

Review Article

Low Concentration Ethanol Prevents Oxidative Stress-Induced Cardiac Cells Injury Under Hyperglycemic Conditions by Inhibiting the SAPK/JNK Signaling Pathway and ROS Generation

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Abstract

The purpose of this study was to determine the effects and mechanisms of ethanol on oxidative stress-induced cardiac H9c2 cells mitochondrial injury under hyperglycemic conditions. Under hyperglycemic conditions, ethanol pretreatment (10-100 μ M) prevented H₂O₂-induced mitochondria swelling, as well as decreased cell viability and Respiratory Control Ratio (RCR) in the H9c2 cells. It also prevented TMRE fluorescence intensity loss and DCF fluorescence intensity increase under hyperglycemic conditions. These effects of ethanol were reversed by the SAPK/JNK agonist, anisomycin. Finally, treatment of H9c2 cells with 33mM glucose significantly enhanced Akt and ERK phosphorylation, which was not affected by ethanol. However, ethanol decreased the phosphorylation of SAPK/JNK under hyperglycemic conditions. Collectively, these findings indicate that under hyperglycemic conditions, that ethanol prevents oxidative stress-induced mitochondrial injury in cardiac H9c2 cells by preventing ROS generation via inhibiting the SAPK/JNK signaling pathway.

Keywords: Ethanol; Hyperglycemia; Oxidative stress injury; SAPK/JNK

Abbreviations

$\Delta\Psi_m$: Mitochondrial Membrane Potential; OCR: Oxygen Consumption Rate; RCR: Respiratory Control Ratio; SAPK/JNK: Stress-Activated Protein Kinase/c-Jun N-terminal Kinase

Introduction

Although many studies suggest that alcohol consumption is closely associated with cardiovascular disease in diabetic populations [1,2], several studies have demonstrated that moderate consumption of alcohol has a direct cardioprotective effect on myocardium in various experimental models [3-5]. The beneficial effect of ethanol on ischemic heart disease was proposed to be attributable to its positive effects on antioxidant capacity [6], lipid profile (Lindberg and Amsterdam, 2008) and the coagulation system [7]. Since several signaling pathways have been proposed to be essential for ethanol-induced cardioprotection against oxidative injury in nondiabetic people [4,7,8], these pathways may be also involved in ethanol-induced cardioprotective effects under diabetic conditions.

The mitochondrial Permeability Transition Pore (mPTP) opening has been proposed as a critical determinant of myocardial oxidative stress [9-11]. Zhou et al. [4] reported that ethanol at low doses can prevent oxidant-induced mPTP opening by activating Phosphatidylinositol 3-Kinase (PI3K)/v-akt-murine thymoma viral onco-gene homolog (Akt) signaling pathway. Although the signal factors such as Akt and extracellular signal-regulated kinase (ERK) have been proposed to be linked to the inhibition of the mPTP opening in nondiabetic models, the exact signaling mechanism by

which cardioprotective interventions prevent mitochondrial oxidant injury in diabetic patients remains unclear [12,13].

In the current study, we hypothesized that ethanol prevents oxidative stress induced-mPTP opening in cardiac H9c2 cells cultured in high glucose conditions. We also sought to define the signaling mechanism by which ethanol prevents the mitochondrial oxidative stress injury.

Materials and Methods

Cell culture

The rat heart tissue-derived H9c2 cardiac myoblast cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100U penicillin/streptomycin at 37°C in a humidified 5% CO₂-95% air atmosphere.

Chemicals and antibodies

Ethanol was purchased from Sigma Chemical (St. Louis, MO); SAPK/JNK agonist anisomycin was purchased from LC Laboratories (LC, USA); Tetramethylrhodamine ethyl ester (TMRE) was from Molecular Probes (Eugene, OR, USA); 2',7'-dichlorofluorescein diacetate (H2DCF-DA) was from Beyotime (Shanghai, China); and all antibodies were from Cell Signaling Technology (Beverly, MA, USA).

Cell viability assays

For cell viability assays, cells were plated in 96-well dishes (10000

cells/well) and grown over 24h. Then cells were treated with different doses of glucose. Cell viability was assessed using the CCK-8 assay (Beyotime, Shanghai, China) according to the manufacturer's instruction.

