Nogo-A Mediated Endoplasmic Reticulum Stress During Myocardial Ischemic-Reperfusion Injury in Diabetic Rats

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Received: 1 July 2022 / Accepted: 2 March 2023 / Published online: 25 March 2023
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Abstract
Among the three isoforms encoded by neurite outgrowth inhibitor proteins has been intensely investigated as a central nervous system inhibitor. Although neurite outgrowth inhibitor protein-A (Nogo-A) expression is increased in plasma of patients who have experienced a coronary heart disease, its role in heart disease is not well elucidated. In this study, we discovered a significant increase in Nogo-A expression in diabetic myocardial ischemia reperfusion (MI/R) injury conditions. Accelerated Nogo-A and MI/R injury in diabetic rats was attenuated by tauroursodeoxycholic acid treatment and knockdown of Nogo-A per se is sufficient to decrease endoplasmic reticulum (ER) stress as well as prevents cardiomyocyte apoptosis. We hypothesized that decreased Nogo-A levels might reducing diabetic MI/R injury. Nogo-A interacted with C/EBP homologous protein, suggesting a role for Nogo-A in ER stress during diabetic MI/R. In conclusion, Nogo-A mediated ER stress plays a major role in diabetic MI/R injury, and pathologically altered Nogo-A expression mediates diabetic MI/R injury, suggesting Nogo-A as a novel target for the treatment of diabetic MI/R injury in clinical settings.

Keywords Myocardial ischemia/reperfusion injury · Diabetes · Endoplasmic reticulum stress

Introduction
Diabetes mellitus (DM) is one of the leading causes of morbidity and mortality in afflicted individuals. The International Diabetes Federation data have shown that there are more than 463 million people with DM worldwide, and it could rise to over 700 million or more by 2045 [1]. Acute myocardial infarction (AMI) is the result of occlusion of coronary artery, which obstructs the blood flow to the myocardium, a clinical scenario widely known as ischemia. Although, timely and adequately re-establishing blood supply after myocardial ischemia, known as myocardial ischemia–reperfusion (MI/R) of the coronary artery, is mandatory to salvage or prevent the demise of ischemic myocardium, reperfusion itself causes additional ischemic damage. DM is the major risk factor for developing cardiovascular diseases, including AMI, which is associated with poor prognosis and eventually higher mortality [2].

The endoplasmic reticulum (ER) stress is a complex membranous network found in all cells where it plays an important role in calcium homeostasis, proteins folding, and lipid biosynthesis [3–5]. A wide variety of stressors, including oxidative stress and ischemia, disrupt ER function, which leads to protein misfolding and unfolded protein response (UPR) [6, 7]. Two of the most inducible ER proteins are glucose-regulated protein 94 (GRP94, gp96 or HSP90B1) and GRP78 (immunoglobulin binding protein or BIP), which are hallmarks of both pathological and physiological UPR [8]. Protein disulfide isomerase family 6 (PDIA6) is mainly localized to the ER and functions as an oxidoreductase to catalyze disulfide bond formation and as a chaperone to assist in protein folding and inhibit aggregation of unfolded substrates [9]. Cumulating evidence suggests that C/EBP homologous protein (CHOP) is involved in the pathogenesis of diabetes, in response to glucotoxicity, lipotoxicity, as well as oxidative stress and islet amyloid derived from IAPP [10]. Diabetic CHOP knockout mice seemed to be protected from MI/R, CHOP has been implicated in exaggerated reactive oxygen species (ROS) production by
upregulation of the UPR-regulated oxidative protein folding machinery in the ER, which is directly contributing to ROS generation through the oxidation of disulfide bonds [11]. However, the mechanism of CHOP mediated ER stress in diabetic MI/R injury remains largely unknown.

