Original Paper

The Opening of ATP-Sensitive K⁺ Channels Protects H9c2 Cardiac Cells Against the High Glucose-Induced Injury and Inflammation by Inhibiting the ROS-TLR4-Necroptosis Pathway

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Key Words

ATP-sensitive K⁺ channel • Reactive oxygen species • Toll-like receptor 4 • Necroptosis • High glucose • H9c2 cardiac cells

Abstract

Background/Aims: Hyperglycemia activates multiple signaling molecules, including reactive oxygen species (ROS), toll-like receptor 4 (TLR4), receptor-interacting protein 3 (RIP3, a kinase promoting necroptosis), which mediate hyperglycemia-induced cardiac injury. This study explored whether inhibition of ROS-TLR4-necroptosis pathway contributed to the protection of ATP-sensitive K⁺ (K_{ATP}) channel opening against high glucose-induced cardiac injury and inflammation. **Methods:** H9c2 cardiac cells were treated with 35 mM glucose (HG) to establish a model of HG-induced insults. The expression of RIP3 and TLR4 were tested by western blot. Generation of ROS, cell viability, mitochondrial membrane potential (MMP) and secretion of inflammatory cytokines were measured as injury indexes. **Results:** HG increased the expression of TLR4 and RIP3. Necrostatin-1 (Nec-1, an inhibitor of necroptosis) or TAK-242 (an inhibitor of TLR4) co-treatment attenuated HG-induced up-regulation of RIP3. Diazoxide (DZ, a mitochondrial K_{ATP} channel opener) or pinacidil (Pin, a non-selective K_{ATP} channel opener) or N-acetyl-L-cysteine (NAC, a ROS scavenger) pre-treatment blocked the up-regulation of TLR4 and RIP3. Furthermore, pre-treatment with DZ or Pin or NAC, or co-treatment with TAK-242 or Nec-1 attenuated HG-induced a decrease in cell viability, and increases in ROS generation, MMP loss and inflammatory cytokines secretion. However, 5-hydroxy decanoic acid (5-HD, a mitochondrial K_{ATP} channel blocker) or glibenclamide (Gli, a non-selective K_{ATP} channel blocker) pre-treatment did not aggravate HG-induced injury and inflammation. **Conclusion:**

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K_{ATP} channel opening protects H9c2 cells against HG-induced injury and inflammation by inhibiting ROS-TLR4-necroptosis pathway.

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Introduction

Accumulating evidence has shown that in the adaptive responses to pathophysiological stresses, such as ischemia [1-3] and hyperglycemia [4-7], ATP-sensitive K^+ (K_{ATP}) channels function as the high-fidelity metabolic sensors, which couple intracellular metabolic state to membrane excitability [8, 9] and play a homeostatic role ranging from blood glucose regulation to cardioprotection [3, 7, 10]. Cardiomyocytes contain K_{ATP} channels in both the sarcolemma (surface membrane) [11] and mitochondria [12, 13]. It has previously been suggested that the K_{ATP} channels play fundamental roles in protecting the heart from injury related to ischemia [1-3]. In addition, the opening of K_{ATP} channels also contributes to the regulation of cardiac mitochondrial function [14] and cardioprotection induced by ischemic preconditioning [14-16]. Noteworthy, several reports have shown a correlation between dysfunction in the K_{ATP} channel gating and insulin secretory disorder [17, 18], such as neonatal diabetes [18]. Additionally, a previous study has indicated diabetes mellitus (DM) is associated with the dysfunction of cardiovascular K_{ATP} channels [17]. More recently, we have demonstrated that high glucose (35 mM glucose, HG) significantly decreases the expression level of K_{ATP} channels and that the opening of K_{ATP} channels protects against the HG-induced injuries, including over-production of reactive oxygen species (ROS) in H9c2 cardiac cells [7]. However, what are the influences of these important channels on the HG-triggered intracellular signaling mechanisms is far less understood.

To date, multiple factors, such as ROS [7, 19-21], chronic inflammation [20, 22], cell death (including apoptosis and necroptosis) [7, 19-21], and activity of several signaling molecules, for example, toll-like receptors (TLRs) [22, 23], have been demonstrated to implicate in the hyperglycemia-induced cardiac injury. Importantly, the above factors can interact or crosstalk, consisting of the complicated intracellular signal mechanisms. For example, we have revealed a positive interaction between ROS and receptor-interacting protein 3 (RIP3, a mediator of necroptosis) in the HG-treated cardiac cells [21]. Necroptosis (also called programmed necrosis) [24], a newly identified programmed cell death, is a caspase-independent death program. RIP3-mediated necroptosis has now been recognized to be a common cell death pathway implicated in cytokine-, virus-, and genotoxic stress-induced cell death. Liu et al. and our team have shown that the expression level of RIP3 is up-regulated in diabetic rat hearts [25] and the HG-treated cardiac cells [21]. Increasing evidence indicates that necroptosis is activated by several pathways, such as tumor necrosis factor- α (TNF- α) [26, 27], TLRs [28, 29] and ROS [21, 30]. Since HG has been shown to induce ROS generation, TNF- α and TLR4 expression as well as necroptosis, and there is a link between ROS, TNF- α and necroptosis in human FADD-deficient Jurkat cells [31], thus we speculated that ROS-TLR4-necroptosis pathway may be an important intracellular signaling mechanism which contributed to the HG-induced cardiac injury and inflammation. Furthermore, we explored whether the inhibition of this pathway contributes to the protective effect of K_{ATP} channels against the HG-induced injury and inflammation in H9c2 cardiac cells. Here, we reported that treatment of H9c2 cardiac cells with HG significantly increased ROS generation, and the expression levels of TLR4 and RIP3; TAK-242 (an inhibitor of TLR4) and Necrostatin-1 (Nec-1, a specific inhibitor of necroptosis) attenuated the HG-induced up-regulation of the expression level of RIP3; NAC, a scavenger of ROS, ameliorated the increased expression levels of TLR4 and RIP3 induced by HG. These results indicated that there is a link between ROS, TLR4 and RIP3 in the HG-treated cardiac cells. Importantly, treatment of the cells with diazoxide (DZ, a mitochondrial K_{ATP} channel opener) or pinacidil (Pin, a non-selective K_{ATP} channel opener) alleviated the HG-induced ROS overproduction and an increase in the expression levels of TLR4 and RIP3, along with an increase in cell viability, decreases

