A novel damage mechanism: Contribution of the interaction between necroptosis and ROS to high glucose-induced injury and inflammation in H9c2 cardiac cells

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Abstract. Recently, a novel mechanism known as ‘programmed necrosis’ or necroptosis has been shown to be another important mechanism of cell death in the heart. In this study, we investigated the role of necroptosis in high glucose (HG)-induced injury and inflammation, as well as the underlying mechanisms. In particular, we focused on the interaction between necroptosis and reactive oxygen species (ROS) in H9c2 cardiac cells. Our results demonstrated that the exposure of H9c2 cardiac cells to 35 mM glucose (HG) markedly enhanced the expression level of receptor-interacting protein 3 (RIP3), a kinase which promotes necroptosis. Importantly, co-treatment of the cells with 100 µM necrostatin-1 (a specific inhibitor of necroptosis) and HG for 24 h attenuated not only the increased expression level of RIP3, but also the HG-induced injury and inflammation, as evidenced by an increase in cell viability, a decrease in ROS generation, the attenuation of the dissipation of mitochondrial membrane potential and a decrease in the secretion levels of inflammatory cytokines, i.e., interleukin (IL)-1β and tumor necrosis factor (TNF-α). Furthermore, treatment of the cells with 1 mM N-acetyl-L-cysteine (a scavenger of ROS) for 60 min prior to exposure to HG significantly reduced the HG-induced increase in the RIP3 expression level, as well as the injury and inflammatory response described above. Taken together, the findings of this study clearly demonstrate a novel damage mechanism involving the positive interaction between necroptosis and ROS attributing to HG-induced injury and inflammation in H9c2 cardiac cells.

Introduction

Accumulating evidence indicates that hyperglycemia is recognized as the most important factor inducing almost all cardiovascular complications associated with chronic diabetes, such as diabetic cardiomyopathy (DCM) (1–4). Multiple factors have been demonstrated to participate in hyperglycemia-induced cardiac injury, such as reactive oxygen species (ROS) generation (3–5), apoptosis (3,6–8) and the activity of several signaling molecules, including mitogen-activated protein kinase (MAPK) (3,4,6,9), p53 (7,10) and nuclear factor-kB (NF-kB) (11,12). In addition, inflammation is also involved in high glucose (HG)-induced cardiomyocyte injury (13). More recently, we indicated that the NF-kB and interleukin-1β (IL-1β) pathways are implicated in the HG-elicited injury and inflammation in H9c2 cardiac cells (13). However, the mechanisms responsible for the deteriorative effects of hyperglycemia on cardiomyocytes are complex, and are not yet fully understood. Thus, to explore the detailed mechanisms underlying hyperglycemia-induced cardiomyocyte injury is important for the prevention and treatment of diabetic cardiovascular complications.

Recently, a novel mechanism known as ‘programmed necrosis’ or necroptosis has been considered as another important mediator of cell death in the heart (14). Similar to apoptosis, necroptosis is tightly regulated by distinct molecules, but leads to the typical morphological characteristics of necrosis, such as defects in membrane integrity and inflammation, thus combining the features of both mechanisms (14–16). In vitro studies have indicated that the tumor necrosis factor-α (TNF-α)-dependent formation of a complex between receptor-interacting protein (RIP1) and another kinase, RIP3 is an essential step for inducing necroptosis (15,17,18). In this
process, RIP3 appears to play an important role, controlling RIP1 phosphorylation, a necessary step in necroptosis (15,18).

Increasing evidence has demonstrated that necroptosis is involved in a number of pathological processes in cardiovascular diseases (19-27). In hearts affected by ischemia/reperfusion (I/R), RIP1 and RIP3 expression and phosphorylation have been shown to be increased, and the necroptosis inhibitor, necrostatin-1 (Nec-1) reduces the infarct size (23-25). RIP3 expression has also been shown to be enhanced in hearts affected by ischemia and RIP3 deficiency protects mouse heart function (26). In addition, Luede et al revealed that RIP3 mediates the inflammatory response in mice with myocardial infarction (26). Collectively, the above-mentioned studies suggest that necroptosis is implicated in ischemic cardiac lesions (23-26) and inflammation (27). However, the exact role of necroptosis in diabetic cardiac injury and inflammation remains unclear.

