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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 μM) prevented H_2O_2-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

ΔΨm: 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 Phosphatidyl Inositol 3-Kinase (PI3K)/v-akt-murinet hymoma 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% CO_2-95% 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

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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 (ΔΨm)

ΔΨm 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 ΔΨm 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 20μM 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 (40μg) 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 (Ser^{473}), ERK (Thr^{202}/Tyr^{204}) and SAPK/JNK (Thr^{183}/Tyr^{185}). 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 H_2O_2 (550μM) for 20min. Ethanol (1-1000 μM) was given 1 h before exposure to H_2O_2. Anisomycin (4μM) 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 ± 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 ΔΨm and cell viability were tested to evaluate the oxidative stress injury of cells cultured in different glucose conditions. Compared to the control group, the ΔΨm and cell viability decreased in the H_2O_2 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 μM) 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 H_2O_2 showed a significant decrease in cell viability which was prevented by 10 and 100 μM 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 H_2O_2 decreased the TMRE fluorescence, which was

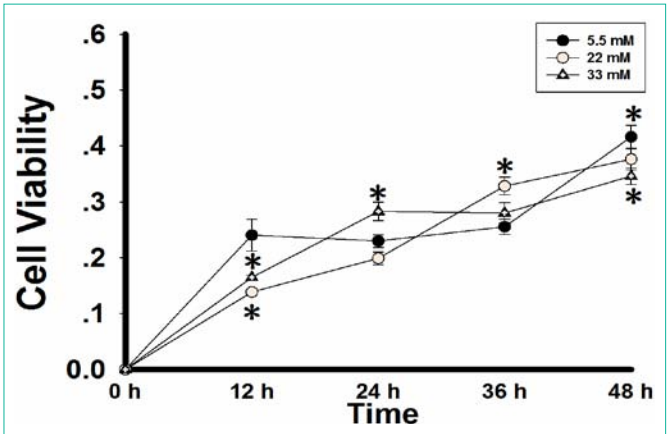

Figure 1: Assay of cell viability in H9c2 cells exposed to different concentrations of glucose by CCK-8 kit. Cells were cultured in culture dishes for 24h and then treated with 5.5, 22 and 33 mM glucose for different time periods. Each bar is the mean ± SD. of at least 6 experimental observations. *P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

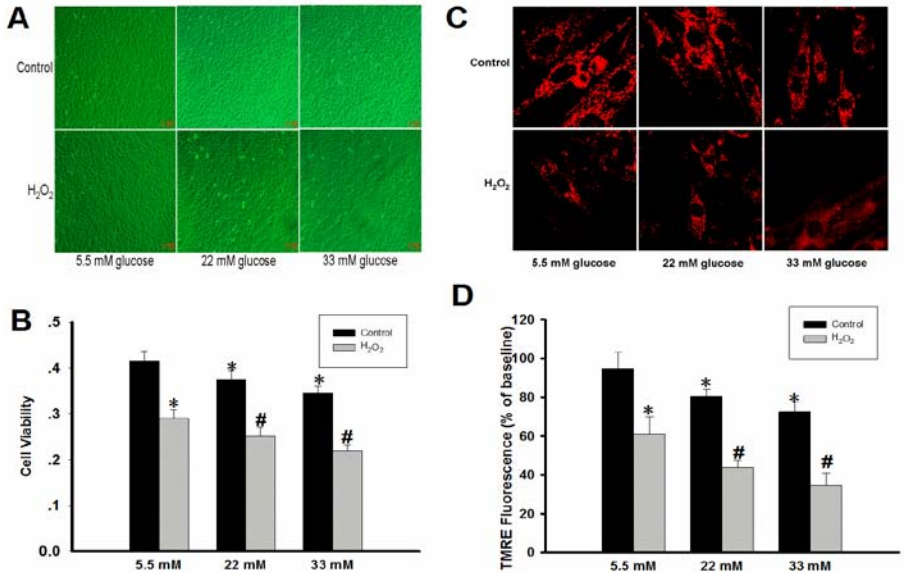

Figure 2: Assay of the cell viability (A, B) and ΔΨm (C, D) in H9c2 cells treated with H_2O_2 (550μM). Cells were cultured in culture dishes for 24h and then treated with 5.5, 22 and 33 mM glucose for 48h before exposure to H_2O_2 (550μM) for 20min. Each bar is the mean ± SD. of at least 6 experimental observations. *P <0.05 versus Control; #P <0.05 versus H_2O_2.