Neurite outgrowth inhibitor proteins (Nogo) belong to reticulon protein family, which is characterized by the ER targeting motif at the carboxy terminal [12]. Nogo gene encodes three splicing isoforms, Nogo-A, Nogo-B and Nogo-C, they lack an N-terminal signal sequence and are predominantly localized to the ER [13]. Nogo-A is the longest protein in Nogo family, and expressed in many tissues, including heart [14]. Nogo-A is well characterized as a potent inhibitor of axonal regeneration and plasticity in the central nervous system, however, the role of Nogo-A in non-nervous tissues is essentially unknown. A previous study has shown that Nogo-A expression was shown to be significantly increased in plasma from patients who have experienced a coronary heart disease is associated with increase ROS levels and promote apoptosis [15]. However, Nogo-A is expressed in cardiomyocytes whether related to ER stress and its precise functions in diabetic ischemia cardiomyopathy remain poorly understood. Accordingly, the aims of the present study were to identify the role of Nogo-A during MI/R in diabetic hearts and examine a key role of Nogo-A in mediated ER stress.

Materials and Methods

Animals Model

Adult SD rats (2-month-old, 200–220 g) were obtained from the Laboratory Animal Services Centre of Wuhan University. During the study, the animals were housed in an animal room (temperature: 20 ± 2 °C, humidity: 60% ± 5%, 12-h light/dark cycle). With free access to standard rat chow and tap water. All institutional and national guidelines for the care and use of laboratory animals were followed and approved by the appropriate institutional committees. All the rats were randomly equally divided into six groups: normal + sham group (NS), normal + I/R group (NI/R), DM + sham (DS) group, DM + I/R group (DI/R), DM + sham + TUDCA (DS + TUDCA), and DM + I/R + TUDCA group (DI/R + TUDCA).

The model of MI/R was established as previously described [16]. Briefly, the animals were anesthetized with pentobarbital sodium (50 mg/kg), placed face up and fixed on the operating table, and connected to electrocardiogram wires. After tracheal intubation and connection to a small animal respirator (tidal volume of 8–12 mL, 1:2 breathing, respiratory rate 70–80 times/min), the chest was opened between the left third and fourth rib exposing the heart. A 6–0 nylon suture was placed around the left anterior descending coronary artery. All groups were made ischemic for 45 min by ligating the artery, and this was followed by reperfusion for 3 h by loosening the ligature. Arrhythmias were monitored during ischemia–reperfusion by electrocardiogram. ST-segment elevation and widening of R wave indicated ischemia. A 50% drop-off in ST-segment elevation was indicative of successful reperfusion.

The induction of diabetes followed the previously published steps [17]. Diabetic rat model was established by intraperitoneal injections of streptozotocin (STZ, Sigma, USA) 60 mg/kg dissolved in a citrate buffer (0.1 mol/L, pH 4.5), whereas normal control rats were injected with the same volume of citrate buffer. Three days after the STZ administration, blood samples were collected from the tail vein and the rats with fasting blood glucose continued to be higher than or equal to 16.7 mmol/L, the diabetes model was successfully prepared. To clarify whether in diabetic hearts Nogo-A upregulation was secondary to overactivation of ER stress, rats received an intraperitoneally injection of taurocholate acid (TUDCA, Sigma, USA) (100 mg/kg) for consecutive 7 days before surgery. During the experiment, the dosage was adjusted according to the body weight of the rats, the indicators were monitored regularly.

Cell Culture, High Glucose (HG) Procedure and Hypoxia-Reoxygenation (HGH/R) Injury

The H9c2 cell lines were cultured in DMEM medium containing 10% fetal bovine serum in a humidified incubator with 5% CO₂ in air at 37 °C and then serum deprived for 16 h to achieve synchronization. H9c2 cells were transfected with the short hairpin interfering RNA against Nogo-A (shNogo-A, 50 nmol/L) or CHOP (shCHOP, 50 nmol/L) using Entranstar™-R4000 for 6 h to knock down endogenous Nogo-A and CHOP or a scramble RNA (shRNA) as the negative control. Six hours after shRNA treatment, cells were kept in normal medium for additional 12 h to allow gene manipulation. Next, H9c2 cells were cultured with ER stress inducer Tunicamycin (TM, Sigma, USA), 10 μmol/L, which was added 6 h before building H/R models. The model of high glucose (HG) and hypoxia-reoxygenation (HGH/R) was established as previously described [18]. Briefly, H9c2 cells were subjected 50% glucose (30 mM) for the HG procedure. Culture dishes were placed inside humidified incubator with 5% CO₂ in air at 37 °C for 24 h, unless noted otherwise. Following, the hypoxia reoxygenation (H/R) procedure was performed. For hypoxia exposure, cells were maintained under anoxic conditions in chambers gassed with a mixture of 94% N₂, 5% CO₂, and 1% O₂ at 37 °C for 4 h. For reoxygenation, plates were removed from the anoxic chamber to a normoxic chamber for 2 h. All the cells were randomly equally divided into ten groups: low glucose...
5 mM (LG) + normal group (LG/N), LG + hypoxia-reoxygenation (H/R) group (LGH/R), high glucose (HG) + normal group (HG/N), HG + H/R group (HGH/R), scramble group, shNogo-A group, shCHOP group, TM + scramble group, TM + shNogo-A group, TM + shCHOP group.

