Original Article

Cell Transplantation
2018, Vol. 27(8) 1222–1234
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DOI: 10.1177/0963689718779361
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Hydrogen Sulfide Protects Human Cardiac Fibroblasts Against H_2O_2-induced Injury Through Regulating Autophagy-Related Proteins

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Abstract

Autophagy, an intracellular bulk degradation process of proteins and organelles, can be induced by myocardial ischemia in the heart. However, the causative role of autophagy in the survival of human cardiac fibroblasts and the underlying mechanisms are incompletely understood. Oxidative stress can induce autophagy in cultured cells upon hydrogen peroxide (H_2O_2) exposure. Because hydrogen sulfide (H_2S) regulates reactive oxygen species (ROS) and apoptosis, we hypothesize that H_2S may have a cardioprotective function. To examine our hypothesis, we investigated the regulation of autophagy by the H_2S donor sodium hydrosulfide (NaHS), using a cell model of human cardiac fibroblasts from adult ventricles (HCF-av) that suffered from endoplasmic reticulum (ER) stress by H_2O_2. In the present study, we found that the apoptosis and autophagy were induced along with ER stress by H_2O_2 in the primary cultured HCF-av cells. In contrast, H_2S suppressed HCF-av cell apoptosis and autophagic flux, in part directly by inhibiting ROS production and preserving mitochondrial functions.

Keywords

autophagy, cardiac fibroblasts, hydrogen sulfide, lysosome, endoplasmic reticulum

Introduction

The normal heart is a highly organized structure comprising four major cell types: cardiomyocytes (CMs), cardiac fibroblasts (CFs), endothelial cells (ECs), and vascular smooth muscle cells (VSMCs)[1]. The proportion of each cell type varies in different species, but overall CFs occupy the myocardium predominantly, accounting for approximately two-thirds of the cell populations. CFs synthesize and organize collagens, fibronectins, and other interstitial components to maintain cardiac integrity during physiologic proliferation and extracellular matrix (ECM) turnover, as well as cardiac remodeling. Because the adult mammalian heart has a negligible regenerative capacity, cardiac injury provides a great challenge for the reparative mechanisms after the loss of CMs, resulting in the formation of a collagen-based scar. Due to their abundance, CFs play an important role during normal and pathologic wound healing following myocardial ischemia, heart failure, and atrial fibrillation[2,3]. Therefore, fibroblasts represent an attractive therapeutic candidate for heart disease.

Autophagy is a dynamic process of intracellular bulk degradation. The cytosolic proteins and organelles are sequestered into double-membrane vesicles, called autophagosomes, to be fused with lysosomes for degradation[4]. Primarily, autophagy is a survival mechanism that allows a starving cell or a cell deprived of growth factors to survive. Theoretically, autophagy serves to regulate protein and organelle abundance and quality. Autophagy occurs at basal levels in the normal condition but is substantially increased in

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Submitted: January 9, 2018. Revised: April 21, 2018.
Accepted: April 27, 2018.

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several heart diseases, such as acute and chronic ischemia, heart failure, and cardiac hypertrophy[5–7]. Furthermore, nutritional status, hormonal factors, and other conditions like temperature, oxygen concentration, and cell density are also involved in autophagy regulation[8,9].

Although H_2S has been considered as a noxious gas with wide-ranging cytotoxic effects, the accumulating scientific evidence shows that H_2S plays a pivotal role in cellular signaling and functions, similar to nitric oxide (NO) and carbon monoxide (CO). Our previous preliminary study found that the exogenous H_2S donor, sodium hydrosulfide (NaHS), has potent anti-inflammatory effects in a heart that has suffered from acute myocardial infarction *in vivo*, which may be in part due to the limitation of the recruitment of $CD11b^+Gr\text{-}1^+$ myeloid cells[10,11]. Moreover, we also investigated whether NaHS prevented TGF-β1-induced proliferation, migration, regulation of cell growth, transformation to myofibroblasts, and collagen synthesis in human cardiac fibroblasts-to-myofibroblasts assay[12]. Despite the importance of fibroblasts in cardiac pathologies, the direct effects of exogenous H_2S on autophagy in human CFs upon oxidative stress have not been well elucidated. In the present study, we attempted to determine whether the exogenous H_2S protected human cardiac fibroblasts-adult ventricular (HCF-av) against hydrogen peroxide (H_2O_2)-induced endoplasmic reticulum (ER) stress. We used this *in vitro* model to mimic the ER stress injury to the heart and focused on apoptosis and autophagy. We found that H_2S markedly inhibited apoptosis and autophagic flux following ER stress induced by H_2O_2, supporting that H_2S could be used as a new therapeutic reagent for treating oxidative-related diseases.

Materials and Methods

Cell Culture

HCF-av cells were obtained from ScienCell Research Laboratories (Cat# 6310, San Diego, USA) and cultured in fibroblast medium (FM) supplemented with 2% fetal bovine serum (FBS), 1% fibroblast growth supplement (FGS), and 1% penicillin/streptomycin solution (P/S) according to the manufacturer's protocol. The cells were maintained in a humidified, 37°C incubator with 5% CO_2 and 95% air. Cells were subcultured when they became more than 90% confluent. Cells were used for all the *in vitro* ER stress induction and treatment, measurement of reactive oxygen species (ROS) production, measurement of mitochondrial membrane potential ($\Delta\psi$), and activity of the lysosomal compartment experiments[12].

Animal Study, Transverse Aortic Constriction (TAC) Protocol, and DATS Administration

Male C57BL/6 J mice (10 weeks old) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Science (SLAC, Shanghai, China). Animal care and experimental procedures were approved by the Ethics Committee on Animal Research of Hubei University of Medicine and the Institutional Animal Care and Use Committee of Cleveland Clinic. The TAC procedure was described previously[13]. Briefly, the mice were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). To create pressure overload of the heart, the chest was opened via minithoracotomy to expose the aortic arch and TAC procedure was performed in 12-week-old mice by placing a 7-0 silk suture around the aortic arch between the brachiocephalic trunk and the left carotid artery. The suture was ligated around a 27-gauge blunt needle and the needle was quickly removed after ligation. Animals that did not survive after the surgeries were excluded from further experiments.

For H_2S therapy, the diallyl trisulfide (DATS) was obtained from LKT Laboratories (St. Paul, MN, USA) and stored at −20°C before use. The mice were injected intraperitoneally once per day for 12 weeks after TAC with DATS (200 μg/kg) or vehicle (1% DMSO). The dose of DATS was used for the mice on the basis of previous experience investigating DATS in murine models of cardiac ischemia/reperfusion injury[13].

In Vitro *ER Stress Induction and Treatment*

The HCF-av cells were cultured in serum-free FM for 16 h before treatment and then were challenged with H_2O_2 (100 μM, Sigma-Aldrich, St. Louis, MO, USA) for 24 h to mimic ER stress injury[14–16] in the presence or absence of the exogenous NaHS (100 μM, Sigma-Aldrich). The untreated cells were served as the control group and were used in the following experiments.

Measurement of ROS Production

For measurement of intracellular ROS, the dihydroethidium (DHE, Sigma-Aldrich) was used to monitor ROS production upon different treatments in accordance with the manufacturer's protocol. Briefly, the subconfluent cells were pretreated with or without NaHS (100 μM) for 30 min and then subjected to H_2O_2 (100 μM) treatment for 24 h. Cells were incubated with the DHE (5 μM) at 37°C for 30 min and the fluorescence was observed with a Nikon fluorescence microscope (TE-2000U, Nikon, Melville, NY, USA).

Measurement of Mitochondrial Membrane Potential ($\Delta\psi$)

For measurement of mitochondrial membrane potential (MMP), a mitochondria-specific cationic dye JC-1 (100 nM, Life Technologies, Carlsbad, CA, USA) was used to monitor the MMP under different treatments according to the manufacturer's protocol. Briefly, the HCF-av cells were treated with or without H_2O_2 and then were incubated with JC-1 for 10 min in pre-warmed culture medium. Subsequently, the cells were washed three times with pre-warmed culture medium and the MMP was observed with

a Nikon fluorescence microscope (TE-2000U, Nikon). Both red and green fluorescence emissions were analyzed after JC-1 staining with Image J software (developed at the National Institutes of Health, Bethesda, MD, USA).

