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Mitochondrial translocation of Nur77 induced by ROS contributed to cardiomyocyte apoptosis in metabolic syndrome

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ABSTRACT

Metabolic syndrome is a major risk factor for cardiovascular diseases, and increased cardiomyocyte apoptosis which contributes to cardiac dysfunction after myocardial ischemia/reperfusion (MI/R) injury. Nur77, a nuclear orphan receptor, is involved in such various cellular events as apoptosis, proliferation, and glucose and lipid metabolism in several cell types. Apoptosis is positively correlated with mitochondrial translocation of Nur77 in the cancer cells. However, the roles of Nur77 on cardiac myocytes in patients with metabolic syndrome remain unclear. The objective of this study was to determine whether Nur77 may contribute to cardiac apoptosis in patients with metabolic syndrome after I/R injury, and, if so, to identify the underlying molecular mechanisms responsible. We used leptin-deficient (ob/ob) mice to make metabolic syndrome models. In this report, we observed that, accompanied by the substantial decline in apoptosis inducer Nur77, MI/R induced cardiac dysfunction was manifested as cardiomyopathy and increased ROS. Using the neonatal rat cardiac myocytes cultured in a high-glucose and high-fat medium, we found that excessive H_2O_2 led to the significant alteration in mitochondrial membrane potential and translocation of Nur77 from the nucleus to the mitochondria. However, inhibition of the relocation of Nur77 to mitochondria via Cyclosporin A reversed the changes in membrane potential mediated by H_2O_2 and reduced myocardial cell injury. Therefore, these data provide a potential underlying mechanism for cardiac dysfunction in metabolic syndrome and the suppression of Nur77 translocation may provide an effective approach to reduce cardiac injury in the process.

1. Introduction

Metabolic syndrome mediated by insulin resistance consists of the clustering of central obesity, dyslipidemia (i.e. high TG, low HDL–C), hyperglycemia, hypertension and urinary protein [1]. Ever-increasing MS correlates positively with the increased mortality and morbidity of cardiovascular diseases in the USA [2,3]. For example, MS can increase the risk for Ischemic heart disease (IHD) by two to three times [4]. Furthermore, evidences suggest that MS was found to aggravate MI/R injury in metabolic dysfunction animal models mediated by obesity [5]. Thus, it is scientifically significant to explore the relationship between MS and increased IHD, which may provide another potential point in the treatments of heart diseases.

Nur77 (NR4A1, NUR77), a transcriptional factor of NR4R family, was first found in PC12 cancer cells [6]. Cooperating with such various growth factors as EGF and NGF, Nur77 participates in cell proliferation, differentiation and maturity [7]. Moreover, relocation of Nur77 to the mitochondrial membrane is involved in the apoptosis of several kinds of tumor cells after apoptotic stimulations [8–10]. The mechanism linking Nur77 to cell apoptosis lies in the release of Cytochrome C induced by change in membrane permeability after Nur77 translocation to the mitochondrial membrane [11]. The role, however, of Nur77 in cardiomyocyte apoptosis mediated by I/R injury is poorly understood.

Evidence has accumulated indicating that increased reactive oxygen species (ROS) mediated by MS tend to play an important role in MI/R injury and cardiomyocyte apoptosis [5–12]. ROS, the main source of which is mitochondria during energy metabolism, can trigger the non-selective channel PTP and then affect the membrane potential in mitochondria, eventually leading to cell death [13,14]. Nevertheless, whether accumulation of ROS in cardiac myocytes is required for the translocation of Nur77 to mitochondria is not known. Furthermore, whether the inhibition of Nur77

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translocation can reduce ROS mediated cardiomyopathy in MS is poorly understood. Therefore, we tested the hypothesis that MS-induced ROS may regulate the expression and translocation of Nur77 to the mitochondrial membrane and inhibition of Nur 77 relocation reduces cardiomyocyte injury.

2. Methods

2.1. Animal models and reagents

Adult male leptin-deficient (ob/ob) mice and their male wild-type littermate controls (WT) were purchased from the Department of Pathology, and one-day-old Sprague–Dawley (SD) rat pups were purchased from the Experimental Animal Center of the Fourth Military Medical University. All experiments were performed in adherence with the National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by the Committee on Animal Care of the Fourth Military Medical University.