**Lactic Dehydrogenase (LDH), Creatine Kinase-MB (CK-MB) Activities**

Levels of cardiovascular biomarkers including LDH (Jilin, China), CK-MB (Jilin, China) were detected using a commercially available kit. Briefly, H9c2 cells were seeded in 96-well plates. Following treatment, centrifuge the cell culture plate at 1000 x g for 5 min, take 80 μL of the culture supernatant, add it to a new 96-well plate, and the supernatant were collected for detection of LDH according to the manufacturer's instructions. The determination of CK-MB is the same as above. Serum was collected after MI/R in each group to detect the levels of LDH and CK-MB. The determination of rat is the same as above. All operations were carried out in strict accordance with the kit instructions.

**Malondialdehyde (MDA) Production and Superoxide Dismutase (SOD) Activity**

The production of MDA, a marker of lipid peroxidation, was determined to assess oxidative injury using a commercial MDA assay kit (Nanjing, China). Briefly, H9C2 cells were seeded in 6-well plates for 24 h prior to various treatments. Following treatment, H9C2 cells were washed with ice-cold PBS and incubated with RIPA lysis buffer (Nanjing, China) for 10 min at 4 °C. The lysates were centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatant were collected for detection of MDA according to the manufacturer's instructions. The determination of SOD (Nanjing, China) is the same as above. All operations were carried out following the manufacture’s instructions.

**Cell Viability**

Cell viability was determined by cell counting kit-8 (Dojindo, Japan) in 96-well plates according to manufacturers’ instructions. In brief, the CCK-8 solution was added to each well after the treatments and incubated for 3 h. The absorbance at 450 nm was measured using a microplate reader. The mean optical density of 6 wells in each group was used to calculate the percentage of cell viability. All operations were carried out following the manufacture’s instructions.

**Cardiac Functional Assessment**

Cardiac function was monitored by animal ultrasound system with rat ultrasound by measuring the left ventricular ejection fraction percentage (% EF) and fractional shortening percentage (% FS) recorded on a polygraph (RM-6240C, China) when rats were anaesthetized by 40 mg/kg pentobarbital sodium according to our previous publications [16]. The measurements of two-dimensional and M-mode echocardiographic were analysed with a GE vivid 7 high-resolution in vivo-imaging system (VisualSonics, Canada). % EF was calculated as [(EDvol-ESvol/EDvol] × 100%, and FS was calculated as [(LVID, d-LVID, s)/LVID, d] × 100.

**Myocardial Infarct Size**

The myocardial infarct size was measured as previously described [16], to evaluate the size of the infarct area (IA), risk area (AAR) and left ventricle (LV), six rats in each group. The left femoral vein of the rat was separated, and 2 ml of 2% Evans Blue (Sigma, USA) was injected into the heart through the left femoral vein immediately after 3 h of reperfusion. Animals were sacrificed, the heart was taken out and washed with phosphate buffer saline and then placed in a − 70 °C for 15 min. Hearts were sliced into 2-mm-thick sections and incubated with freshly prepared 1% TTC (Servicebio, China) solution at 37 °C for 15 min. TTC stained the viable part red, and the infarct part remained pale. The infarct size was analyzed by the image analysis system (Image-J). The percentage of area at risk versus left ventricle (AAR/LV × 100%) and infarct area versus area at risk (IA/ AAR × 100%) were calculated.