Activity of the Lysosomal Compartment

LysoTracker Deep Red is an ideal fluorescent acidotropic probe that selectively labels vacuoles with low internal pH. Thus, it can be used to label and track functional lysosomes. Briefly, the cells were treated with H_2O_2 in the presence or absence of NaHS and were then incubated with the LysoTracker Deep Red (70 nM, Life Technologies) in a pre-warmed medium at 37°C for 30 min. Subsequently, the solution was replaced with fresh medium, and the cells were observed using a fluorescence microscope (TE-2000U, Nikon). The activity and intracellular distribution of cathepsin B, a predominant lysosomal protease, was assessed with Magic Red Cathepsin B Detection Kit (Immunochemistry Technologies, LLC, Bloomington, MN, USA). The cells were stained with MagicRed Cathepsin B substrate for 30 min at 37°C and then washed twice with phosphate buffered saline (PBS). Finally, the cells were stained with DAPI (1 μg/ml, Sigma-Aldrich) for 10 min and observed with a fluorescence microscope (TE-2000U, Nikon).

Cell Apoptosis Assay

The cell apoptosis was detected with propidium iodide (PI)/Annexin V assay kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instruction. Briefly, the cells were washed twice with cold PBS and resuspended in 1 × binding buffer at a concentration of 1 × 106 cells/ml. Next, 100 μl cell suspension (1 × 105 cells) was transferred to a 1 ml tube and stained with 5 μl FITC-Annexin V reagents for 30 min and then the cells were stained with 10 μl PI for 10 min at room temperature. Finally, 400 μl 1 × binding buffer was added to each tube. Flow cytometry was performed with the FACScanto II flow cytometer (Becton Dickinson, Mountain View, CA, USA) with excitation at 488 nm. Fluorescent emission of FITC was measured at 515–545 nm and that of DNA–PI complexes at 564–606 nm. Cell debris was excluded from the analysis by an appropriate forward light scatter threshold setting. Compensation was used wherever necessary.

Transmission Electron Microscopy (TEM)

For TEM analysis, the cells were rinsed in PBS and fixed with 2.5% glutaraldehyde in PBS (pH 7.4) for 1 h at 4°C. The cells were washed three times with PBS and then were post-fixed in 1% osmium tetroxide (OsO_4) with 1% potassium ferricyanide. Next, the cells were washed with PBS and dehydrated in a gradient of alcohol (30%, 50%, 70%, and 90%) before embedding in epon. TEM was performed with a Philips CM10 (Andover, MA, USA) at 80 kV on ultra-thin sections (100 nm on 200 mesh grids) stained with uranyl acetate and counterstained with lead citrate.

Western Blot Analysis

Western blot analysis was performed as previously described[10]. Briefly, the cells were washed twice with ice-cold PBS and proteins were extracted using lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 1% Triton X-100, 0.1% SDS, and 100 mM phenylmethylsulfonyl fluoride). The extracted proteins were separated in SDS-polyacrylamide gels and transferred to PVDF membranes (PVDF, Millipore, Burlington Massachusetts, USA). The membranes were washed three times for 10 min each time with TBST and incubated with primary antibodies at 4°C overnight. The primary antibodies used in this study are listed below: activated caspase 3 p17 (Bioworld; 1:1000 dilution Dublin, OH, USA), BiP (Cell Signaling Technology; 1:1000 dilution Danvers, MA, USA), C/EBP homologous protein (CHOP) (Cell Signaling Technology; 1:1000 dilution), LC3 (Sigma-Aldrich; 1:1000 dilution), Beclin1 (Abcam; 1:1000 dilution, Cambridge, UK), P62/SQSTM1 (Cell Signaling Technology; 1:1000 dilution), Puma (Cell Signaling Technology; 1:1000 dilution), and Ubiquitin (Cell Signaling Technology; 1:1000 dilution). The membranes were washed with TBST followed by incubation with indicated horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA). Detection was performed using enhanced chemiluminescence (ECL) Western blotting detection reagent (G&E) and the data were quantified by densitometry.

Proteasome activity assay. Proteasome activity was measured by aminomethylcoumarin (AMC)-linked synthetic peptide substrates: Ac-Gly-Pro-Leu-Asp-AMC and Suc-Leu-Leu-Val-Tyr-AMC (Proteasome Substrate Pack, Enzo Life Sciences, Farmingdale NY, USA). Proteins were extracted from treated or untreated cells with lysis buffer (50 mM HEPES pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and 2 mM ATP). Next, 200 μl of lysate containing equal amounts of protein (5 μg) were incubated for 30 min at 37°C in a dark environment with 2.5 μl of each substrate. The reaction was stopped by stop buffer (ice-cold 96% ethanol). The proteasome activity was detected by Tecan Infinite M200 Plate Reader (380 nm excitation and 460 nm emission, Männedorf, Switzerland).

Statistical analysis. All experiments were carried out in triplicate under identical conditions and data were represented as means ± standard error of the mean (SEM). For animal studies, experiments were performed in duplicate and each group included three mice. Statistical analysis was performed with SPSS software (IBM Corp., Armonk, NY, USA). Different groups were compared by one-way analysis

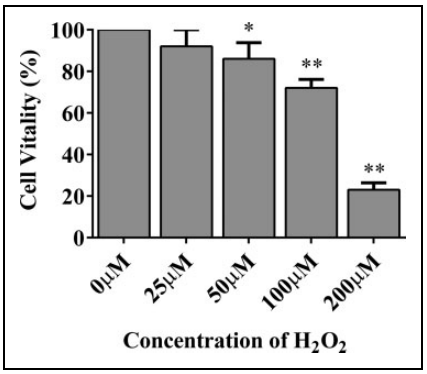

Figure 1. The effect of H_2O_2 on HCF-av cell vitality. The HCF-av cells were treated with different concentrations of H_2O_2 for 24 h and then the cell vitality was determined by CCK-8 kit ($n = 3$). *P < 0.05, **P < 0.01 vs. control.

of variance (ANOVA), followed by Tukey's or Bonferroni post-hoc test when applicable. Comparisons between the two groups were assessed by the t test. A P value less than 0.05 was considered significant.

Results

The Effect of H_2O_2 on Cell Proliferation of HCF-av Cells

HCF-av cells were treated with H_2O_2 at different concentrations (0–200 μM) for 24 h. Cell vitality was measured by a Cell Counting Kit (CCK-8, Dojindo, Rockville, MD, USA) according to the manufacturer's protocol. H_2O_2 exhibited cytotoxicity in HCF-av cells in a dose-dependent manner. There was no significant loss of vitality with 0 or 25 μM H_2O_2 in HCF-av cells. In contrast, decreases of nearly 14%, 28%, and 77% cell