in the dissipation of mitochondrial membrane potential (MMP) and the secretion levels of inflammatory cytokines (IL-1 β and TNF- α).

Materials and Methods

Materials

Rhodamine 123 (Rh 123), 2',7'-dichlorofluorescein diacetate (DCFH-DA), N-acetyl-L cysteine (NAC, a scavenger of ROS) and necrostatin-1 (Nec-1) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-RIP3 antibody (Catalog Number: ab152130) and Anti-TLR4 antibody (Catalog Number: ab22048) were purchased from Abcam Inc. (Cambridge, MA, USA); anti- β -actin antibody (Catalog Number: GTX109639) was purchased from GeneTex (Irvine, CA, USA). The Cell Counting Kit-8 (CCK-8) was offered by Dojindo Laboratories (Kumamoto, Japan). IL-1 β and TNF- α ELISA kits were purchased from Cusabio Biotech Co. Ltd (Wuhan, China). Horseradish peroxidase (HRP) conjugated secondary antibody and BCA protein quantification kit were purchased from KangChen Bio-tech (Shanghai, China). Diazoxide (DZ, a mitochondrial K_{ATP} channel opener), pinacidil (Pin, a non-selective K_{ATP} channel opener), 5-hydroxydecanoic acid (5-HD, a mitochondrial K_{ATP} channel blocker) and glibenclamide (Gli, a non-selective K_{ATP} channel blocker) were purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). TAK-242 (an inhibitor of TLR4) was obtained from Invivogen (San Diego, CA, USA). The enhanced chemiluminescence (ECL) solution was purchased from KeyGen Biotech (Nanjing, China). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were purchased from Gibco BRL (Grand Island, NY, USA). H9c2 cardiac cells were supplied by Sun Yat-sen University Experimental Animal Center (Guangzhou, China).

Cell culture and treatments

H9c2 cardiac cells, derived from rat embryonic ventricular cardiomyocytes, were grown in DMEM, supplemented with 10% FBS in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. The culture medium was replaced with fresh medium every 2-3 days. When grown to about 80% confluency, the cells were expanded to new culture plates.

In order to evaluate the role of ROS-TLR4-necroptosis pathway in the HG-induced injury and inflammation, H9c2 cells were pre-treated with 1 mM NAC for 60 min before exposure to HG for 24 h or co-treated with 30 μ M TAK-242 or 100 μ M Nec-1 and HG for 24 h. To assess the effects of blocking K_{ATP} channels on the HG-induced injury and inflammation, H9c2 cardiac cells were treated with 100 μ M 5-HD or 1 mM Gli for 30 min before a 24 h HG exposure. To investigate whether the protective effect of K_{ATP} channel opening was associated with the inhibition of ROS-TLR4-necroptosis pathway, H9c2 cardiac cells were treated with 100 μ M DZ or 50 μ M Pin for 30 min before exposing to HG for 24 h.

Western blot analysis

H9c2 cardiac cells were seeded in 60-mm culture dishes. After being subjected to the indicated treatments, the cells were harvested by scraping and centrifugation (16,000 $\times$ g for 15 min at 4 °C) after lysing with RIPA buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) at 4 °C for 30 min. Protein concentration was determined using the BCA protein quantification kit. Loading buffer was added to the cytosolic extracts and after boiling for approximately 5 min, equal amounts of supernatant from each sample were loaded on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes followed by blocking of the membranes with fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T) containing 5% fat-free milk] for approximately 90 min at room temperature. Then the membranes were incubated with either anti-TLR4 or anti- β -actin or anti-RIP3 antibody (1:1,000 dilution) in freshly prepared TBS-T with 3% fat-free milk overnight with slow agitation at 4 °C temperature. Following 3 washes with TBS-T, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:2,500 dilution) in TBS-T with 3% fat-free milk for 90 min at room temperature. Then the membranes were washed 3 times with TBS-T solution for 15 min. The immunoreactive signals were visualized by using ECL detection. In order to quantifying the protein expression, the X-ray films were scanned and analyzed with Image J 1.47i software. The experiment was repeated 5 times.

