Inhibition of low-molecular mass protein-7 alleviates hyperglycemia-induced myocardial apoptosis by repairing metabolism disorder and inflammation

Xing Shui, Ze-Qi Wen, Ze-Feng Chen, Zhuo-Shan Huang, Lei-Le Tang, Zhen Wu, Yun-Yue Zhao, Lin Chen

Abstract

Diabetic cardiomyopathy (DCM) is characterized as ventricular dysfunction and poor prognosis. The pathogenesis is unclear. Here, we explored the role of low-molecular mass protein-7 (LMP7) in DCM development. DCM model was established in primary cultured rat cardiomyocytes by stimulation with elevated glucose. The cardiomyocytes were induced using different concentrations of glucose for different times to optimize the conditions, and cells were assigned into four groups: normal control group (NG), high glucose group (HG), ONX-0914 group (HG + ONX-0914 to selectively inhibit LMP7), and solvent control group followed by analysis of the mRNA and protein expression levels of LMP7, glucose transporter type 4 (GLUT4), insulin receptor substrate-1 (IRS-1), and CD36 by qRT-PCR and western blot, respectively. Inflammatory cytokines were assessed by ELISA and cell viability was examined by CCK8 assay along with analysis of myocardial apoptosis by flow cytometry. LMP7 was increased in a time-dependent manner in hyperglycemic cardiomyocytes, accompanied with decreased cell viability. Compared with NG group, GLUT4 and IRS-1 expression was significantly decreased in HG group (P \leq 0.01) with elevated CD36 expression ($P \le 0.01$), which were all reversed in ONX-0914 group (P \leq 0.01). Secretion of IL-6 and TNF- α , and apoptosis were significantly decreased in ONX-0914 group than those in HG group ($P \le 0.01$). LMP7 activation by hyperglycemia may regulate glucose and fatty acid transporter through the impaired insulin signaling pathway. LMP7 inhibition could alleviate hyperglycemia-induced myocardial apoptosis by repairing metabolic disorder and inflammation.

Keywords: diabetic cardiomyopathy, LMP7, apoptosis, metabolic disorder, inflammation

Introduction

Diabetes mellitus (DM) has been regarded as a major public health problem and a global burden to human health and economies. Cardiovascular complications are the main cause of mortality in patients with diabetes. The Framingham heart study showed that the risk of heart failure increases 2.4-fold in men and 5-fold in women with diabetes compared to the non-diabetic population after adjustment for other risk factors [1]. Diabetic cardiomyopathy (DCM) is a specific cardiovascular complication characterized by subclinical cardiac diastolic dysfunction in the early stages, later by clinical heart failure with normal ejection fraction, and finally with reduced ejection fraction, independent of hypertension, coronary heart disease, and significant valvular disease. However, the mechanism underlying the development of DCM remains unclear. This is because of the involvement of multiple factors, including systemic metabolic disorders, abnormal insulin metabolic signaling, maladaptive immune modulation, and inflammation [2].

Low-molecular mass protein-7 (LMP7) is a multifunctional immunoproteasome subunit with chymotrypsin hydrolysis activity. In addition to processing proteins for major histocompatibility complex-1 presentation, LMP7 regulates T cell differentiation, macrophage activation, and

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production of inflammatory cytokines [3]. The findings that LMP7 deficiency increased mice's resistance to obesity, as well as improved glucose intolerance and insulin sensitivity indicate that LMP7 may participate in the regulation of metabolic disorders [4]. Interestingly, mounting evidence indicates a potential interaction between inflammatory processes and metabolic disorders in the heart failure in diabetic patients [5]. More importantly, LMP7 is up-regulated in DCM [6]. However, the exact molecular mechanism by how LMP7 involves in DCM is unclear [6]. The present study explored the role of LMP7 in DCM at the cellular level by establishing a DCM model in cardiomyocytes stimulating with an elevated concentration of glucose. ONX-0914, which specifically blocks LMP7, was used to detect the potential effect of LMP7 on cardiomyocytes. Our study could provide a new insight concerning the role of LMP7 in crosstalk between metabolic disorder and inflammation in the pathogenesis of DCM.

MATERIALS AND METHODS Cell culture and treatment

Primary neonatal rat cardiomyocytes were derived from 1-to-3-day-old Sprague-Dawley rats obtained from the Laboratory Animal Center of Sun Yat-sen University (Guangdong, China). The study was approved by the Experimental Animal Ethics Committee of Sun Yat-sen University. Neonatal rat cardiomyocytes were isolated and cultured. Briefly, hearts were dissected and digested in trypsin (0.125%) and collagenase II (0.1%) at 37°C. The cell suspension was cultured in DMEM (5.5 mmol/L; Gibco, Grand Island, NY, USA) supplemented with 10% FBS in a 5% CO₂ humidified incubator at 37°C. Due to differential cell adhesion, the fibroblasts were eliminated from the cell suspension by preplating for 90 min. Subsequently, the cells were seeded in 6-well plates (1×10⁶ cells/well).