A cell isolation kit and JC-1 detection kit were purchased from Nanjing KeyGEN Biotech Co., China and Beyotime CO., China, respectively. Cyclosporin A, Collagenase I Trypsin, Glucose, Mannitol, sodium palmitate were obtained from Sigma–Aldrich, Inc., USA, with skimmed BSA from MP Bio. CO., USA.

2.2. Surgical preparation of animals and determination of cardiac function

Mice were anesthetized with 2% isoflurane, and MI/R was produced by temporarily exteriorizing the heart via left thoracic incision and placing a 6-0 silk suture slipknot at the distal 1/3 of the left anterior descending coronary artery. After 30 min of ischemia, the slipknot was released, and the myocardium was reperfused for 3 h (for apoptosis, WB, and Superoxide Production) or 24 h (for cardiac function). Sham-operated mice underwent the same surgical procedures except that the suture placed under the left coronary artery was not tied. Cardiac function was determined by echocardiography (VisualSonicsVeVo 770 imaging system) 24 h after coronary occlusion, with EF as an indicator.

2.3. Primary culture of neonatal rat cardiac myocytes

Single cardiac myocytes were isolated from the hearts of Sprague–Dawley rats using a standard enzymatic technique (equal volume mixtures of 0.1% Collagenase I and 0.25% Trypsin), and then isolated via the differential adherence method, planted on 6-well plates. The cells were cultured for 5–7 days before the addition of relative reagents.

2.4. Preparation of 500 nM sodium palmitate in a high-glucose and high-fat medium

0.01397 g sodium palmitate were dissolved in 5 ml PBS at 90 °C and then cooled to 60 °C at room temperature. This solution was added quickly to PBS–BSA (20% BSA), dissolved, filter sterilized and stored at 4 °C. The complete mixture was added at a dilution of 1:9 (High-glucose DMEM cell medium) to the plates up to a final density of 500 nM. The control group was treated with free-sodium palmitate BSA solutions and glucose–mannitol solutions (20 mmol/L).

2.5. Determination of myocardial injury

Myocardial injury was determined by TUNEL staining and serum CK levels. TUNEL staining was performed by using the Roche apoptosis kit (Roche Applied Science) and as described previously [15]. Serum CK levels were tested in the department of Pathology of Xijing Hospital.

2.6. Immunofluorescence, JC-1 and confocal microscopy

Cells were permeabilized in 0.1% Triton X-100 for 5 min, blocked with 10% goat serum in PBS for 1 h, and incubated with mouse anti-α-actinin (Sigma, St. Louis, USA, 1:1000) at 4 °C overnight. The next day, slides were incubated for 1.5 h at room temperature with Cy3-conjugated secondary antibodies. Nuclei were stained with DAPI.

JC-1 dye was used to detect the cellular mitochondrial membrane potential ($\Delta\psi m$). At a high $\Delta\psi m$, the accumulation of JC-1 to the mitochondrial membrane forms JC-1 aggregates producing red fluorescence. Alternatively, green fluorescence is generated by JC-1 monomers. Neonatal heart cells were cultured and then JC-1 staining was performed according to the manufacturer's instructions.

2.7. Determination of superoxide production in cardiac tissue

ROS Fluorescent Probe-DHE was used for frozen heart sections. DHE (10 mol/L) was applied to each tissue section, and then coverslipped. The slides were incubated in a light-protected humidified chamber at 37 °C for 30 min. Ethidium fluorescence (excitation at 490 nm, emission at 610 nm) was examined by fluorescence microscopy.