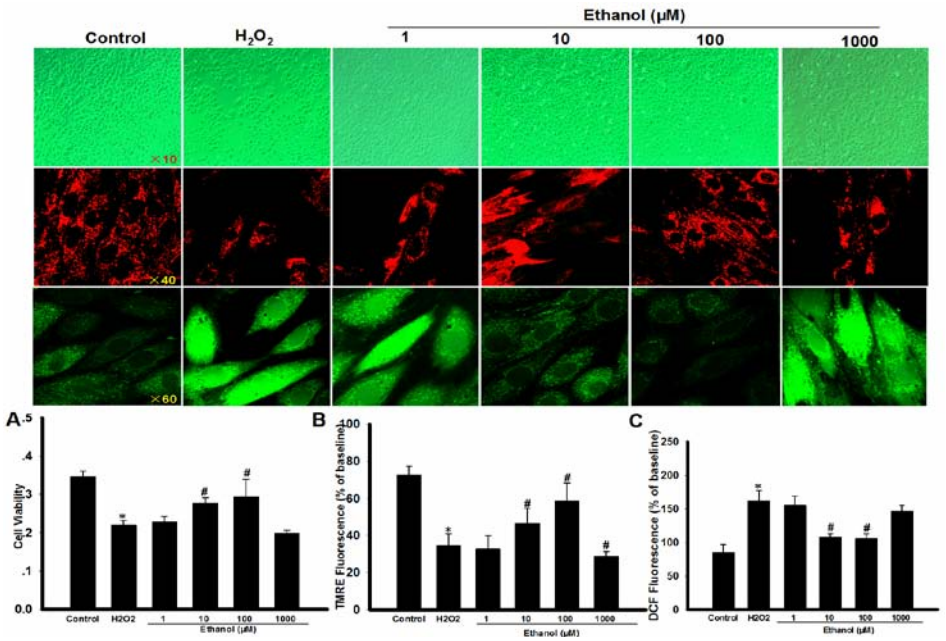

Figure 3: Assay of the cell viability (A), ΔΨm (B) and ROS production (C) in H9c2 cells treated with different concentrations of ethanol (1-1000 μM) after exposure to high levels of glucose (33mM) and H_2O_2 (550μM). Cells were cultured in culture dishes for 24h and then exposed to 33mM glucose for 48h. The cell viability was tested 1h after exposure to ethanol and then 20min to H_2O_2. Each bar is the mean ± SD of at least 6 experimental observations. *P <0.05 versus Control; #P <0.05 versus H_2O_2.

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

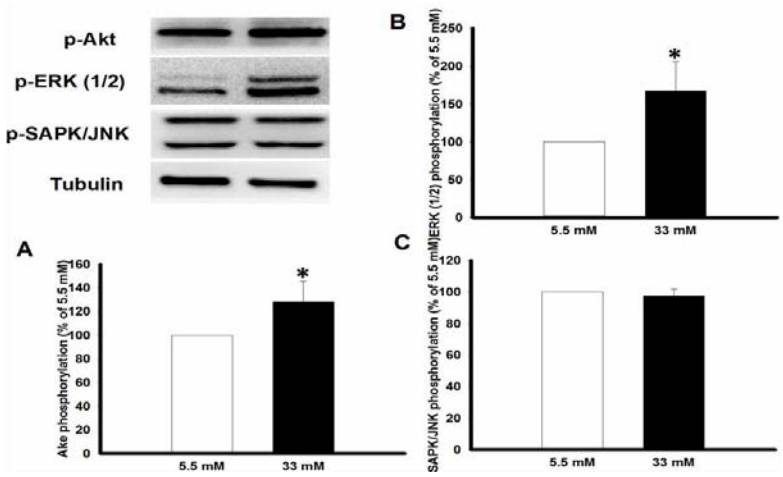

Figure 4: Western blot analysis of Akt phosphorylation at Ser^{473} (A), ERK1/2 phosphorylation at Thr^{202}/Tyr^{204} (B), and SAPK/JNK phosphorylation at Thr^{183}/Tyr^{185} (C) in cardiac H9c2 cells. Cells were cultured in culture dishes for 24h and then treated with 5.5 and 33 mM glucose for 48h respectively. Each bar is the mean ± SD. of at least 4 experimental observations. *P <0.05 versus Control (5.5mM).