**Immunohistochemical Staining**

The part of the cardiac apex, which is infarct area was collected for the immunohistochemical staining. Immunohistochemical staining was performed according to the manufacturer’s instruction, kit was purchased from Servicebio in China. Briefly, the deparaffinized and rehydrated specimens were incubated overnight at 4 °C with monoclonal antibody, and then washed and incubated with HRP goat anti rabbit IgG antibody for 30 min. The sections were stained with DAB solution for 1 min and the nucleus was counterstained with hematoxylin [19].

**Apoptosis Levels**

In vitro study [20], after H/R, cells were collected and resuspended in binding buffer and incubated with fluorescein isothiocyanate conjugated annexin V and propidium Iodide for 15 min in the dark. All manipulation strictly followed the
manufacturer’s instructions. Cellular fluorescence was measured using a FACS Calibur flow cytometry (BD Biosciences, USA). The data obtained from the cell population were analyzed using Cell Quest Pro software (BD Biosciences, USA).

**Western Blot and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)**

The part of the cardiac apex, which is infarct area was collected for the follow-up experiments. Western blot analysis was performed as described previously [16] tissue or cells were lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail. After quantifying protein concentration, lysates were subjected to western blot analysis using specific antibodies against Nogo-A (Santa Cruz Biotechnology, CA), GRP94 (Cell Signaling Technology, USA), GRP78 (Cell Signaling Technology, USA), PDIA6 (Abcam, UK), CHOP (Cell Signaling Technology, USA), Bax (Cell Signaling Technology, USA), Bcl-2 (Cell Signaling Technology, USA), cleaved caspase-3 (Cell Signaling Technology, USA), GAPDH (Cell Signaling Technology, USA). We repeated each Western blot analysis using protein from three different and separate experiments. The specific protein bands were analyzed using Odyssey Application Software 3.0 to obtain the integrated intensities, followed by linear regression of the intensity data. Total RNA extracted by Trizol (ThermoFisher, China) and PureLink RNA Mini Kit (ThermoFisher, China) was reverse-transcribed using TIANScript II RT Kit (Tiangen Biotech, China) following the instructions of the manufacturers. qRT-PCR was carried out using RealMasterMix (SYBR Green, China). Three parallel wells were set for each group. The relative quantifications of Nogo-A and CHOP expressions were normalized to GAPDH. The primers used in this study are listed in Table 1. The GAPDH was used as the internal reference, and the $2^{-\Delta\Delta CT}$ method was used for transcriptional change evaluation. The formula was expressed as $\Delta\Delta CT = \Delta CT_{\text{experimental group}} - \Delta CT_{\text{control group}}$ in which $\Delta CT = CT_{\text{target gene}} - CT_{\text{internal reference}}$.

**Statistical Analysis**

Data are shown as the mean ± SEM. Comparisons between multiple groups were made by one-way ANOVA followed by the Tukey test. GraphPad Prism 8 software was used for statistical analyses. $P$ values < 0.05 were considered statistically significant.

**Results**

**Diabetic Hearts are More Vulnerable to MI/R Injury**

After 8 weeks of STZ-induced diabetes, the rats showed characteristic symptoms of type 1 diabetes including polydipsia and polyphagia. In all experimental groups, body weight was measured at 1 to 8 weeks after STZ injection (Fig. 1A). Fasting blood glucose level in all experimental groups was measured at 1 to 8 weeks (Fig. 1B). Bodyweight was lower, whereas the blood glucose level was higher than that in the age-matched non-diabetic rats ($P < 0.05$). To investigate the effects of diabetes status on MI/R injury, we next measured serum biochemical markers in each experimental group. Compared with the NI/R group, serum LDH (Fig. 1C) as well as CK-MB (Fig. 1D) levels were significantly increased in the DI/R group at baseline ($P < 0.05$). Echocardiography showed a decreased left ventricular ejection fraction and left ventricular fractional shortening in diabetic MI/R rats compared with NI/R rats ($P < 0.05$) (Fig. 1E-G). Diabetic rats subjected to MI/R showed larger infarct sizes ($P < 0.05$) (Fig. 1H). These results show that diabetic hearts are more vulnerable to MI/R injury.

