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GRP78 effectively protect hypoxia/reperfusion-induced myocardial apoptosis via promotion of the Nrf2/HO-1 signaling pathway

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Abstract

Myocardial infarction is a major cause of death worldwide. Despite our understanding of the pathophysiology of myocardial infarction and the therapeutic options for treatment have improved substantially, acute myocardial infarction remains a leading cause of morbidity and mortality. Recent findings revealed that GRP78 could protect myocardial cells against ischemia reperfusion injury-induced apoptosis, but the exact function and molecular mechanism remains unclear. In this study, we aimed to explore the effects of GRP78 on hypoxia/reperfusion (H/R)-induced cardiomyocyte injury. Intriguingly, we first observed that GRP78 overexpression significantly protected myocytes from H/R-induced apoptosis. On mechanism, our work revealed that GRP78 protected myocardial cells from hypoxia/reperfusion-induced apoptosis via the activation of the Nrf2/HO-1 signaling pathway. We observed the enhanced expression of Nrf2/HO-1 in GRP78 overexpressed H9c2 cell, while GRP78 deficiency dramatically antagonized the expression of Nrf2/HO-1. Furthermore, we found that blocked the Nrf2/HO-1 signaling by the HO-1 inhibitor zinc protoporphyrin IX (Znpp) significantly retrieved H9c2 cells apoptosis that inhibited by GRP78 overexpression. Taken together, our findings revealed a new mechanism by which GRP78 alleviated H/R-induced cardiomyocyte apoptosis in H9c2 cells via the promotion of the Nrf2/HO-1 signaling pathway.

KEYWORDS

apoptosis, GRP78, HO-1, myocardial hypoxia/reperfusion, Nrf2

1 | INTRODUCTION

Myocardial infarction, which is defined by pathology as myocardial cell death due to prolonged ischemia that results from a perfusion imbalance between supply and demand, is a major cause of death worldwide (Benjamin et al., 2017). Patients with acute myocardial infarction may present with typical ischemic-type chest discomfort or with dyspnea, nausea, unexplained weakness, or a combination of these symptoms, and ultimately leads to cordial failure or death (Benjamin et al., 2017). Despite our understanding of the

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Abbreviation: AREs, antioxidant response element; CO, carbon monoxide; H/R, hypoxia/reperfusion; HO, heme oxygenase; Nrf2, NFE2-related factor 2; ROS, reactive oxygen species; UPR, unfolded protein response.

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pathophysiology of patients with myocardial infarction and the therapeutic options for treatment have improved substantially, acute myocardial infarction remains a leading cause of morbidity and mortality worldwide.

Among multiple pathological features, myocardial cell death is one of the most important types of damage resulting from myocardial infarction (Altamirano, Wang, & Hill, 2015; Turer & Hill, 2010; Yellon & Hausenloy, 2007). Perfusion imbalance could cause prolonged ischemia that in turn lead to myocardial cell death. Partial or complete occlusion of a coronary artery deprives oxygen and nutrients of the downstream tissues and recovery of blood supply can interrupt the hypoxia and ischemic insult, while restoration of oxygen delivery triggers a second wave of insult, termed reperfusion injury (Murphy & Steenbergen, 2008; Turer & Hill, 2010; Yellon & Hausenloy, 2007). Plenty of events, including accumulation of reactive oxygen species (ROS), inflammation, disruption of calcium handling, and metabolic derangements (Altamirano et al., 2015; Bravo et al., 2013; Hausenloy et al., 2016; Hausenloy & Yellon, 2016; Wang & Hill, 2015), are participated in the pathogenesis of hypoxia/reperfusion (H/R). For example, the accumulation of ROS can induce the unfolded protein response (UPR), a cellular mechanism evolved to achieve protein-folding stress (Austin, 2009; Doroudgar & Glembotski, 2013; Walter & Ron, 2011). And growing evidence have indicated that UPR in cardiomyocytes is activated by ischemic or hypoxia/reperfusion injury (Bi et al., 2018). GRP78, a master regulator of the UPR and Xbp1s, the most highly conserved branch of the UPR and protective in response to cardiac H/R injury, is upregulated after H/R (Bi et al., 2018). Recently, reports demonstrated that ischemia/reperfusion (I/R) induction of GRP78 stimulated Akt signaling pathway and protected against oxidative stress, which could protect cells from I/R damage (Bi et al., 2018), in cardiomyocytes. However, the exact functions and molecular mechanisms underlying the GRP78 on regulating myocardial I/R injury remains unclear.