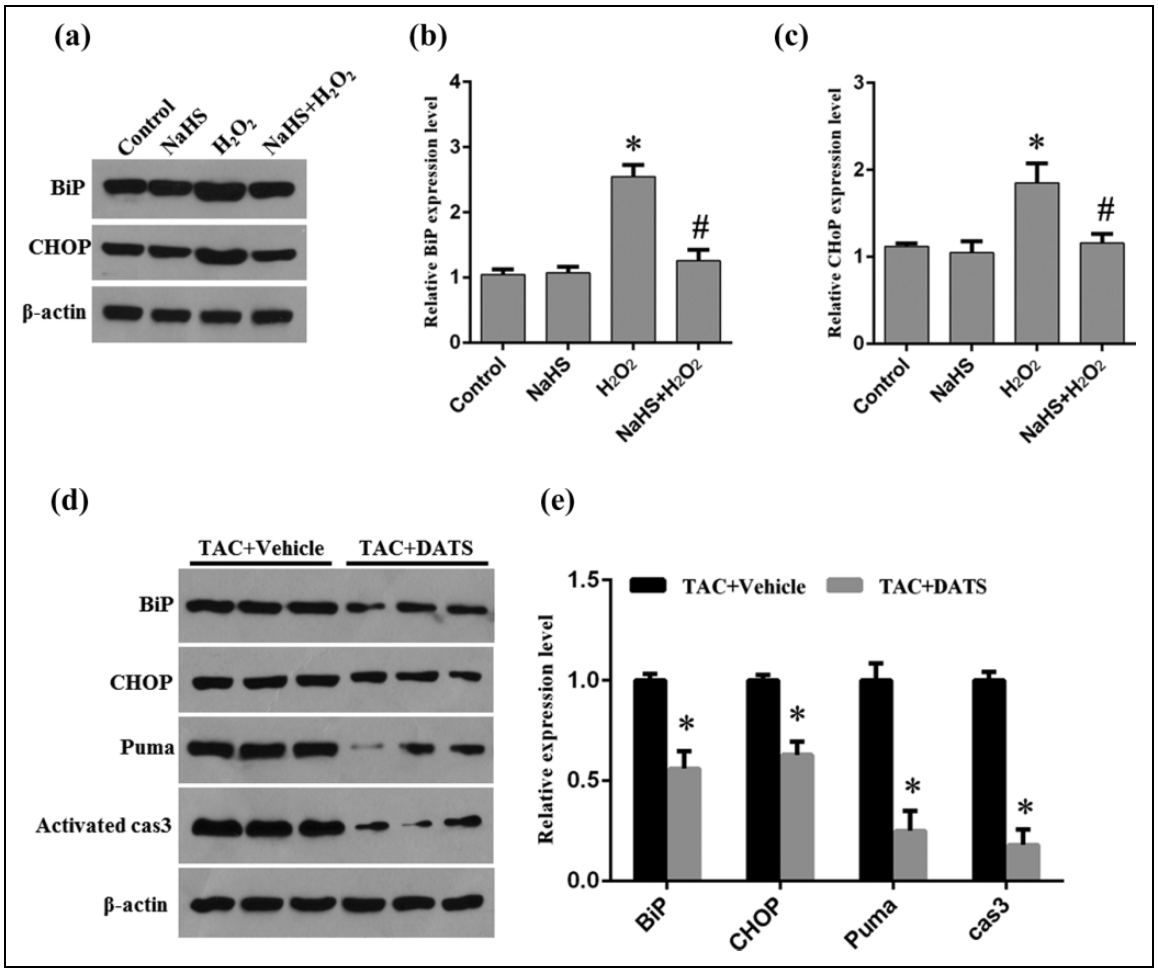

Figure 2. H_2S ameliorates H_2O_2-induced ER stress in HCF-av cells. (a) Western blot analysis of HCF-av cells upon different treatments was performed to detect BiP and CHOP. β-actin served as the loading control. (b,c) Quantitative analysis of the changes of BiP and CHOP in treated cells. Data represent mean ± SEM ($n = 3$. *P < 0.05 vs. control; #P < 0.05 vs. NaHS). (d) Representative Western blot analysis for BiP, CHOP, Puma, and caspase 3 expression in hearts from vehicle- and DATS-treated mice. β-actin served as the loading control. (e) Quantitative analysis of the changes of BiP and CHOP in treated cells. Data represent mean ± SEM ($n = 3$. *P < 0.05 vs. TAC + Vehicle).

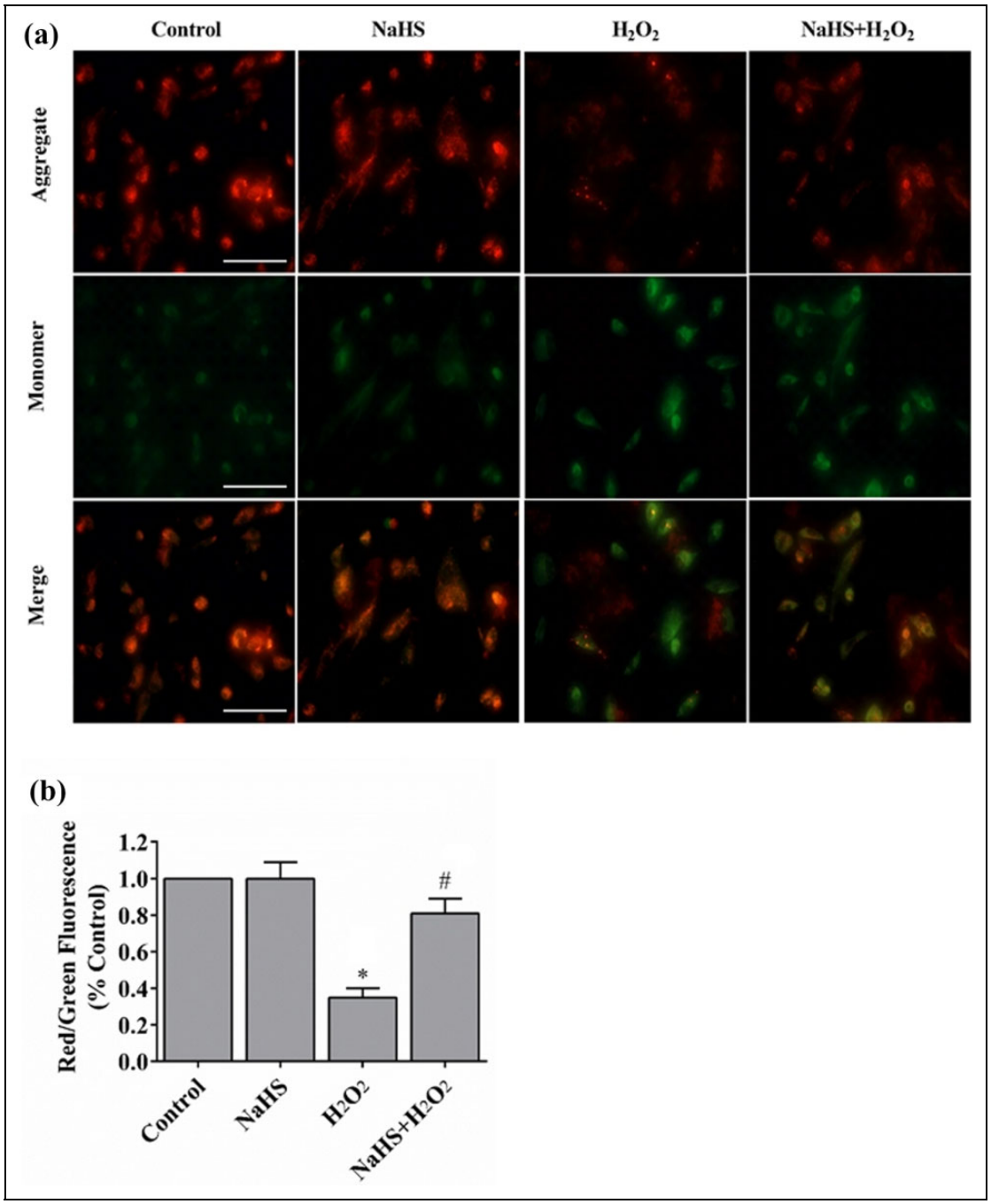

Figure 3. H_2S restores H_2O_2-induced reduction of $\Delta\psi$. (a) The $\Delta\psi$ loss was determined by the lipophilic cationic probe JC-1. Red signal indicated JC-1 in mitochondria. Green signal indicated cytosolic JC-1. Magnification, ×400. (b) Quantitative analysis of membrane potential ($n = 3$). *$P < 0.01$ vs. control; #$P < 0.01$ vs. NaHS; #$P < 0.01$ vs. H_2O_2.

vitality occurred in HCF-av cells exposed to 50 μM, 100 μM, and 200 μM H_2O_2 for 24 h, respectively (Figure 1). Therefore, we used 100 μM H_2O_2 for the next experiments.