**The Expression of Nogo-A is Increased in STZ-Induced Diabetic MI/R Injury**

To understand the pathological role of Nogo-A in the diabetic MI/R injury, we initially investigated the expression of Nogo-A in STZ-induced diabetic MI/R injury. As shown in Fig. 1A and B, the expression of Nogo-A in rat myocardial tissue was detected by immunohistochemistry. Compared with NS group, the level of Nogo-A was increased in DS, NI/R, and DI/R group ($P < 0.05$). Compared with DS and NI/R group, the Nogo-A protein level was increased in DI/R group ($P < 0.05$). Western blot result confirmed the increased Nogo-A protein level in the DI/R group ($P < 0.05$) (Fig. 2C, D). To elucidate the role of the Nogo-A in the process of ER stress, we investigated the key factors in the ER pathway by Western blotting. The levels of GRP94, GRP78, PDIA6 and CHOP were increased in the DI/R group compared with

**Table 1** Primer sequences for qRT-PCR

| Species | Gene   | Forward | Reverse  |
|---------|--------|---------|----------|
| Rat     | Nogo-A | GAGCTCTCTCCTT | AGCTGTTGGCCTT |
|         |        | TTCGCCCTT   | CTCTCTCACTAC |
|         | CHOP   | GACATAGACCT | TGGTCCTGTCCT |
|         |        | CAGTCGTCGT | CTGTGCTGCT |

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the NI/R group ($P < 0.05$) (Fig. 2C, E–H). Together, these results showed that in diabetic rats Nogo-A and ER stress were changed during MI/R injury, suggesting that Nogo-A and ER stress may have the role in the pathogenesis of ischemia-related cardiac diseases in diabetes.

Accelerated Nogo-A and MI/R Injury in Diabetic Rats was Attenuated by TUDCA Treatment

To investigate whether there is a link between the Nogo-A expression level in diabetic MI/R injury and ER stress. We found that the diabetic rats treated with TUDCA (ER stress inhibitor) had greatly reduced MI/R injury ($P < 0.05$) (Fig. 3A, B), and Nogo-A levels was decreased after MI/R ($P < 0.05$) (Fig. 3C) as assessed by western blot. The ER markers GRP94, GRP78, PIDA6 and CHOP also decreased...
as comparing with DI/R group after MI/R ($P < 0.05$) (Fig. 3C) as assessed by western blot. In addition, we investigated the MI/R induced cardiomyocytes apoptosis in diabetic rats and found that was alleviated by TUDCA treatment as assessed by the measurement of Tunel assay ($P < 0.05$) (Fig. 3I, J) and Bax, Bcl-2 and cleaved caspase-3 ($P < 0.05$) (Fig. 3K–N). Suggesting that inhibition of ER stress by TUDCA, a pharmacological inhibitor of ER stress, reduce Nogo-A expression levels and attenuated MI/R injury in diabetic rats.

**TUDCA Treatment Restored Cardiac Function and Reduced Infarct Size in Diabetic Rats**

We next measured cardiac function and myocardial infarct size. Echocardiography showed a increased left ventricular EF and left ventricular FS in DI/R + TUDCA group compared with DI/R group ($P < 0.05$) (Fig. 4A–C). DI/R + TUDCA group showed less infarct sizes ($P < 0.05$) (Fig. 4D). These results show that TUDCA treatment restored cardiac function and reduced infarct size in diabetic rats.

**H/R Injury of H9c2 Cells Aggravated Under High-Glucose Conditions**

To verify the in vitro model of MI/R injury in diabetes, H9c2 cardiomyocytes were treated with 50% glucose (30 mM) and followed by hypoxia reoxygenation procedure. We measured cells viability, LDH release and the biochemical markers of oxidative stress including MDA production, SOD activity. We found that HGH/R group resulted in a sharp increase in LDH and MDA ($P < 0.05$) (Fig. 5A and D). Furthermore, as
shown in (Fig. 5B, C), H9c2 cells viability and SOD activity were significantly lower in the HGH/R fgroup (P < 0.05). These results suggested that increased susceptible of H9c2 cells in high glucose-induced hypoxia-reoxygenation injury. The above results are consistent with the in vivo model.