One of the most important cellular defense mechanisms against the deleterious effects of environmental insults is mediated by the transcription factor nuclear factor, erythroid 2 like 2 (Nrf2). Nrf2 is a member of a small family of basic leucine zipper (bZIP) proteins that contains seven conserved Nrf2-ECH homology (Neh) domains enables Nrf2 to binding to the ARE sequence and then regulates a number of cytoprotective genes expression (Itoh, Tong, & Yamamoto, 2004), many of which were involved in response to injury and inflammation. It is noteworthy that, in addition to against oxidative stress or electrophiles, the Nrf2 signaling pathway is associated with the protective function that against I/R-induced myocardial injury (Duan et al., 2017). Studies demonstrated that upregulation of Nrf2 in astrocytes suppressed apoptosis (Xia et al., 2017), and Nrf2 can enter the nucleus to regulate genes expression that playing a vital role in oxidative stress and H/R-induced myocardial apoptosis (Itoh et al., 2004; Padmanabhan et al., 2006; Satoh et al., 2006; Talalay, 2000; Wang, Hayes, Higgins, Wolf, & Dinkova-Kostova, 2010). One important target gene of Nrf2 is heme oxygenase (HO). HO is a rate-limiting and microsomal enzyme that catalyzed the oxidative degradation of free heme to biliverdin, free

iron, and carbon monoxide (CO; Yang et al., 2016). There are three distinct isoforms of heme oxygenase: HO-1, HO-2, and HO-3. HO-1 is ubiquitously distributed and strongly induced by oxidative, nitrosative, osmotic, and hemodynamic stresses (Yang et al., 2016). Under oxidative stress, Nrf2 induces HO-1 expression and HO1 protects cells from oxidative stress through heme catabolites.

In the present study, we examined the function of GRP78 on protecting myocardial cells from H/R-induced injury. Our work revealed that GRP78 overexpression can disrupt the H/R-induced Bcl-2/Bax/caspase3 apoptosis signaling pathway. On mechanism, we found that the protective function of GRP78 is mediated by the Nrf2/HO-1 signaling pathway regulating. Our work may provide a deep insight into the myocardial infarction.

2 | METHODS

2.1 | Cell culture

H9c2 rat embryonic primary myocardial cells were purchased from the American Type Culture Collection (Rockville, MD) and cultured in Dulbecco's modified Eagle's medium (DMEM) high-glucose medium (Hyclone, Logan, UT) containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humidified incubator and a 5% CO_2 atmosphere. The culture medium was replaced every 2 days. Cells of passages 3–4 were used in this study.

2.2 | Adenovirus production and infection

Overexpression of GRP78 was performed by using adenovirus infection (Bi et al., 2018), and ad-NON (scramble) was used as a control. To engineer GRP78 expressing adenovirus, a flag tag was inserted after the signal sequence (AA 1-19) in wild type mouse GRP78. The cDNA was then cloned into a shuttle and used for adenovirus production (Bi et al., 2018). Adenovirus was produced in HEK-293A cells and concentrated by ultracentrifugation.

Adenovirus infection was established by exposing H9c2 cells to the adenovirus for 6 hr multiplicity of infection (MOI, 10–50), and following by restoration of standard culture medium. We routinely obtained an almost 70% infection rate. Then, cells were cultured and subjected to H/R treatment, as described below.

2.3 | Treatment

H9c2 cells were divided into five groups: control group, H/R 36 hr group, ad-NON + H/R 36 hr group, ad-GRP78 + H/R 36 hr group, ad-GRP78 + H/R 36 hr+ Znpp. Zinc Protoporphyrin IX (Znpp, an HO-1 inhibitor) was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in 0.2 M NaOH (PH 7.4), and subsequently diluted to 3 mM before use (Zhao et al., 2016). To induce H/R injury, the culture

medium was exchanged to DMEM without glucose and incubated in an atmosphere of 93% N2, 2% O$_2$, and 5% CO$_2$.

2.4 | Cell viability assay

Cell viability was evaluated using cell counting kit-8 (CCK-8) (Dojindo, Mashiki-machi, Japan), according to manufacturer's instruction. In brief, control and GRP78 overexpressing H9c2 cells were plated in 96-well plates and treated H/R. To determine the cell viability, the absorbance was measured at 450 nm using a microplate reader (MQX 200, BioTek Instruments, Winooski, VT).

2.5 | Flow cytometry assay of apoptosis

H9c2 cells were dissociated by trypsinization, centrifuged at 2,500g for 5 min, washed, and resuspended at approximately 1×10^6 cells/ml in phosphate buffered saline (PBS). For flow cytometry analysis, the cells were fixed in 75% ice-cold ethanol overnight at 4°C, digested with 50 ug/ml RNase, and stained with 50 mg/ml propidium iodide (PI) for 15 min at room temperature. Cellular apoptosis was then assayed by flow cytometry (FACS-Calibur; BD Biosciences, Franklin Lakes, NJ) using an Annexin V-FITC apoptosis detection kit (Beyotime Biotechnology, China). The cells were stained with PI and Annexin V-FITC for 10 min at room temperature in the dark. Fluorescence was read at emission wavelengths of 530 and 585 nm and cell apoptosis were analyzed by a FACS Calibur flow cytometer (BD, San Jose, CA).

