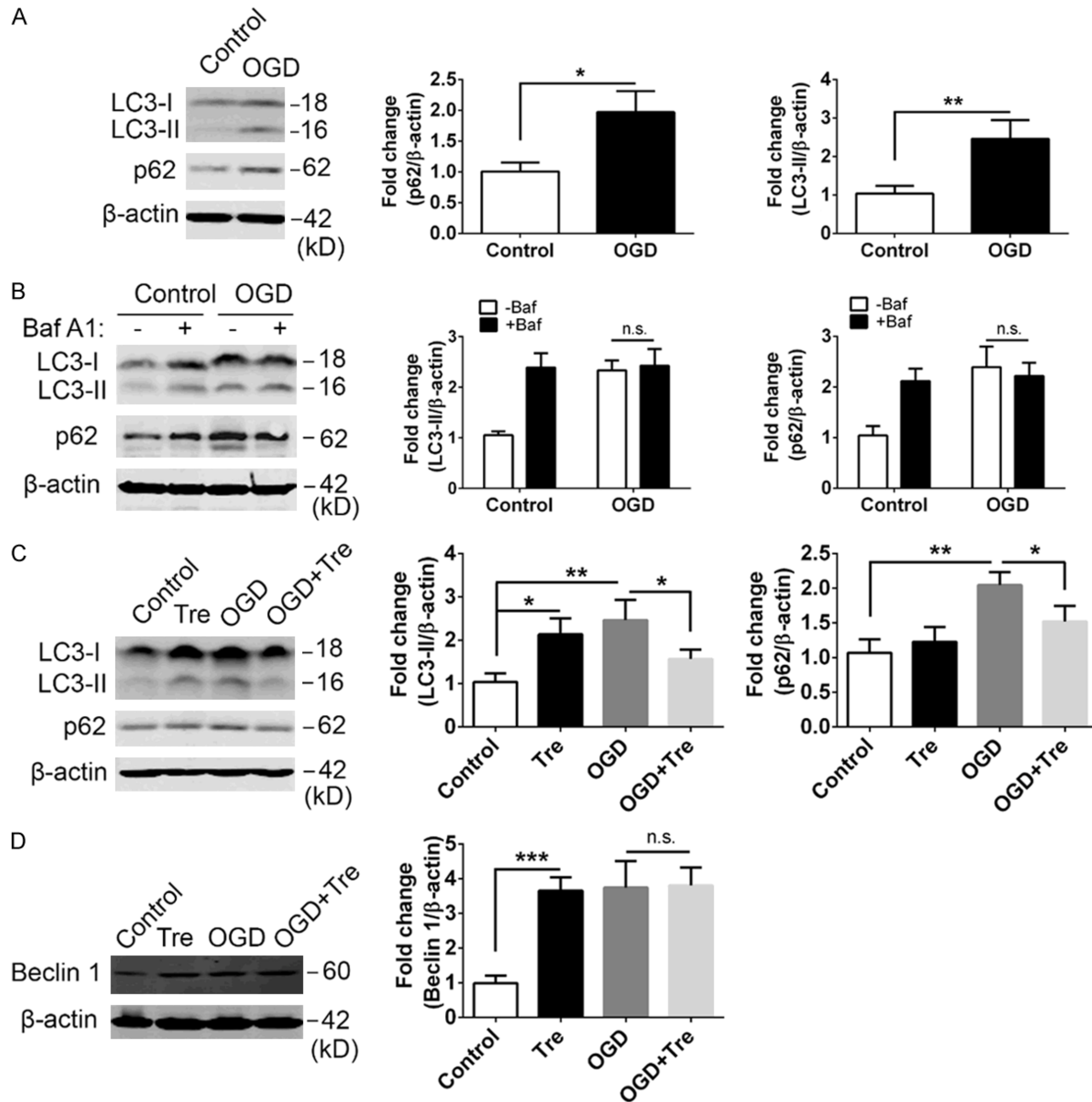

Figure 2. Trehalose improved autophagic flux damaged by OGD in myocardial cells. (A) H9c2 cells were exposed to OGD for 6 h and the protein levels of LC3 and p62 were determined by western blot and quantified. (B) H9c2 cells were exposed to OGD for 6 h and Bafilomycin A1 (Baf A1) (100 nM) was added for 2 h before cells were harvested. The protein levels of LC3 and p62 were determined by western blot and quantified. (C) H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD and the protein levels of LC3 and p62 were determined by western blot and quantified. (D) H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD and the protein levels of Beclin 1 was determined by western blot and quantified. Statistical analysis was performed by the two-tailed unpaired t-test (A), or by two-way ANOVA followed by Bonferroni's test (B), or by one-way ANOVA followed by Dunnett's t-test (in C and D). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs the indicated groups; n.s., no significance. Data represent mean ± SD for combined data from three independent experiments. OGD for oxygen glucose deprivation; LC3 for microtubule-associated protein light chain 3; ANOVA for Analysis of Variance; SD for standard deviation.