H_2S Reduces ER Stress Induced by H_2O_2 and TAC

The ER stress was induced by H_2O_2 in cultured HCF-av cells, which was assessed by the ER stress protein markers immunoglobulin binding protein (BiP) and CHOP. H_2O_2 challenge provoked a significantly increased Expression of BiP and CHOP compared with the control cells (Figure 2(a–c)). Interestingly, H_2S treatment effectively abrogated ER stress by reducing the expression levels of BiP and CHOP induced by H_2O_2. To further confirm our results, we evaluated the effects of H_2S on the ER stress in heart tissues from mice after TAC. As shown in Figure 2(d,e), the ER

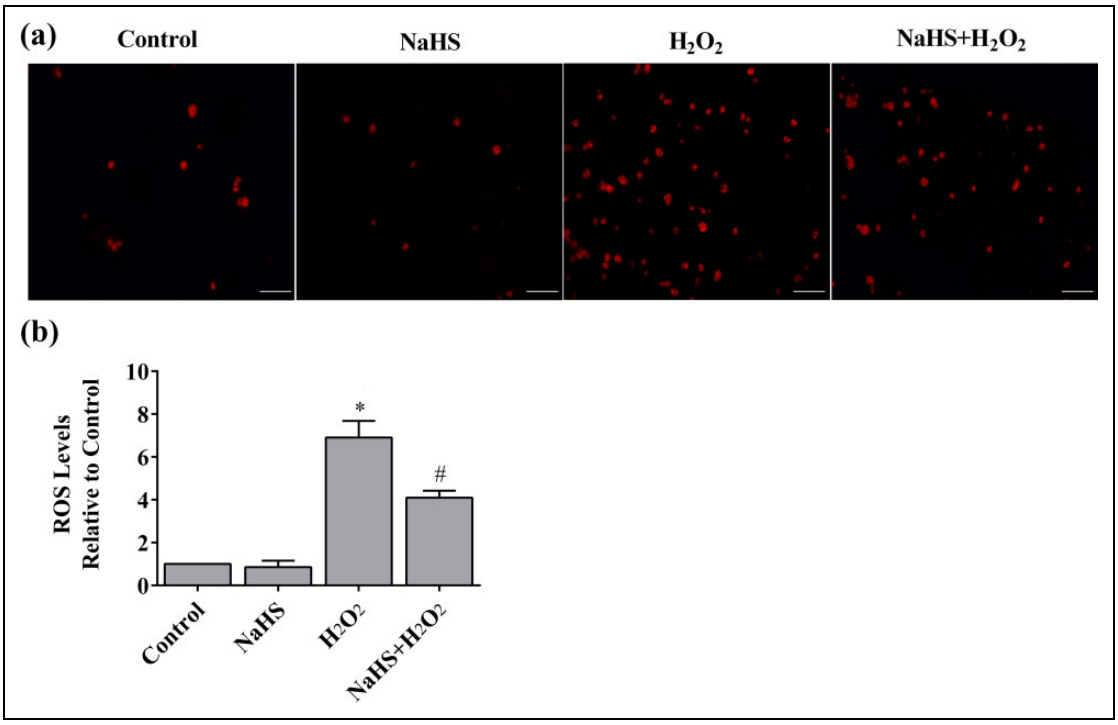

Figure 4. H_2S suppresses superoxide anion production induced by H_2O_2. (a) Intracellular superoxide anion production was detected with dihydroethidium and observed by fluorescent microscopy. (b) The fluorescent signal was measured and quantified ($n = 6$). $^{*}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. NaHS; $^{\#}P < 0.01$ vs. H_2O_2.

stress-related markers (BiP, CHOP, and Puma) and caspse-3 were significantly induced by TAC. Strikingly, BiP, CHOP, Puma, and caspase-3 was markedly reduced by H_2S after TAC. Altogether, these results demonstrate that H_2S protected heart cells against ER stress.

H_2S Prevents Loss of MMP Induced by H_2O_2

Mitochondrial function is highly susceptible to oxidative damage. Therefore, we investigated whether H_2S protected mitochondria from H_2O_2-induced ER stress. Mitochondria in control cells stained with JC-1 exhibited bright orange fluorescence. However, when cells were exposed to H_2O_2, they showed fewer and less intense JC-1 fluorescence in mitochondria (Figure 3(a)), which was greatly improved by pretreatment of H_2S (Figure 3(b)). These results suggested that H_2S could prevent the loss of mitochondrial $\Delta\psi$ upon oxidative stress.

H_2S Suppresses ROS Production Induced by H_2O_2

To determine the effect of H_2S on H_2O_2-induced ROS production from ER and mitochondria, DHE, a specific fluorescent probe for O_2^-, was used to track cellular ROS generation (Figure 4(a)). HCF-av cells were subjected to H_2O_2 treatment and ROS production was significantly enhanced compared with the control. Conversely, this elevation was markedly suppressed by pretreatment of cells with H_2S (Figure 4(a,b)). No significant difference in ROS production was observed with NaHS treatment alone. These results indicated that H_2S abrogated ROS production in HCF-av cells.

H_2S Attenuate Cell Apoptosis Induced by H_2O_2

ROS production is known to promote apoptosis. To evaluate the effect of H_2S on ER stress-induced apoptosis, HCF-av cells were subjected to different treatments and the cell death was analyzed by flow cytometry. As shown in Figure 5(a,b), oxidative stress induced by H_2O_2 resulted in significant cell death (Annexin V^+/PI^+ cells) compared with the control cells. By contrast, pretreatment of NaHS dramatically reduced cell death induced by H_2O_2. These results were consistent with the level of activated caspase 3, a cell apoptotic marker (Figure 5(c,d)).

H_2S Ameliorates Lysosomal Activity in HCF-av Cells Induced by H_2O_2

To investigate the role of lysosomal activity on cell apoptosis and damage of ER or mitochondria, HCF-av cells were subjected to different treatments and then incubated with LysoTracker Deep Red, which labeled the highly acidic lysosomal vacuoles and monitored activity of the vacuolar H^+-ATPase (v-ATPase). We found that H_2O_2 exposure markedly increased the LysoTracker red staining. However,