Confocal imaging of mitochondrial membrane potential ($\Delta\Psi_m$)

$\Delta\Psi_m$ was measured by loading cardiomyocytes with Tetramethylrhodamine Ethyl Ester (TMRE). TMRE is a cell permeable, cationic, nontoxic, fluorescent dye that specifically stains live mitochondria. TMRE is accumulated specifically by the mitochondria in proportion to membrane potential. A number of studies have measured $\Delta\Psi_m$ by imaging cardiac cells loaded with TMRE [4,14]. Briefly, cardiac cells cultured in a specific temperature-controlled culture dish (120,000 cells/dish) were incubated with TMRE (100nM) in a standard Tyrode solution containing (in mM) NaCl 140, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5 and glucose 5.8 (pH 7.4) for 10min. Cells were then mounted on the stage of an Olympus FV 1000 laser scanning confocal microscope. The red fluorescence was excited with a 543-nm line of argon-krypton laser line and imaged through a 560-nm-long path filter. Temperature was maintained at 37°C.

Confocal imaging of intracellular ROS

H9c2 cells (120,000 cells/dish) were plated in specific temperature-controlled culture dishes and grown over 24h. Then cells were treated with different doses of glucose. Cells were incubated with 20 μ M H2DCF-DA in a standard Tyrode solution containing (in mM) NaCl 140, KCl 6, $MgCl_2$ 1, $CaCl_2$ 1, HEPES 5 and glucose 5.8 (pH 7.4) for 20 min. After washing with phosphate buffered saline (PBS), the cells were incubated in new standard Tyrode solution for ROS measurements. Cells were then mounted on the stage of an Olympus FV 1000 laser scanning confocal microscope. The DCF fluorescence was excited at 480nm and collected at 530nm. Temperature was maintained at 37°C throughout the experiment.

Western blot analysis

Equal amounts of protein lysates (40 μ g) were loaded and electrophoresed on SDS-polyacrylamide gel and transferred to a PVDF membrane. Membranes were probed with primary antibodies that recognize phosphorylation of Akt (Ser⁴⁷³), ERK (Thr²⁰²/Tyr²⁰⁴) and SAPK/JNK (Thr¹⁸³/Tyr¹⁸⁵). Each primary antibody binding was detected with a secondary antibody and visualized by the Enhanced Chemiluminescence (ECL) method. Equal loading of samples were confirmed by reprobing membranes with anti-Tubulin antibody. The images were recorded on a computer and were quantified by Image J.

Experimental protocols

To determine the effect of hyperglycemia on cardiac H9c2 cells, cells were cultured with glucose (5.5, 22, 33 mM) DMEM from 12 to 48 h separately. To examine the effect of ethanol on cardiac H9c2 cells under oxidative stress and hyperglycemic conditions, cells were cultured with high glucose (33mM) for 48h before exposure to H₂O₂ (550 μ M) for 20min. Ethanol (1-1000 μ M) was given 1 h before exposure to H₂O₂. Anisomycin (4 μ M) was given 10min before exposure to ethanol. To examine the effect of ethanol on Akt (or ERK, SAPK/JNK) phosphorylation, cells were exposed to ethanol for 1h.

Statistical analysis

Data were collected from repeated experiments and are presented as mean $\pm$ SD. One-way ANOVA and the Student's t test were used for statistical analysis. Differences were considered to be significant at P < 0.05.

Results

Hyperglycemia increases cardiac H9c2 cells mitochondrial injury

Hyperglycemia is a major risk factor for cardiovascular disease. To determine the effects of high doses of glucose on H9c2 cells, we first examined the change of cell viability. As shown in Figure 1, compared to the cells cultured in 5.5mM glucose, treatment of cells with 33mM glucose for 48h decreased the cell viability markedly, implying that hyperglycemia increases H9c2 cells injury. In addition, the $\Delta\Psi_m$ and cell viability were tested to evaluate the oxidative stress injury of cells cultured in different glucose conditions. Compared to the control group, the $\Delta\Psi_m$ and cell viability decreased in the H₂O₂ group, implying that oxidative stress increases mitochondrial injury in different glucose conditions. The data suggests that oxidative stress-induced cell injury is increased in a glucose dependent manner (Figure 2A-2D).