crease in the presence of Baf A1 (**Figure 2B**). Those data suggest that OGD treatment blocked the autophagic flux instead of enhancing autophagic activity in myocardial cells. Interestingly, trehalose treatment decreased the upregulation of LC3 as well as p62 induced by OGD (**Figure 2C**), indicating trehalose could decrease the accumulation of autophagosomes and autophagic substrates under OGD conditions. The enhanced protein level of Beclin 1 induced by OGD was not affected by trehalose treatment (**Figure 2D**), indicating trehalose may not affect autophagy induction through Beclin 1. Together, these data suggest-

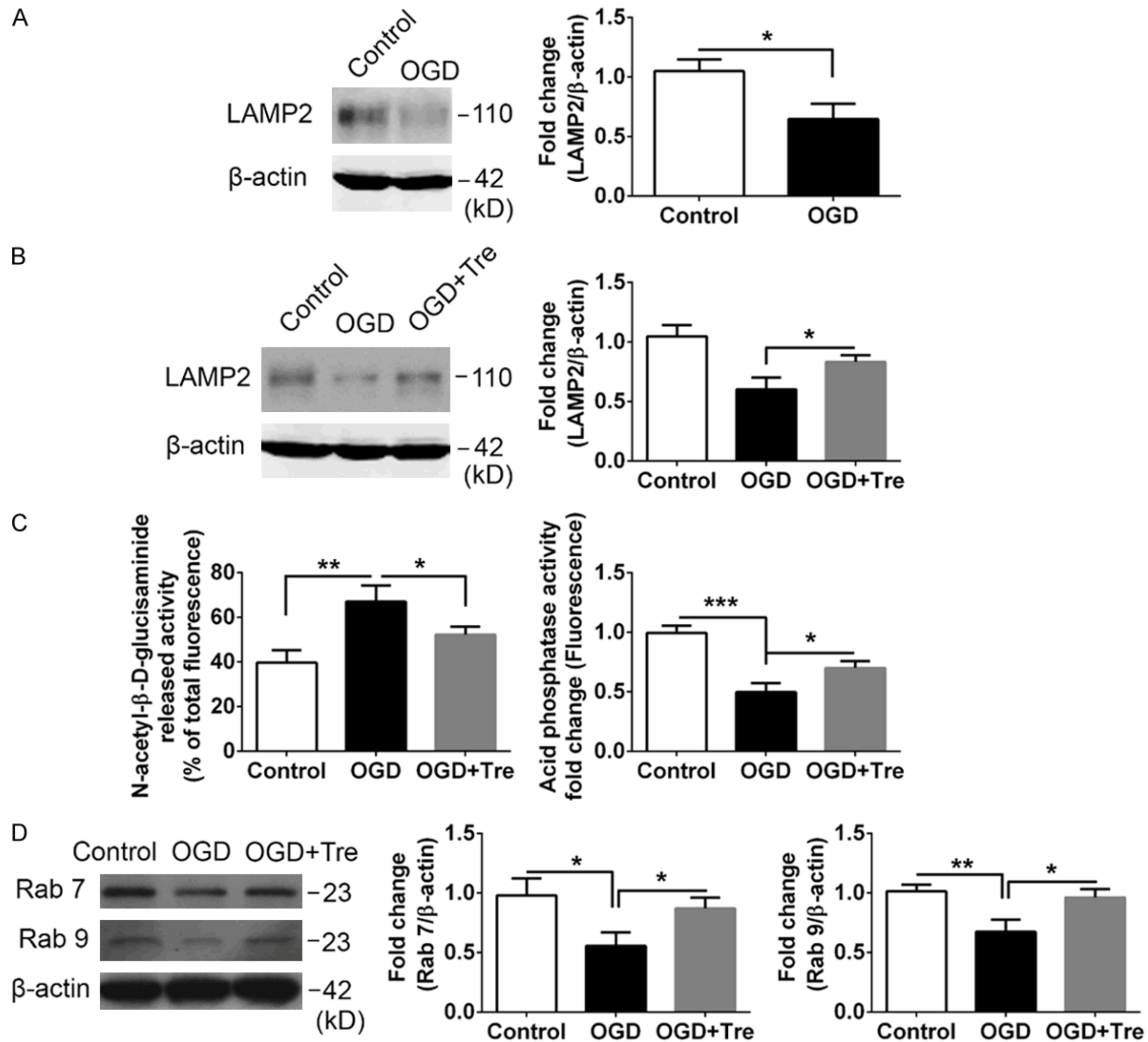

Figure 3. Trehalose increased LAMP2 levels decreased by OGD and protects lysosomal integrity in myocardial cells. (A) H9c2 cells were exposed to OGD for 6 h and the protein levels of LAMP2 was detected by western blot and quantified. (B) H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD and the protein levels of LAMP2 was determined by western blot and quantified. (C) H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD. Lysosomes were isolated and the released N-acetyl-β-D-glucosaminidase (NAG) in supernatant and acid phosphatase activity was determined with the indicated kits and quantified. (D) H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD and the protein levels of Rab 7 and Rab 9 were determined by western blot and quantified. Statistical analysis was performed by the two-tailed unpaired t-test (A) or by one-way ANOVA followed by Dunnett's t-test (in B-D). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ vs the indicated groups. Data represent mean ± SD for combined data from three independent experiments. LAMP2 for lysosomal associated membrane protein 2; OGD for oxygen glucose deprivation; ANOVA for Analysis of Variance; SD for standard deviation.

ed that trehalose could improve the autophagic flux through decreasing the accumulation of p62 and LC3 induced by OGD treatment in myocardial cells.

Trehalose ameliorated OGD-induced lysosomal damage

To investigate the potential mechanism by which trehalose protects autophagic flux, we focused on the lysosome as it is the final destiny of the autophagosomes. OGD treatment significantly decreased the level of lysosomal associated membrane protein 2 (LAMP2, **Figure 3A**), a marker that is localized on the lysosomal membrane, indicating that OGD treatment decreased lysosomal numbers in myocardial cells. Trehalose treatment partially increased the protein level of LAMP2 under OGD conditions (**Figure 3B**). To determine the integ-

