MEDICAL SCIENCE MONITOR

MOLECULAR BIOLOGY

e-ISSN 1643-3750
© Med Sci Monit, 2014; 20: 1017-1023
DOI: 10.12659/MSM.890897

Received: 2014.04.21
Accepted: 2014.06.09
Published: 2014.06.19

Reactive oxygen species contribute to simulated ischemia/reperfusion-induced autophagic cell death in human umbilical vein endothelial cells

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Source of support: This research was supported by National Natural Science Foundation of China with grant No. 81100153, 81260043 and Application Technology Research and Demonstration Promotion Special Program of Hainan province No. ZDXM2014065 and Natural Science Foundation of Hainan province with grant No. 310130.

Background: Autophagy is important for cells to degrade protein aggregates and organelles. Our preliminary study suggests that ischemia/reperfusion in rabbit hearts promoted autophagic myocardial injury, resulting in no-reflow phenomenon. In this study, we sought to further understand the mechanism and outcome of the upregulation of autophagy in ischemia/reperfusion.

Material/Methods: We employed a simulated ischemia/reperfusion (sI/R) model in human umbilical vein endothelial cells (HUVECs) *in vitro*, in the presence or absence of antioxidants.

Results: Our study confirms that sI/R induces autophagy in HUVECs as measured by increased expression of Beclin 1 and microtubule-associated protein 1 light chain 3 (LC3), electron microscopic analysis, and special biofluorescent staining with monodansylcadaverine. This sI/R-induced autophagy was also accompanied by increased levels of p65 protein expression and cell death. In addition, we detected the accumulation of reactive oxygen species (ROS) after sI/R. Moreover, with the application of ROS scavengers that block the release of ROS, we were able to demonstrate that inhibition of autophagy increases cell survival.

Conclusions: The study suggests that ROS accumulation is involved in the sI/R-induced autophagic cell death in HUVECs.

MeSH Keywords: Autophagy • Reactive Oxygen Species • Reperfusion Injury

Full-text PDF: http://www.medscimonit.com/download/index/idArt/890897

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Background

Autophagy is an adaptive mechanism that enables eukaryotic cells to capture cytoplasmic components for degradation within lysosomes. The process of autophagy begins with sequestration of a portion of the cytoplasm and intracellular organelles in a double membrane structure known as the autophagosome. Autophagy contributes to cell survival by removing damaged organelles and protein aggregates under certain stress conditions. It also provides an alternative energy source during starvation by recycling amino acids and fatty acids for ATP production. However, excessive activation of autophagy can be detrimental, even to the point of inducing cell death [1]. Increasing evidence suggests that autophagy is activated in heart diseases such as congenital heart disease, cardiac hypertrophy, heart failure, and myocardial ischemia/reperfusion (I/R) injury [2,3]. Myocardial I/R induces multiple cellular conditions that favor autophagy [4]. During myocardial ischemia, hypoxia, depletion of ATP, and organelle injury may all act to induce autophagy, and autophagy serves a critical function in ischemic cardiomyocytes to maintain energy production by generating free amino acids and necessary fatty acids [5]. During reperfusion, although the energy crisis is relieved, other factors such as inflammation, oxidative stress, mitochondrial damage, and endoplasmic reticulum stress trigger autophagy instead [6].

Oxidative stress is an important factor to induce autophagy. During periods of oxidative stress, reactive oxygen species (ROS) are produced, including singlet oxygen, superoxide, hydroxyl radical, and hydrogen peroxide. High levels of ROS can induce cell death, which has previously been explained as the result of apoptosis that occurs through the activation of caspase [7]. Emerging evidence, however, indicates that cellular oxidative stress also induces autophagic cell death [8,9]. However, the specific signaling mechanism by which ROS induces autophagy remains poorly understood [8].

