Mst1-Nrf2 regulates myocardial injury in type 2 diabetic mice through autophagy

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Abstract

Purpose This paper presents the effect of Mst1-Nrf2 on myocardial injury in type 2 diabetic mice through autophagy.

Method C57BL/6N wild-type mice, Mst1 knockout mice and Mst1 knockout mice interfered by Keap1-Nrf2 pathway inhibitors are divided into wild group, Mst1 group, Mst1-Nrf2 group, diabetic cardiomyopathy group (DCM), DCM+Mst1 group and DCM+ Mst1-Nrf2 group. Each group contains 5 mice. Mice in DCM group, DCM+Mst1 group and DCM+Mst1-Nrf2 group are given high-glucose and high-fat diet for 4 weeks, then intraperitoneally injected 40mg/kg streptozotocin(STZ). Meanwhile, high-glucose and high-fat diet is continued for 8 weeks. Body weight and fasting blood glucose are measured every week. Mice in Mst1-Nrf2 group and mice in DCM+Mst1-Nrf2 group are given interference of Keap1-Nrf2 pathway inhibitors, and intraperitoneally injected for 4 weeks at a dose of 5mmol/L.

Results After 8 weeks of STZ injection, the body weight of mice in DCM+Mst1 group and mice in DCM+Mst1-Nrf2 group increases and fasting blood glucose level decreases, the differences are statistically significant ($P < 0.01$). Compared with the wild group, the fasting glucose level of mice in Mst1 group and Mst1-Nrf2 group decreases after 8 weeks ($P < 0.01$). Compared with the wild group, the degree of myocardial fibrosis in Mst1 group and Mst1-Nrf2 group is significantly reduced, while the level of myocardial fibrosis in DCM group is significantly increased ($P < 0.01$). After 8 weeks of STZ injection, the degree of myocardial fibrosis in DCM+Mst1 group and DCM+ Mst1-Nrf2 group is significantly reduced. Compared with the wild group, the expression of myocardial Beclin1 protein in Mst1 group and Mst1-Nrf2 group is significantly increased ($P < 0.01$); Compared with DCM group, myocardial Beclin1 protein expression is significantly increased in DCM+Mst1 group and DCM+Mst1-Nrf2 group ($P < 0.01$). Compared with the wild group, the expression of p62 protein in myocardium of Mst1 group and Mst1-Nrf2 group is significantly decreased ($P < 0.01$). Compared with DCM group, the expression of p62 protein in myocardium of DCM+Mst1 group and DCM+Mst1-Nrf2 group is decreased significantly ($P < 0.01$). Compared with the wild group, myocardial Nrf2 protein expression in Mst1 group and Mst1-Nrf2 group is significantly increased ($P < 0.01$). Compared with DCM group, myocardial Nrf2 protein expression of mice in DCM+Mst1 group and DCM+ Mst1-Nrf2 group is significantly increased ($P < 0.01$).

Conclusion Mst1-Nrf2 promotes autophagy in type 2 diabetic mice and inhibits the progression of diabetic cardiomyopathy.

Introduction

Diabetic cardiomyopathy (DCM) is independent of hypertension, coronary heart disease and heart valvular disease, and is caused by glucose metabolism disorder with heart structure and function disorders as the main pathological changes^[1]. In recent years, a variety of molecular mechanisms have jointly promoted diabetic cardiomyocyte injury and impaired cardiac function, among which the changes of cell homeostasis, such as apoptosis and autophagy, have attracted widespread attention^[2]. At the

same time, changes in gene regulation are also of great concern. Therefore, revealing the pathogenesis of diabetic cardiomyopathy from a new perspective and providing theoretical basis for the accurate diagnosis and prevention of DCM have become a hotspot of international attention^[3].

Diabetic cardiomyopathy is a cardiovascular complication caused by diabetes. More and more experimental evidence indicates that apoptosis and autophagy play a key role in the cardiovascular complications of diabetes mellitus^[4]. The enhancement of apoptosis is the initiating factor of cardiovascular complications of diabetes mellitus. Therefore, a thorough understanding of the relationship between apoptosis and autophagy in the pathogenesis of diabetic cardiomyopathy is of great significance in the early prevention and treatment of diabetic cardiomyopathy^[5].

Mst1 (Mammalian ste20-like Kinase1) is a serine/threonine protein kinase and is a key molecule of Hippo signaling pathway. Mst1 is a protein kinase activated during apoptosis. Mst1 is widely expressed in all tissues of the human body, and it regulates apoptosis and organ size. Upregulation of Mst1 results in decreased autophagy level and increased protein aggregation^[6,7].