rity of lysosome, we isolated lysosomes and examined the release activity of lysosomal enzyme, N-acetyl-β-D-glucosaminidase (NAG) and acid phosphatase activity. OGD increased NAG activity in the supernatant and decreased the acid phosphatase activity of lysosome, indicating that the lysosome was impaired under OGD conditions. Importantly, trehalose dramatically inhibited NAG release and rescued the decreased acid phosphatase activity (**Figure 3C**), indicating that the lysosome integrity impaired by OGD was partially rescued by trehalose treatment. Interestingly, trehalose also partially reversed the decreased levels of Rab 7 and Rab 9 (**Figure 3D**), which are involved in endosomal trafficking. As endosomes and lysosomes are tightly connected, those data suggested that trehalose could affect the endosome-lysosome pathway. Overall, these results suggested that trehalose could protect the lysosome from OGD-induced damage, and thus may restore the impaired autophagic flux.

Trehalose inhibited the production of pro-inflammatory cytokines induced by OGD in myocardial cells

Previous studies showed that trehalose could inhibit the production of pro-inflammatory cytokines in different pathological conditions [22-24]. To further explore the protective effects of trehalose on OGD-induced MI model in myocardial cells. We detected the release of necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-8 in OGD model treated with trehalose or not. Trehalose could significantly decrease the release of those cytokines upregulated by OGD treatment (**Figure 4A**), indicating that trehalose could inhibit the production of inflammatory cytokines induced by OGD in myocardial cells. Meanwhile, the increased ROS level and the production of malondialdehyde (MDA) were inhibited by trehalose treatment (**Figure 4B**). The reduced activity of the anti-oxidative enzyme super oxide dismutase (SOD) induced by OGD was also increased with trehalose treatment (**Figure 4B**). These results suggested that trehalose inhibited OGD-induced oxidative response in myocardial cells.