2.6 | Immunofluorescence, 5-ethynyl-2′-deoxyuridine staining, and terminal deoxynucleotidyl transferase dUTP nick end labeling assay

Immunofluorescence was implemented in the 4% paraformaldehyde-fixed H9c2 cells. And then cells were incubated with anti-GRP78 antibody (1:400; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-Nrf2 antibody (1:400; Santa Cruz Biotechnology) overnight at 4°C. After washed five times with PBS for 1 hr the next day, H9c2 cells were incubated with the secondary antibodies (AlexaFluor488 and AlexaFluor594, 1:200) for 2 hr at room temperature. Then the cells were washed with PBS again for five times for 1 hr before being stained by 4′,6-diamidino-2-phenylindole for 2 min. After three further washes, the dishes were observed with a fluorescence microscope. Negative controls were prepared by omitting the primary antibodies. For EdU (5-ethynyl-2'-deoxyuridine) assay, H9c2 cells were seeded in 96-well plates and treated with EdU 6 hr. Subsequently EdU incorporated cells were subjected to staining with a commercial EdU staining kit (#C10310-2; RiboBio, Guangzhou, China).

The apoptosis profile of the H9c2 cells was evaluated using a commercially available situ cell death detection kit (Roche Diagnostics) to test the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL).

2.7 | Western blot

The total protein of H9c2 cells was extracted using radio-immunoprecipitation assay lysis buffer containing phenylmethylsulfonyl fluoride (PVDF) and protease inhibitor cocktail (Beyotime, China). After determination of the total protein concentration by bicinchoninic acid assay, equal amounts of protein lysate (20 g in each lane) were loaded and separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels and transferred onto PVDF membranes (Millipore, Madison, WI). The membranes were blocked for 2 hr with 5% skim milk in TBS containing 1‰ Tween-20, then incubated with primary antibodies against GRP78, Nrf2, HO-1, Bcl-2, Bax, caspase-3, cleaved caspase-3, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; all antibodies described here purchased from Santa Cruz Biotechnology, Santa Cruz) at 4°C overnight. The membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1.5 hr at room temperature the next day. The bands were visualized with ECL detection reagents (Thermo Fisher Scientific) and the density of the bands was read by Quantity One software (Bio-Rad, CA).

2.8 | RNA extraction and real-time quantitative polymerase chain reaction

Total RNA was extracted from cultured cells by Trizol reagent according to the manufacturer's instruction (Takara, Dalian, China). RNA was quantified using NanoDrop Spectrophotometer and equal amount RNAs were subjected to reverse transcription for complementary DNA (cDNAs) with a Takara PrimeScript RT-PCR Kit (Takara, Dalian, China). Real-time polymerase chain reaction (PCR) was done using the TB Green Fast qPCR Mix (Takara), and the reference gene GAPDH was used as the internal control. The primers used in this study were listed as follows:

> GRP78 forward, 5′-CATCACGCCGTCCTATGTCG-3′;
> GRP78 reverse: 5′-CGTCAAAGACCGTGTTCTCG-3′;
> NRF2 forward: 5′-TCAGCGACGGAAAGAGTATGA-3′;
> NRF2 reverse: 5′-CCACTGGTTTCTGACTGGATGT-3′;
> HO-1 forward: 5′-AAGACTGCGTTCCTGCTCAAC-3′;
> HO-1 reverse: 5′-AAAGCCCTACAGCAACTGTCG-3′;
> GAPDH forward: 5′-CTGGGCTACACTGAGCACC-3′;
> GAPDH reverse: 5′-AAGTGGTCGTTGAGGGCAATG-3′.

2.9 | Statistical analysis

Continuous variables were expressed as mean ± standard error. One-way analysis of variance was performed for multiple comparisons using GraphPad Prism software, version 5.0 (GraphPad, La Jolla, CA). $p \leq .05$ indicated a statistically significant difference.

3 | RESULTS

3.1 | Adenovirus vector-mediated GRP78 overexpressing in H9c2 cells

To explore the exact function of GRP78 on protecting myocardial cells from H/R induced injury, we first constructed a GRP78 over-expressing cell line by adenovirus infection. GRP78 overexpressing adenovirus vector was constructed by a cloned flag-tagged wild type mouse GRP78 cDNA into the shuttle (Bi et al., 2018). Adenovirus was produced in HEK-293A cells and concentrated by ultracentrifugation. Then H9c2 cells were exposed to the adenovirus for 6 hr (MOI 10-50) for adenovirus infection, and following by restoration of standard culture medium (Watson et al., 2003). We routinely obtained an almost 70% infection rate. As the data are shown in Figure 1a–d, adenovirus infection successfully overexpressed GRP78 in H9c2 cells.

3.2 | GRP78 overexpression significantly protected myocytes from hypoxia-induced apoptosis

It was reported that, in cultured neonatal rat ventricular myocytes, simulated I/R injury can induce apoptotic cell death and strong activation of the UPR and GRP78 (Bi et al., 2018). To verify whether H/R injury can also stimulate GRP78 expression, we first grouping the cells into control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr four groups, and examined the GRP78 expression by immunoblot and qRT-PCR. However, it was different from I/R injury in rat ventricular myocytes, the GRP78 expression was not induced in H9c2 cells after H/R treatment, as shown in Figure 1b–d.