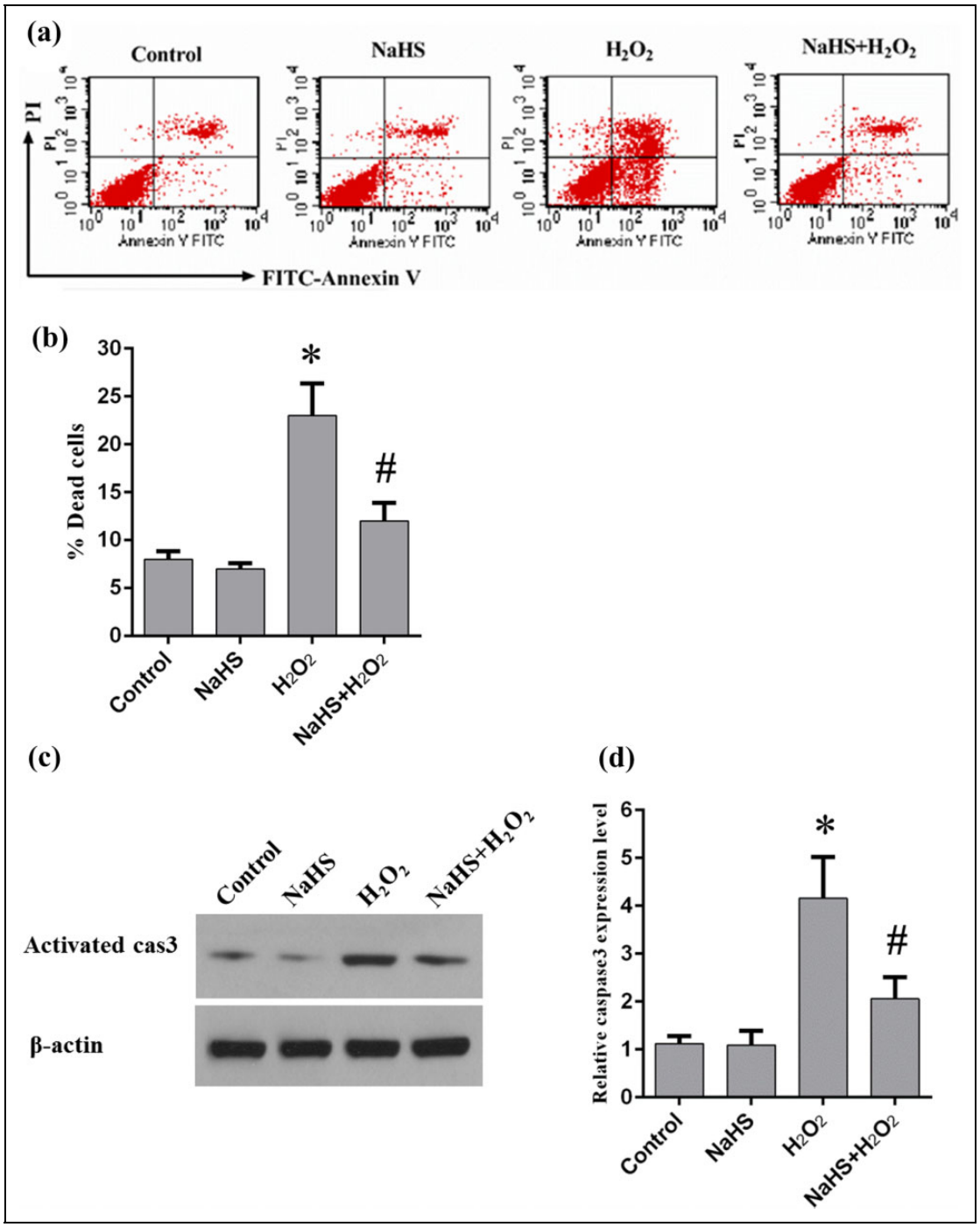

Figure 5. H_2S attenuates cell apoptosis in HCF-av cells induced by H_2O_2. Cell death analysis of treated cells was performed by flow cytometry with Annexin V/PI double staining (a,b). Representative images and quantitative analysis were shown in (c) and (d), respectively. Data represent mean ± SEM ($n = 3$; $^{*}P < 0.05$ vs. control cells, $^{\#}P < 0.05$ vs. indicated cells).

this fluorescent signal was significantly decreased by pretreatment of NaHS (Figure 6(a,b)).

H_2S Prevents H_2O_2-Induced Autophagy

To investigate whether the autophagy was activated in the period following increased lysosomal activity in HCF-av cells undergoing oxidative stress, the expression level of cathepsin B was examined using Magic Red staining. As shown in Figure7(a), H_2O_2-induced ER stress caused approximately three-fold increase in fluorescence intensity of cathepsin B in HCF-av cells compared to the control cells. Consistent with this finding, cells treated with H_2O_2 displayed an increased abundance of multilamellar

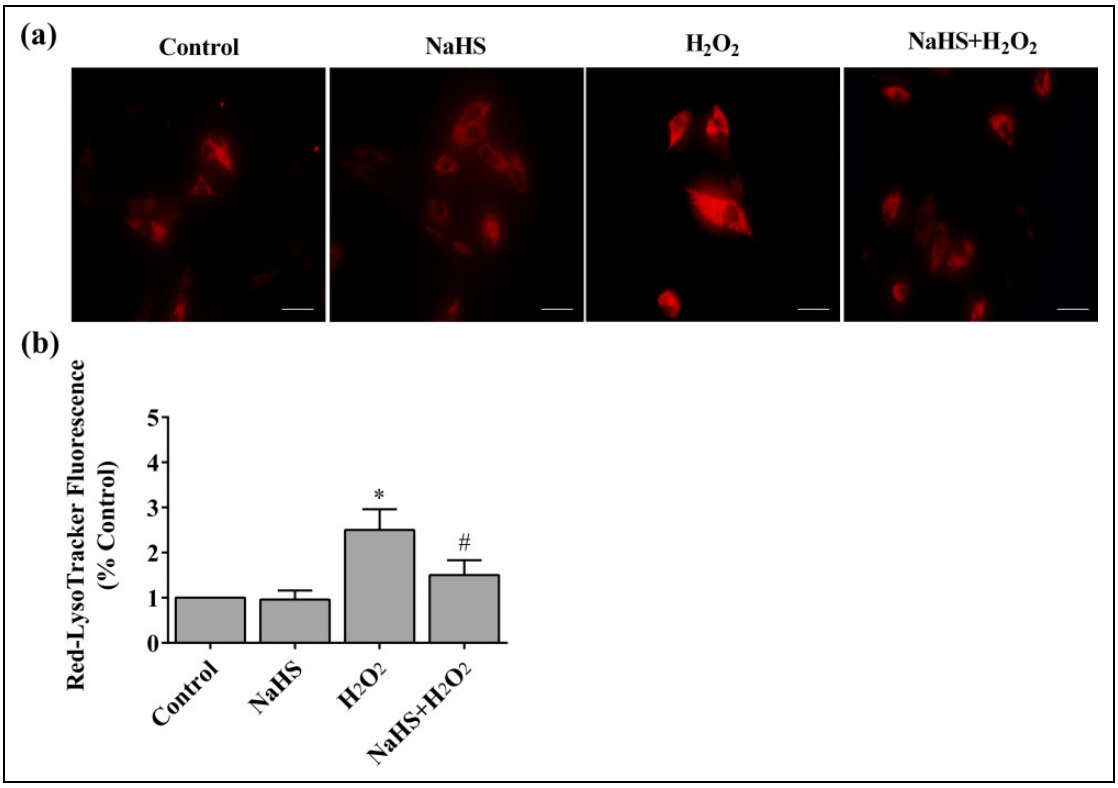

Figure 6. H_2S ameliorates lysosomal activity in HCF-av cells induced by H_2O_2. (a) Cells were subjected to different treatments and then stained with 70 nM LysoTracker® Deep Red (magnification, ×400). (b) The fluorescent signal (red) was measured and quantified ($n = 6$). $^{*}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. NaHS; $^{\#}P < 0.01$ vs. H_2O_2.

autophagosomes (Figure 7(b)). Strikingly, these phenomena were significantly diminished when the cells were treated with NaHS (Figure 7(c,d)).

H_2S Regulates the Expression of LC3-II, Beclin1, and P62 During Autophagy

The expression levels of LC3-I/II, Beclin1, and P62 play vital roles for autophagic activity[17–19]. To further investigate the role of H_2S-regulated autophagy induced by H_2O_2, HCF-av cells were subjected to different treatments and the autophagy-related proteins were detected. As shown in Figure 8(a), the expression levels of Beclin1, LC3-II/LC-I ratio, and P62 were robustly increased when cells were treated with H_2O_2. Meanwhile, the increases of these key proteins were diminished in cells pretreated with H_2S (Figure 8(a–d)). Because p62 plays a key role in both autophagy and the ubiquitin proteasome system, we further investigate the effects of H_2S on the ubiquitin proteasome system upon H_2O_2 treatment in HCF-av cells. As shown in Figure 8(e–g), the proteasome activity and ubiquitin expression did not change when cells were treated with or without H_2S plus H_2O_2. These results suggested that H_2S could regulate the autophagic activity but not proteasome activity in HCF-av cells under H_2O_2 treatment.

Discussions

Recent studies indicated that H_2S was a powerful endogenous second messenger, capable of modulating a variety of physiological or pathophysiological events in mammalian cells and tissues[20,21]. These results prompted us to investigate the potential role of H_2S as a cardioprotective reagent. Previous studies indeed suggest that H_2S was a potent cardioprotective signaling molecule reagent for heart disease[22,23]. Current studies have shown that H_2S can regulate the activation of ion channel, and upregulate antioxidant, anti-apoptotic, and anti-inflammatory signaling pathways[10,24–26]. In the present study, we evaluated effects of NaHS on the *in vitro* ER stress cell model. H_2O_2 is one kind of ROS and has been widely used in experiments to mimic the situation of oxidative stress. Different concentrations of H_2O_2 have been widely used in different cell types, and different cell types have showed different responses to oxidative stress induced by H_2O_2[27]. In the present study, the HCF-av cells were exposed to H_2O_2 to mimic *in vivo* ER stress. BiP expression level was robustly increased, which revealed ER stress induced by H_2O_2 in HCF-av cells (Figure 2). This result is consistent with the accumulation of CHOP (also known as growth-arrest and DNA damage inducible gene 153) in HCF-av cells induced by H_2O_2, which was a transcription factor and activated at multiple levels during ER