2.8. Cell separation and Western blot analysis

Proteins were separated on SDS–PAGE gels, transferred to PVDF (Millipore, MA, USA) and incubated overnight at 4 °C with antibodies directed against Rabbit anti-Nur77 (sc-5569, Santa Cruz, USA, 1:200), mouse anti-β-actin, mouse anti-VDAC (Cell Signaling Technology, Danvers, MA, USA, 1:1000), rabbit anti-H3 (Santa Cruz Biotechnology, 1:500). After washing blots to remove excessive primary antibody binding, blots were incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz, USA, 1:2000). Antibody binding was detected via enhanced chemiluminescence (Millipore). Film was scanned with Chemi-DocXRS (Bio-Rad Laboratory, Hercules, CA). Immunoblot band intensity was analyzed with Lab Image software.

2.9. Data analysis

All values in the text and figures are presented as means ± SEM. The statistical analysis was performed using the SPSS 14.0 (SPSS, USA). Values of $P < 0.05$ were considered statistically significant, as determined using Student's unpaired *t*-test or two-way ANOVA.

3. Results

3.1. MS exacerbated MI/R induced injury accompanied by decreased Nur77

We administrated ob/ob mice as the animal model of MI/R injury. The results show that compared with WT mice, ob/ob mice experienced a significant decline in cardiac function after MI/R injury ($P < 0.05$, Fig. 1A and B). We also measured the apoptosis and necrosis via TUNEL and serum CK, respectively. MI/R induced cardiac injury was enhanced in MS mice, as evidenced by increases in both TUNEL positive cells and serum CK levels ($P < 0.05$, Fig. 1C–E). Meanwhile, the generations of ROS in MS