ROS are highly reactive molecules that have been considered to function both as second messengers of TNF-α-elicited cell death and modulators of signaling pathways (28,29). Since both ROS and necroptosis have been reported to be involved in cell death and inflammation, the interaction between ROS and necroptosis has recently attracted attention. Classically, the execution of necroptosis is believed to involve the generation of ROS and mitochondrial dysfunction (30,31). On the other hand, RIP3 has been demonstrated to be a key regulator in energy metabolism-associated ROS generation, which partially accounts for the ability of RIP3 to promote necrosis (16,32). In addition, RIP3 has been repeatedly reported to regulate ROS production (18,26,33). Of note, a more recent study demonstrated that in BV6/TNF-α-treated Jurkat cells, there was a positive interaction between necroptosis and ROS, as on the one hand, radical scavengers reduced necroptosis, but on the other hand, ROS generation was decreased by the knockdown of RIP1 or RIP3 (34). Since both necroptosis and ROS play critical roles in cell death in the heart (14), it would of interest to explore whether there is a positive interaction between necroptosis and ROS in hyperglycemia-induced cardiac injury and inflammation in order to provide a novel mechanistic explanation for diabetic cardiac lesions.

In this study, we report that HG induces necroptosis-dependent cardiac injury and inflammation. Furthermore, there was a positive interaction between necroptosis and ROS generation, which plays important roles in HG-induced injury and inflammation in H9c2 cardiac cells.

Materials and methods

Materials. Anti-RIP3 antibody (cat. no. ab152130) was purchased from Abcam (Cambridge, MA, USA); anti-GAPDH antibody (cat. no. 10494-1-AP) was purchased from Proteintech Group, Inc. (Wuhan, China). Dulbecco’s modified Eagle’s medium (DMEM) medium and fetal bovine serum (FBS) were purchased from Gibco-BRL (Grand Island, NY, USA). The BCA protein quantification kit and horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody were obtained from KangChen Bio-tech (Shanghai, China). N-acetyl-L-cysteine (NAC), rhodamine 123 (Rh123), Nec-1 and 2,7’-dichlorofluorescein diacetate (DCFH-DA) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Enhanced chemiluminescence (ECL) solution was purchased from KeyGen Biotech Co., Ltd. (Nanjing, China). The Cell Counting Kit-8 (CCK-8) was offered by Dojindo Laboratories (Kumamoto, Japan). IL-1β and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China). The H9c2 cardiac cells were supplied by the Sun Yat-sen University Experimental Animal Center (Guangzhou, China).

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

In the control group, H9c2 cardiac cells were incubated with 5.5 mM glucose. To observe the effects of glucose at 35 mM glucose (HG) on the expression level of RIP3, the cells were exposed to HG for 3, 6, 9, 12 and 24 h (Fig. 1). In order to examine the effect of necroptosis on HG-induced injury, the H9c2 cells were co-processed with different concentrations (75, 100, 200, 400, 600 and 800 µM) of Nec-1 (a specific inhibitor of necroptosis) and HG for 24 h. To determine whether there was an interaction between necroptosis and ROS, the H9c2 cells were treated with 1 mM NAC (a scavenger of ROS) for 60 min prior to HG exposure.

Western blot analysis. After being subjected to the indicated treatments, the H9c2 cardiac cells were harvested and lysed with RIPA buffer containing 1 mM phenylmethanesulfonyl fluoride (PMSF) at 4°C for 30 min. The 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 subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes followed by the 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. The membranes were then incubated with either anti-RIP3 or anti-GAPDH (1:1,000 dilution) antibody in freshly prepared 1% BSA and TNF-α enzyme-linked immunosorbent assay (ELISA) kits were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China). The H9c2 cardiac cells were supplied by the Sun Yat-sen University Experimental Animal Center (Guangzhou, China).

Cell viability assay. CCK-8 assay was applied to detect the viability of the cells. The H9c2 cells were digested and seeded in a 96-well growth-medium plate at a concentration of 1x10⁴ cells/ml and incubated at 37°C. After the indicated treatments, the cells were washed twice with phosphate-buffered...
saline (PBS). Subsequently, 10 µl CCK-8 test solution and 90 µl DMEM were added to each well, and the cells were incubated at 37°C for 2.5 h. The absorbance value (OD value) at the 450 nm wavelength was measured using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The means of the optical density (OD) of 3 wells in the indicated groups were used to calculate the percentage of cellular activity according to the following formula: cell viability (%) = (OD/OD control group) x100%. The experiment was repeated 5 times.

Measurement of the secretion levels of IL-1β and TNF-α by ELISA. The H9c2 cells were seeded in 96-well growth-medium plates. After the indicated treatments, the levels of IL-1β and TNF-α in the culture supernatant were evaluated by ELISA according to the manufacturer’s instructions (Cusabi BioTech Co., Ltd.). The experiment was performed 5 times.