Nuclear factor erythroid-derived factor 2-related factor (Nrf2) is an antioxidant transcription factor Kelch-like ECH-associated protein 1 (Keap1), which acts as a negative regulator of Nrf2 and maintains intracellular Nrf2 levels by mediating Nrf2 ubiquitination^[8]. Due to the important role of Nrf2-Keap1 signaling pathway in cell defense and the interaction between Nrf2-Keap1 signaling pathway and apoptosis, autophagy and other signaling pathways, Nrf2-Keap1 signaling pathway has become a research hotspot. Nrf2 is a "switch" that switches on endogenous antioxidant responses, activating genes that reduce oxidative stress^[9,10].

We found that Nrf2 expression increased after Mst1 deletion, and overexpressed Nrf2 also enhanced the protective effect of Mst1 deletion on cardiomyocyte survival and mitochondrial homeostasis. In addition, Nrf2 overexpression can also protect diabetic cardiomyocytes from apoptosis damage. Overall, our data confirm that Mst1 activation and Nrf2 down-regulation can induce apoptosis and cellular oxidative stress.

Materials And Methods

Chemicals and reagents. Keap1-Nrf2-IN₁ (cat.no.HY-126245/CS-0100953) was purchased from MedChemExpress company. STZ was purchased from Sigma. The primary antibodies against Bcl-2, Bax, LC3 and Beclin1 were obtained from Santa Cruz Biotechnology, Inc. The primary antibodies against p62, Nrf2 and Mst1 were obtained from Abcam. Blood lipid, high density lipoprotein, total cholesterol kit purchased from Nanjing built company.

Animals. Healthy Mst1 knockout mice (approval no. SCXK(SU)2018-0003, KOCMP-22633-Stk4) weighing 18-20 g were used in the present study (Qiqihar Medicine University Animal Center). Mice were housed on

a 12h dark-light cycle at $23\pm 1^{\circ}\text{C}$. The study protocol was approved by the Institutional Animal Research Committee (approval no. QMU-AECC-2021-205).

Diabetic mouse modeling and experimental animal grouping. Modeling method of type 2 diabetic mouse: After 4 weeks of high-sugar and high-fat diet, mice were intraperitoneally injected with STZ 40mg/kg. One week later, blood glucose was detected. If the model was not successful, the second injection was continued, and the average blood glucose was 11.1 mmol/L was the standard for successful model construction. After successful modeling, the mice were fed with high sugar and fat for 12 weeks for further experiments^[11,12].

The experimental animals were randomly divided into the following 6 groups, with 5 animals in each group and a total of 30 animals:

1. wild Type mice group : 5 mice in WT group;
2. Mst1 knockout mice group : 5 mice in Mst1^{-/-} group;
3. Mst1 knockout mice with Keap1-Nrf2 pathway inhibitor interference group: 5 mice in Mst1^{-/-}+Nrf2 group;
4. Diabetic mice group: 5 mice in DM group;
5. Mst1 knockout diabetic mice group : 5 mice in DM+ Mst1^{-/-} group;
6. Diabetic mice with Mst1 knockout interfered by Keap1-Nrf2 pathway inhibitors: 5 mice in DM+Mst1^{-/-}+Nrf2 group;

Myocardial fibrosis was observed by HE staining. HE staining is one of the staining methods to show fibers in tissue and can be used to observe myocardial fibrosis levels. The heart tissues of mice in each group were placed in 4% paraformaldehyde and fixed to denature and solidify the tissue proteins, which were dehydrated, transparent, embedded and paraffin sected. The dried tissue sections were dewaxed by xylene twice, 5min each. Xylene was removed with anhydrous ethanol twice, 10min each time. Then the tissue sections were soaked in 95%, 85%, 80% and 75% ethanol for 5min each. Dye with hematoxylin for 5min and rinse with water for 1min. Return 1% dilute ammonia to blue for 30s, and rinse with water for 1min; After returning to blue, soak in ethanol for dehydration, 1min each time; Deethanol in xylene for 2min, twice in total; Finally, the sheet was sealed with neutral resin and placed at 70⁰C dried in the oven. The images were observed under a light microscope. After HE staining, the nucleus of normal myocardium was blue, cytoplasm, muscle fibers, collagen fibers and red blood cells were red in different shades, and the tissue structure was clear^[13,14,15].

The morphology and number of cardiac autophagosomes were observed by transmission electron microscopy. According to the group of intervention, the mouse heart samples, with double scalpel parallel cut into the size of large grain of myocardial tissue, with physiological saline wash blood. Gently blot the surface liquid and add electron microscope fixative (2.5% glutaraldehyde and phosphate buffer), 4⁰C

refrigerator for 24 hours. Transmission electron microscopy (TEM) was used to observe the morphology and number of myocyte autophagosomes. Three fields were randomly selected for each sample^[16,17].