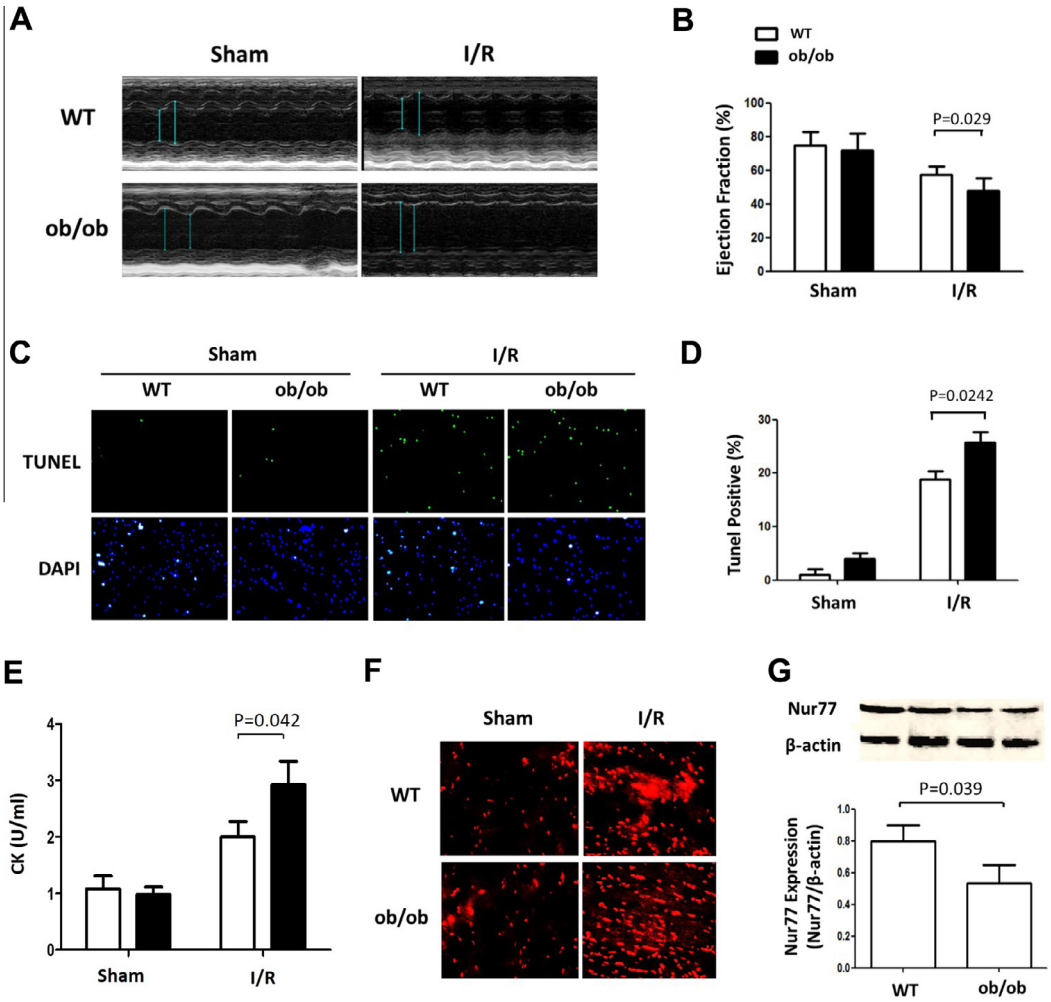

Fig. 1. MS exacerbated MI/R induced injury, and reduced the expression of Nur77in whole myocyte. Compared with WT mice, ob/ob mice experienced a significant decline in cardiac function after MI/R injury (A and B). Both TUNEL positive cells (C and D) and serum CK levels (E) were increased. The generations of ROS in ob/ob mice were elevated after I/R injure (F). Nur77 expression was decreased in ob/ob mice (G).

animals were elevated ($P < 0.05$, Fig. 1F). In contrast, Nur77 expression was decreased dramatically, opposite to the previous hypothesis ($P < 0.05$, Fig. 1G).

3.2. ROS led to Nur77 translocation in the neonatal heart cells cultured in a high-glucose and high-fat medium

Neonatal heart cells cultured in a high-glucose and high-fat medium (Fig. 2A) were subjected to MI/R injury to determine whether Nur77 was involved in enhanced MI/R injury mice subjected to MS. The addition of H_2O_2 into cultured neonatal cells stimulated I/R cardiomyopathy due to the concomitant excessive production of ROS.

The results suggest that H_2O_2 not only induced increased apoptosis ($P < 0.001$, Fig. 2B and C) and CK levels ($P < 0.05$, Fig. 2D) in the supernatant, but destroyed $\Delta\psi m$ followed by a rise in membrane permeability (Fig. 2E). H_2O_2 had no effect on the expression of Nur77 in myocardial cells (Fig. 2F). Furthermore, the relocation of Nur77 from nucleus to mitochondria, not its expression, increased dramatically after H_2O_2 simulation in a high-glucose and high-fat medium (Fig. 2G and H). Collectively, these data demonstrate that ROS was found to mediate the translocation of Nur77 in the neonatal heart cells subjected to MS.

3.3. Inhibiting relocation of Nur77 to mitochondria resulted ROS-induced cardiomyocyte injury in a high-glucose and high-fat medium

To further demonstrate the involvement of Nur77 relocation in enhanced MI/R injury by MS, we used the mitochondrial membrane stabilizer Cyclosporin A. Compared with the H_2O_2 group, Cyclosporin A protected neonatal heart cells against ROS mediated I/R injury, manifested as reduced apoptosis and necrosis ($P < 0.05$, Fig. 3A–C). Cyclosporin A also maintained normal mitochondrial membrane potential after H_2O_2 injury in a high-glucose and high-fat medium (Fig. 3D). Furthermore, Cyclosporin A was able to inhibited Nur77 transport from the nucleus to the mitochondria ($P < 0.01$, Fig. 3F), but had no effect on the Nur77 expression in the whole cell and in nucleus (Fig. 3E and G).

4. Discussion

Cardiomyocyte apoptosis has been demonstrated to be reversible and preventable, exemplified as decreased infarct size and improved cardiac systolic function [16].

However, the mechanisms for MI/R induced cardiomyocyte apoptosis are unclear although various biologically active factors, such as ROS and chemokines, play a major role in the process