To further evaluate whether GRP78 involved in protecting cardiomyocyte from H/R-induced injury, corresponding cells were subjected to cell viability analysis by CCK-8 assay. As shown in Figure 2a, significantly decreased cell viability of cardiomyocytes was observed in H9c2 cells 36 hr after H/R treatment, which was restored in GRP78 over-expressing group, but H/R treatment and GRP78 had no effect on H9c2 cells proliferation, which was measured by EdU staining (Figure 2b,c). Next, we determined the effects of H/R treatment and GRP78 on H9c2 cells apoptosis. We observed a significantly increased cell apoptosis of cardiomyocytes 36 hr after H/R treatment, which was reduced in GRP78 overexpressing group (Figure 2d,e). Furthermore, we also examined the expression of apoptosis-related markers cleaved-Caspase3, Bax, and Bcl-2 in corresponding cells by immunostaining. The results revealed that compared with the control group, the expression of Bax and cleaved-caspase3 was notably increased, whereas Bcl-2 expression was decreased in H/R 36 hr group (Figure 2f–h); while ad-GRP78 transfected group markedly reduced the expression of Bax and cleaved-caspase3, but increased Bcl-2 expression to normal level (Figure 2f–h), indicated that GRP78 indeed protected d myocardial cell from H/R induced apoptosis.
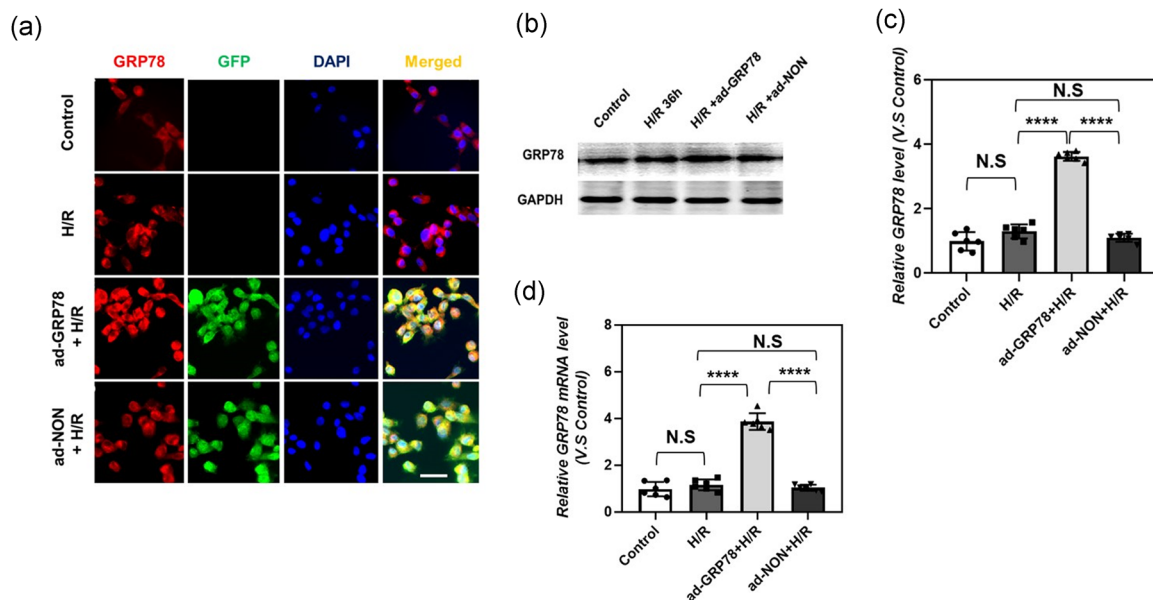