The many methods that are useful to monitor the formation of autophagosomes include electron micrographs, Monodansylcadaverine (MDC) staining, the expression Beclin1, and the conversion of the microtubule-associated protein light chain 3 (LC3) by Western blotting [10]. Electron micrography is a classic tool used to evaluate autophagosomes morphologically. MDC is helpful to indicate autophagic vacuoles, in that it accumulates in acidic cell compartments and enables these vacuoles to be visualized. The amount of LC3-II correlates well with the number of autophagosomes, therefore the conversion from endogenous LC3-1 to LC3-II detected by immunoblotting serves as a credible marker for the activity of autophagy. Beclin 1 (Atg6) is the first mammalian protein described that mediates autophagy. By forming a complex with class III phosphatidylinositol 3-kinases (PI3K), Beclin 1 up-regulates autophagy by participating in autophagosome formation and mediating the localization of other autophagic proteins to pre-autophagosomal membranes [11]. Therefore, use of multiple methods to explore autophagy yields more convincing results [10].

Endothelial cells are sensitive to changes in the microenvironment (e.g., ischemia and hypoxia). With the merits of being convenient to manipulate and easy to culture, human umbilical vein endothelial cells (HUVECs) are very popular in cardiovascular disease research [12,13]. In this study, we utilized an *in vitro* simulated ischemia/reperfusion (sI/R) model in HUVECs, along with antioxidants as ROS scavengers, to investigate the association between the generation of ROS, autophagy, and cell death.

Material and Methods

Cell culture

Human umbilical vein endothelial cells (HUVECs) were obtained from ScienCell Research Laboratories (Carlsbad, CA, USA) and cultured in endothelial cell medium (ECM) supplemented with endothelial cell growth supplement (ECGS), 5% fetal bovine serum (FBS), and penicillin/streptomycin (P/S) solution (ScienCell, Carlsbad, CA, USA) in an incubator with 5% CO2 set at a temperature of 37°C. For our experiments, cells of passages 4–6 were grown until confluence. Antioxidants NAC (N-acetyl-L-cysteine) and BHA (Butylated Hydroxyanisole) were dissolved in sterile H2O and ethanol to obtain 306.37 mM and 50 mM stock solutions, respectively.

HUVEC-simulated ischemia/reperfusion protocol

sI/R was achieved by incubating HUVECs for 30 min in an "ischemic buffer" containing 118 mM NaCl, 24 mM NaHCO3, 1.0 mM NaH2PO4, 2.5 mM CaCl2, 1.2 mM MgCl2, 20 mM sodium lactate, 16 mM KCl, and 10 mM 2-deoxyglucose (pH adjusted to 6.2) as reported previously, followed by reoxygenation for 24–72 h [14]. Reoxygenation was accomplished by replacing the ischemic buffer with normal medium under normal oxygen conditions.

Oxidative stress and antioxidant treatment *in vitro*

HUVECs were treated with butylated hydroxyanisole (BHA, 0.1 mM or 0.2mM), N-acetyl-L-cysteine (NAC, 5 mM or 10 mM), or hydrogen peroxide (H_2O_2, 0.5 mM) at the onset of simulated ischemia to provide antioxidant treatment or produce oxidative stress, respectively.

Reagents

The antibodies we used in this study were purchased from the following vendors: p-NF-κB p65 (Ser536), Beclin1, and LC3B

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were from Cell Signaling Technology (Beverly, MA); NF-κB p65 and β-Actin were from Santa Cruz Biotechnology (Santa Cruz, CA). BHA was purchased from Alfa Aesar. All other chemicals were from Sigma.

Lipid peroxidation determination

Malondialdehyde (MDA), an end-product of lipid peroxidation, was determined by measurement of chromogen generated from the reaction of MDA with 2-thiobarbituric acid as described previously [15]. Briefly, we collected 2×10^6 HUVECs/mL in 15-mL tubes and either exposed them to sI/R or left them unexposed as controls, followed by centrifugation (×1600 g) for 10 min. The cell pellets were re-suspended in 0.5 ml of Tris-HCl, pH 7.4 and lysed using a sonicator (W-220; Ultrasonic, Farmingdale, NY) for 5 s on ice. The protein concentration of the cell suspension was determined using a BCA assay (Pierce). A 100-µl aliquot of supernatant was assayed for MDA according to the lipid peroxidation assay kit protocol (Beyotime Institute of Biotechnology). The absorbance of the sample was then read at 586 nm, and the concentration of MDA was determined using a standard curve.