Measurement of the intracellular ROS level. The intracellular level of ROS was detected 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 µM) 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, Tokyo, Japan). ImageJ 1.47i software was applied 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, Rh123, 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 Rh123 from the mitochondria and a decrease in intracellular green fluorescence. The H9c2 cardiac cells were plated in 24-well plates. After the indicated treatments, the cells were washed 3 times with PBS. The H9c2 cells were incubated with 1 µM Rh123 at 37°C for 30 min in an incubator and washed 3 times with PBS. The Rh123 fluorescence was then measured over the entire field of vision using a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). The MFI of Rh123 from 5 random fields was analyzed using ImageJ 1.47i software and was regarded as an index of the level of MMP. The experiment was carried out 5 times.

Statistical analysis. All data are expressed as the means ± SEM. Differences between groups were determined by one-way analysis of variance (ANOVA) using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) followed by the least significant difference (LSD) post hoc comparison test. Differences were considered statistically significant at a P-value <0.05.

Results

Inhibitor of necroptosis attenuates the HG-induced upregulation of RIP3 expression in H9c2 cardiac cells. Based on the results from our primary dose-response experiment (data not shown), 35 mM glucose was selected as an effective injury-inducing concentration for H9c2 cardiac cells in our recent studies (3,13). In this study, to examine the effect of HG (35 mM glucose) on the protein expression of RIP3 in H9c2 cardiac cells, a time-response experiment to evaluate the protein expression level of RIP3 was performed. As shown in Fig. 1A and B, after the cells were exposed to HG for 3, 6, 9, 12 and 24 h, the protein expression level of RIP3 was markedly increased (P<0.01), reaching the maximum level at 24 h. Of note, co-treatment of the H9c2 cardiac cells with 100 µM Nec-1 (a specific inhibitor of necroptosis) and HG for 24 h considerably blocked the upregulation of RIP3 expression induced by HG (Fig. 1C and D; P<0.01). Alone 100 µM Nec-1 did not alter the basal expression level of RIP3.

Scavenger of ROS ameliorates the HG-induced upregulation of RIP3 expression in H9c2 cardiac cells. Since Schenk et al (34) have indicated that ROS is involved in the regulation of BV6/ TNF-α-induced necroptosis in Jurkat cells, in this study, we evaluated the role of ROS in the HG-induced upregulation of RIP3 expression in H9c2 cardiac cells. As shown in Fig. 2, treatment of the cells with 1 mM NAC (a scavenger of ROS) for 60 min prior to exposure to HG for 24 h markedly inhibited the
increased protein expression level of RIP3. NAC at 1 mM alone did not affect the basal expression level of RIP3 in H9c2 cardiac cells. The above-mentioned results indicate that ROS participates in the HG-induced upregulation of RIP3 expression.

**Necroptosis is involved in HG-induced oxidative stress in H9c2 cardiac cells.** As shown in Fig. 3A-b and B, exposure of the cells to 35 mM glucose (HG) for 24 h significantly increased the intracellular generation of ROS. However, co-treatment of the cells with 100 µM Nec-1 and HG for 24 h markedly attenuated the increased generation of ROS (Fig. 3A, panel c and B). Alone 100 µM Nec-1 did not affect the basal intracellular generation of ROS. These results indicated that necroptosis contributes to the HG-induced overproduction of ROS in cardiac cells.

**Necroptosis and ROS are implicated in HG-induced cytotoxicity to H9c2 cardiac cells.** Consistent with previous studies (3,9,13), the exposure of H9c2 cardiac cells to HG for 24 h markedly induced cytotoxicity, leading to a decrease in cell viability. Co-treatment of the cells with HG and 75, 100, 200, 400, 600, 800 µM of Nec-1 for 24 h considerably reduced HG-induced cytotoxicity, as evidenced by an increase in cell viability (Fig. 4A), and at the concentration of 100 µM, Nec-1 exhibited the most prominent anti-cytotoxic effect. Thus, 100 µM was used as the effective concentration of Nec-1 in the following experiments. Alone, Nec-1 at 75, 100, 200, 400, 600 and 800 µM did not significantly affect cell viability (Fig. 4B). Similar to the protective effects of Nec-1 against HG-induced cytotoxicity, pre-treatment of the H9c2 cardiac cells with 1 mM NAC also antagonized the HG-induced cytotoxicity, leading to an increase in cell viability (Fig. 4C). Alone, 1 mM NAC did not significantly alter cell viability. The above-mentioned data suggest that necroptosis and ROS mediate cytotoxicity in HG-exposed H9c2 cardiac cells.