FIGURE 1 Adenovirus infection mediated GRP78 overexpressing in H9c2 cells. (a) Typical photomicrographs illustrated immunoflourescence staining in H9c2 cells in control, H/R 36 hr, ad-GRP78 + H/R 36 hr and ad-NON + H/R 36 groups. GRP78, GFP, and DAPI were used as markers for GRP78 protein expression, adenovirus expression, and nuclei, respectively. (b) Western blot showed the protein level of GRP78 in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells. (c) Quantitative analysis of GRP78 protein expression related to Figure 1b. (d) Quantitative analysis of GRP78 mRNA expression by qRT-PCR in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells. DAPI, 4′,6-diamidino-2-phenylindole; GFP, green fluorescence protein; mRNA, messenger RNA; qRT-PCR, quantitative real-time polymerase chain reaction
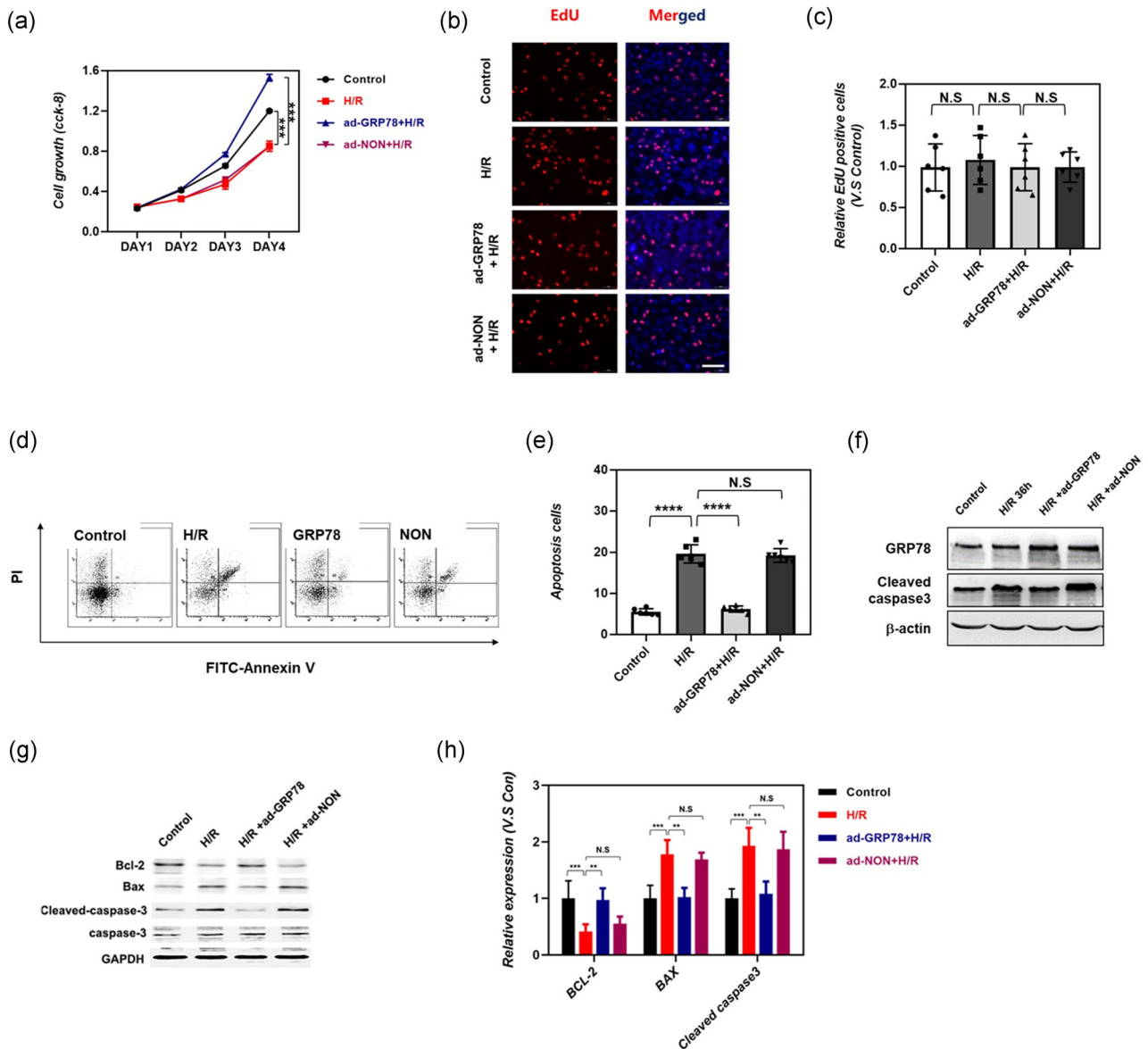
FIGURE 2 Overexpression of GRP78 in H9c2 significantly protected myocytes from hypoxia-induced apoptosis. (a) Cell growth in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells measured by CCK-8. (b) Representative EdU staining in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells was shown. (c) Quantitative analysis of EdU positive cells related to Figure 2b. (d) Apoptosis of H9c2 cells assessed by flow cytometry to detect FITC-Annexin V staining. (e) The relative apoptosis ratio was calculated with Annexin V-positive apoptotic cells. $N = 5$ per group, Data are mean ± SEM, ****$p < .0001$, N.S represent no significance; (f) Western blot showed caspase3 cleavage in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells. (g) Western blot showed caspase3 cleavage, BCL-2, and BAX expression in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells. (h) Quantitative analysis of caspase3 cleavage, BCL-2, and BAX expression in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells related to Figure 2g. CCK-8, cell counting kit 8; EdU, 5-ethynyl-2'-deoxyuridine; SEM standard error of mean

3.3 | GRP78 overexpression enhanced the Nrf2/HO-1 signaling pathway in H/R injured myocardial cells