Knockdown of Nogo-A Attenuated ER Stress in Myocardial H9c2 Cells During HGH/R

We wondered whether Nogo-A knockdown could relieve TM-induced ER stress and apoptosis in H9c2 cells during HGH/R. The qRT-PCR results showed that contrasted with
the scramble group, the expression level of Nogo-A was significantly decreased after Nogo-A transfection, indicating successful transfection \((P < 0.05)\) (Fig. 6A). We found that knockdown of Nogo-A attenuated TM-induced ER stress under HGH/R group, indicating that H9c2 cells ER stress was activated in response to TM stimulation and was alleviated by knockdown of Nogo-A, as reflected by decreased expression of GRP94, GRP78, PIDA6 and CHOP \((P < 0.05)\) (Fig. 6B–G). In addition, we investigated the role of Nogo-A in TM-induced H9c2 cells apoptosis and found that proapoptotic protein, cleaved caspase-3 and Bax, was decreased, with increased Bcl-2, anti-apoptotic protein after knockdown Nogo-A \((P < 0.05)\) (Fig. 6H–K) and attenuated apoptosis \((P < 0.05)\) (Fig. 6L, M). Collectively, these data indicate that Nogo-A contributes to TM overloading induced ER stress and plays a critical role in H9c2 cells injury and apoptosis under HGH/R.

**Knockdown of CHOP Downregulated Nogo-A and Alleviated Apoptosis Induced by TM in Myocardial H9c2 Cells During HGH/R**

Finally, to understand how Nogo-A contributes to ER stress and apoptosis in diabetic MI/R injury, we examined the interaction between Nogo-A and CHOP, a key ER stress transcriptional factor leading to the activation of apoptosis. The qRT-PCR results showed that contrasted with the scramble group, the expression level of CHOP was significantly decreased after CHOP transfection, indicating successful transfection \((P < 0.05)\) (Fig. 7A). In the previous experiments we have found that CHOP was extremely increased in diabetic MI/R both in vivo and vitro. We knocked down CHOP specific shRNA in H9c2 cells, then exposed to high glucose for 24 h and stimulated with ER stress inducer TM for 6 h, followed by 4 h of hypoxia and 2 h of reoxygenation showed that knockdown
of CHOP significantly suppressed Nogo-A expression ($P < 0.05$) (Fig. 7B–D), indicating that there may be a positive feedback loop between CHOP and Nogo-A. In addition, CHOP depletion also protected cardiomyocytes from HGH/R-induced apoptosis ($P < 0.05$) (Fig. 7B and Fig. 7E–I). Taken together, our data suggest that CHOP has a positive feedback to Nogo-A and down regulation CHOP can protect diabetic heart from apoptosis.

**Discussion**

Nogo family proteins are profoundly involved in multiple cellular processes especially the morphology and functional science of ER. Our present study found that Nogo-A is a determinant player in ER stress and mediated cardiomyocyte apoptosis during MI/R in diabetes. There are several lines supporting our notions. First, Nogo-A protein is increased in diabetic MI/R heart and also increased in HGH/R stimuli-induced cardiomyocytes. Second, accelerated Nogo-A and MI/R injury in diabetic rats was attenuated by TUDCA treatment and knockdown of Nogo-A per se is sufficient to decrease ER stress as well as prevents cardiomyocyte apoptosis. Third, we identified Nogo-A may be a target gene of CHOP, in vitro knockdown of CHOP protected cardiomyocytes against apoptosis after HGH/R, which positively regulated Nogo-A and is upregulated during MI/R in diabetes.