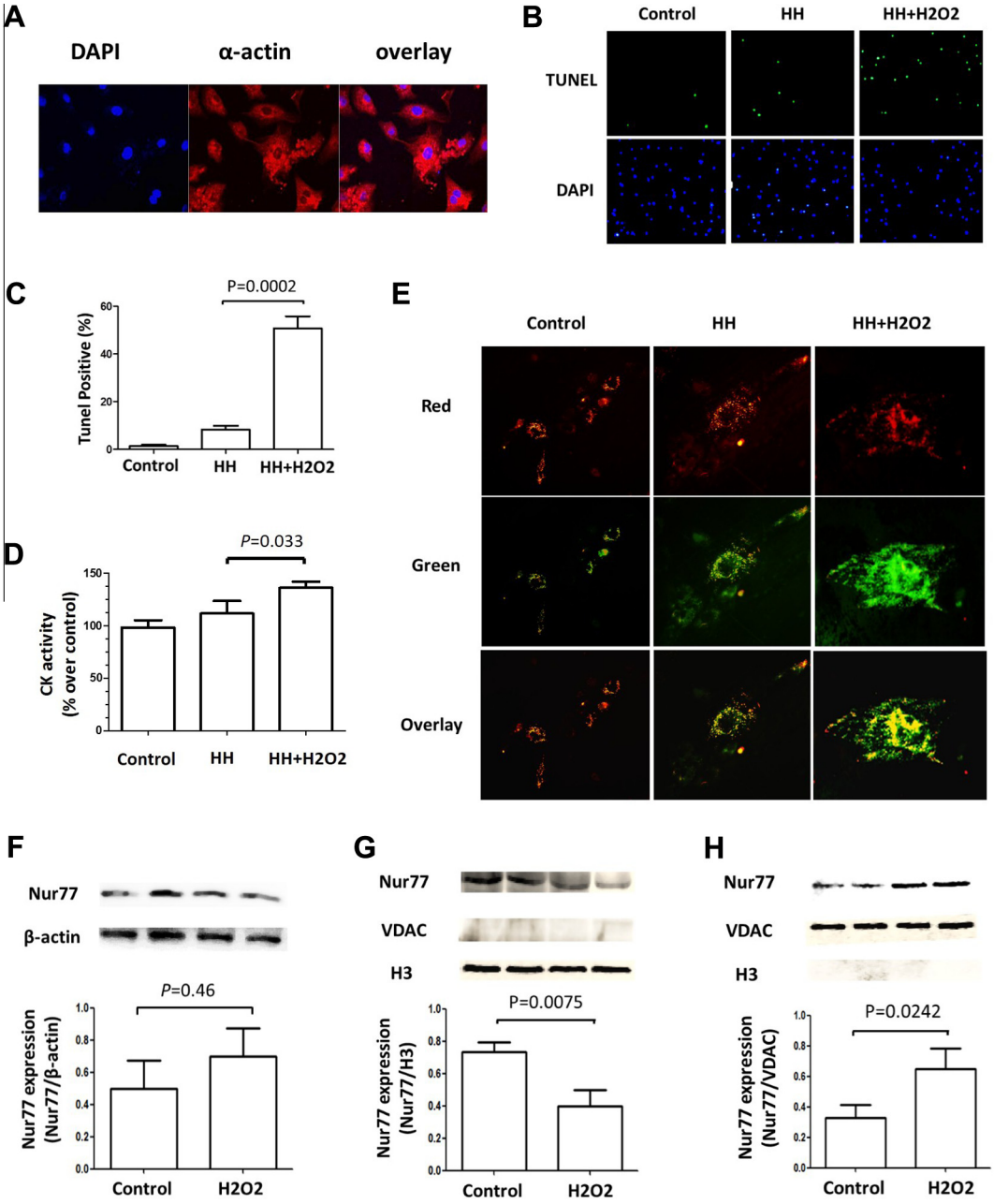

Fig. 2. H_2O_2 induced the change of mitochondrial membrane potential ($\Delta\psi m$) and Nur77 translocation in the neonatal heart cells cultured in a high-glucose and high-fat medium. Immunofluorescent staining for neonatal heart cells: α-actin (Cy3, red),nuclei (DAPI, blue) (A). H_2O_2 increased apoptosis (B and C) and necrosis (D) in the neonatal heart cells cultured in a high-glucose and high-fat medium. H_2O_2 exacerbated the change of $\Delta\psi m$ in the neonatal heart cells cultured in a high-glucose and high-fat medium (E): high $\Delta\psi m$ showed red fluorescence, low $\Delta\psi m$ showed green fluorescence. H_2O_2 had no effect on the expression of Nur77 in myocardial cells (F). H_2O_2 decreased the level of Nur77 in nucleus (G) and increased the level of Nur77 in mitochondria (H). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