Cell viability assay

The CCK-8 assay was applied to detect the cellular viability. H9c2 cardiac cells were digested and seeded in 96-well growth-medium plate at a concentration of 1×10^4 cells/ml and incubated at 37 °C. After the indicated treatments, the cells were washed twice with PBS. Then 10 μ l CCK-8 test solution and 90 μ l DMEM were added to each well, and the cells were incubated at 37 °C for 2.5 h. The absorbance value (*A* value) at the 450 nm wavelength was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The means of the *A* value of 3 wells in the indicated groups were used to calculate the percentage of cellular viability according to the following formula: cell viability (%) = ($A_{\text{treatment group}} / A_{\text{control group}}$) $\times 100\%$. The experiment was repeated 5 times.

Measurement of intracellular ROS level

The intracellular level of ROS was monitored using the redox-sensitive fluorescent dye DCFH-DA. Briefly, the culture medium was removed and the cells were washed 3 times with PBS. The cells were incubated with DCFH-DA (10 μ M) which was diluted by serum-free medium at 37 °C during the last 20 min. The cells were then washed 5 times with PBS and the relative amount of fluorescent product was captured using a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). Image J 1.47i software was apply to analyze the mean fluorescence intensity (MFI) of DCFH-DA, which indirectly showed the level of cell ROS. The experiment was carried out 5 times.

Measurement of mitochondrial membrane potential (MMP)

MMP was assessed using a fluorescent dye, Rh 123, an indicator of mitochondrial polarization that preferentially enters the mitochondria based on the highly negative MMP. The depolarization of MMP leads to the loss of Rh 123 from the mitochondria and a decrease in intracellular green fluorescence. H9c2 cardiac cells were planted in 24-well plates. After the indicated treatments, the cells were washed 3 times with PBS. Then the cells were incubated with 1 μ M Rh 123 at 37 °C for 30 min in an incubator and then washed 3 times with PBS. Rh 123 fluorescence was measured over the entire field of vision using a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). The MFI of Rh 123 from 5 random fields was analyzed using Image J 1.47i software and was regard as an index of the level of MMP. The experiment was carried out 5 times.

Measurement of the concentrations of IL-1 β and TNF- α

H9c2 cells were seeded in 96-well growth-medium plates. After the indicated treatments, the levels of IL-1 β and TNF- α in the culture supernatant were evaluated by ELISA according to the manufacturer's introductions (Cusabio Biotech Co. Ltd). The concentrations of IL-1 β and TNF- α were changed into absorbance (*A*) monitored at 450 nm wavelength using a microplate reader. The means of the *A* value of 3 wells in the indicated groups were used to calculate the percentage of induction of IL-1 β and TNF- α according to the following formula: percent induction (%) = ($A_{\text{treatment group}} / A_{\text{control group}}$) $\times 100\%$. The experiment was performed for 5 times.

Statistical analysis

All data are expressed as the means $\pm$ SEM. Differences between groups were determined by one-way analysis of variance (ANOVA) using SPSS 17.0 software (SPSS, Inc, Chicago, IL, USA) followed by the least significant difference (LSD) post hoc comparison test. Differences were considered statistically significant at *P*-value <0.05.

Results

HG promotes the expression levels of TLR4 and RIP3 in H9c2 cardiac cells

In order to explore the effects of HG on the TLR4 and RIP3 expression levels in H9c2 cardiac cells, Firstly, a time-response experiment was performed, i.e. the cells were exposed to HG for 1, 3, 6, 9, 12, 24, 36 and 48 h, respectively. As shown in Fig. 1A and B, after exposure to HG for 6 h, the expression level of TLR4 began to increase, reaching the maximum level at 24 h. Then the TR4 expression level gradually decreased at 36 and 48 h, but was still significantly higher than the one in the control group (*P*<0.01). Similarly, the expression

level of RIP3 was significantly increased after the cells were exposure to HG for 1 h, and the maximum increase in expression levels was observed after exposure to HG for 24 h (Fig. 1C and D). Secondly, a dose-response experiment was carried out. The cells were exposed to 15, 20, 25, 30, 35, 40, and 45 mM glucose for 24 h, respectively. Exposure of the cells to 20~45 mM glucose up-regulated the expression level of TLR4, and the maximum increase was observed at 35 and 40 mM points (Fig. 1E and F); while exposure of the cells to 15~45 mM glucose all significantly increased the expression level of RIP3 ($P<0.05$), reaching the maximum level at 30 mM point ($P<0.01$, Fig. 1E and G). These results indicated that HG induces the activation of TLR4 pathway and necroptosis in H9c2 cardiac cells.

The inhibitor of necroptosis and TLR4 inhibitor ameliorate the HG-induced an increase in the expression level of RIP3 in H9c2 cardiac cells

In agreement with the above results, HG increased the expression level of RIP3 in H9c2 cardiac cells. However, co-treatment of the cells with 100 μ M necrostatin-1 (Nec-1, a specific inhibitor of necroptosis) and HG for 24 h significantly attenuated the increased RIP3 expression induced by HG (Fig. 2A and B). 100 μ M Nec-1 alone did not influence the basal expression level of RIP3. To clarify whether TLR4 is involved in the HG-induced cardiac necroptosis, the effect of TAK-242 (an inhibitor of TLR4) on the expression level of RIP3 was observed. As shown in Fig. 2C and D, co-treatment of the cells with 30 μ M TAK-242 and HG for 24 h dramatically attenuated the increased expression level of RIP3 induced by HG. Alone, 30 μ M TAK-242 did not alter the basal expression level of RIP3. These results suggested that Nec-1 can inhibit the HG-induced necroptosis which is activated by TLR4.