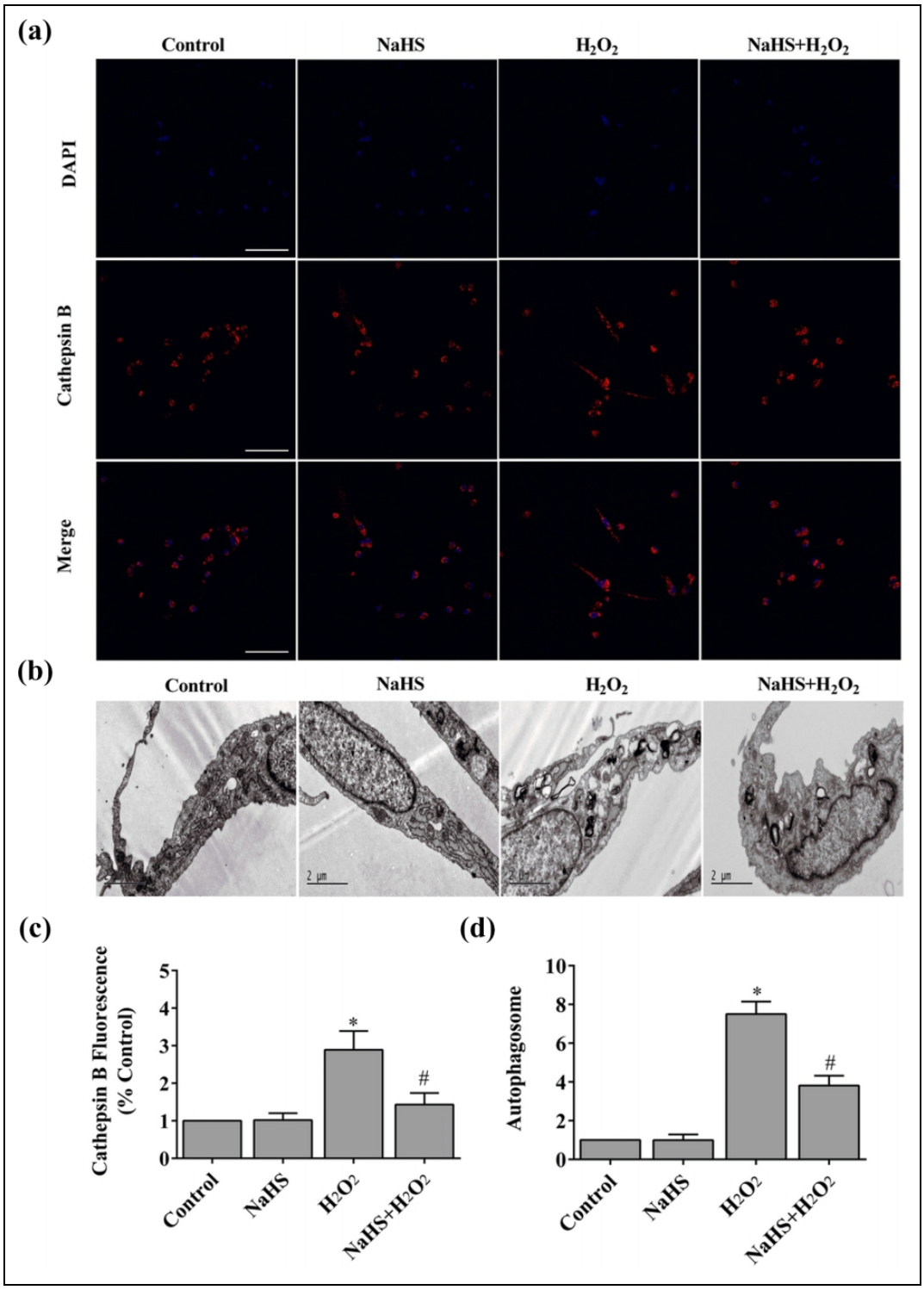

Figure 7. H_2S blocks autophagy in HCF-av cells induced by H_2O_2. (a) Cells were subjected to different treatments and then stained with Magic Red® Cathepsin B Detection Kit (magnification, ×200). (b) Representative TEM micrographs upon H_2O_2 treatment. (c) The fluorescence (red) intensity of cathepsin B was measured and quantified ($n = 6$). (d) Quantification of the autophagosome ($n = 4$). $^{*}P < 0.01$ vs. control; $^{\#}P < 0.01$ vs. NaHS; $^{\#}P < 0.05$ vs. H_2O_2.

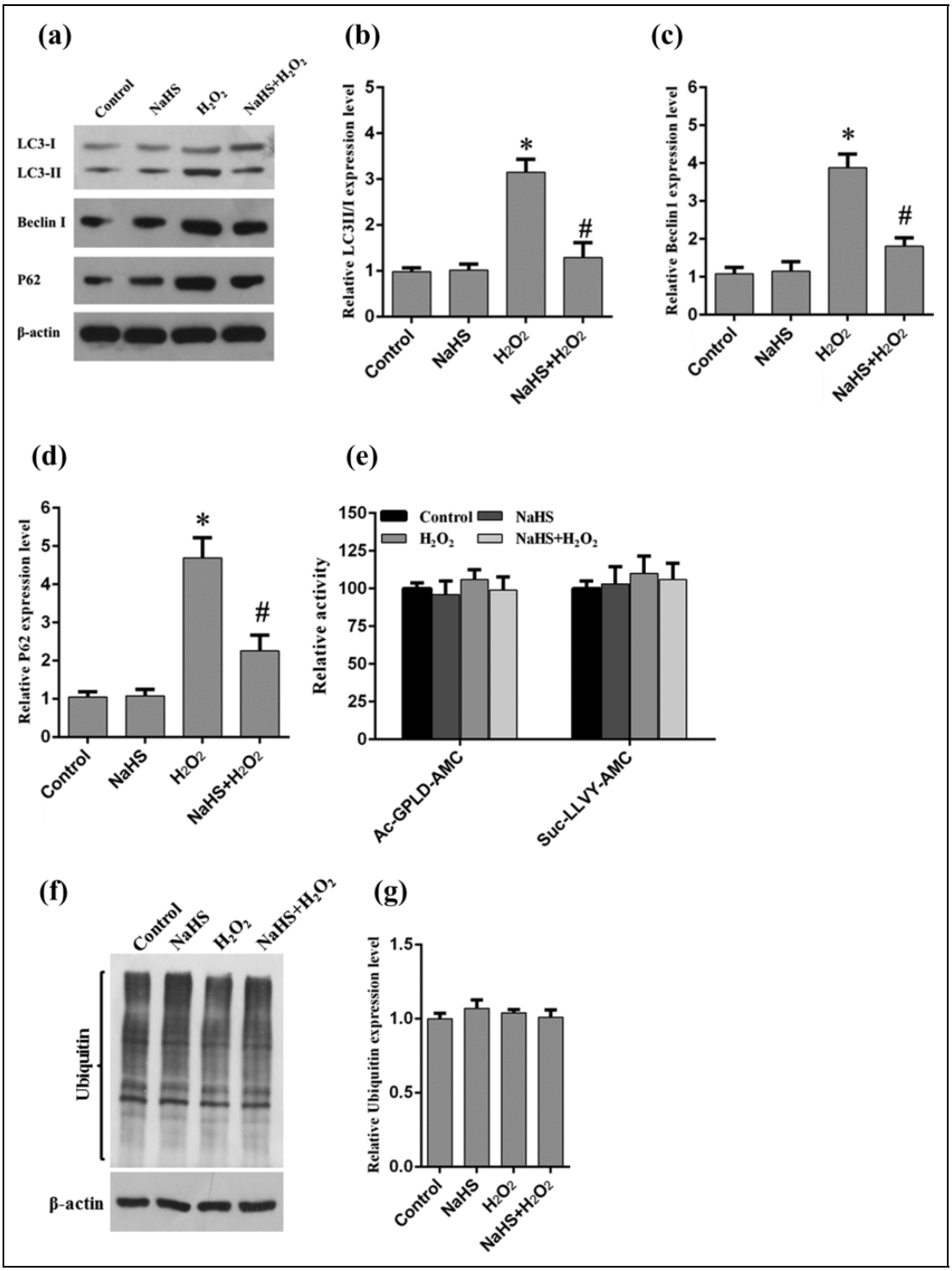

Figure 8. H_2S regulates the expression of LC3-II, Beclin1, and P62 during autophagy. (a) Western blot analysis of HCF-av cells upon different treatments was performed to detect LC3I/II, Beclin 1, and P62. (b–d) Quantitative analysis of the changes of LC3I/II, Beclin1, and P62 in treated cells ($n = 3$). $^{*}P < 0.01$ vs. control; $^{*}P < 0.01$ vs. NaHS; $^{\#}P < 0.05$ vs. H_2O_2. (e) Proteasome activity was measured using AMC-linked substrate peptides under different treatments ($n = 3$). (f) Representative Western blot analysis for ubiquitin expression. β-actin served as the loading control. (g) Quantitative analysis of the changes of ubiquitin in treated cells ($n = 3$).

stress[28–30]. Because ER stress is closely related to cell apoptosis, we have found that ER stress significantly elevated the activated caspase 3 level in HCF-av cells induced by H_2O_2 (Figure 5). Importantly, the activated caspase 3 level is abrogated by H_2S treated HCF-av cells induced by H_2O_2.

