Original Article

Interleukin-33 prevents high glucose-induced apoptosis in H9c2 cardiac cells by inhibiting reaction oxygen species-activated JNK and p38 MAPK pathway

Dan Liu1,2, Tuohua Mao2,3, Xifeng Lv4, Qizhu Tang2,5

1Department of Radiology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China; 2Cardiovascular Research Institute of Wuhan University, Wuhan 430060, Hubei Province, China; 3Department of Endocrinology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China; 4Department of Nephrology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China; 5Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

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Abstract: Interleukin-33 (IL-33), a new member of IL-1 family, has been shown protective effects on many cardiovascular diseases. The present study was to investigate the protective effects of IL-33 against high glucose (HG)-induced apoptosis in H9c2 cardiac cells and explore the potential mechanisms. H9c2 cardiac cells were exposed to HG with or without IL-33 pre-treatment. Cell viability was assessed by methyl thiazolyl tetrazolium (MTT) assay. Cell apoptosis was detected by flow cytometry. Reactive oxygen species (ROS) was assessed by measuring 2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) oxidation. The protein expressions of cleaved (c)-caspase-3, phosphorylated (p)-JNK and p-p38 MAPK were determined by Western blotting. Exposure of cells to HG resulted in a loss in cell viability and an increase in apoptosis. HG induced ROS production and phosphorylation of JNK and p38 MAPK. Treatment with IL-33 attenuated HG-induced oxidative stress, prevented JNK and p38 MAPK phosphorylation and attenuated cell apoptosis. Our study demonstrates that IL-33 prevents HG-induced H9c2 cardiac cell apoptosis by attenuating ROS formation and inhibiting the activation of JNK and p38 MAPK.

Keywords: IL-33, high glucose, apoptosis, ROS, JNK, p38 MAPK

Introduction

Diabetes mellitus (DM) has become a major threat to human health in the 21st century. Hyperglycaemia is the most important feature of DM. Diabetic cardiomyopathy (DCM) is an important complication of diabetes, which can cause a significant rise of mortality in diabetic patients. It is widely accepted that the diabetic heart is associated with left ventricular diastolic dysfunction, cardiomyocyte hypertrophy, myocardial interstitial fibrosis, increased apoptosis and upregulation of oxidative stress [1]. The mechanisms involved in hyperglycaemia induced myocardial injury are still incompletely clear. Many factors have been reported to be responsible for DCM, such as mitochondrial dysfunction, abnormal cellular metabolism, impaired regulation of intracellular calcium, etc [2, 3].

Oxidative stress is an imbalance between reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) generation and their clearance [4]. Heart is highly susceptible to oxidative damage due to its low contents of antioxidants [3]. It has been confirmed that sustained hyperglycemia can induce ROS generation in diabetics. ROS are important contributors to the pathogenesis and the development of DCM; ROS scavenging by antioxidants prevented hyperglycemia induced cardiac cell death [5, 6]. ROS can induce or mediate the activation of the mitogen-activated protein kinases (MAPKs) [7, 8], which have a pivotal role in cardiovascular disease, including DCM. MAPKs contain three subgroups: the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs, and all of them are involved in both cell growth and cell death [9]. In recent years, accumulating evidence has
demonstrated that inhibitors of MAPKs prevent the damaging effects of HG on cultured cardiomyocytes [10, 11].

Interleukin-33 (IL-33) is the most recently discovered member of the IL-1 superfamily [12]. It is broadly expressed in many tissues including the heart. It has dual function both as a traditional cytokine and as a nuclear factor regulating gene transcription [13, 14]. It has recently been linked to many diseases, such as inflammatory diseases including hypersensitive diseases, cardiovascular diseases and neurodegenerative diseases [15-17]. Researchers have proven that IL-33 can attenuate myocardial infarction after ischaemia/reperfusion (I/R) in vivo and protect cardiomyocytes from anoxia/reoxygenation (A/R)-induced injury in vitro. The protect effects of IL-33 may be related with the regulation of IAP family proteins, reduction of cleaved (c)-caspase-3 and inhibition of the PKCβ/JNK pathway [18, 19]. In DM model, it has been demonstrated that the level of myocardial IL-33 is decreased which results in the increased sensitivity of the diabetic myocardium to I/R. Reduction in IL-33 results in a chronic activation of PKCβII which results in exaggeration of myocardial injury [20]. Administration of recombinant IL-33 to genetically obese diabetic mice led to reduced adiposity, reduced fasting glucose and improved glucose and insulin tolerance [21]. Accordingly, we can speculate that IL-33 maybe has the beneficial effect on cardiomyocyte injury induced by hyperglycemia. Therefore, we designed the experiment to investigate the effect of IL-33 on high glucose (HG)-induced H9c2 cardiac cell apoptosis and explore the possible mechanisms.