The scavenger of ROS attenuates the up-regulation of TLR4 and RIP3 expression induced by HG in H9c2 cardiac cells

To investigate the role of ROS in the HG-induced an increase in the expression levels of TLR4 and RIP3, the cells were treated with 1 mM NAC (a scavenger of ROS) for 60 min prior to exposure to HG for 24 h. The results of western blot assay showed that pre-treatment of the cells with NAC attenuated the up-regulation of the expression levels of TLR4 and RIP3 induced by HG (Fig. 3). Alone, NAC did not alter the basal expression levels of TLR4 and

Fig. 1. High glucose (HG) promotes the expression levels of toll-like receptor 4 (TLR4) and receptor-interacting protein 3 (RIP3) in H9c2 cardiac cells. H9c2 cardiac cells were treated with 35 mM glucose for 1, 3, 6, 9, 12, 24, 36 and 48 h and were treated with 15, 20, 25, 30, 35, 40 and 45 mM glucose for 24 h, respectively. (A, C and E) TLR4 and RIP3 expression levels were tested by western blot assay. (B, D, F and G) Densitometric analysis of the expression levels of TLR4 and RIP3 in (A), (C) and (E). Data are shown as the means $\pm$ SEM ($n=5$). * $p<0.05$ vs the control group (the cells were treated with 5.5 mM glucose for 24 h); ** $p<0.01$ vs the control group.

Fig. 2. The inhibitor of necroptosis and toll-like receptor 4 (TLR4) inhibitor ameliorate the high glucose (HG)-induced an increase in the expression level of receptor-interacting protein 3 (RIP3) in H9c2 cardiac cells. (A) H9c2 cardiac cells were treated with HG in the absence or presence of 100 μ M Nec-1 for 24 h. (C) The cells were treated with or without 30 μ M TAK-242 and HG for 24 h. (A and C) RIP3 expression levels were detected by western blot assay. (B and D) Densitometric analysis of RIP3 expression levels in (A and C). The data are shown as the means $\pm$ SEM (n=5). ** $p < 0.01$ vs the control group; ## $p < 0.01$ vs the HG-treated group.

RIP3. These results indicated that ROS is involved in the HG-induced activation of TLR4-necroptosis pathway in H9c2 cardiac cells.

The opening of K_{ATP} channels attenuates the HG-induced an increase in the expression levels of TLR4 and RIP3 in H9c2 cardiac cells

In order to explore the effects of the opening of K_{ATP} channels on the HG induced up-regulation of TLR4 and RIP3 expression levels, H9c2 cardiac cells were treated with 100 μ M DZ (a mitochondrial K_{ATP} channel opener) or 50 μ M Pin (a non-selective K_{ATP} channel opener) for 30 min prior to exposure to HG for 24 h. Results of western blot analysis showed that pre-treatment of the cells with 100 μ M DZ or 50 μ M Pin for 30 min prior to exposure to HG considerably attenuated the up-regulation of the expression levels of TLR4 (Fig. 4A and B) and RIP3 (Fig. 4A and C) induced by HG. Besides, there was no significant difference between DZ and Pin in the inhibitory effect on the up-regulation of TLR4 and RIP3 expression levels induced by HG. Alone, treatment of the cells with 100 μ M DZ or 50 μ M Pin did not alter the basal expression levels of TLR4 and RIP3. These results indicated that the HG-induced activation of TLR4-necroptosis pathway can be suppressed by the opening of K_{ATP} channels in H9c2 cardiac cells.

Inhibition of ROS-TLR4-necroptosis pathway activation is implicated in the protection of the opening of K_{ATP} channels against the HG-induced cytotoxicity in H9c2 cardiac cells

Due to the above results that ROS activated the TLR4-necroptosis pathway in the HG-treated H9c2 cardiac cells, we further assessed the role of ROS-TLR4-necroptosis pathway activation in the HG-induced cytotoxicity. The cells were pre-treatment with 1 mM NAC for 60 min before exposure to HG for 24 h or co-treated with 30 μ M TAK-242 or 100 μ M

Fig. 3. Reactive oxygen species (ROS) contributes to the high glucose (HG)-induced an increase in the expression levels of toll-like receptor 4 (TLR4) and receptor-interacting protein 3 (RIP3) in H9c2 cardiac cells. H9c2 cardiac cells were treated with HG in the absence or presence of pre-treatment with 1 mM NAC (a scavenger of ROS) for 60 min prior to exposure to HG for 24 h. (A) TLR4 and RIP3 expression levels were detected by western blot assay. (B and C) Densitometric analysis of the TLR4 and RIP3 expression levels in (A). Data are shown as the means $\pm$ SEM (n=5). ** $p < 0.01$ vs the control group; ## $p < 0.01$ vs the HG-treated group.