Ethanol prevents oxidative stress-induced cardiac H9c2 cell injury under hyperglycemic conditions

To determine the effects of ethanol (1-1000 μ M) on oxidative stress-induced cardiac H9c2 cell injury under hyperglycemic conditions, we first examined the cell viability change by CCK-8 test. As shown in Figure 3A, compared to the control group, cells treated with H₂O₂ showed a significant decrease in cell viability which was prevented by 10 and 100 μ M ethanol under hyperglycemic conditions, confirming that ethanol prevents oxidative stress-induced cardiac cells injury. As mPTP opening has been proposed as an early indicator of mitochondrial damage [4,9,15], we further examined whether ethanol could inhibit oxidative stress-induced mPTP opening by monitoring changes in TMRE fluorescence with confocal microscopy under hyperglycemic conditions. Figure 3B shows that H₂O₂ decreased the TMRE fluorescence, which was

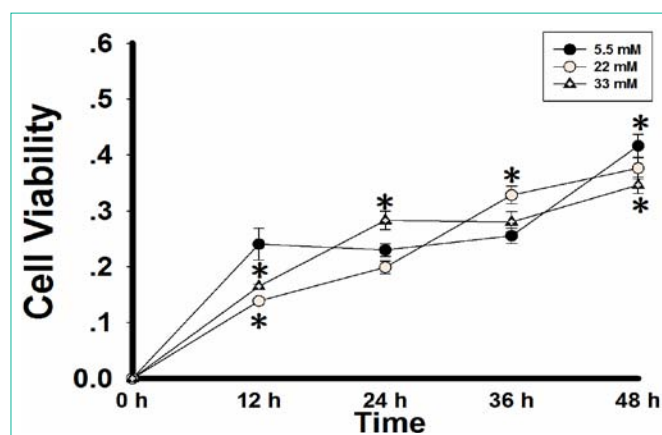


Figure 1: Assay of cell viability in H9c2 cells exposed to different concentrations of glucose by CCK-8 kit. Cells were cultured in culture dishes for 24h and then treated with 5.5, 22 and 33 mM glucose for different time periods. Each bar is the mean $\pm$ SD. of at least 6 experimental observations. *P < 0.05 versus Control (5.5mM).

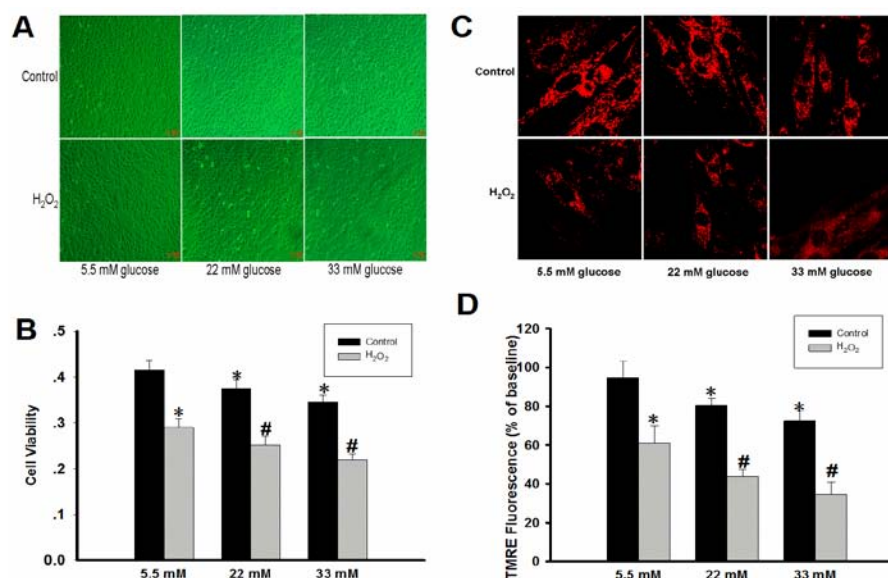


Figure 2: Assay of the cell viability (A, B) and $\Delta\Psi_m$ (C, D) in H9c2 cells treated with H₂O₂ (550 μM). Cells were cultured in culture dishes for 24h and then treated with 5.5, 22 and 33 mM glucose for 48h before exposure to H₂O₂ (550 μM) for 20min. Each bar is the mean $\pm$ SD. of at least 6 experimental observations. *P < 0.05 versus Control; #P < 0.05 versus H₂O₂.