Material and methods

Cell culture and treatments

The H9c2 cell line derived from rat cardiac tissue was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in Dulbecco's modified essential medium (DMEM; Hyclone; Logan, Utah) containing glucose (5.6 mmol/l) supplemented with 10% fetal bovine serum (FBS; Hangzhou Sijiqing BiologicaI Engineering Materials Co., Ltd.; Hangzhou, China), 100 U/ml of penicillin G and 100 μg/ml streptomycin in humidified air (5% CO₂) at 37°C. After reaching 80% confluence, the cells were serum-starved for 12 h in DMEM and then given different treatments.

H9c2 cardiac cells were treated with 30 mmol/l glucose to establish the cell injury model induced by HG. In order to control the osmotic effects of HG, the normal culture media containing 5.6 mmol/l glucose was supplemented with 24.4 mmol/l mannitol. To explore the effect of IL-33 (Prospec; Rehovot, Israel), cells were treated with various concentrations of IL-33 (0.2-5 ng/ml) for 1 h prior to exposure to HG. To investigate the functional mechanism of IL-33, cells were pretreated with N-acetyl-L-cysteine (NAC, a ROS scavenger, 1000 μmol/l; Sigma-Aldrich Inc.; Shanghai, China), SP600125 (a selective inhibitor of JNK, 10 μmol/l; Sigma-Aldrich Inc.) or SB203580 (a selective inhibitor of p38 MAPK, 3 μmol/l; Sigma-Aldrich, Inc.) for 1 h prior to exposure to HG.

Cell viability assay

Cell viability was evaluated using methyl thiazolyl tetrazolium (MTT; Amresco Inc.; Solon, OH) method. The cells were seeded in 96-well plates at a density of 1×10⁵ cells/ml and challenged with HG for 24 h in the absence or presence of pre-treatment with IL-33, NAC, SP600125 or SB203580. After that, 20 μl MTT solution (5 mg/ml) was added to each well. After 4 h incubation, the medium was carefully aspirated and the purple formazan crystals were solubilized with 150 μl Dimethyl Sulphoxide (DMSO; Amresco Inc.). Optical density (OD) was measured at 570 nm. The relative ratio of cell viability was calculated according to the formula: Cell viability (%) = (OD treatment group/OD control group) ×100%.

H9c2 cell apoptosis assay

Cell apoptosis was detected by flow cytometry with an Annexin V-FITC/propidium iodide (PI) assay (Keygen Biotechnology; Nanjing, China). After the indicated treatments, cells were dispersed with 0.25% trypsin (Amresco Inc.) and rinsed twice with phosphate-buffered saline (PBS; Wuhan goodbio technology CO., LTD; Wuhan, China). Subsequently, they were resuspended in 300 μl binding buffer after centrifugation (4°C, 1500 r/min) for 5 min. After that,
cells were incubated with 5 μl annexin V-FITC and 5 μl PI for 15 min at room temperature in the dark. Flow cytometry was performed on a fluorescence-activated cell sorter (FACS) scan flow cytometer (BD Biosciences; San Jose, Calif) within 1 h after staining. Excitation wavelength is 488 nm, and emission wavelength is 530 nm.

Measurement of intracellular ROS accumulation

2', 7'-dichlorodihydrofluorescein diacetate (DCFH-DA) (Beyotime Institute of Biotechnology; Haimen, China) and flow cytometry were used to detect intracellular ROS level. After the designated treatment, cells were isolated and incubated with serum-free medium containing DCFH-DA (at a final concentration of 10 μmol/l) at 37°C for 20 min. Subsequently, the cells were washed thrice with culture medium in order to eliminate the residual DCFH-DA, and then mixed with 300 μl culture medium. The 2', 7'-dichlorodihydrofluorescein (DCF) fluorescence was detected by flow cytometer, with excitation at 488 nm and emission at 525 nm. The ROS level was represented by the DCF intensity.