Nrf2/HO-1 signaling is an important regulator for cellular defense against the deleterious effects of environmental insults including oxidative stress, and GRP78 knockdown enhances apoptosis via the downregulation of oxidative stress (Chang, Huang, Li, & Chen, 2012). Therefore, we

reasonably to suspect that the function of GRP78 on against H/R induced injury may be mediated by regulating Nrf2/HO-1 signaling. To verify this possibility, we determined the effect of GRP78 on Nrf2/HO-1 signaling, and downregulated of Nrf2 and HO-1 was observed in H/R treatment group, but dramatically increased by GRP78 overexpression on both mRNA and protein level (Figure 3a–c), and according to the immuno-fluorescence staining, enhanced expression of Nrf2 showed the exactly colocalized with ad-GRP78 expression (Figure 3d), suggested that GRP78
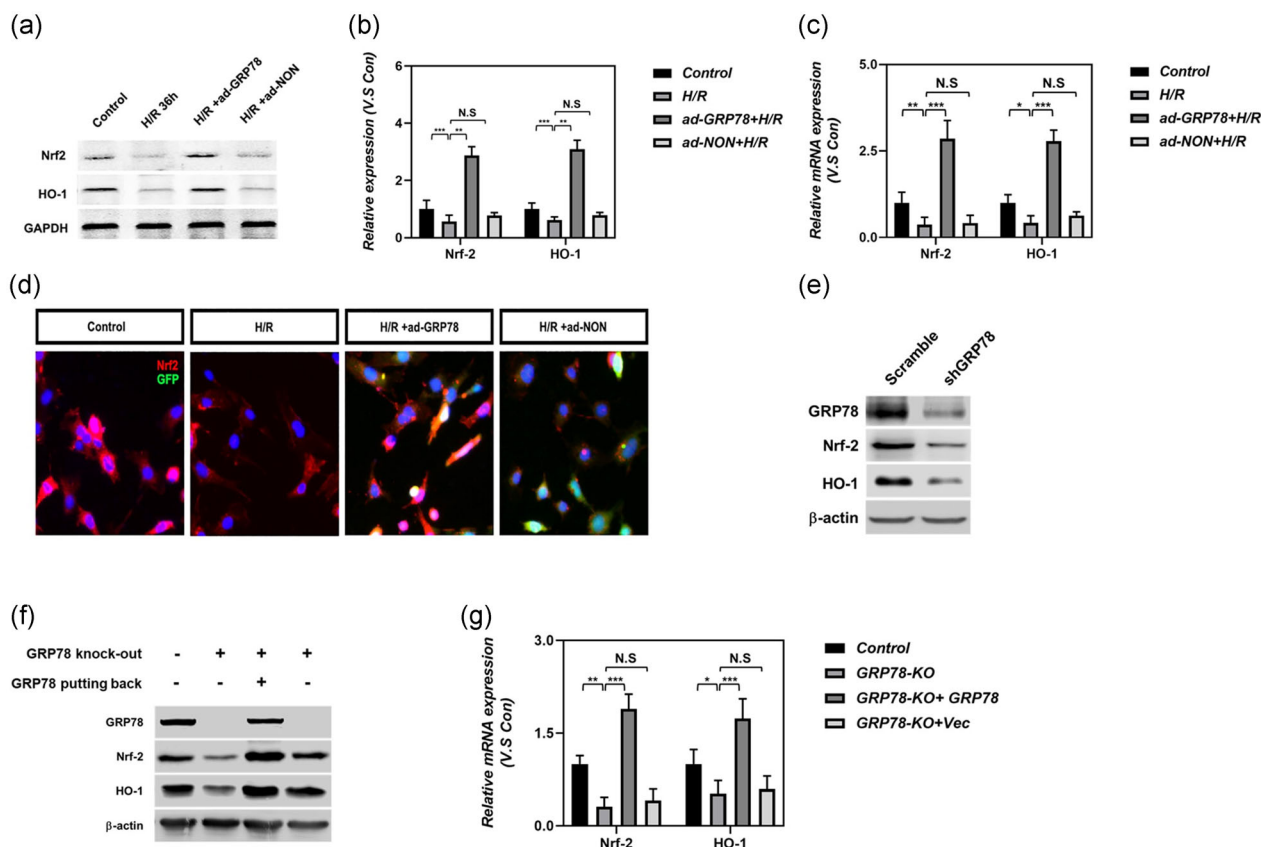
FIGURE 3 GRP78 overexpression activates the Nrf2/HO-1 signaling pathway. (a) Representative immunoblot of NRF2 and HO-1 expression is shown in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr four groups and GAPDH was used as a loading control. (b) Bar graph shows the analysis of Nrf2 and HO-1 protein level in the four groups, and Nrf2/HO-1 were augmented by GRP78 overexpression after H/R 36 hr as an inducer. (c) Quantitative analysis of mRNA expression of NRF2 and HO-1 in control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells. (d) A representative of immunofluorescence with NRF2, GFP, DAPI in the control, H/R 36, ad-GRP78 + H/R 36, and ad-NON + H/R 36 hr cells. (e) Western blot showed the GRP78, NFR2, and HO-1 expression in control and GRP78 knockdown cells. (f) Western blot showed the GRP78, NFR2, and HO-1 expression in control, GRP78 knockout, and GRP78 knockout with GRP78 putting back cells, respectively. (g) Quantitative analysis of mRNA expression of NRF2 and HO-1 in control, GRP78 knockout, and GRP78 knockout with GRP78 putting back cells, respectively. DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; SEM standard error of mean

upregulated Nrf2 and HO-1 expression. Consistently, we knockdown and knockout GRP78 expression in H9c2 cells and reduced Nrf2 and HO-1 expression was detected in GRP78 deficient cells, while GRP78 putting back significantly restored the expression of Nrf2 and HO-1 respectively (Figure 3e–g). These results indicated that GRP78 overexpression could activate the Nrf2/HO-1 signaling pathway.