While timely reperfusion has proved to be an invaluable tool, MI/R injury represents a mechanism that may limit its effectiveness. We and others have previously shown that the diabetic myocardium is more susceptible to ischemia–reperfusion injury, possible causes are increased oxidative stress, inflammation response, apoptosis, ER stress or mitochondrial dysfunction, but further mechanisms are still to be studied [16, 21, 22]. ER stress is an important feature leading to the cardiac dysfunction after ischemic heart diseases in diabetes. The activation of the mild stress appears to have a cardioprotective role, ER-associated degradation is activated by UPR to clear irreparably misfolded proteins. It is increasingly recognized that ER stress and UPR pathways contribute to the pathogenesis of metabolic diseases such as diabetes [23]. However, when severely stress the
Fig. 6 Knockdown of Nogo-A attenuated HGH/R-induced ER stress and apoptosis in H9c2 cells. A Effect of shNogo-A on Nogo-A expression level after transfection. B The expression of Nogo-A, GRP94, GRP78, PDIA6 and CHOP were analyzed by Western blot. C–G The bar graph of Nogo-A, GRP94, GRP78, PDIA6 and CHOP proteins expression. H The proteins expression of cleaved caspase-3, Bax and Bcl-2 were detected by Western blot. I–K The bar graph of cleaved caspase-3, Bax and Bcl-2 proteins expression. L Cell apoptosis was assessed by flow cytometer with Annexin FITC and PI. M Quantification of apoptotic cells. Values are expressed as means ± SEM. *P < 0.05 compared to scramble, #P < 0.05 compared to shNogo-A, ▲P < 0.05 compared to TM + scramble, n = 6
UPR fails to reduce ER stress and restore homeostasis, ER stress causes cell dysfunction and apoptosis. Thus, the environment in the ER must be optimal for efficient synthesis and folding of these important proteins. In this setting, ER stress lead to cardiovascular complications. It has been suggested that Nogo serves an important role in ER homeostasis may contribute to myocardial I/R damage [22, 24]. In this study, we found that both Nogo-A protein level and ER stress markers response were increased in diabetic MI/R injury heart, and treat with TUDCA can protect diabetic MI/R heart. It is worth noting that TUDCA did not reduce the level of ER stress in diabetes. The possible reason is that ER stress is adaptive and has a protective effect in diabetes. There are many possible causes of a relationship between ER stress and diabetic myocardial vulnerability increases. Other studies have shown that diabetes-induced.

**Fig. 7** Knockdown of CHOP attenuated HGH/R-induced apoptosis in H9c2 cells. A Effect of shCHOP on CHOP expression level after transfection. B The proteins expression of Nogo-A, CHOP, cleaved caspase-3, Bax and Bcl-2 were assessed by western blot. C–E The densitometry analyses western blots are shown. F Flow cytometry was performed to analyze cardiomyocytes apoptosis after double labeling with FITC and propidium iodide. G Quantification of apoptotic cells. Values are expressed as means±SEM. *P<0.05 compared to scramble, **P<0.01 compared to scramble; #P<0.05 compared to shCHOP; *P<0.05 compared to TM+ scramble, n=6
ER dysfunction through neuregulin-1 and O-linked beta-N-acetylglucosamine [25, 26]. Other study shows that hyperglycemia-induced ER stress in rats and significantly lowered the expression of glucose transporter proteins, misfolded insulin was shown to cause diabetes in both mouse models and humans [27]. Recent studies indicated that in perioperative diallyl trisulfide treatment effectively ameliorates MI/R injury in type 1 diabetic setting by suppressed PERK/eIF2α/ATF4/CHOP-mediated ER stress level, thus reducing myocardial apoptosis and eventually preserving cardiac function [28]. This is consistent with our recent in vivo studies showing that ER stress markers increase and heart injury aggravation in diabetic rats during MI/R. At present, there is no satisfactory way to cure or mitigate diabetic MI/R injury, hence, a deeper understanding of the underlying molecular mechanisms of this disease is essential to the development of new effective therapies.