Mitochondria played pivotal roles in the two types of cell death: apoptosis and necrosis[31]. However, autophagy, a cellular stress response, is involved in a variety of diseases and has recently been proposed as a third distinct mode of cell death. Autophagy is a dynamic process involving the rearrangement of subcellular membranes to sequester cytoplasm and organelles which are delivered to the lysosome or vacuole, and then the sequestered cargo is degraded and recycled[4]. Accumulated evidence indicates that autophagy may constitute an important physiological response to cardiac stresses, ischemia, or pressure overload, which are frequently encountered in patients with coronary artery disease, hypertension, aortic valvular disease, and congestive heart failure. The accumulation of autophagosomes has been noted in cardiac biopsy tissues of patients with these disorders, rodent models of these cardiac diseases, and isolated stressed cardiomyocytes[4]. Autophagy participated in the constitutive turnover of mitochondria in oxidative tissues, and removal of damaged organelles[32,33]. One of the conclusions of our study is that the protective mechanism of H_2S may be involved in stabilization of the mitochondria in H_2O_2-induced cell death. Changes of the mitochondrial permeability transition (MPT) and loss of $\Delta\psi$ triggered autophagic scavenging. Our findings suggested that H_2O_2 exposure reduced JC-1 aggregates in HCF-av cells, indicating mitochondria $\Delta\psi$ was decreased. Conversely, pretreatment of H_2S restored the mitochondria $\Delta\psi$ induced by H_2O_2. Furthermore, autophagic flux was significantly increased following H_2O_2 exposure, as shown by the multilamellar autophagosomes and the increased LC3-II/LC-I ratio, Beclin1, and p62 protein level. In contrast, exogenous H_2S completely abrogates these phenomena.

In eukaryotic cells, the lysosome is a major organelle that contains a lot of enzymes, which can degrade essentially any subcellular component by hydrolases such as proteins, lipids, nucleic acids, and carbohydrates[34]. Lysosomal enzymes also play a role in the activation of certain types of caspase, which are involved in cell apoptosis. Lysosomes have been referred to as "suicide bags," as they contribute to autophagic cell death[35,36]. Moreover, ROS can induce lysosomal permeabilization before mitochondrial dysfunction. Although oxidative stress induces many alterations within the cell, mitochondria may be the first organelle to be demerged by ROS. Lysosomal enzymes have been found to act on mitochondria and promote mitochondrial ROS generation, creating a feedback loop and leading to more lysosomal permeabilization. Our studies determined the effect of H_2S on the autophagic activity induced by H_2O_2. Autophagy was involved in the delivery of autophagosomes and their contents to lysosomes and accomplished the catabolic processes of autophagy. We found that cellular lysosomal activation and the expression level of cathepsin B are both increased in cells with ER stress induced by H_2O_2, but are diminished by NaHS treatment.

Recent studies have shown that H_2S is a strong promoter of angiogenesis[37,38] and stimulates cell replication, migration, and tube formation[39]. Furthermore, H_2S also promotes angiogenesis *in vivo*[40] and the proangiogenic effects of H_2S on chronic vascular disease have been reported[39]. However, the protective role of H_2S in ER stress and autophagy induced by oxidative stress in the heart is still obscure. A previous study reported that H_2S attenuates oxidative stress in the heart through activation of nuclear factor E2-related factor (Nrf2)[41], because Nrf2 regulates a large number of gene expressions for enzymes that serve to detoxify pro-oxidative stressors[42], such as GPx1 and HO-1, via binding to the antioxidant response element found in the gene's promoter region[40]. Maybe that is why H_2S can prevent ER stress, autophagy, and heart cell apoptosis induced by H_2O_2. In the current study we demonstrated that H_2S treatment dramatically repressed ER stress marker and autophagy marker expression and cell apoptosis induced by H_2O_2 using an *in vitro* model (HCF-av cells). Furthermore, the *in vivo* model also suggested that ER stress-related markers and heart cell apoptosis were significantly blocked by H_2S treatment (Figure 2(d)). Further investigations are needed to determine the precise mechanism by which H_2S prevents ER stress, autophagy, and cell apoptosis induced by oxidative stress in the heart.

In summary, H_2S pretreatment efficiently protects HCF-av cells from H_2O_2-induced ER stress, apoptosis, and autophagy, which maintains mitochondria membrane integrity and prevents the activation of caspase 3. Our study suggests that H_2S could potentially be a therapeutic reagent for suppressing ER stress in the heart.

Author Contributions

Ao Feng and Chen Ling contributed equally to this work.

Ethical Approval

Animal care and experimental procedures were approved by the Ethics Committee on Animal Research of Hubei University of Medicine and the Institutional Animal Care and Use Committee of Cleveland Clinic.

Statement of Human and Animal Rights

Male C57BL/6 J mice (10 weeks old) were purchased from the Shanghai Laboratory Animal Center of the Chinese Academy of Science (SLAC, Shanghai, China).

Statement of Informed Consent

Statement of Informed Consent is not applicable for this article.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by the National Nature Science Foundation of China (No. 81500237) and Special Foundation for Knowledge Innovation of Hubei Province (Nature Science Foundation) (No. 2017CFB563).