3.4 | GRP78 overexpression protected myocardial cells from H/R induced apoptosis through the Nrf2/HO-1 signaling pathway

To further evaluate the function of GRP78 on against H/R induced injury was indeed mediated by regulating Nrf2/HO-1, the HO-1 inhibitor Zinc Protoporphyrin IX (Znpp, 3 mM) were used in these experiments. Immunoblot and qRT-PCR showed that Znpp treatment certainly decreased the expression of HO-1 (Figure 4a,b). Subsequently, we determined the effect of Znpp treatment on H9c2

apoptosis after H/R injury. As expected, the apoptosis ratio was dramatically increased in the Znpp treatment group after H/R injury (Figure 4c,d). Similar to the results from TENEL assay and flow cytometry analysis, significantly upregulation of Bax and cleaved-caspase3 and downregulated Bcl-2 expression was detected in Znpp-treated group, compared with ad-GRP + H/R 36 hr group (Figure 4e), indicated that Znpp indeed induced apoptosis in GRP78 over-expressing H9c2 cells 36 hr after H/R treatment, and Nrf2/HO-1 is a downstream signaling pathway of GRP78 in regulating apoptosis. Taken together, our results suggested that the Nrf2/HO-1 signaling pathway mediated the protective function of GRP78 overexpression on against hypoxia-induced H9c2 apoptosis.

4 | DISCUSSION

GRP78 also referred to as BiP, is a central regulator of endoplasmic reticulum (ER) function due to its roles in protein folding and
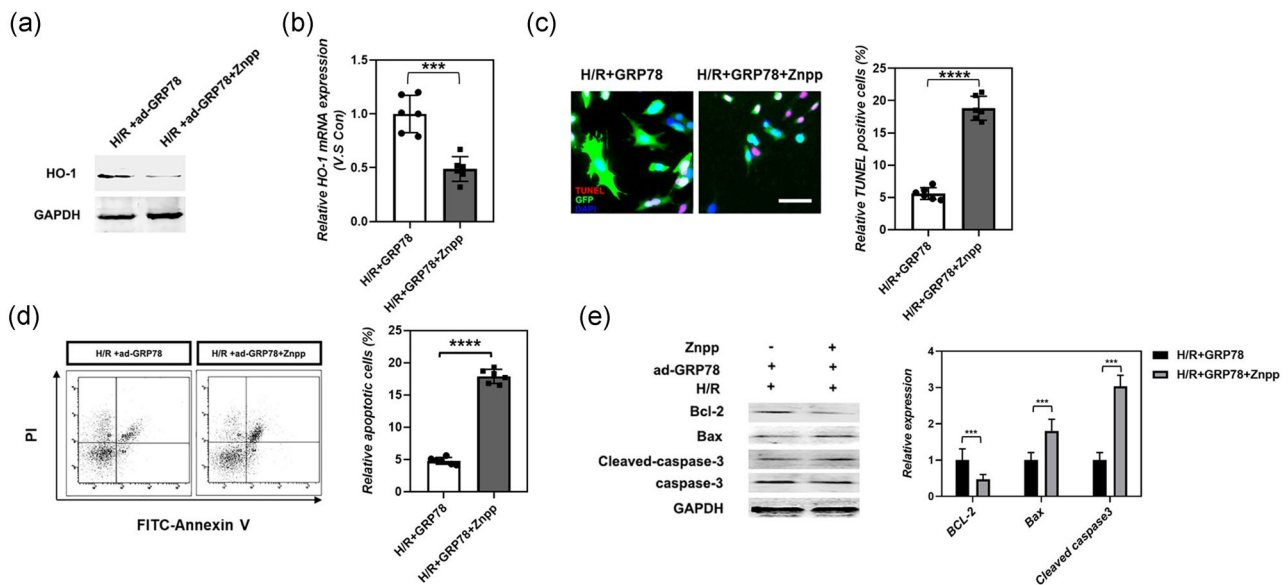
FIGURE 4 Nrf2/HO-1 mediated the effect of GRP78 on the apoptotic pathway. (a) A representative immunoblot of HO-1 in ad-GRP78 + H/R 36 and ad-GRP78 + H/R 36 hr + Znpp two groups and GAPDH was used as a loading control. (b) The bar graph showed the analysis of HO-1 mRNA level in the ad-GRP78 + H/R 36 hr and ad-GRP78 + H/R 36 hr + Znpp cells. (c) A representative of immunofluorescence with TUNEL, GFP, DAPI in the ad-GRP78 + H/R 36 hr and ad-GRP78 + H/R 36 hr+ Znpp cells. (d) Apoptosis of ad-GRP78 + H/R 36 hr and ad-GRP78 + H/R 36 hr + Znpp cells was assessed by flow cytometry to detect annexin V-FITC staining, and relative apoptosis ratio was calculated with Annexin V-positive apoptotic cells in this two groups. (e) Representative immunoblot of apoptotic proteins is showed in the two groups and GAPDH was used as a loading control, and bar graph showed western blot analysis for the level of Bax, Bcl-2, and cleaved caspase 3, $N = 5$ per group, Data are mean ± SD, ****$p < .0001$. DAPI, 4′,6-diamidino-2-phenylindole; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GFP, green fluorescence protein; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; SD, standard deviation