Western blotting

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

WST-1 cell viability assays

Dye conversion of 4-[3-(4-idophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3- benzene disulfonate to formazan (WST-1 Cell Proliferation and Cytotoxicity Assay Kit, Beyotime institute of Biotechnology) was used to assess the cell viability of the HUVECs. HUVECs were plated at 7500 cells/well into 96-well tissue culture plates and then were allowed to attach for 12 h. Then the media was switched to ischemic buffer for 30 min followed by replacement with normal media in the absence or presence of BHA (0.1 mM) and NAC (5 mM). WST-1 reagent was added in 24-h increments up to 72 h, following the manufacturer's instructions, and absorbance was recorded at 450 nm wavelength. The experiments were conducted in triplicate at least 3 times.

Immunofluorescence confocal laser microscopy

MDC was proposed as a marker for autophagic vacuoles. HUVECs were cultured on poly-L-lysine coated coverslips overnight. Then the media was switched to ischemic buffer for 30 min followed by replacement with normal media in the absence or presence of BHA (0.1 mM) and NAC (5 mM) for 24 h as described, and then rinsed with PBS. The samples were then stained with 50 µM MDC at 37°C for 1 h. After incubation, the cells were fixed for 15 min with ice-cold 4% paraformaldehyde at 4°C and washed twice with PBS. Images were viewed and captured blindly by 2 observers using a Leica TCS SP5 confocal laser microscope.

Electron microscopy

Electron microscopic analysis was performed as published previously [16], with minor modifications. Briefly, HUVECs were fixed for 30 min with ice-cold 3% glutaraldehyde in 0.1 M cacodylate buffer, embedded in Epon, and processed for transmission electron microscopy using standard procedures. Representative areas were chosen for ultra-thin sectioning and examined on transmission electron microscope at X6000 magnification.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences between 2 groups were analyzed using the *t*-test. The data from more than 2 groups were evaluated by 1-way ANOVA followed by the Newman-Keuls test. $P<0.05$ is considered statistically significant.

Results

In vitro sI/R produces oxidative stress in HUVECs

Our previous *in vivo* studies showed that areas of high p65 activity in rabbit hearts corresponded with the main zones of ROS production after myocardial I/R [16]. We therefore sought to determine whether ROS directly activates p65. HUVEC treatment with H_2O_2 (0.5 mM) induced p65 phosphorylation at Ser536, as well as Beclin 1 protein levels, but did not increase the total level of p65 protein (Figure 1A). Quantitative analysis of the immunoblotting bands indicated p65 phosphorylation at Ser536 increased 4.61-fold compared to control within as little as 15 min of supplementation of H_2O_2. The maximum effect was observed at 4 h of incubation, which was 10.59-fold that of control. Additionally, protein levels of Beclin1 increased with H_2O_2 treatment at 15 min, 30 min, and 1, 2, and 4 h of incubation, with peak protein expression occurring at 2 h, which was 5.07-fold that of control (Figure 1B). To find out whether *in vitro* sI/R culture also produces oxidative stress in HUVECs, we performed a thiobarbituric acid test to determine the level of malondialdehyde (MDA)

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production, an indicator of lipid peroxidation as the result of oxidative stress. sI/R significantly potentiated the production of MDA by 2.49-fold compared to that of control (P<0.05) (Figure 1C).