[17]. For example, the severity of MS-related cardiomyocyte apoptosis is correlated positively to ROS generation [18]. Under physiological conditions, the cellular low-level ROS participates in some cell signaling [19]. Therefore, excessive ROS elimination does not necessarily preserve cardiac myocytes against apoptosis. Flaherty found that SOD was unable to further protect cardiac myocytes of AMI patients treated with PCI [20]. Another case is that smoking AMI patients did not experience improved cardiac function after long-term alpha-tocopherol and beta-carotene antioxidant treatments [21]. Therefore, it is important to find the factors critical to MI/R mediated injury.

We originally found that MS resulted in a decline in Nur77 levels. More interesting still, relocation of Nur77 to the mitochondrial membrane increased substantially in the MS related cardiac myocytes injury after H_2O_2 stimulation. Consistent with studies by Li et al., Nur77 translocation, not its expression, mediated MI/R induced cardiomyocyte apoptosis [11–22]. We reported that MS-related ROS induced cardiomyocyte injury was mediated by Nur77 relocation and inhibited by the mitochondrial membrane stabilizer Cyclosporin A with no change in Nur77 release from the nucleus. These data support our hypothesis that Nur77 translocation significantly contributes to the MS-related cardiomyocyte injury subject to MI/R.

Together with Nurr1 (NR4A2) and NOR-1 (NR4A3), nucleus orphan receptor Nur77 consists of NR4A family. Nur77, a transcriptional factor with no legend, can be found in such various organs