assembly and in regulating UPR and Xbp1s, targeting misfolded protein for degradation, controlling the activation of ER stress sensors (Li & Lee, 2006). Multiple evidence shows that stress induction of GRP78 represents an important prosurvival component of the UPR. In cardiac H/R injury, plenty of events, including accumulation of ROS, inflammation, disruption of calcium handling, and metabolic derangements (Altamirano et al., 2015; Bravo et al., 2013; Hausenloy & Yellon, 2016; Hausenloy et al., 2016; Wang & Hill, 2015), are participated in the pathogenesis of H/R and can induce UPR, which is a crucial cause for myocardial cell death (Austin, 2009; Doroudgar & Glembotski, 2013; Walter & Ron, 2011). In addition, reports demonstrated that I/R induction of GRP78 stimulated the Akt signaling pathway and protected against oxidative stress, which could protect cells from I/R damage (Bi et al., 2018). In the present study, we also demonstrated that GRP78 overexpression significantly inhibited hypoxia-induced apoptosis in cultured H9c2 cells. The findings reasonably lead us to suspect that gene therapy based on GRP78 is a very promising method in myocardial infarction treatment.

Recently, Bi et al. (2018) reported that simulated I/R led to robust induction of GRP78 at both mRNA and protein levels, consistent with previous finding (Doroudgar, Thuerauf, Marcinko, Belmont, & Glembotski, 2009; Martindale et al., 2006; Thuerauf et al., 2006). Unlike these reports, the protein levels of GRP78 were not induced by H/R injury in our case, but we demonstrated GRP78 indeed has a protective function in H/R injury. We did not

detect the enhanced expression of GRP78 in H9c2 cells after H/R may due to the model we used with a longer reperfusion time (36 hr) that the robust induction of GRP78 expression cannot be detected significantly, or the difference in the treatment method we used.

On molecular mechanism, we found GRP78 exerts the protective function on against H/R injury through upregulating the Nrf2/HO-1 signaling pathway. We demonstrated that GRP78 overexpression dramatically increased the Nrf2 and HO-1 expression in H9c2 cells after H/R treatment, and the HO-1 inhibitor could significantly block the protective function of GRP78. We suspected that the Nrf2/HO-1 signaling pathway is downstream of GRP78 in the situation that H/R induced myocardial cell apoptosis. Unfortunately, we did not figure out how GRP78 regulates the Nrf2/HO-1 signaling pathway. Literature has reported that GRP78 overexpression suppressed I/R-induced cardiomyocyte injury via the inhibition of ER stress. GRP78 is a key protein of ER stress that detaches from three ER stress sensors: inositol requiring enzyme-1α (IRE1α), double-stranded RNA-dependent protein kinase-like ER kinase (PERK) and activating transcription factor 6 (ATF6), under stress conditions, and resulting in the activation of these sensors and subsequently triggering the UPR signal (Bukau, Weissman, & Horwich, 2006; Pham, Seong, Ngabire, Oh, & Kim, 2017). GRP78 essentially interacts with these three transmembrane molecules and retains them on the ER membrane surface, however, when misfolded proteins accumulate, GRP78 preferentially interacts with hydrophobic patches on the

ER-resident misfolded proteins, thereby releasing the three signaling arms of the UPR and leading to ER-associated protein degradation. GRP78 expression level is closely associated with the intensity of ER stress (Pham et al., 2017). And ER stress can produce reactive oxygen species (ROS) accumulation and lipid peroxidation of the ER membrane, and Nrf2/HO-1 is a crucial sensor and regulator of ROS. The correlation of GRP78, ER-stress, ROS, and Nrf2/HO-1 signaling may be a compelling field we would explore in the future.

5 | CONCLUSION

In the present study, we revealed a GRP78-Nrf2/HO-1 signaling pathway that is crucial for protecting myocardial cells from H/R induced apoptosis. We found that GRP78 overexpression inhibits the apoptosis induced by H/R treatment, and on the mechanism, which is dependent upon upregulated Nrf2/HO-1 expression. In this study we provide a novel mechanism insight into the protective role of GRP78 in myocardial H/R injury and modulation of GRP78 signaling represents a novel target in myocardial H/R injury and one with potential therapeutic relevance.

CONFLICT OF INTERESTS
The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS
F. Y. and J. X. designed the study. H. J., S. L., and F. X. performed the experiments and collected the data. F. Y. and Y. L. analyzed and interpreted the experimental data. F. Y. and J. X. prepared the manuscript.

DATA AVAILABILITY STATEMENT
All data generated or analyzed in this study are included in this published article.

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