Discussion

In the present study, we examined the role of trehalose in an OGD model in myocardial cells. Trehalose has been shown to have potential neuroprotective effects in various neuronal diseases. Recently, it has also been shown to reduce cardiac remodeling after myocardial infarction through autophagy activation [20]. However, the role of trehalose in autophagy induction is under debate, and the role of trehalose in OGD-induced damage in myocardial cells is still unknown. Here, we present evidence showing that trehalose protects OGD-induced apoptosis in myocardial cells. We further showed that trehalose improved the impaired autophagic flux induced by OGD through protecting the lysosomal integrity. Finally, we showed that trehalose inhibited the production of pro-inflammatory cytokines and the inflammatory response induced by OGD. Our study provided new evidence for the functions of trehalose in the OGD model in myocardial cells.

Trehalose is a natural compound. The protective role of trehalose in neuronal diseases such as brain ischemia and neurodegenerative diseases has been widely recognized [17]. Generally, the effects of trehalose are mainly believed to induce autophagy and/or inhibit the aggregation of proteins, which are not completely contradicted by each other, as autophagy could also promote the clearance of aggregated proteins itself. However, some earlier studies observed that the upregulation of autophagy didn't distinguish autophagic induction from the inhibition of autophagic flux, which has also upregulated LC3-II [25, 26]. In the present study, we found that OGD-induced upregulation of LC3-II didn't further increased when the fusion of autophagosome and lysosome was blocked by Bafilomycin A1. Consistently, the increased autophagic substrate p62 in OGD conditions didn't further increased in the present of Bafilomycin A1. These data strongly suggested OGD-induced upregulation of LC3 was a dictator of the impairment of autophagic flux, not the upregulation of autophagy activity. Interestingly, trehalose treatment decreased the accumulation of autophagosome as well as the upregulated p62, which suggested that OGD-induced accumulation of autophagosomes along with substrate p62 was partially degraded by the autophagy-lysosome pathway.

Autophagosomes finally fused with endosomes or lysosomes for degradation. Previous studies showed that mutations of the lysosome localized protein gene (*LAMP2*, lysosome-associat-