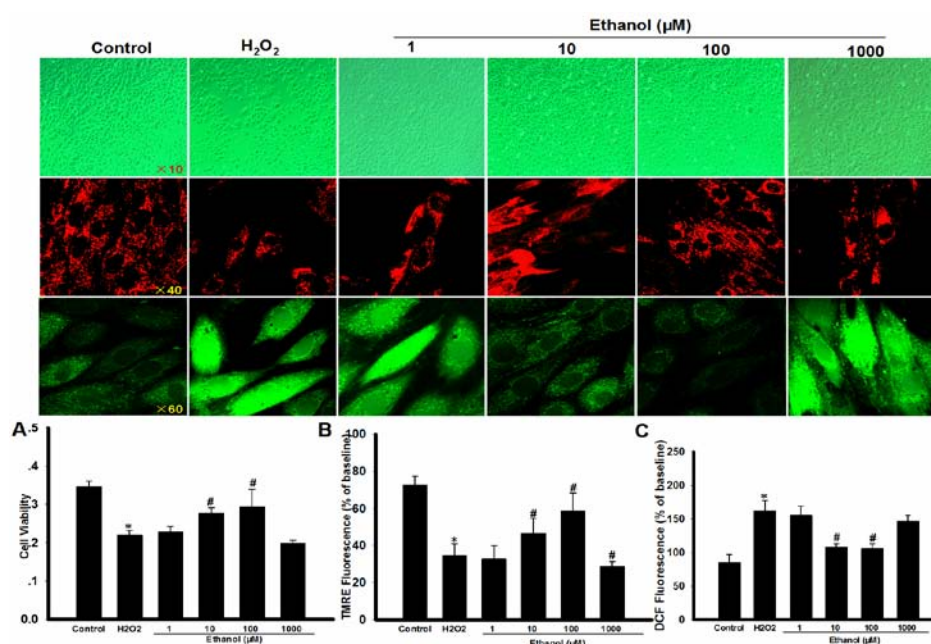


Figure 3: Assay of the cell viability (A), $\Delta\Psi_m$ (B) and ROS production (C) in H9c2 cells treated with different concentrations of ethanol (1-1000 μM) after exposure to high levels of glucose (33 mM) and H₂O₂ (550 μM). Cells were cultured in culture dishes for 24h and then exposed to 33 mM glucose for 48h. The cell viability was tested 1h after exposure to ethanol and then 20min to H₂O₂. Each bar is the mean $\pm$ SD of at least 6 experimental observations. *P < 0.05 versus Control; #P < 0.05 versus H₂O₂.

inhibited by ethanol in a dose dependent manner, implying that ethanol may prevent oxidative stress-induced mPTP opening under hyperglycemic conditions. Excess ROS emission leads to oxidative stress and this has been implicated in numerous pathological conditions. Oxidative stress markedly increased intracellular ROS generation, which was reversed also by ethanol (Figure 3C). These data indicate that ethanol could prevent oxidative stress-induced H9c2 cardiac cell/mitochondrial injury by inhibiting mPTP opening

via decreasing ROS concentration under hyperglycemic conditions.

PI3K/Akt signaling pathway may not be involved in ethanol-induced cardioprotection

To evaluate whether PI3K/Akt signaling pathway could play a role in ethanol-induced cardioprotection in cells cultured in high glucose, Akt activity was analyzed by Western blot analysis. As shown in Figure 4A and Figure 5A, compared to the control group, cells cultured in high dose glucose for 48h were able to increase