References

1. Fan D, Takawale A, Lee J, Kassiri Z. Cardiac fibroblasts, fibrosis and extracellular matrix remodeling in heart disease. Fibrogenesis Tissue Repair. 2012;5(1):15.
2. Cunnington RH, Wang B, Ghavami S, Bathe KL, Rattan SG, Dixon IM. Antifibrotic properties of c-Ski and its regulation of cardiac myofibroblast phenotype and contractility. Am J Physiol Cell Physiol. 2011;300(1):C176–C186.
3. Becher PM, Gotzhein F, Klingel K, Escher F, Blankenberg S, Westermann D, Lindner D. Cardiac function remains impaired despite reversible cardiac remodeling after acute experimental viral myocarditis. J Immunol Res. 2017;6590609. doi: 10.1155/2017/6590609
4. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell. 2008;132(1):27–42.
5. Cao DJ, Wang ZV, Battiprolu PK, Jiang N, Morales CR, Kong Y, Rothermel BA, Gillette TG, Hill JA. Histone deacetylase (HDAC) inhibitors attenuate cardiac hypertrophy by suppressing autophagy. Proc Natl Acad Sci USA. 2011;108(10):4123–4128.
6. Ma X, Liu H, Foyil SR, Godar RJ, Weinheimer CJ, Hill JA, Diwan A. Impaired autophagosome clearance contributes to cardiomyocyte death in ischemia/reperfusion injury. Circulation. 2012;125(25):3170–3181.
7. Pistoia V, Pezzolo A. Involvement of HMGB1 in resistance to tumor vessel-targeted, monoclonal antibody-based immunotherapy. J Immunol Res. 2016;3142365. doi: 10.1155/2016/3142365
8. Klionsky DJ. Autophagy: from phenomenology to molecular understanding in less than a decade. Nat Rev Mol Cell Biol. 2007;8(11):931–937.
9. Maiuri MC, Zalckvar E, Kimchi A, Kroemer G. Self-eating and self-killing: crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol. 2007;8(9):741–752.
10. Zhang Y, Li H, Zhao G, Sun A, Zong NC, Li Z, Zhu H, Zou Y, Yang X, Ge J. Hydrogen sulfide attenuates the recruitment of cd11b(+)gr-1(+) myeloid cells and regulates bax/bcl-2 signaling in myocardial ischemia injury. Sci Rep. 2014;4:4774.
11. Wu T, Li H, Wu B, Zhang L, Wu SW, Wang JN, Zhang YE. Hydrogen sulfide reduces recruitment of cd11b+gr-1+ cells in mice with myocardial infarction. Cell Transplant. 2017;26(5):753–764.
12. Zhang Y, Wang J, Li H, Yuan L, Wang L, Wu B, Ge J. Hydrogen sulfide suppresses transforming growth factor-beta1-induced differentiation of human cardiac fibroblasts into myofibroblasts. Sci China Life Sci. 2015;58(11):1126–1134.
13. Polhemus D, Kondo K, Bhushan S, Bir SC, Kevil CG, Murohara T, Lefer DJ, Calvert JW. Hydrogen sulfide attenuates cardiac dysfunction after heart failure via induction of angiogenesis. Circ Heart Fail. 2013;6(5):1077–1086.
14. Anderson KJ, Russell AP, Foletta VC. Ndrg2 promotes myoblast proliferation and caspase 3/7 activities during differentiation, and attenuates hydrogen peroxide – but not palmitate-induced toxicity. FEBS Open Bio. 2015;7(5):668–681.
15. Zhu Y, Zhao KK, Tong Y, Zhou YL, Wang YX, Zhao PQ, Wang ZY. Exogenous NAD(+) decreases oxidative stress and protects H_2O_2-treated RPE cells against necrotic death through the up-regulation of autophagy. Sci Rep. 2016;5(6):26322.
16. Philip L, Shivakumar K. cIAP-2 protects cardiac fibroblasts from oxidative damage: an obligate regulatory role for ERK1/2 MAPK and NK-kappaB. J Mol Cell Cardiol. 2013;9(62):217–226.
17. Pattingre S, Tassa A, Qu X, Garuti R, Liang XH, Mizushima N, Packer M, Schneider MD, Levine B. Bcl-2 antiapoptotic proteins inhibit Beclin 1-dependent autophagy. Cell. 2005;122(6):927–939.
18. Kabeya Y, Mizushima N, Ueno T, Yamamoto A, Kirisako T, Noda T, Kominami E, Ohsumi Y, Yoshimori T. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 2000;19(21):5720–5728.
19. Kanamori H, Takemura G, Goto K, Maruyama R, Tsujimoto A, Ogino A, Takeyama T, Kawaguchi T, Watanabe T, Fujiwara T, Fujiwara H, Seishima M, Minatoguchi S. The role of autophagy emerging in postinfarction cardiac remodelling. Cardiovasc Res. 2011;91(2):330–339.
20. Greabu M, Totan A, Miricescu D, Radulescu R, Virlan J, Calenic B. Hydrogen sulfide, oxidative stress and periodontal diseases: a concise review. Antioxidants. 2016;5(1):1–13.
21. Sun J, Aponte AM, Menazza S, Gucek M, Steenbergen C, Murphy E. Additive cardioprotection by pharmacological postconditioning with hydrogen sulfide and nitric oxide donors in mouse heart: S-sulfhydration vs. S-nitrosylation. Cardiovasc Res. 2016;110(1):96–106.
22. Predmore BL, Lefer DJ. Hydrogen sulfide-mediated myocardial pre- and post-conditioning. Expert Rev Clin Pharmacol. 2011;4(1):83–96.
23. Ma SF, Luo Y, Ding YJ, Chen Y, Pu SX, Wu HJ, Wang ZF, Tao BB, Wang WW, Zhu YC. Hydrogen sulfide targets the cys320/cys529 motif in kv4.2 to inhibit the Ito potassium channels in cardiomyocytes and regularizes fatal arrhythmia in myocardial infarction. Antioxid Redox Signal. 2015;23(2):129–147.
24. Calvert JW, Elston M, Nicholson CK, Gundewar S, Jha S, Elrod JW, Ramachandran A, Lefer DJ. Genetic and pharmacologic hydrogen sulfide therapy attenuates ischemia-induced heart failure in mice. Circulation. 2010;122(1):11–19.
25. Calvert JW, Jha S, Gundewar S, Elrod JW, Ramachandran A, Pattillo CB, Kevil CG, Lefer DJ. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. Circ Res. 2009;105(4):365–374.
26. Zanardo RC, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. Hydrogen sulfide is an endogenous modulator of

leukocyte-mediated inflammation. FASEB J. 2006;20(12): 2118–2120.
27. Djordjevic VB, Zvezdanovic L, Cosic V. [Oxidative stress in human diseases]. Srp Arh Celok Lek. 2008;136(Suppl 2): 158–165.
28. Zinszner H, Kuroda M, Wang X, Batchvarova N, Lightfoot RT, Remotti H, Stevens JL, Ron D. CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. Genes Dev. 1998;12(7):982–995.
29. Liu T, Zhou Y, Liu YC, Wang JY, Su Q, Tang ZL, Li L. Coronary microembolization induces cardiomyocyte apoptosis through the LOX-1-dependent endoplasmic reticulum stress pathway involving JNK/P38 MAPK. Can J Cardiol. 2015; 31(10):1272–1281.
30. Ryoo HD. Long and short (timeframe) of endoplasmic reticulum stress-induced cell death. FEBS J. 2016;283(20): 3718–3722.
31. Hotchkiss RS, Strasser A, McDunn JE, Swanson PE. Cell death. N Engl J Med. 2009;361(16):1570–1583.
32. Yu Y, Sun G, Luo Y, Wang M, Chen R, Zhang J, Ai Q, Xing N, Sun X. Cardioprotective effects of notoginsenoside R1 against ischemia/reperfusion injuries by regulating oxidative stress- and endoplasmic reticulum stress- related signaling pathways. Sci Rep. 2016;2(6):21730.
33. Xu X, Pang J, Chen Y, Bucala R, Zhang Y, Ren J. Macrophage migration inhibitory factor (MIF) deficiency exacerbates aging-induced cardiac remodeling and dysfunction despite improved inflammation: role of autophagy regulation. Sci Rep. 2016;3(6):22488.
34. Klionsky DJAK, Abe A, Abedin MJ, Abeliovich H, Acevedo Arozena A, Adachi H, Adams CM. Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). Autophagy. 2016;12(1):1–222.
35. Lockshin RA, Zakeri Z. Programmed cell death and apoptosis: origins of the theory. Nat Rev Mol Cell Biol. 2001;2(7): 545–550.
36. Leist M, Jaattela M. Four deaths and a funeral: from caspases to alternative mechanisms. Nat Rev Mol Cell Biol. 2001;2(8): 589–598.
37. Szabo C, Papapetropoulos A. Hydrogen sulphide and angiogenesis: mechanisms and applications. Br J Pharmacol. 2011; 164(3):853–865.
38. Szabo C. Hydrogen sulphide and its therapeutic potential. Nat Rev Drug Discov. 2007;6(11):917–935.
39. Papapetropoulos A, Pyriochou A, Altaany Z, Yang G, Marazioti A, Zhou Z, Jeschke MG, Branski LK, Herndon DN, Wang R, Szabo C. Hydrogen sulfide is an endogenous stimulator of angiogenesis. Proc Natl Acad Sci USA. 2009;106(51): 21972–21977.
40. Cai WJ, Wang MJ, Moore PK, Jin HM, Yao T, Zhu YC. The novel proangiogenic effect of hydrogen sulfide is dependent on Akt phosphorylation. Cardiovasc Res. 2007;76(1):29–40.
41. Calvert JW, Jha S, Gundewar S, Elrod JW, Ramachandran A, Pattillo CB, Kevil CG, Lefer DJ. Hydrogen sulfide mediates cardioprotection through Nrf2 signaling. Circ Res. 2009; 105(4):365–374.
42. Fisher CD, Augustine LM, Maher JM, Nelson DM, Slitt AL, Klaassen CD, Lehman-McKeeman LD, Cherrington NJ. Induction of drug-metabolizing enzymes by garlic and allyl sulfide compounds via activation of constitutive androstane receptor and nuclear factor e2-related factor 2. Drug Metab Dispos. 2007;35(6):995–1000.