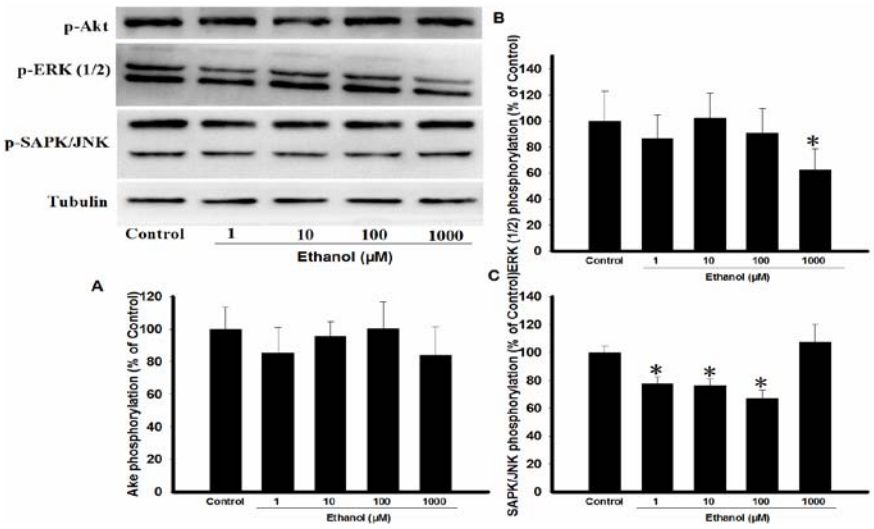

Figure 5: Western blot analysis of Akt phosphorylation at Ser^{473} (A), ERK1/2 phosphorylation at Thr^{202}/Tyr^{204} (B), and SAPK/JNK phosphorylation at Thr^{183}/Tyr^{185} (C) in cardiac H9c2 cells. Cells were cultured in culture dishes for 24h and then exposed to 33mM glucose for 48h. Cells were treated with ethanol (1-1000 μM) for 1h. Each bar is the mean ± SD. of at least 4 experimental observations. *P <0.05 versus Control; #P <0.05 versus H_2O_2.

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 Thr^{202}/Tyr^{204} 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, 1000μM 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, ΔΨm, 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 μM) significantly prevented oxidative stress-induced cell injury with the peak at 100μM under hyperglycemic conditions. This finding could imply that light but not heavy consumption of alcohol may beneficial to the diabetic heart.

According to a study by Zhou et al. low concentrations of ethanol fed to mice can increased 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 (•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.

Acknowledgements

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References

1. Munukutla S, Pan G, Deshpande M, Thandavarayan RA, Krishnamurthy P, Palaniyandi SS. Alcohol Toxicity in Diabetes and Its Complications: A Double Trouble? Alcoholism, clinical and experimental research. 2016; 40: 686-697.
2. Long GH, Cooper AJ, Wareham NJ, Griffin SJ, Simmons RK. Healthy behavior change and cardiovascular outcomes in newly diagnosed type 2 diabetic patients: a cohort analysis of the ADDITION-Cambridge study. Diabetes care. 2014; 37: 1712-1720.
3. Toma A, Paré G, Leong DP. Alcohol and Cardiovascular Disease: How Much is Too Much? Current atherosclerosis reports. 2017; 19: 13.
4. Zhou K, Zhang L, Xi J, Tian W, Xu Z. Ethanol prevents oxidant-induced mitochondrial permeability transition pore opening in cardiac cells. Alcohol and alcoholism (Oxford, Oxfordshire). 2009; 44: 20-24.
5. Kloner RA, Rezkalla SH. To drink or not to drink? That is the question. Circulation. 2007; 116: 1306-1317.
6. Lorenzo O, Ramírez E, Picatoste B, Egido J, Tuñón J. Alteration of energy substrates and ROS production in diabetic cardiomyopathy. Mediators of inflammation. 2013; 2013: 461967.
7. Brien SE, Ronksley PE, Turner BJ, Mukamal KJ, Ghali WA. Effect of alcohol consumption on biological markers associated with risk of coronary heart disease: systematic review and meta-analysis of interventional studies. BMJ (Clinical research ed). 2011; 342: d636.
8. Lindberg ML, Amsterdam EA. Alcohol, wine, and cardiovascular health. Clinical cardiology. 2008; 31: 347-351.
9. Campos JC, Queliconi BB, Bozi LHM, Bechara LRG, Dourado PMM, Andres AM, et al. Exercise reestablishes autophagic flux and mitochondrial quality control in heart failure. Autophagy. 2017; 13: 1304-1317.
10. Lv D, Bai Z, Yang L, Li X, Chen X. Lipid emulsion reverses bupivacaine-induced apoptosis of h9c2 cardiomyocytes: PI3K/Akt/GSK-3β signaling pathway. Environmental toxicology and pharmacology. 2016; 42: 85-91.
11. Cui SX, Xu JM, Hao ZM, Yang LM, Wei T. Puerarin Inhibits Oxidative Stress-Induced Cardiac H9c2 Cell Injury Through Suppressing SAPK/JNK Activation. Latin American Journal of Pharmacy. 2015; 34: 1319-1327.
12. Zhou HZ, Karliner JS, Gray MO. Moderate alcohol consumption induces sustained cardiac protection by activating PKC-epsilon and Akt. American journal of physiology Heart and circulatory physiology. 2002; 283: H165-H174.
13. Zhang SY, Chen G, Wei PF, Huang XS, Dai Y, Shen YJ, et al. The effect of puerarin on serum nitric oxide concentration and myocardial eNOS expression in rats with myocardial infarction. Journal of Asian natural products research. 2008; 10: 373-381.
14. Xu J, Bian X, Liu Y, Hong L, Teng T, Sun Y, et al. Adenosine A(2) receptor activation ameliorates mitochondrial oxidative stress upon reperfusion through the posttranslational modification of NDUFV2 subunit of complex I in the heart. Free radical biology & medicine. 2017; 106: 208-218.