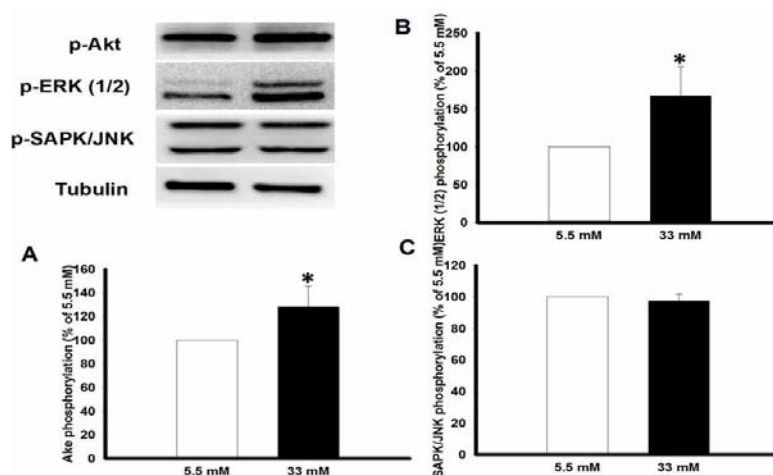


Figure 4: Western blot analysis of Akt phosphorylation at Ser⁴⁷³ (A), ERK1/2 phosphorylation at Thr²⁰²/Tyr²⁰⁴ (B), and SAPK/JNK phosphorylation at Thr¹⁸³/Tyr¹⁸⁵ (C) in cardiac H9c2 cells. Cells were cultured in culture dishes for 24h and then treated with 5.5 and 33 mM glucose for 48h respectively. Each bar is the mean $\pm$ SD. of at least 4 experimental observations. *P < 0.05 versus Control (5.5mM).

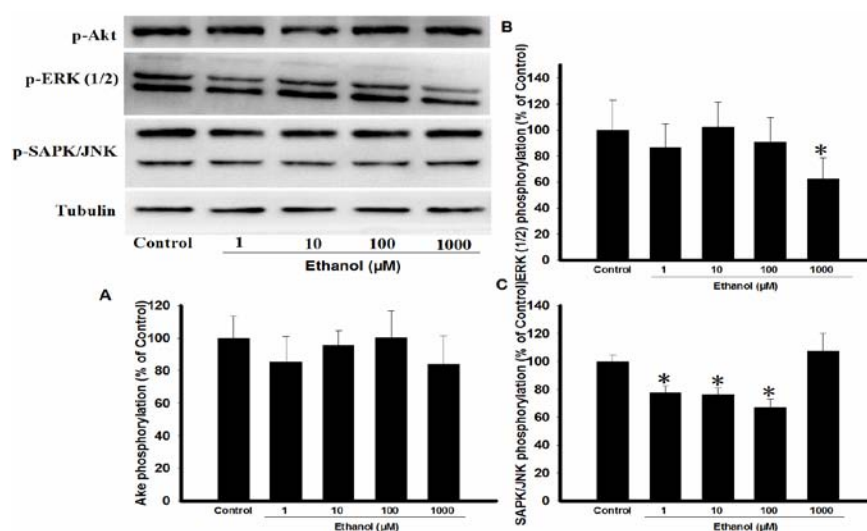


Figure 5: Western blot analysis of Akt phosphorylation at Ser⁴⁷³ (A), ERK1/2 phosphorylation at Thr²⁰²/Tyr²⁰⁴ (B), and SAPK/JNK phosphorylation at Thr¹⁸³/Tyr¹⁸⁵ (C) in cardiac H9c2 cells. Cells were cultured in culture dishes for 24h and then exposed to 33mM glucose for 48h. Cells were treated with ethanol (1-1000 μ M) for 1h. Each bar is the mean $\pm$ SD. of at least 4 experimental observations. *P < 0.05 versus Control; #P < 0.05 versus H₂O₂.

Akt phosphorylation significantly, which could not be changed by different doses of ethanol, implying that PI3K/Akt signaling pathway may have nothing to do with the protective effect of ethanol in this model.

The effect of ERK signaling pathway on ethanol-induced cardioprotection

To determine the potential role of ERK signaling pathway in the cardioprotective effect of ethanol, we first tested if high dose glucose could enhance ERK phosphorylation at Thr²⁰²/Tyr²⁰⁴ in H9c2 cardiac cells. As shown in Figure 4B, compared to the cells treated with 5.5mM glucose, cells treated with 33mM glucose showed a significant higher level of ERK phosphorylation, implying that cells may start automatic protective mechanism by activating ERK during hyperglycemic conditions. However, this effect could not be affected by ethanol, suggesting that ERK signaling pathway may not play a role in the

cardioprotective effect of ethanol (Figure 5B). In contrast, 1000 μ M ethanol significantly decreased ERK phosphorylation, suggesting that high dose ethanol may increase cell injury by inhibiting ERK activity (Figure 5B).