sI/R enhances the expression of Beclin1, p65 and increases LC3II/LC3I ratio, which are associated with the release of ROS

sI/R significantly enhanced protein expression of Beclin 1 (Figure 2A). Quantitative analysis of Western blotting demonstrated 24-h sI/R media incubation enhanced Beclin 1 expression by 3.66-fold compared to control (P<0.05) (Figure 2B). Furthermore, electron micrographs revealed the development of autophagosomes induced by sI/R containing digested cytoplasmic materials (Figure 2C). Meanwhile, sI/R-induced p65 protein expression was significantly suppressed by antioxidants BHA (an O2- scavenger) and NAC (a thiol antioxidant), both in a dose-dependant manner (Figure 2D). sI/R increased p65 protein expression 8.53-fold compared to that of control, which decreased by 71% and 75.5% with 0.1 mM and 0.2 mM BHA, respectively, and by 34.5% and 85.6% with 5 mM and 10 mM NAC, respectively (P<0.05) (Figure 2E). Intriguingly, along with increased expression of p65, the LC3II/LC3I ratio increased 5.6-fold after sI/R compared to control. In addition, media supplementation with BHA and NAC markedly inhibited LC3-I to LC3-II conversion, coinciding with suppression of p65 expression. Quantitative analysis indicated that maximal suppression, by 78.9% and 79.5%, was achieved with 0.1 mM BHA and 10 mM NAC, respectively (P<0.05).

ROS scavengers inhibit sI/R-induced autophagy in HUVECs

sI/R culture-induced autophagy in HUVECs was further confirmed by MDC assay. Staining with MDC, which accumulates in acidic cell compartments enriched in lipids, enabled us to assess the formation of autophagic vacuoles. Stimulated autophagy led to a brightly punctate staining pattern around cell nuclei. As shown in Figure 3A, in the setting of sI/R, HUVECs demonstrated bright green fluorescent staining surrounding the nucleus. By quantitative calculation, the number of autophagic cells with punctate structures significantly increased following 24 h of sI/R culture, suggesting that sI/R induced the accumulation of autophagic vacuoles in these cells. Conversely, the number of visible autophagic vacuoles was decreased by the presence of either BHA or NAC. Treatment of HUVECs with BHA (0.1 mM) or NAC (5 mM) significantly reduced sI/R-enhanced accumulation of MDC in the cytoplasmic vacuoles by 29.4% and 48.1% compared to sI/R alone (P<0.05) (Figure 3B).

Oxidative stress generated by sI/R threatens cell survival

sI/R enhanced cell death in a time-dependent manner. As revealed by statistical analysis, sI/R led to the death of HUVECs from 29.6% at 24 h to 64.8% at 48 h and 77.5% at 72 h compared to 0 h (Figure 4). To verify whether the sI/R-related