Fig. 4. The opening of K_{ATP} channels attenuates the high glucose (HG)-induced an increase in the expression levels of toll-like receptor 4 (TLR4) and receptor-interacting protein 3 (RIP3) in H9c2 cardiac cells. H9c2 cardiac cells were treated with HG in the absence or presence of pre-treatment with 100 μ M DZ or 50 μ M Pin for 30 min prior to exposure to HG. (A) TLR4 and RIP3 expression levels were tested by western blot analysis. (B and C) Densitometric analysis of the TLR4 and RIP3 expression levels in (A). Data are shown as the means $\pm$ SEM (n=5). ** $p < 0.01$ vs the control group; ## $p < 0.01$ vs the HG-treated group.

Nec-1 and HG for 24 h. Similar to the anti-cytotoxicity effect of the opening of K_{ATP} channels [7], the results of CCK-8 assay (Fig. 5) showed that pre-treatment of the cells with NAC or co-treatment of the cells with TAK-242 or Nec-1 dramatically blocked the HG-induced cytotoxicity, leading to an increase in cell viability, indicating the involvement of ROS-TLR4-necroptosis pathway activation in the HG-induced cytotoxicity. In addition, we observed the effects of K_{ATP} channel blockers, including 5-hydroxy decanoic acid (5-HD, a mitochondrial K_{ATP} channel blocker) or glibenclamide (Gli, a non-selective K_{ATP} channel blocker) on the HG-induced cardiac cytotoxicity. As shown in Fig. 5, pre-treatment of the cells with 100 μ M 5-HD or 1 mM Gli for 30 min did not aggravate the HG-induced cytotoxicity ($P > 0.05$). Taken together, our results suggested that inhibiting the activity of ROS-TLR4-necroptosis may be one of the mechanisms underlying the anti-cytotoxicity effect of the opening of K_{ATP} channels which attenuated ROS generation [7] and the expression levels of TLR4 and RIP3 (Fig. 4).

Inhibition of ROS-TLR4-necroptosis pathway activation is involved in the protection of the opening of K_{ATP} channels against the HG-induced oxidative stress in H9c2 cardiac cells

In agreement with our recent studies [7, 19-21], after H9c2 cardiac cells were exposed to HG for 24 h, the intracellular production of ROS was significantly increased (Fig. 6Ab and

Fig. 5. The opening of K_{ATP} channels and the inhibition of ROS-TLR4-necroptosis pathway attenuate the high glucose (HG)-induced cytotoxicity in H9c2 cardiac cells. H9c2 cardiac cells were treated with HG in the absence or presence of pre-treated with 100 μ M DZ or 50 μ M Pin or 100 μ M 5-HD or 1 mM Gli for 30 min or 1 mM NAC for 60 min prior to exposure to HG or co-treated with 30 μ M TAK-242 or 100 μ M Nec-1 and HG for 24 h. Cell counting kit-8 (CCK-8) assay was used to exam the cell viability. Data are shown as the means $\pm$ SEM (n=5). ** p <0.01 vs the control group; ## p <0.01 vs the HG-treated group.

Fig. 6. The opening of K_{ATP} channels and the inhibition of ROS-TLR4-necroptosis pathway attenuate the HG-induced oxidative stress in H9c2 cardiac cells. (A) After the cells were treated with the indicated treatments, intracellular ROS generation was measured by 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining followed by photofluorography. (Aa) Control group. H9c2 cardiac cells were treated with (Ab) 35 mM glucose (HG) for 24 h; (Ac) 100 μ M DZ or (Ad) 50 μ M Pin for 30 min prior to exposure to HG for 24 h; (Ae) 1 mM NAC for 60 min prior to exposure to HG for 24 h; (Af) treated with 30 μ M TAK-242 or (Ag) 100 μ M Nec-1 and HG for 24 h; (Ah) treated with 100 μ M DZ or (Ai) 50 μ M Pin for 30 min followed by 24 h of DMEM culture; (Aj) treated with 1 mM NAC for 60 min followed by 24 h of DMEM culture; (Ak) treated with 30 μ M TAK-242 or (Al) 100 μ M Nec-1 and DMEM for 24 h; (Am) treated with 100 μ M 5-HD or (An) 1 mM Gli for 30 min prior to exposure to HG for 24 h; (Ao) treated with 100 μ M 5-HD or (Ap) 1 mM Gli for 30 min followed by 24 h of DMEM culture. (B) Quantitative analysis mean fluorescence intensity (MFI) in (Aa-p) using Image J 1.47i software. Data are presented as the means $\pm$ SEM (n=5). ** p <0.01 vs the control group; ## p <0.01 vs the HG-treated group.