**Necroptosis and ROS are linked to HG-induced mitochondrial insults in H9c2 cardiac cells.** Exposure of the cells to HG for 24 h markedly induced mitochondrial damage, leading to a loss of MMP (Fig. 5A, panel b and B). However, co-treatment of the cells with 100 µM Nec-1 and HG for 24 h or treatment of the cells with 1 mM NAC for 60 min prior to exposure to HG for 24 h markedly attenuated the HG-induced dissipation of MMP (Fig. 5A, panels c and d, and B). Alone, 100 µM Nec-1 and 1 mM NAC did not significantly affect the MMP of the cells. These results indicate that necroptosis and ROS are involved in HG-induced mitochondrial damage.

**Necroptosis and ROS mediate the HG-induced secretion of pro-inflammatory cytokines in H9c2 cardiac cells.** As shown in Fig. 6, after the cells were treated with HG for 24 h, the secretion levels of IL-1β (Fig. 6A) and TNF-α (Fig. 6B) were markedly increased, as compared with the control group (P<0.01). However, co-treatment of the cells with 100 µM Nec-1 and HG for 24 h or treatment of the cells with 1 mM NAC for 60 min prior to exposure to HG for 24 h markedly alleviated the increased production of IL-1β and TNF-α, revealing that necroptosis and ROS contribute to the HG-induced inflammatory response in H9c2 cardiac cells.
Figure 4. Necroptosis and reactive oxygen species (ROS) participate in high glucose (HG)-induced cytotoxicity to H9c2 cardiac cells. Cell viability was detected by Cell Counting Kit-8 (CCK-8) assay. (A) The cells were exposed to 35 mM glucose (HG) for 24 h with or without 75, 100, 200, 400, 600 and 800 µM necrostatin-1 (Nec-1). (B) The cells were incubated in the absence or presence of 75, 100, 200, 400, 600 and 800 µM Nec-1. (C) The cells were treated with HG for 24 h with or without co-treatment with 100 µM Nec-1 or pre-treatment with 1 mM NAC for 60 min. Data are presented as the means ± SEM (n=5). **P<0.01 vs. control group; ##P<0.01 vs. the HG-exposed group; #P<0.05 vs. the HG-treated group.

Figure 5. Necroptosis and reactive oxygen species (ROS) mediate the high glucose (HG)-induced dissipation of mitochondrial membrane potential (MMP) in H9c2 cardiac cells. (A) After the cells were subjected to the indicated treatments, MMP was examined by staining with the fluorescent dye, rhodamine 123 (Rh123), followed by photofluorography. (A) Panel a, control group; panel b, H9c2 cardiac cells exposed to 35 mM glucose (HG) for 24 h or with (panel c) 100 µM necrostatin-1 (Nec-1) and HG for 24 h or (panel d) 1 mM N-acetyl-L-cysteine (NAC) for 60 min prior to exposure to HG for 24 h; panel e), cells were treated with 100 µM Nec-1 for 24 h; panel f, cells were treated with 1 mM NAC for 60 min. (B) Quantitative analysis of the mean fluorescence intensity (MFI) in panels a-f in (A) using ImageJ 1.47i software. Data are presented as the means ± SEM (n=5). **P<0.01 vs. control group; ***P<0.01 vs. the HG-exposed group.
Discussion

To date, four forms of cell death have been defined and confirmed: necrosis, apoptosis, autophagy and necroptosis (35-37). Among these, apoptosis, necrosis and autophagy have been demonstrated to contribute to the development of DCM (3,6,9,22,38,39). However, the role of necroptosis in hyperglycemia-induced cardiac injury remains incompletely understood, although Liu et al reported that the expression level of RIP3 was upregulated in diabetic rats (22). In this study, to the best of our knowledge, we demonstrate for the first time that necroptosis plays important roles in HG-induced cardiac injury (cytotoxicity, oxidative stress and dissipation of MMP) and inflammation. Therefore, necroptosis may represent a promising novel target for therapeutic strategies in DCM. Moreover, the findings of this study suggested that there is a positive interaction between necroptosis and ROS production, which may be a novel mechanism underlying HG-elicted cardiac injury and inflammation.