15. Perrelli MG, Tullio F, Angotti C, Cerra MC, Angelone T, Tota B, et al. Catestatin reduces myocardial ischaemia/reperfusion injury: involvement of PI3K/Akt, PKCs, mitochondrial KATP channels and ROS signalling. Pflugers Archiv: European journal of physiology. 2013; 465: 1031-1040.

16. Bhatheja S, Panchal HB, Ventura H, Paul TK. Obesity Cardiomyopathy: Pathophysiologic Factors and Nosologic Reevaluation. The American journal of the medical sciences. 2016; 352: 219-222.

17. Harjutsalo V, Feodoroff M, Forsblom C, Groop PH. Patients with Type 1 diabetes consuming alcoholic spirits have an increased risk of microvascular complications. Diabetic medicine: A journal of the British Diabetic Association. 2014; 31: 156-164.

18. Idewaki Y, Iwase M, Fujii H, Ohkuma T, Ide H, Kaizu S, et al. Association of Genetically Determined Aldehyde Dehydrogenase 2 Activity with Diabetic Complications in Relation to Alcohol Consumption in Japanese Patients with Type 2 Diabetes Mellitus: The Fukuoka Diabetes Registry. PloS one. 2015; 10: e0143288.

19. Kikuchi Y, Iwase M, Fujii H, Ohkuma T, Kaizu S, Ide H, et al. Association of severe hypoglycemia with depressive symptoms in patients with type 2 diabetes: the Fukuoka Diabetes Registry. BMJ open diabetes research & care. 2015; 3: e000063.

20. Karwi QG, Bice JS, Baxter GF. Pre- and post- conditioning the heart with hydrogen sulfide (H_2S) against ischemia/reperfusion injury *in vivo*: a systematic review and meta-analysis. Basic research in cardiology. 2018; 113: 6.

21. Korge P, Ping P, Weiss JN. Reactive oxygen species production in energized cardiac mitochondria during hypoxia/reoxygenation: modulation by nitric oxide. Circulation research. 2008; 103: 873-880.

22. Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. Diabetes. 2002; 51: 1938-1948.

23. Zhou H-Z, Karliner JS, Gray MO. Moderate alcohol consumption induces sustained cardiac protection by activating PKC-epsilon and Akt. American journal of physiology Heart and circulatory physiology. 2002; 283: H165-H174.

24. Gómez-Santos C, Ferrer I, Reiriz J, Viñals F, Barrachina M, Ambrosio S. MPP+ increases alpha-synuclein expression and ERK/MAP-kinase phosphorylation in human neuroblastoma SH-SY5Y cells. Brain research. 2002; 935: 32-39.

25. Hong L, Xi J, Zhang Y, Tian W, Xu J, Cui X, et al. Atrial natriuretic peptide prevents the mitochondrial permeability transition pore opening by inactivating glycogen synthase kinase 3β *via* PKG and PI3K in cardiac H9c2 cells. European journal of pharmacology. 2012; 695: 13-19.

26. Liu LH, Fang CX, Deng YG, Zhou H. C-JUN N-terminal kinase pathway mediates high glucose-induced proliferation of cardiac fibroblasts in rats. Basic & Clinical Medicine. 2013; 33: 199-204.

27. Hosoki A, Yonekura S, Zhao QL, Wei ZL, Takasaki I, Tabuchi Y, et al. Mitochondria-targeted superoxide dismutase (SOD_2) regulates radiation resistance and radiation stress response in HeLa cells. Journal of radiation research. 2012; 53: 58-71.