To ensure the optimum experiment time, cardiomyocytes were treated with different concentrations of glucose applied for different times. As described in the previous studies [7-9], cardiomyocytes were cultured in normal glucose (NG: 5.5 mmol/L) or high glucose (HG: 25 mmol/L) medium for 24 and 48 h. Mannitol (19.5 mmol/L) was dissolved in DMEM containing 5.5 mmol/L glucose and included as an osmotic control.

Cell viability analysis

To evaluate the viability of cardiomyocytes, the aforementioned cardiomyocytes were incubated in 96-well plates (3000 cell/well), followed by the use of the Cell Counting Kit 8 (CCK-8; HY-KO301, MedChemExpress, San Rafael, CA, USA). The absorbance was measured with the Multiskan microplate reader at 450 nm. And the cell viability of 5.5 mmol/L glucose group was set at 100%, while the viability of other groups was expressed as a percentage relative to this group.

Subsequently, cardiomyocytes were cultured with the HG concentration of 25 mmol/L (HG group) for 48 h to model DCM at the cellular level. ONX-0914 was dissolved in dimethylsulfoxide (DMSO, 1%) using the concentration of 200 nM, consistent with a previous study [10]. Cardiomyocytes were treated with ONX-0914 for 48 h under hyperglycemia (ONX-0914 group). The glucose concentration of 5.5 mmol/L was used for the normal control (NG group), and a glucose concentration of 25 mmol/L in the presence of 1% DMSO was used for the solvent control (Mock group).

qRT-PCR

Total RNA was extracted using TRIZOL reagent, and then converted into cDNA using a PrimeScript RT reagent Kit (DBI-2220, Germany). Real-time PCR was conducted with the Viia 7 system (Applied Biosystems, Foster City, CA, USA) with 2×AllinOne Q-PCR Mix (AOPR-1200, GeneCopoeia, Rockville, MD, USA). Gene expression was normalized to the mRNA content of glyceraldehyde-3-phosphatedehydrogenase (GAPDH). Primers for target genes are summarized in Table 1.

ELISA

Levels of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF α) were determined using corresponding commercial ELISA kits (RayBiotech, Peachtree Corners, GA, USA) in accordance with the manufacturer's instructions.

Western blot

Protein samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. After blockage with the non-specific background, the membrane was incubated with primary antibodies at 4°C overnight, the membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h.

Flow cytometry

Cardiomyocytes were collected and suspended in 300 μ mol binding buffer, then mixed with 5 L Annexin V-fluorescein isothiocyanate (FITC)

(Dongjin Road, Tokyo, Japan) for 15 min, and then mixed with 5 L propidium iodide (PI) solution for 5 min at room temperature. Flow cytometry was used to detect apoptosis. All experiments were repeated three times.

Statistical analyses

All statistical analyses were conducted using SPSS 25.0 software (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± standard deviation (SD). Differences between or within groups were assessed with Student's t-tests and one-way or two-way analysis of variance (ANOVA). A P-value < 0.05 indicated a significant difference.

RESULTS

LMP7 is increased in a time-dependent manner in cardiomyocytes during hyperglycemia

To assess the relationship between LMP7 and cardiomyocytes during hyperglycemia, cardiomyocytes were cultured for 24 h and 48 h in cell culture medium with different glucose concentrations. According to Figure 1A and 1B, the mRNA and protein expression levels of LMP7 were detected using western blot and qRT-PCR, respectively. When compared with baseline, LMP7 was obviously up-regulated in the HG group at mRNA and protein levels (both P<0.05). More importantly, the expression of LMP7 mRNA and protein at 48 h was significantly up-regulated than those at 24 h (both P<0.05). CCK-8 analysis revealed that the viability of cardiomyocytes in the HG group at 48 h was significantly decreased in comparison to that in the HG group at 0 h and 24 h (both P<0.01). The collective data suggested that LMP7 could increase in a time-dependent manner in cardiomyocytes during hyperglycemia. 48 hours was selected as the optimum time for the subsequent experiments.

LMP7 regulates GLUT4, CD36, and IRS1 expression in cardiomyocytes under hyperglycemia

To understand the role of LMP7 in the uptake of glucose, we tested whether administration of ONX-0914, a specific inhibitor of LMP7, could influence the expression of glucose transporter 4 (GLUT4), CD36, and insulin receptor substrate1 (IRS-1) on the membranes of cardiomyocytes. When compared with control group (NG group), the protein expression level of GLUT4 was reduced when treated using high glucose (HG group) (Figure 2A). After administration of ONX-0914, the protein expression of GLUT4 was significantly upregulated compared that in the HG group. In accordance with western blot, GLUT4 mRNA expression in HG group

was significantly down-regulated in comparison to that in NG group (P < 0.01; Figure 2B). The expression of GLUT4 mRNA in ONX-0914 group was significantly higher than that in HG group (P<0.01).