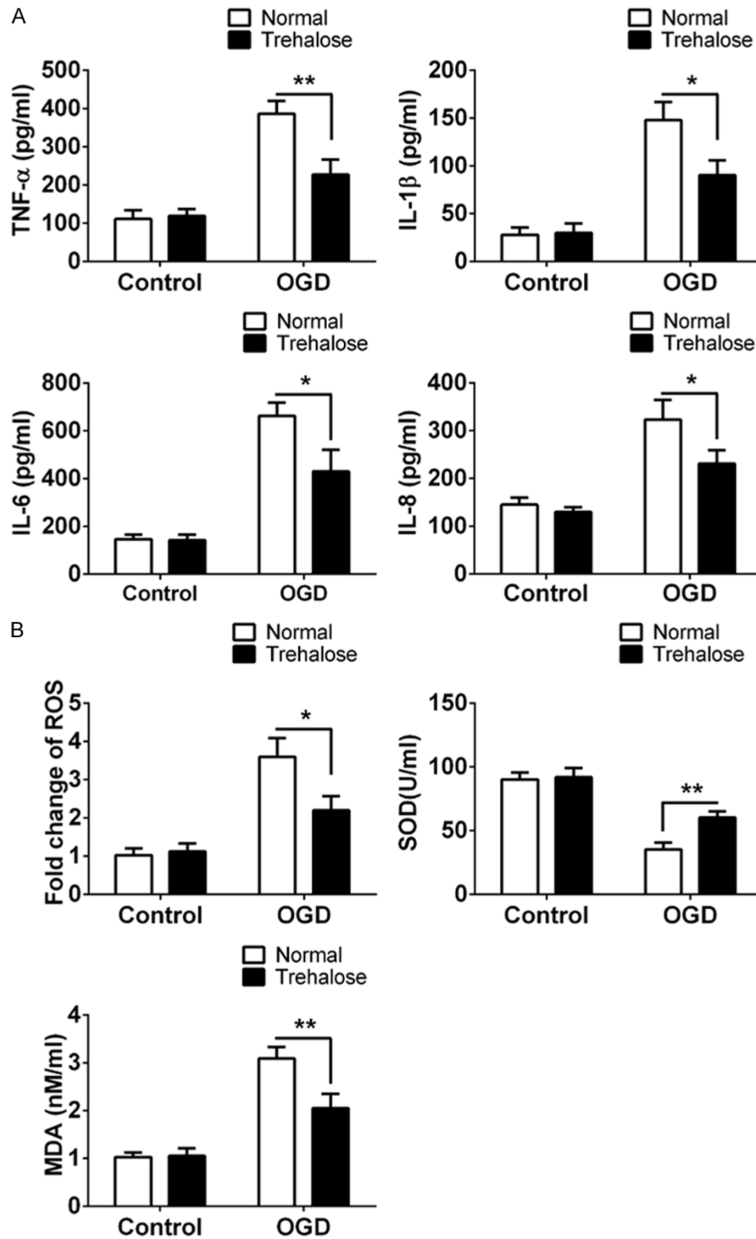

Figure 4. Trehalose inhibited OGD-induced proinflammatory cytokines release. A. H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD and the released inflammatory cytokines including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-8 (IL-8) were detected with enzyme-linked immunosorbent assay (ELISA) kits and the data were quantified. B. H9c2 cells were pre-treated with trehalose (100 mM) for 6 h before OGD and the productions of reactive oxygen species (ROS) and malondialdehyde (MDA) as well as (super oxide dismutase) SOD activity were determined and quantified. Statistical analysis was performed by two-way ANOVA followed by Bonferroni's test. *$P < 0.05$, and **$P < 0.01$ vs the indicated groups. Data represent mean ± SD for combined data from three independent experiments. OGD for oxygen glucose deprivation; ANOVA for Analysis of Variance; SD for standard deviation.

ed membrane protein-2) caused cardiomyopathy in young patients with Danon disease [27] and LAMP-2-deficient mice showed the accumulation of autophagic vacuoles and cardiomyopathy [28], which underscored the key role of lysosome in the pathogenesis of myocardial diseases. Thus, we investigate the effect of trehalose on lysosomes in OGD-treated myocardial cells. Our data showed that OGD significantly decreased the lysosomal marker LAMP2, which indicated that the number of lysosomes was decreased in OGD conditions. Importantly, trehalose treatment rescued the decreased LAMP2 level induced by OGD in myocardial cells, indicating that trehalose could rescue the decreased number of lysosomes. Furthermore, OGD-induced release of lysosomal enzyme NAG was inhibited by trehalose, and OGD-induced decrease of acid phosphatase activity was inhibited by trehalose. Those data strongly suggested that trehalose treatment improved the impaired lysosomal integrity and increased lysosomal numbers. Interestingly, trehalose also inhibited the decreasing of endosomal markers, suggesting a general effect of trehalose on the endosome-lysosome pathway. This is consistent with previous study showing that trehalose altered subcellular trafficking towards endosome and lysosome [29].

Myocardial proinflammatory cytokines have been implicated in the pathogenesis of heart infarction for many years. Recently, inflammatory cytokines are regarded as early biomarkers of organ dysfunction and results in deleterious complications for myocardial infarction [30, 31]. The

development of compounds reducing the levels of proinflammatory cytokines is of potential clinical significance. Trehalose has been implicated in inflammation. Recently, trehalose reduced neuroinflammation and inhibition of inflammatory signaling have been reported [22, 32]. We thus proposed that trehalose might also have a protective role in the OGD model in myocardial cells. Indeed, we found that upregulated proinflammatory cytokines induced by OGD were partially inhibited by trehalose pretreatment. Oxidative stress is wildly known to play an essential role in the pathogenesis of OGD conditions in myocardial cells. Trehalose could also decrease the ROS levels caused by OGD. Meanwhile, trehalose increased the activity of the important anti-oxidative enzyme SOD, indicating trehalose also has a role in anti-oxidative response.

The present study found that trehalose protected the myocardial cells from OGD-induced damage. Trehalose improved the impaired autophagic flux through protecting the lysosomal integrity. Trehalose also inhibited the inflammatory and oxidative responses induced by OGD. Our study provided the novel evidence for the protective role of trehalose in the OGD model of myocardial cells and shed light on the potential usage of trehalose in myocardial infarction treatment in the clinic.

Acknowledgements

This work was supported by the Natural Science Foundation of Hunan Province (Grant NO. 12JJ3108 for J.J.) and the Priority Academic Program Development of the Jiangsu Higher Education Institutes (PAPD).

Disclosure of conflict of interest

None.

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