B). The increased ROS production was attenuated by the treatment with 100 μ M DZ (Fig. 6Ac and B) or 50 μ M Pin (Fig. 6Ad and B) for 30 min prior to exposure to HG for 24 h. However, pre-treatment of the cells with 100 μ M 5-HD (Fig. 6Am and B) or 1 mM Gli (Fig. 6An and B) did not aggravate the HG-induced ROS overproduction (P >0.05). To determine whether the activation of ROS-TLR4-necroptosis pathway activation plays a role in the HG-induced oxidative stress, the H9c2 cardiac cells were pre-treated with 1 mM NAC for 60 min before a 24 h HG exposure or co-treated with 30 μ M TAK-242 or 100 μ M Nec-1 and HG for 24 h. It

Fig. 7. The opening of K_{ATP} channels and the inhibition of ROS-TLR4-necroptosis pathway attenuate the high glucose (HG)-induced mitochondrial insults in H9c2 cardiac cells. (A) After the cells were treated with the indicated treatments, MMP was examined using a fluorescent dye, Rhodamine 123 (Rh 123) staining followed by photofluorography. (Aa) Control group. H9c2 cardiac cells were treated with (Ab) 35 mM glucose (HG) for 24 h; (Ac) 100 μ M DZ or (Ad) 50 μ M Pin for 30 min prior to exposure to HG for 24 h; (Ae) treated with 1 mM NAC for 60 min prior to exposure to HG for 24 h; (Af) treated with 30 μ M TAK-242 or (Ag) 100 μ M Nec-1 and HG for 24 h; (Ah) treated with 100 μ M DZ or (Ai) 50 μ M Pin for 30 min followed by 24 h of DMEM culture; (Aj) treated with 1 mM NAC for 60 min followed by 24 h of EMEM culture; (Ak) 30 μ M TAK-242 or (Al) 100 μ M Nec-1 and DMEM for 24 h; (Am) treated with 100 μ M 5-HD or (An) 1 mM Gli for 30 min prior to exposure to HG for 24 h; (Ao) treated with 100 μ M 5-HD or (Ap) 1 mM Gli for 30 min followed by 24 h of DMEM culture. (B) Quantitative analysis of the mean fluorescence intensity (MFI) in (Aa-p) using Image J 1.47i software. Data are presented as the means $\pm$ SEM (n=5). ** p <0.01 vs the control group; ## p <0.01 vs the HG-treated group.

was showed that pre-treatment with NAC (Fig. 6Ae and B) or co-treatment with TAK-242 (Fig. 6Af and B) or Nec-1 (Fig. 6Ag and B) dramatically reduced the increased intracellular ROS generation induced by HG, suggesting the contribution of the activation of ROS-TLR4-necroptosis pathway to the HG-induced oxidative stress and the involvement of inhibition of this pathway in the protective effect of opening K_{ATP} channels against the oxidative stress.

Inhibition of ROS-TLR4-necroptosis pathway activation contributes to the protection of the opening of K_{ATP} channels against the HG-induced mitochondrial insults in H9c2 cardiac cells

In agreement with our previous studies [7, 19-21], exposure of H9c2 cardiac cells to HG for 24 h significantly induced mitochondrial damage, as indicated by a loss of MMP (Fig. 7b and B). Moreover, pre-treatment of the cells with 100 μ M DZ (Fig. 7Ac and B) or 50 μ M Pin (Fig. 7Ad and B) for 30 min before exposure to HG for 24 h attenuated the HG-induced a loss of MMP. However, pre-treatment of the cells with 100 μ M 5-HD (Fig. 7Am and B) or 1 mM Gli (Fig. 7An and B) did not aggravate the HG-induced MMP loss (P >0.05). For the purpose of exploring the role of the activation of ROS-TLR4-necroptosis pathway in the HG-induced dissipation of MMP, H9c2 cardiac cells were treated with 1 mM NAC for 60 min before exposure to HG for 24 h, or co-treated with 30 μ M TAK-242 or 100 μ M Nec-1 and HG for 24 h. As shown in Fig. 7, pre-treatment of the cells with NAC (Fig. 7Ae and B) or co-treatment of the cells with TAK-242 (Fig. 7Af and B) or Nec-1 (Fig. 7Ag and B) and HG

Fig. 8. The opening of K_{ATP} channels and the inhibition of ROS-TLR4-necroptosis pathway attenuate the high glucose (HG)-induced secretion of interleukin (IL)-1 β and tumor necrosis factor- α (TNF- α) in H9c2 cardiac cells. H9c2 cardiac cells were treated with HG for 24 h in the absence or presence of pre-treatment with 100 μ M DZ or 50 μ M Pin or 100 μ M 5-HD or 1 mM Gli for 30 min or 1 mM NAC for 60 min prior to exposure to HG or co-treatment with 30 μ M TAK-242 or 100 μ M Nec-1 and HG for 24 h. Enzyme-linked immunosorbent assay (ELISA) was performed to detect the secretion levels of (A) IL-1 β and (B) TNF- α in cell supernatants. Data are shown as the means $\pm$ SEM (n=5). ** p <0.01 vs the control group; ## p <0.01 vs the HG-treated group.

markedly blocked a loss of MMP induced by HG, demonstrating that the activation of ROS-TLR4-necroptosis pathway plays a critical role in the HG-induced mitochondrial damage in H9c2 cardiac cells and that the inhibition of this pathway participates in the protection of opening of K_{ATP} channels against the HG-induced dissipation of MMP.