CD36 is an important fatty acid transporter on the membrane of cardiomyocytes. To detect the effect of LMP7 on the uptake of fatty acid in primary neonatal cardiomyocytes under hyperglycemia, the expression of CD36 was analyzed and found that its mRNA and protein levels on the membrane of cardiomyocytes were significantly upregulated in HG condition when compared with control group (P < 0.05). However, after treatment of ONX-0914, the expression of CD36 were obviously reduced (P < 0.01; Figure 2A and 2C)

On the contrary, the levels of IRS-1 (both mRNA and protein) on the membrane of primary neonatal cardiomyocytes were both significantly reduced after HG treatment, while the results were reversed by administration of ONX-0914 (both P < 0.01; Figure 2A and 2D)

LMP7 regulates proinflammatory cytokines secretion under hyperglycemia

To evaluate the effect of LMP7 on proinflammatory cytokines under the HG condition, IL-6 and TNF- α levels were analyzed. As illustrated in Figure 3, the levels of both IL-6 and TNF- α were significantly increased in HG group in comparison to control group. However, when compared with those in the HG group, the levels of IL-6 and TNF- α were reduced by ONX-0914 treatment (all P<0.01).

Inhibition of LMP7 reduces myocardial apoptosis in vitro

The Annexin V-FITC/PI assay was conducted to explore the effect of LMP7 on myocardial apoptosis in the HG condition. Exposure of primary neonatal cardiomyocytes to HG for 48 h resulted an apoptosis rate of 29.9 \pm 3.3% in cardiomyocytes (P<0.01; Figure 4). After treatment of cardiomyocytes with ONX-0914, the percentage of apoptotic cardiomyocytes was significantly reduced to 15.9 \pm 2.9% (P<0.01).

DISCUSSION

Immunoproteasome subunit LMP7 has been reported to play multifunctional roles in immune regulation, inflammatory responses, oxidative stress, cell differentiation, and metabolism, and is involved in several diseases, including autoimmune or inflammatory diseases, cancers, and metabolic disorders [3]. However, the exact molecular mechanism by how LMP7 involves in DCM remains unclear. In the present study, we explored the role

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of LMP7 involved in cardiomyocyte injury under hyperglycemia. LMP7 was increased in a timedependent manner in cardiomyocytes under hyperglycemia, consistent with previous studies showing increased LMP7 in mesangial cells cultured in elevated glucose conditions [11] and in the heart [6], suggesting that it might be involved in the pathogenesis of DCM..

Proinflammatory cytokines such as TNF- α and IL-6 can lead to cardiac oxidative stress and arterial dysfunction, ultimately leading to cardiac remodeling, fibrosis, and diastolic dysfunction [12]. Accumulating evidence indicates that LMP7 mediates proinflammatory molecules expression by regulating the pathway of nuclear factor-kappa activation. Deficiency of the В (NF-кB) immunoproteasome influences NF-kB signaling [13]. Atherogenic diet can significantly upregulate LMP7 expression in atherosclerotic plaques, involving IκBα degradation and NF-κB activation [14]. microRNA-451 directly targets LMP7 expression to inhibit NF-kB activity, and downregulates the transcription of proinflammatory molecules [11]. Cao et al. described that genetic ablation and pharmacological inhibition of LMP7 reduced IL-6 in cardiac tissue slices induced by treatment with deoxycorticosterone acetate-salt [15]. Li et al. also demonstrated that ablation of LMP7 attenuated Ang ${\rm I\!I}$ -induced IL-6 in atrial tissue [16]. Other studies have revealed that the expression of immunoproteasome LMP7 is increased in response to several factors including interferon-gamma, TNF- α , oxidative stress, and IL-1 [17-19]. Our findings demonstrated that inhibition of LMP7 could attenuate the levels of TNF- α and IL-6, indicating that LMP7 could be an inflammatory target for monitoring and treatment of DCM.

Ablation and pharmacological inhibition of LMP7 could attenuate aortic wall remodeling and vascular smooth muscle cell apoptosis induced by Ang II infusion [20]. Resveratrol could significantly suppress pressure overload-induced cardiac hypertrophy, fibrosis, and apoptosis in mice by the inhibition of LMP7 [21]. Administration of ONX-0914 to inhibit LMP7 significantly attenuated the clinical experimental symptoms of encephalomyelitis in mice [22]. ONX-0914 also reportedly provided neuroprotection by inhibiting T lymphocyte infiltration and decreasing Th17 cell differentiation, accompanied with decreased proinflammatory cytokines, which resulted in a significant decrease in infarct volume, indicating that it might be an anti-inflammatory therapy for ischemic stroke [23]. Our study found that inhibition of LMP7 by ONX-0914 upregulated levels

of GLUT4 and IRS-1 and decreased CD36 expression.