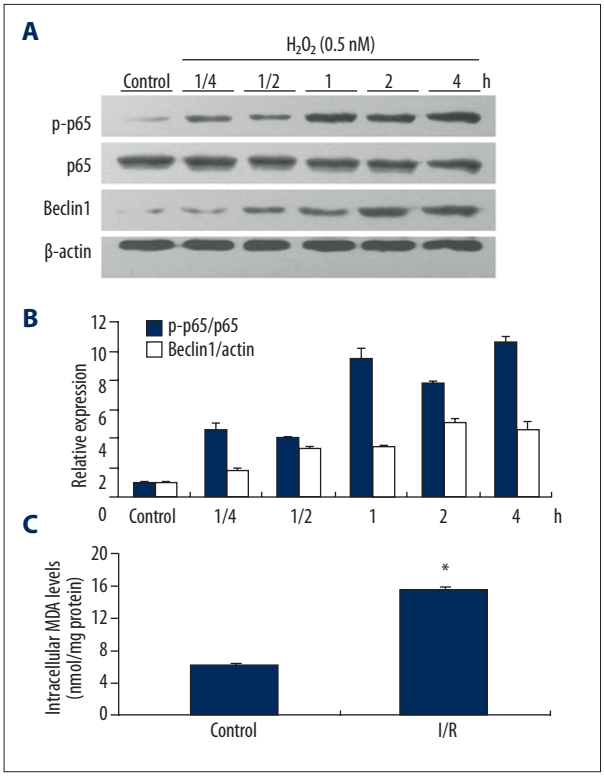

Figure 1. sI/R culture generates ROS in HUVECs. (**A**) HUVECs treated with 0.5mM H_2O_2 for the indicated times (0, 1/4, 1/2, 1, 2, and 4 h) were subjected to Western blotting for p-p65, total p65, and Beclin1. β-actin served as loading control. (**B**) Densitometry values for p-p65/total p65 and Beclin1/β-actin from (**A**) were expressed as multiples of control values normalized to 1. (**C**) Oxidative stress of HUVECs was analyzed after sI/R for 4 h by assessing the level of lipid peroxidation product MDA. *, P<0.05. Results were obtained from 3 independent experiments carried out in duplicate and expressed as multiples of the levels in control cells cultured in normal culture media. Data are presented as mean ±SD.

reduction in cell survival was due to oxidative stress, we assessed the effect of antioxidants on cell viability. BHA (0.1 mM) treatment significantly decreased sI/R-induced cell death by 49.1% and 46.2% in 48 h and 72 h, respectively. Accordingly, NAC (5 mM) treatment significantly rescued sI/R-induced cell death by 58.4% and 51.6% in 48 h and 72 h, respectively.

Discussion

Autophagy plays an important role in cell growth, development, and homeostasis by maintaining a balance between the synthesis, degradation, and subsequent recycling of cellular products. However, excessive autophagy is detrimental. I/R induce multiple cellular conditions that favor autophagy [6].

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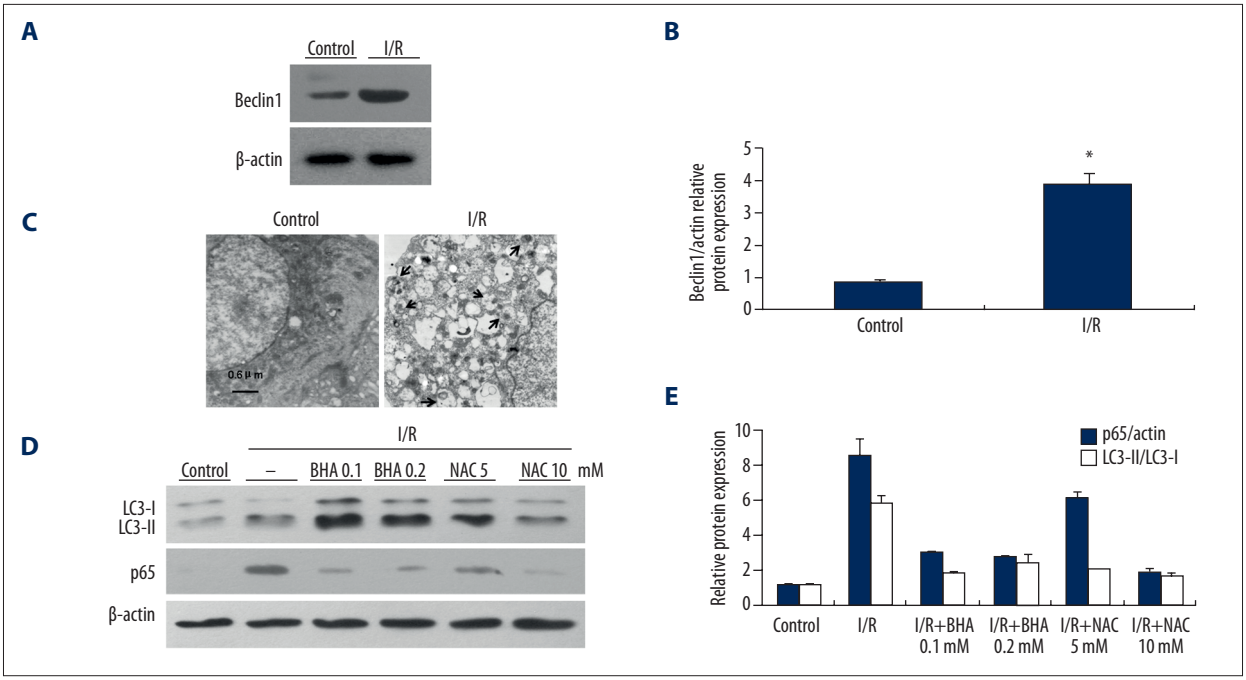