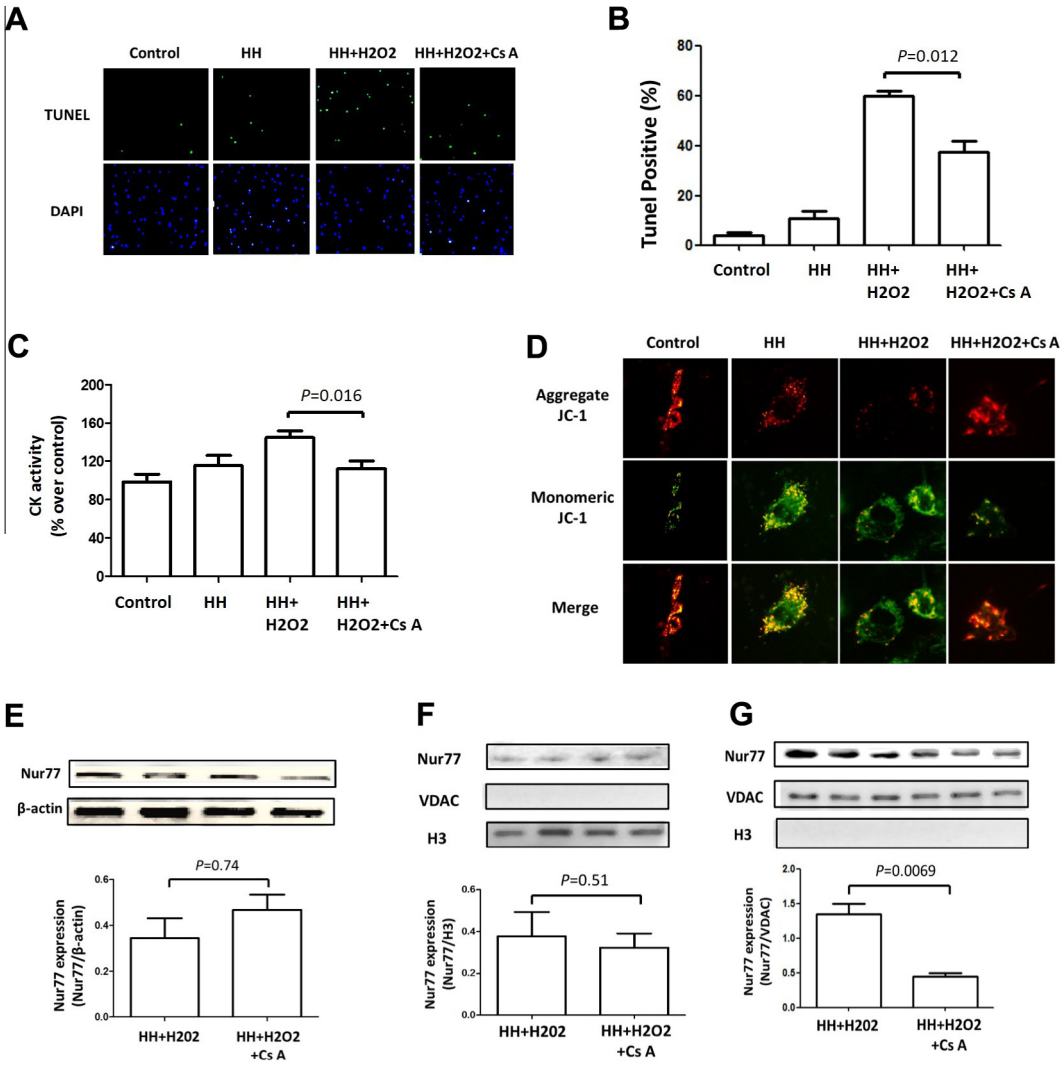

Fig. 3. Cyclosporin A inhibited the relocation of Nur77 to mitochondria and decreased cardiomyocyte injury induced by ROS in a high-glucose and high-fat medium. Cyclosporin A protected neonatal heart cells against H_2O_2 in reduced apoptosis (A and B) and CK level (C) in a high-glucose and high-fat medium. Cyclosporin A protected normal $\Delta\psi m$ after H_2O_2 injury in a high-glucose and high-fat medium (D). Cyclosporin A had no effect on the Nur77 expression in the whole cell (E) and the translocation of Nur77 from the nucleus (F), but inhibited the mitochondrial translocation of Nur77 (G).

as the pituitary gland, muscles, liver, lungs, and the nervous system [10]. After the growth factor stimulation, Nur77 performs as an early transcriptional factor to regulate cell proliferation, which participates in cell apoptosis under the influence of apoptotic factors [8–11]. Recently, under the effect of apoptosis-inducing agents, Nur77 migrated to the mitochondrial membrane rather than targeted at the sites in the nucleus, eventually leading to cell apoptosis [11]. It has been demonstrated that the heterodimer of Nur77 and RXRs can migrate and target Bcl-2 in mitochondrial membrane, precipitating cell apoptosis [23]. Summarily, the effects of Nur77 on cell activities depend on what Nur77 locates and targets.

Interestingly, Nur77 expression varies after different stimulations in different cell types. For example, elevated Nur77 expression participated in apoptosis of T lymphocytes in cancer [24]. Nevertheless, Nur77 translocation to cytoplasm, rather than to mitochondrial membrane, triggered the combinations of other apoptosis-inducing factors to mitochondria and then ignited cell apoptosis [25].

Mitochondria play a major role in regulating the physiological functions and apoptosis in cardiac myocytes. MI/R injury is associated with the concomitant damages to mitochondrial structures and functions, manifested as increased membrane permeability [26]. In this study, we show that Nur77 relocation to mitochondria in MS related myocyte injury after MI/R simulation was related to mitochondrial apoptotic pathway. Furthermore, Cyclosporin A, via inhibiting the open of PTP, not only reduced the ROS-mediated Nur77 translocation, but protected cardiac myocytes against I/R injury, which confirms that Nur77 contributes significantly to mitochondrial apoptotic mechanisms.

In conclusion, though our study has some limitations, it strongly suggests that Nur77 translocation participated in MI/R induced apoptotic injury of cardiac myocytes subjected to MS, and ROS mediated Nur77 relocation to mitochondria in this process. Therefore, inhibiting the migration of Nur77 to mitochondria can protect cardiac myocytes subjected to MS against I/R injury and become a potential target for IHD treatments.

Conflict of interest

The authors declare no conflict of interest.

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