Here, we first reported that a novel ER stress marker, Nogo-A, which was highly expressed in diabetic ischemic heart and suppressed by CHOP. Nogo-A is an important neurite growth-regulatory protein in the adult and developing nervous system, and are involved in neuroendocrine secretion or membrane trafficking and apoptotic processes [29]. Our above results on Nogo-A and others’ previous studies on Nogo-B or Nogo-C indicate that the increased Nogo proteins during ischemic heart diseases may contribute to cardiac dysfunction through regulating cardiomyocyte apoptosis. To better understand the pathophysiological significance of Nogo-A in the diabetic heart, we generated the Nogo-A knockout model in vitro. Our in vivo functional study Nogo-A in rats provides solid evidence supporting our hypothesis, that Nogo-A is extremely increased after MI/R in diabetic rats. Then, we found that depletion of Nogo-A protected cardiomyocyte apoptosis in HGH/R H9c2 cells, largely decreased HGH/R injury, and most importantly, decreased ER stress after HGH/R, suggesting that Nogo-A may serve as a target for ER stress, and can treatment of ischemia-related cardiac diseases. In addition, Nogo-A expression is increased in human dilated cardiomyopathy and ischemic hearts as well as involved in glucose homeostasis [14, 15, 30]. It was also stated that Nogo-A knockdown inhibits H/R-induced activation of mitochondrial-dependent apoptosis in cardiomyocytes [14], unlike our study, they showed that knockdown of Nogo-A inhibited H/R-induced cleaved caspase-3 cleavage without affecting ER stress. This different in high glucose model may account for the different results. Our present study of the protective effect of Nogo-A knockdown in the H9c2 cells is in general agreement with Nogo family protein Nogo-C knockout mouse model [31]. Although Nogo-A knockdown protected the cardiomyocytes from HGH/R damage, it showed no cardiac phenotype at basal level, suggesting that Nogo-A is either dispensable for normal functions or there are redundant pathways in the heart. These possibilities should be examined to further clarify the role of Nogo-A.

How Nogo-A mediates ER stress remains unclear. The findings from this study expand our understanding of the regulatory role of ER stress in diabetic MI/R induced apoptosis. We focused on the regulation of Nogo-A by CHOP, given that excessive CHOP is linked to maladaptive ER stress. We have shown that knockdown of Nogo-A prevented H/R induced activation of CHOP under high glucose. It is known that CHOP deletion can protect cardiomyocytes from apoptosis through inhibition PERK/CHOP pathway [11]. In vivo, CHOP deficiency attenuated apoptosis, inflammation, fibrosis in fat-induced liver injury [32], targeted disruption of the CHOP gene significantly prevented Ins2C96Y–induced diabete by decreasing ER stress-mediated apoptosis in β cells [33]. These data suggest a critical role of CHOP in disease development. Here, we found that knockdown of CHOP suppressed Nogo-A expression and Nogo-A-mediated ER stress and apoptosis in diabetic MI/R injury, suggesting Nogo-A expression is regulated by CHOP. These data together suggest a mechanism that Nogo-A induces CHOP expression, forming a positive feedback manner to synergistically stimulate ER stress in diabetic heart.

There are some limitations to our study. First, although we found abnormal increase in Nogo-A in diabetic MI/R injury in both in vivo and in vitro models, further research is needed to investigate the role of Nogo-A homologues Nogo-B and Nogo-C in diabetic MI/R injury explore its deep mechanism. Second, we have only made a mechanism on the cell line, and further research requires use of primary cultures of neonatal rat cardiomyocytes. However, this study at least demonstrates the feasibility of conducting such studies on mammalian cardiomyocytes, and uses these findings to further understand the relationship between Nogo-A and ER stress-signaling pathways in diabetic cardiomyocytes and provided a theoretical and experimental basis to put these findings into further understanding of the mechanisms in the myocardial systems under both physiological and pathologi cal conditions.

Conclusions

In summary, this study demonstrated that Nogo-A expression significantly increased in diabetic MI/R injury, and knockdown of Nogo-A using shRNA can be relieved ER stress and apoptosis. Furthermore, CHOP has a positive feedback to Nogo-A and down-regulation CHOP can protect diabetic MI/R heart from apoptosis. Our findings provide a novel mechanism involved in the pathophysiology of MI/R injury in diabetes. To promoting the prevention and treatment of diabetic MI/R injury, more comprehensive work on Nogo-A need to be conducted in the future.
Author contributions  All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by YX, WL, WL, HT, JT, RC and ZX. The first draft of the manuscript was written by Yan Leng and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding  This work was supported by Grants from the National Natural Science Foundation of China (Grant Nos: 81970722, 81901947).

Data Availability  The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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