Western blot assay for expression of protein

The H9c2 cardiac cells were washed by PBS and then lysed in RIPA lysis buffer (Wuhan goodbio technology CO., LTD). After standing for 30 min at 4°C, the mixture was centrifuged at 12000 r/min for 10 min. The supernatant was collected. Protein concentrations were determined using the bicinchoninic acid protein assay kit (Wuhan goodbio technology CO., LTD). Equal quantities of proteins were submitted to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride membrane. After blocking the membrane with Tris-buffered saline-Tween 20 (TBST, 0.1% Tween 20) containing 5% fat-free milk for 1 h at room temperature, the membrane was washed and then incubated overnight with primary antibodies against c-caspase-3 (1:1000 dilution; Cell Signaling Technology; Danvers, MA), phosphor (p)-JNK (1:500 dilution; Santa Cruz Biotechnology; Santa Cruz, CA), p-p38 MAPK (1:1000 dilution; Cell Signaling Technology) or β-actin (1:500 dilution; Santa Cruz Biotechnology) for protein loading control at 4°C. After washed trice with TBST for 5 min each, the membranes were incubated with 1:3000 dilution horseradish peroxidase-conjugated secondary antibodies (KPL; Gaithersburg, Maryland) for 1 h at room temperature. Then, immune complexes were detected using the enhanced chemiluminescence (ECL; Wuhan goodbio technology CO., LTD) method, and immunoreactive bands were quantified using Alpha Imager (Alpha Innotech, San Leandro, CA).

Statistical analysis

All data were presented as the mean ± standard deviation. Differences between groups were analyzed by one-way analysis of variance (ANOVA) with the SPSS 17.0 statistical package, and followed by LSD test (equal variances assumed) or Dunnett's T3 test (equal
variances not assumed). A $P$ value less than 0.05 was considered to be statistically significant.

**Results**

**Cell viability**

The H9c2 cardiac cells in control group were considered 100% viability. Viability of cells treated with HG decreased to 62.06% ± 3.58% ($P<0.01$). When the cells were pretreated with 1 ng/ml IL-33 (67.41% ± 3.57%, $P<0.05$) or 5 ng/ml IL-33 (77.90% ± 2.87%, $P<0.01$) prior to exposure to HG, the cell viability increased significantly compared to HG treated group. 5 ng/ml IL-33 was used in the subsequent study. Pre-treatment of cells with NAC, SP600125 or SB203580 before exposure to HG also prevented HG-induced decreases in cell viability ($P<0.01$) (**Figure 1A**).

There were no significant differences in cell viability between control samples and cells treated with the indicated concentrations of IL-33, mannitol, NAC or MAPK inhibitors alone (**Figure 1B**).

*Figure 2. H9c2 cardiac cells were challenged with HG in the absence or presence of pre-treatment with IL-33, NAC, SP600125 or SB203580 and harvested at 24 h after exposure to HG. n=3; **, $P<0.01$ compared with control group; ##, $P<0.01$ compared with the HG-treated group.*
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Quantitation of apoptosis

Compared with the control group, HG treated group had a significantly higher cell apoptotic ratio (22.01% ± 3.09% vs. 1.21% ± 0.48%, \( P < 0.01 \)). However, the increased percentage of the apoptotic cells was reduced by the pre-treatment with IL-33, SP600125, SB203580 or NAC (\( P < 0.01 \)) (Figure 2).

Expression of c-caspase-3

Exposure of H9c2 cardiac cells to HG resulted in an increase in c-caspase-3 expression (\( P < 0.01 \)), while the expression of c-caspase-3 was decreased in the IL-33, SP600125, SB203580 or NAC pre-treatment group compared with that in the HG group (\( P < 0.05 \)) (Figure 3). The results are in line with the finding of cell apoptosis.

Evaluation of oxidative stress

There was a marked increase in production of ROS in the HG group compared to the control group (\( P < 0.01 \)). However, the increased amount of ROS induced by HG was reduced by IL-33. NAC exerted a similar ROS-decreasing effect (\( P < 0.01 \)) (Figure 4).

Expression levels of p-JNK and p-p38 MAPK

In the HG group, expression levels of p-JNK and p-p38 MAPK were significantly increased compared to those in the control group (\( P < 0.01 \)). However, pre-treatment with IL-33 or NAC decreased the phosphorylation of JNK and p38 MAPK (\( P < 0.05 \)) (Figure 5).

Discussion

DCM is a poorly understood disease and the underlying mechanisms remain to be completely elucidated. Cardiomyocyte apoptosis has been increasingly recognized as a contributing factor in the pathogenesis of DCM. Recent studies indicate the protective effect of IL-33 in various diseases including atherosclerosis, obesity, glucose homeostasis disorders [22-25]. The present study was conducted to investigate the protective effects of IL-33 against HG-induced cardiomyocyte apoptosis and explore the potential mechanisms.