The effect of SAPK/JNK signaling pathway on ethanol-induced cardioprotection

Next, we examined the action of SAPK/JNK signaling pathway on ethanol-induced cardioprotection. Compared to the control group, treatment of cells with 33 mM glucose did not increase SAPK/JNK phosphorylation markedly, but that with ethanol did decrease it, implying that ethanol may reduce oxidative stress-induced cardiac cell damage by down regulating the activity of SAPK/JNK (Figure 4C and 5C). In support of this contention, the effects of ethanol on cell viability, $\Delta\Psi_m$, ROS generation and mitochondrial respiration were reversed by the SAPK/JNK agonist anisomycin, implying that ethanol

may reduce oxidative stress- and high glucose-induced mitochondrial injury by down regulating the activity of SAPK/JNK (Figure 5C-5E).

Discussion

Increasing evidence has accumulated demonstrating an increased risk of myocardial dysfunction in diabetic patients [16-19]. Studies have also demonstrated a direct link between diabetes and ischemic heart disease, especially for myocardial infarction [16]. Although reperfusion is an effective way to treat myocardial infarction, it may produce irreversible damage to the heart through a well-defined molecular event called ischemia/reperfusion (I/R) injury [20]. Oxidative stress has been implicated as a major cause of I/R injury [21]. Previous studies demonstrated that ethanol at low doses can prevent oxidant-induced mPTP opening by upregulating the PI3K/Akt signaling pathway [4]. However, the anti-oxidative stress effects of ethanol during hyperglycemic conditions remain unclear.

The H9c2 cell line derived from embryonic rat hearts maintains some features of cardiac myocytes and has been used extensively in *in vitro* studies [4,9,22]. As diabetic cardiomyopathy is directly related to hyperglycemia, high dose glucose was used to induce cardiac H9c2 cell diabetic injury [22]. In this study, cells treated with high dose glucose for an extended time period had increased cell injury. Further studies demonstrated that ethanol (10-100 μ M) significantly prevented oxidative stress-induced cell injury with the peak at 100 μ M under hyperglycemic conditions. This finding could imply that light but not heavy consumption of alcohol may be beneficial to the diabetic heart.

According to a study by Zhou et al. low concentrations of ethanol fed to mice can increase expression of Akt three- to fivefold, whereas PKC inhibition prevented increases in Akt kinase activity [23]. Since Reperfusion Injury Salvage kinases (RISK) such as Akt and ERK have been proposed to be linked to the prevention of mitochondrial injury [4,24,25], we tested whether these signaling factors are involved in the protective effect of ethanol against oxidative stress- and hyperglycemia-induced mitochondrial injury. Our study demonstrates that PI3K/Akt and ERK pathway signaling may not be involved in the protective effect of ethanol under hyperglycemic conditions, but these factors may play a role in the cell protective effect against hyperglycemia. SAPK/JNK, a member of MAPK family, has been reported to be a target of cardioprotection against oxidative stress and could inhibit mPTP opening [24]. The JNK pathway plays a key role in the proliferation of myocardial fibroblasts induced by high glucose. Inhibiting the JNK pathway may prevent the occurrence and development of diabetic myocardial fibrosis [26]. Our study provided evidence, which suggests that ethanol may prevent oxidative stress-induced mitochondrial injury through inhibiting the activity of SAPK/JNK under hyperglycemic conditions.

Mitochondria are also the primary source of Reactive Oxygen Species (ROS), mainly including superoxide anion radical ($O_2^{\cdot-}$), H_2O_2 and hydroxyl radical ($\cdot OH$) [27]. Although ROS are important signal factors, excess ROS emission leads to oxidative stress, and this has been implicated in numerous pathological conditions, as well as in cardiac I/R injury [24]. Intracellular ROS was measured by staining cells with H2DCF-DA fluorescence. Our findings suggest that ethanol may reduce oxidative stress-induced H9c2 cell injury by inhibiting the SAPK/JNK signaling pathway via inhibiting ROS generation

under hyperglycemic conditions.

Conclusion

In conclusion, the results of the current study demonstrate that low concentration ethanol prevents oxidative stress-induced cardiac H9c2 cell mitochondrial injury by inhibiting SAPK/JNK signaling, which leads to diminished ROS generation under hyperglycemic conditions.

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