Inhibition of ROS-TLR4-necroptosis pathway activation contributes to the protection of the opening of K_{ATP} channels against the HG-induced inflammatory response in H9c2 cardiac cells

In agreement with our recent studies [20, 21], after H9c2 cardiac cells were treated with HG for 24 h, the secretion levels of IL-1 β (Fig. 8A) and TNF- α (Fig. 8B) were markedly increased. However, pre-treatment of the cells with 100 μ M DZ or 50 μ M Pin for 30 min prior to exposure to HG significantly attenuated the increased levels of IL-1 β and TNF- α induced by HG, suggesting the inhibitory effect of the opening of K_{ATP} channels on the secretion levels of IL-1 β and TNF- α . Interestingly, there was no significant difference between DZ and Pin in their inhibitory effects on the production of inflammatory cytokines induced by HG. However, pre-treatment of the cells with 100 μ M 5-HD or 1 mM Gli did not aggravate HG-induced up-regulation of the secretion levels of IL-1 β and TNF- α (P >0.05). Additionally, pre-treatment of the cells with 1 mM NAC for 60 min before exposure to HG for 24 h or co-treatment of the cells with 30 μ M TAK-242 or 100 μ M Nec-1 and HG for 24 h also obviously alleviated the increased secretion levels of IL-1 β and TNF- α (Fig. 8A and B), indicating that the activation of ROS-TLR4-necroptosis pathway contributes to the HG-induced inflammatory response in H9c2 cardiac cells.

Discussion

ROS are highly reactive molecules that have been reported to be a key factor in the pathogenesis of diabetic complications, such as diabetic cardiomyopathy [32]. ROS not only induce cardiac cytotoxicity, apoptosis and mitochondrial damage [33], but also activate some signal pathways, for example, mitogen-activated protein kinase (MAPK) which is involved in the HG-induced injury [33]. However, the relationship between oxidative stress (ROS) and the pathobiology of cardiac injury and inflammation is not completely clear, largely due to a

lack of understanding of the intracellular mechanisms by which ROS interact with the other signal molecules under the HG conditions. Thus to investigate the intracellular mechanisms underlying the role of ROS in activating the signaling cascades is of great importance. In the present study, we provide a novel evidence to show an important intracellular signal pathway: ROS-TLR4-necroptosis, which contributes to the HG-induced cardiac injury and inflammation. Importantly, we further demonstrated that the inhibiting of this important signal pathway contributes to the protective effect of opening of the K_{ATP} channels against the injury and inflammatory response in the HG-treated H9c2 cardiac cells.

ROS from mitochondrial and other cellular sources have been traditionally regarded as toxic by-products of metabolism with the potential to cause damage to lipids, proteins, and DNA [34, 35]. The diabetes-related studies have shown that ROS plays an important role in the hyperglycemia-induced cardiac injury [32, 33]. Several lines of evidence have shown that the development of diabetic cardiovascular complications is associated with some of the ROS-activated signaling pathways [33, 36]. For example, hyperglycemia promotes the development of choroidal neovascularisation by ROS-activated signal transducer and activator of transcription 3 (STAT 3) pathway in retinal pigment epithelial (RPE) cells [36]. A recent study also shows that the ROS-activated MAPK pathway attributes to the HG-induced cardiac cell injury [33]. However, the intracellular mechanisms responsible for the hyperglycemia-induced cardiac injury and inflammation are complicated, several signal molecules, including TLR4 [22, 23] and RIP3 [21, 25] have been shown to participate in the cardiac injury caused by hyperglycemia. But what is the relationship between ROS, TLR4 and RIP3 is still unclear in the HG-treated cardiac cells.

TLRs are an important family of pattern recognition receptors (PRRs) that provoke innate immune response. To date, 11 human and 13 mouse TLRs have been cloned [37]. Among the TLRs, TLR4 is one of the most expressed in cardiomyocytes [38, 39]. The TLR4 expression is increased in a plethora of inflammatory disorders, including atherosclerosis and diabetes [40, 41]. Mohammad et al. [42] demonstrated that the expression level of TLR4 is enhanced in type 1 diabetic NOD mice, correlating with increased proinflammatory cytokines. In addition, activation of TLR4 is associated with insulin resistance in adipocytes [43]. Also, Song et al. [43] indicated the increased TLR4 mRNA expression in differentiating adipose tissue of db/db mice. Collectively, these results suggest a potential role for TLR4 in the pathology of diabetes with limited mechanistic details. However, the data examining the role of ROS in the HG-induced an increase in TLR4 expression in cardiac cells are incompletely clear.

In the recent years, a novel mechanism called “programmed necrosis” or “necroptosis” has been considered as a critical mediator of cell death in the hearts [44]. *In vitro* studies have demonstrated the TNF- α -dependent formation of a complex between RIP1 and another kinase, RIP3 which is an essential step for eliciting necroptosis [45, 46]. In this process, RIP3 have been shown to play an important role, regulating phosphorylation of RIP1, a necessary step in necroptosis [45, 47]. Increasing observations have indicated contribution of RIP3 to ischemic cardiac damage [48, 49] and inflammation [50]. For example, RIP3 expression is increased in ischemic hearts and RIP3 deficiency protects mice hearts against ischemic injury [49]. Furthermore, the expression of RIP3 is enhanced in diabetic rat hearts [25] and the HG-treated cardiac cells [21], suggesting involvement of RIP3 in the hyperglycemia-induced cardiac injury. Of note, the activation of TLR4 has been reported to induce necroptosis, and ROS can also elicit necroptosis in cardiomyocytes [21] and other types of cells [50, 51]. Although HG can induce ROS generation and the expression of TLR4 and RIP3, and there is a link among ROS, TNF- α and necroptosis in human FADD-deficient Jurkat cells [32], it remains unclear whether there is the ROS-TLR4-necroptosis pathway in H9c2 cardiac cells under HG condition.