Consistent with this, we observed that the inhibition of LMP7 reduced hyperglycemia-induced myocardial apoptosis, indicating a potential protective role of LMP7 in the maintenance of cardiac quantities in hyperglycemia. In DM, insulin resistance and hyperglycemia result in oxidative stress, endoplasmic reticulum stress, and impaired Ca²⁺ handling, which leads to myocardial apoptosis in DCM [24]. Guo et al. reported that the protective effect of soluble receptor for advanced glycation end-products on myocardial ischemia/reperfusioninduced apoptosis is associated with improved proteasome activity and elevated B1i, LMP7 mediated by STAT3 activation [25]. We observed that LMP7 participated in regulating the impaired insulin metabolic signaling pathway and production of proinflammatory cytokines. Thus, we speculate that the inhibition of LMP7 reduces apoptosis by improving impaired metabolism and inflammation in the insulin signaling pathway. However, the exact mechanism by how LMP7 involves in the regulation of apoptosis was not investigate in the current study which is a main study limitation. In the future, we plan to investigate how LMP7 regulates apoptosis using gene-knockout cell line or anima model.

In conclusion, our study demonstrated that LMP7 was involved in regulating impaired insulin metabolic signaling as well as inflammation, and pharmacological inhibition of LMP7 could reduce myocardial apoptosis under hyperglycemia, implicating that LMP7 might be a potential target for treating metabolic disorders.

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Disclosure of conflict of interest

None

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Table and Figure legends

 Table 1. The primers sequence of the target genes

Genes	Primer sequence (5'-3')		
GAPDH	Forward:	GCAAGGATACTGAGAGCAAGAG	
	Reverse:	GGATGGAATTGTGAGGGAGATG	
LMP7	Forward:	GGTGATCGAGATTAACCCTTACC	
	Reverse:	CCCATTCCGCAGATAGTATAGC	
GLUT4	Forward:	ACTTAGGGCCAGATGAGAATG	
	Reverse:	GTAAGGGAAGAGAGGGCTAAAG	
IRS-1	Forward:	GACGCTCCAGTGAGGATTTAAG	
	Reverse:	GGAGGATTGCTGAGGTCATTTAG	
CD36	Forward:	ACGACTGCAGGTCAACATAC	
	Reverse:	CGATGGTCCCAGTCTCATTTAG	



Figure 1. Expression levels of LMP7 in cardiomyocytes under hyperglycemia and cell viability.

LMP7 expression levels in different glucose concentrations applied for different times measured using western blot (A) and qRT-PCR (B). (1) Results at baseline (0 h). (2, 6) Cardiomyocytes were cultured with a glucose concentration of 5.5 mmol/L (NG) for 24 h (2) and 48 h (6). (3, 7) Cardiomyocytes were cultured with a glucose concentration of 25 mmol/L (HG) for 24 h (3) and 48 h (7). (4, 8) Cardiomyocytes were cultured with mannitol (HO) for 24 h (4) and 48 h (8). (5,9) Cardiomyocytes were cultured with interferongamma (INF, 40 ng/mL) for 24 h (5) and 48 h (9). #P<0.05 compared with baseline; *P<0.05 compared with (3). (C) Cell viability determined by the CCK-8 assay. **P<0.01 compared with 0 h. ##P<0.01 compared with 24 h.

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Figure 2. Regulation of LMP7 on glucose and fatty acid transporter in cardiomyocytes.

(A) The protein bands of CD36, GLUT4, and IRS1 measured by western blot. (B-D) The mRNA expression of GLUT4 (B), CD36 (C), and IRS1 (D) measured by qRT-PCR. NG: normal glucose group;

HG: high glucose group; Mock: solvent control group; ONX0914: administration of ONX-0914 group. **P<0.01 compared with NG group. ##P<0.01 compared with HG group.



Figure 3. Effect of LMP7 on proinflammatory cytokines.

(A) The concentration of TNF- α in the different groups. (B) The concentration of IL-6 in the different groups. NG: normal glucose group; HG: high glucose group; Mock: solvent control group; ONX0914:

administration of ONX-0914 group. **P<0.01 compared with NG group. ##P<0.01, compared with HG group.





Figure 4. The effect of LMP7 on myocardial apoptosis.

Normal glucose (NG) group (A), high glucose (HG) group (B), solvent control (Mock) group (C), administration of ONX-0914 (ONX0914) group (D), and total apoptosis rate of each group (E). **P<0.01 compared with the NG group. ##P<0.01 compared

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with the HG group.