There is clear evidence linking increased oxidative stress, in particular ROS production, to the development of diabetic cardiovascular complications [26]. In diabetes, alteration of energy substrates can directly or indirectly induce ROS formation in the myocardium. Overproduction of ROS can increase inflammatory, cardiac hypertrophy, cardiac fibrosis and cell death events [1]. Antioxidant treatments have demonstrated beneficial effects in DCM pathology [10, 11, 27]. NAC, precursor of glutathione, is one of well-known broad spectrum antioxidants. Our study revealed that HG decreased H9c2 cardiac cell viability, enhanced cell apoptosis, increased c-caspase-3 expression and promoted ROS formation. NAC diminished HG-induced cytotoxicity and apoptosis in H9c2 cardiac cells. These results were in line with the previous papers.

MAPK family is important mediator of intracellular signaling. It is generally believed that the activation of ERK1/2 is implicated in the regulation of cell growth and survival. The activation of JNK and p38 MAPK mediates cell apoptosis [28]. However, there is some evidence suggesting that ERK1/2 also contributes to cell death of cardiomyocytes [29]. Studies have demonstrated that ROS can mediate the activation of the MAPKs. A lot of cellular stimuli that induce ROS production also in parallel can activate...
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MAPKs [30, 31]. The prevention of ROS accumulation by antioxidants inhibits MAPK activation [27]. Similar to previous studies, our results showed that the inhibitor of JNK or p38 MAPK depressed HG induced cell apoptosis, and NAC blocked the phosphorylation of JNK and p38 MAPK in HG treated H9c2 cells. It implied that JNK and p38 MAPK were downstream signal transduction pathways of ROS and they played an important role in HG-induced deteriorative effects on H9c2 cells.

IL-33 is a new member of the IL-1 family of cytokines that promotes Th2 type immune responses by signaling through the ST2L and IL-1R accessory protein (IL-1RACp) dimeric receptor complex. Furthermore, the biological effects of IL-33 are limited by a soluble decoy form of ST2 (sST2) [32]. Recent data

Figure 4. H9c2 cardiac cells were challenged with HG in the absence or presence of pre-treatment with IL-33 or NAC and harvested at 24 h after exposure to HG. n=3; **, P<0.01 compared with control group; ##, P<0.01 compared with the HG-treated group.

Figure 5. H9c2 cardiac cells were challenged with HG in the absence or presence of pre-treatment with IL-33 or NAC and harvested at 30 min after exposure to HG. n=3; **, P<0.01 compared with control group; #, P<0.05 compared with the HG-treated group; ##, P<0.01 compared with the HG-treated group.
show that IL-33/ST2 pathway exerts protective effects on many cardiovascular diseases, such as atherosclerosis, myocardial infarction and cardiac remodeling [33]. In our study, we found that the H9c2 cell viability increased and cell apoptosis decreased with IL-33 pretreatment before exposure to HG. The beneficial effects of IL-33 on the HG-induced insults in H9c2 cells may be associated with its antioxidant effect. Because we found out IL-33 prevented the HG-induced increase in intracellular ROS generation and NAC could block HG-induced cytotoxicity. We also provided evidence that IL-33 could inhibit the phosphorylation of JNK and p38-MAPK. As previously mentioned, the activation of MAPK was involved in HG-induced cytotoxicity. Accordingly, it can be concluded that the inhibitory effect of IL-33 on the activation of JNK and p38-MAPK may be one of the important mechanisms responsible for its protection against HG-induced deteriorative effects in H9c2 cells. According to the above analysis, MAPKs are downstream signal transduction pathways of ROS, this inhibitory effect of IL-33 on the activation of JNK and p38-MAPK may be associated, at least in part, with its antioxidation.

In conclusion, in the present study we have demonstrated: 1) JNK and p38 MAPK were downstream signal transduction pathways of ROS and they played an important role in HG-induced injuries in H9c2 cardiac cell; 2) IL-33 protects the H9c2 cardiac cell from HG-induced apoptosis by inhibiting ROS-activated JNK and p38 MAPK pathway. Therefore, IL-33 may have therapeutic potential to prevent hyperglycemia enhancing diabetic cardiomyopathy. However, future studies may be conduct to confirm these findings in cardiomyocytes and myocardial tissue.

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Disclosure of conflict of interest

None.

Address correspondence to: Qizhu Tang, Departments of Cardiology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China. E-mail: qztang@whu.edu.cn

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