Figure 2. Antioxidants suppress sI/R-induced upregulation of p65 and LC3II/LC3I ratio. HUVECs were cultured in normal media (control) or sI/R media for 24 h before harvesting. (**A**) Cell lysates were subjected to Western blotting for Beclin1. β-actin served as loading control. (**B**) Densitometry values for Beclin1/β-actin from (**A**) are expressed as multiples of control values normalized to 1. (**C**) Representative electron micrographs of sI/R-induced autophagosomes containing digested cytoplasmic materials are shown. Bar, 0.6 μm. Arrows denote the presence of autophagic vacuoles. (**D**) HUVECs were cultured in normal media (control) or sI/R media in the absence or presence of antioxidants BHA (0.1, 0.2 mM) or NAC (5, 10 mM) for 24 h before harvesting. Cell lysates were subjected to Western blotting for LC3 and p65. β-actin served as loading control. (**E**) Quantitative analysis of LC3-I to LC3-II conversion and p65/β-actin from the immunoreactive bands obtained as (**D**). *, P<0.05.

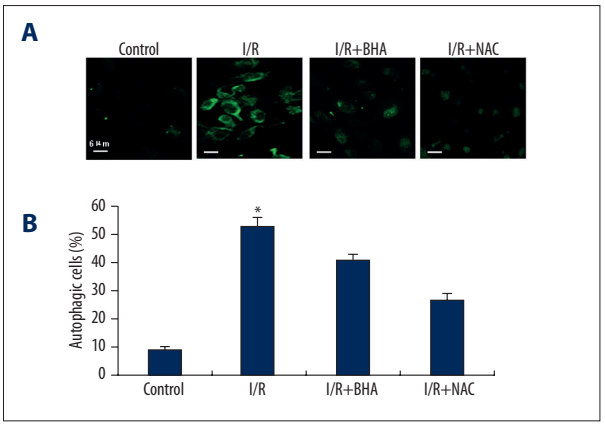

Figure 3. Oxidative stress mediates sI/R-induced autophagy. (**A**) HUVECs were subjected to sI/R in the absence or presence of 1 of 2 ROS scavengers – BHA (0.1 mM) or NAC (5 mM) – for 24 h. Representative images of cells with MDC staining from confocal microscopy are shown (X600 magnification). (**B**) Images in each condition were viewed and captured blindly by 2 observers and the percentages of autophagic cell numbers with MDC-stained dots were quantified accordingly. *, P<0.05. Results were obtained from 3 independent experiments carried out in duplicate. Data are presented as mean ±SD.

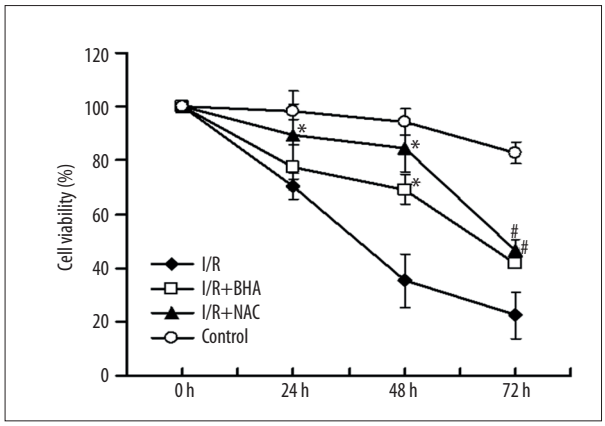

Figure 4. sI/R-generated ROS promotes cell death. Time course of cell viability as determined by WST assay in normal or sI/R media with or without supplements BHA (0.1 mM) or NAC (5 mM). *, P<0.05. Results were obtained from 3 independent experiments carried out in duplicate. Data are presented as mean ±SD.