To confirm this, we used the following strategies: First, it was shown that HG markedly up-regulated the expression level of TLR4 and NAC, a ROS scavenger, attenuated the increased TLR4 expression induced by HG. Second, HG significantly enhanced the expression level of RIP3, a kinase promoting necroptosis and TAK-242 (an inhibitor of TLR4) or Nec-1

(a specific inhibitor of necroptosis) ameliorated the up-regulation of RIP3 expression level by HG. Third, NAC also alleviated the increased RIP3 expression level induced by HG. Taken together, our results clearly demonstrated that there is a link among ROS, TLR4 and RIP3, and that ROS mediates the HG-induced activation of TLR4 and necroptosis. Schenk et al. [31] more recently showed that ROS regulate Smac mimetic/TNF- α -induced necroptosis signaling, which strengthens our findings that ROS plays a critical role in activation of TLR4 and necroptosis induced by HG in H9c2 cardiac cells. Next, our further studies revealed that NAC or TAK-242 or Nec-1 respectively attenuated the HG-induced cardiac injuries and inflammation, as indicated by an increase in cell viability, and a decrease in ROS generation, dissipation of MMP and the secretion levels of IL-1 β as well as TNF- α . To our best knowledge, we provided the first evidence that the ROS-TLR4-necroptosis pathway is an important intracellular mechanism underlying the HG-induced cardiac injuries and inflammation. Interestingly, we demonstrated a positive feedback loop between ROS generation and activity of TLR4, i.e. the inhibitory of NAC on TLR4 activation versus the one of TAK-242 on ROS generation, existing in the HG-treated H9c2 cardiac cells. Although a few studies indicated that ROS [32, 50, 51] or TLR4 [29] induces necroptosis in some types of cells, we speculated that a positive interaction between ROS and TLR4 activation may play a key role in the HG-induced necroptosis, by which causes the cardiac injury and inflammation. To test this hypothesis, further study is needed.

K_{ATP} channels are important sensors and effectors of the metabolic status of cardiomyocytes and prominently involved in the regulation of insulin secretion [17, 18], cardioprotection [1-3] and cellular adaptation to stress, such as oxidation stress [3]. Several reports have shown the relationship between dysfunction in K_{ATP} channel gating and diabetes [17]. A previous study reported that nicorandil, a mitochondrial K_{ATP} channel opener, protects from diabetes through inhibition of the production of ROS in the streptozotocin-treated rats [52]. More recently, we also observed that the HG reduces a decrease in the cardiac expression of K_{ATP} channels and that opening of K_{ATP} channels protects cardiac cells against the HG-induced injury, including ROS production [7]. Based on these findings [7, 21], this study explored the effects of opening of K_{ATP} channels on the HG-stimulated ROS-TLR4-necroptosis pathway and cardiac inflammation. In agreement with our recent data [7], the findings of current study showed that diazoxide (DZ, a mitochondrial K_{ATP} channel opener) or pinacidil (Pin, a non-selective K_{ATP} channel opener) significantly attenuated the HG-induced cardiac injuries, including ROS overproduction. In addition, DZ and Pin also ameliorated the HG-induced cardiac inflammation, evidenced by a decrease in the secretion levels of IL-1 β and TNF- α . Unexpectedly, pre-treatment of the cells with a mitochondrial or a non-selective K_{ATP} channel blocker did not aggravate HG-induced cytotoxicity, oxidative stress, mitochondrial dysfunction and inflammatory response. One tentative explanation is that under the HG condition, the functional activities of K_{ATP} channels had been greatly depressed, thus, the blockers of K_{ATP} channel did not aggravate the injuries and inflammatory response induced by HG. To confirm this hypothesis, further studies are needed. Importantly, our results revealed that DZ and Pin markedly depressed the HG-induced increase in the expression levels of TLR4 and RIP3. Combining with the above results that the ROS-activated TLR4-necroptosis pathway is implicated in the HG-induced cardiac injury and inflammation, these findings suggested that the inhibition of ROS-TLR4-necroptosis pathway may be a critical intracellular mechanism responsible for the cardiac protection effect of opening of K_{ATP} channels and extend the previous studies [7, 21].

Conclusion

In conclusion, here we demonstrate, for the first time, that the opening of K_{ATP} channels protects H9c2 cardiac cells against the HG-induced injury and inflammation by inhibiting the ROS-TLR4-necroptosis pathway which may be a key intracellular mechanism underlying the role of ROS in the hyperglycemia-induced cardiac damages. Mechanistic understanding of diabetic cardiac injury and the cardioprotection effect of opening of cardiac K_{ATP} channels

may provide new insights into the development of strategies for the management of diabetic cardiac injury and inflammation.

Acknowledgments

Wenzhu Zhang and Jun Lan conceived and designed the study; Weijie Liang, Meiji Chen, Dongdan Zheng and Mingcai Song performed the experiments; Jianhao Li analyzed the data; Weijie Liang and Jianqiang Feng wrote the paper. All authors have read and approved the final manuscript.

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Disclosure Statement

All authors declare no conflict of interest.

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