Necroptosis (also known as programmed necrosis) represents a newly indentified mechanism of cell death combining the features of both apoptosis and necrosis. Several types of stimuli, including ligands of death receptors (such as Fas, TRAIL and TNF-α), viral infection and anticaner agents, can induce necroptosis (40). In recent years, necroptosis has been demonstrated to be an important mediator of cell death in the heart (14,23-26). Several studies have indicated that I/R induces an increase in the expression levels of cardiac RIP1 and RIP3, and that Nec-1, an inhibitor of necroptosis, leads to a reduction in myocardial infarct size (23-25). Therefore, necroptosis may be a novel mechanism responsible for cardiac lesions. Recently, the effect of hyperglycemia on necroptosis has attracted attention. A more recent study by Liu et al demonstrated that RIP3 expression was enhanced in diabetic rats; however, the role of necroptosis in hyperglycemia-induced cardiac injury were not determined (22).

In order to clarify this issue, in this study, we first observed the effects of HG on the expression level of RIP3 in cardiomyocytes. Consistent with the results reported by Liu et al (22), we found that the expression level of RIP3 was upregulated in HG-exposed H9c2 cardiac cells. Combining our results and the ones reported by Liu et al, it is suggested that hyperglycemia is a strong stimuli for inducing necroptosis. Second, we examined the effects of Nec-1 on HG-induced cardiac injury (including cytotoxicity, ROS generation and dissipation of MMP). The findings of the present study indicated that Nec-1 markedly attenuated the increased expression of RIP3 by HG, along with the inhibitory effects on HG-induced cardiac injury, as evidenced by an increase in cell viability, a decrease in ROS generation and the attenuation of the dissipation of MMP. These results provide novel evidence that necroptosis contributes to HG-induced cardiomyocyte injury, and extend the findings reported by Liu et al (22).

Another important result of this study relates to the role of necroptosis in HG-induced cardiomyocyte inflammation. Chronic mild inflammation has been considered as one of the features of DCM in humans (41,42). Moreover, in a mouse model of streptozotocin-induced type 1 diabetes, anti-inflammatory therapy represented a potential approach for the therapy of diabetes and its complications (43). Therefore, the further exploration of the mechanisms and the identification of novel therapeutic targets of HG-induced inflammatory response has a promising future. Inflammatory signaling molecules, such as TNF-α, Fas and TRAIL, have been reported to be initiators of necroptosis (40,44). On the other hand, necroptosis has been found to trigger intestinal inflammation, acute pancreatitis, experimental sepsis, salmonella infection and inflammation in atherosclerosis (45,46). Our results demonstrated that the exposure of H9c2 cardiac cells to HG induced an obvious inflammatory response, as evidenced by the increased secretion levels of IL-1β and TNF-α, which was similar to the results of our previous study (13). However, the increased secretion of IL-1β and TNF-α was ameliorated by Nec-1, indicating the involvement of necroptosis in HG-induced inflammation. Of note, necroptosis was triggered by TNF-α (40), and we demonstrated the contribution of necroptosis to the HG-induced increase in TNF-α secretion; thus, we speculated that there was a positive feedback loop between necroptosis and TNF-α in the HG-treated H9c2 cardiac cells. To confirm this hypothesis, further studies are required.
Importantly, it has been shown that there is a positive interaction between necroptosis and ROS generation in BV6/TNF-α-treated Jurkat cells (34). This led us to explore whether there was a similar interaction between necroptosis and ROS generation in HG-exposed cardiac cells. Our results demonstrated that NAC, a ROS scavenger, markedly ameliorated the HG-induced increase in RIP3 expression, accompanied by the inhibition of the HG-induced cardiac injury and inflammation, as indicated by an increase in cell viability and a decrease in ROS generation, the attenuation of MMP dissipation and a decrease in the secretion levels of IL-1β and TNF-α induced by HG. These results clearly demonstrate that a positive feedback loop between necroptosis and ROS production exists in HG-exposed H9c2 cardiac cells, which plays important roles in cardiac injury and inflammation induced by HG. Since the roles of necroptosis and ROS in diabetic cardiac injury have been emphasized by us and others, further experiments using conditional RIP3-knockout mice are warranted in order to clarify the mutual interaction between necroptosis and ROS generation in vivo.

In conclusion, revealing the contribution of necroptosis to HG-induced cardiac injury and inflammation, the present study provides further insight into the mechanisms underlying diabetic cardiovascular complications, such as DCM. Considering the significance of the positive interaction between necroptosis and ROS generation in HG-induced cardiac injury and inflammation, a better understanding of the molecular mechanisms of this interaction will likely have important implications for the development of novel strategies to interfere with necroptosis and ROS generation in patients with diabetes.

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