Autophagy was promoted after myocardial I/R [17]. Our previous study suggested that rabbit myocardial I/R promote autophagic myocardial injury, resulting in a no-reflow phenomenon [18]. Early in 1976, the formation of autophagic

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vacuoles containing damaged organelles in fetal mouse heart using *in vitro* glucose-free hypoxia organ culture followed by resupply of O2 and glucose was reported [19]. Results from the present study with the *in vitro* sI/R model suggested that sI/R enhanced the development and activity of autophagy in HUVECs. During reperfusion, oxidative stress plays important role in triggering autophagy. Even though hypoxia/reoxygenation was not included in the recipe of sI/R in the present study [20] [14], this model effectively aroused the accumulation of ROS and induction of autophagy. Recently, 2-Deoxy-D-Glucose treatment of endothelial cells was reported to induce autophagy by ROS, with an ROS- AMP-activated protein kinase (AMPK) -autophagy pathway implicated [21]. Therefore, 2-Deoxy-D-Glucose, an essential ingredient in our sI/R model, might play the key role.

I/R stimulates cell autophagy and causes cell death in H9c2 cells [22]. Consistently, the present study showed that upregulation of autophagy during sI/R threatened the survival of cells. Aggravation myocardial injury following I/R involved mitochondrial JNK activation [23]. Postulated mechanisms for autophagic cell death include positive feedback between autophagy and oxidative stress. ROS released by mitochondria during reperfusion induce autophagy to degrade catalase and generate hydrogen peroxide. Increased levels of hydrogen peroxide, in turn, accelerate autophagy [4]. Furthermore, ROS induce changes in mitochondrial membrane permeability to trigger autophagy and result in cellular injury [24]. In our current study, autophagic cell death increased in a reperfusion time-dependent manner. However, antioxidants BHA and NAC significantly improved cell survival in the setting of sI/R. Our results indicate that ROS plays an essential role in sI/R-induced autophagy that results in cell damage of HUVECs.

The current study indicates that the protein and phosphorylation levels of p65 increase in a time-dependent manner during sI/R. Considering that ROS itself is a potent inducer of p65 activity, a critical subunit of nuclear factor-κB, it is tempting to speculate that this sI/R-induced ROS-mediated autophagy is p65-dependent. Interestingly, with the treatment of H_2O_2 on HUVECs, the phosphorylation of p65 at Ser365 was elevated, while total level of p65 protein remained the same. This finding suggests that there might be other mechanisms involved in the activation of p65 during sI/R besides the effects of ROS. To address these questions, further investigations are needed.

Beclin1 is essential for the formation of autophagosome. In the present study, sI/R or directly application of 0.5mM H_2O_2 upregulated the protein expression of Beclin1, which suggests that ROS is possibly involved in the Beclin1-mediated autophagy by sI/R. Consistently, using RNAi against Beclin 1 to block autophagy in isolated cardiac myocytes resulted in cell survival subjected to sI/R [25]. ROS/Beclin1 pathway was probed in HUVECs subjected to sI/R. This confirms the previous reports showing that ROS are involved in the initiation of the Beclin-1-mediated pathway of autophagy upon ischemia/reperfusion in myocardium [26].

Limitations exist in our study. To monitor the autophagy in sI/R, a few methods were used in the study, in which the number and activity of lysosomes by MDC is not a reliable indicator of autophagy. Other methods (e.g., GFP-LC3 cleavage assay and long-lived protein degradation) to explore autophagy and to modulate autophagic activity used in the future study will lead us to an improved understanding of the biological functions of autophagy.

Conclusions

The present study indicates that sI/R-induced autophagy resulted in cell death in which ROS accumulation was involved. Understanding the mechanisms of autophagy during I/R may ultimately allow scientists and clinicians to exploit this process in order to improve the clinical prognosis of patients with cardiac I/R injury.

Competing interests

The authors declare that they have no competing interests.

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