Blood glucose, blood lipids, total cholesterol content determination. Blood was collected through the inferior vena cava of mice, centrifuged, and placed in a 4°C refrigerator for 15min. After centrifugation at 3000r/min for 5min, the supernatant was taken. Blood lipid and total cholesterol were detected by automatic biochemical detector. Blood glucose was measured by tail vein of mice^[18].

Western blotting. About 50 mg of cardiac tissue was taken from each group, lysate was fully applied, supernatant was taken after centrifugation at 4 °C, and Bradford was used. After the protein concentration was determined, the protein was quantified and denatured. The proteins were transferred to PVDF membrane by SDS-PAGE electrophoresis. After being sealed at room temperature for 1h, primary antibodies Bcl-2, Bax, Beclin1, Nrf2, Mst1 and β -Actin (1:500) were added, and incubated at 4 °C overnight. TBST was rinsed 3 times, 5min each, and the corresponding secondary antibody (1:10000) was added and incubated at room temperature for 1.5h. TBST was rinsed 2 times, 5min each, and TBS was rinsed 2 times, 5min each. ECL chemiluminescence coloration, electrophoresis imaging system quantitative scanning analysis, the results of β -actin correction^[19,20].

Statistical analysis. All data were presented as the means $\pm$ standard error of the mean. One-way ANOVA followed by a Dunnett's or Student-Newman-Keuls post hoc tests were used for data analysis with GraphPad Prism 5.00 software (GraphPad Software, Inc.). $P < 0.01$ was considered to indicate a statistically significant difference.

Results

Effects of Mst1-Nrf2 on body weight and blood glucose in diabetic cardiomyopathy mice.

In order to determine the effect of Mst1-Nrf2 on diabetic cardiomyopathy in each group of mice, the diabetic mouse model was established after STZ injection and observed. There were significant differences in body weight and blood glucose between 1 and 8 weeks. From 1 to 8 weeks after STZ injection, the body weight of mice in the wild group increased steadily over time, while the body weight of mice in the DCM group increased rapidly in the early stage and decreased with the progress of the disease ($P < 0.05$), and the difference was statistically significant. Compared with the DCM group, the body weight of the DCM+Mst1 knockout group increased after knockout ($P < 0.01$), and the difference was statistically significant. Compared with DCM+Mst1 knockout, the body weight of mice in DCM+Mst1 knockout +Nrf2 group increased after knockout and Nrf2 interference ($P > 0.05$), and there was no statistical significance. (Figure 1)

The fasting blood glucose of mice in the wild group remained stable and within the normal blood glucose range (3.9-6.1mmol /L). Compared with the wild group, the fasting blood glucose of mice in the DCM group was ≥ 11.1 mmol/L, and the fasting blood glucose was maintained at a higher level, ($P < 0.01$), and the difference was statistically significant. Compared with the DCM group, the body weight and blood

glucose of the DCM+Mst1 knockout group decreased after knockout ($P < 0.01$), and compared with DCM+Mst1 knockout, blood glucose in DCM+Mst1 knockout +Nrf2 group decreased after knockout and Nrf2 interference ($P < 0.01$), and the difference was statistically significant.(Figure 1)

Effects of Mst1-Nrf2 on high density lipoprotein, triglyceride and total cholesterol in diabetic cardiomyopathy mice

The contents of triglyceride and total cholesterol increased with the development of diabetes mellitus. Compared with the wild group, the contents of triglyceride and total cholesterol in DCM group were significantly higher ($P < 0.01$), and the difference was statistically significant. Compared with the DCM group, the contents of triglyceride and total cholesterol in the DCM+Mst1 knockout group were decreased ($P < 0.01$), and the difference was statistically significant. Compared with DCM group, the contents of triglyceride and total cholesterol in DCM+Mst1 knockout +Nrf2 group were significantly decreased ($P < 0.01$), and the difference was statistically significant.

The level of high density lipoprotein decreased with the development of diabetes. Compared with the wild group, the content of high density lipoprotein in DCM group was significantly decreased ($P < 0.01$), and the difference was statistically significant. Compared with DCM group, DCM+Mst1 knockout group had an increase in high-density lipoprotein content ($P < 0.01$), and the difference was statistically significant. Compared with DCM group, DCM+Mst1 knockout +Nrf2 group significantly increased the content of HIGH-DENSITY lipoprotein ($P < 0.01$), and the difference was statistically significant.(Figure 1)

Effect of Mst1-Nrf2 on diabetic cardiomyopathy and myocardial fibrosis.

In the wild group, 5 rats showed orderly arrangement of myocardial cell fibers, uniform staining of cytoplasm and nucleus, complete and clear structure. However, in the DCM group, 5 mice showed irregular arrangement of myocardial cells, deep staining of cytoplasm, local myocardial fiber fracture and inflammatory cell infiltration between myocardial cells. However, in the DCM+Mst1 knockout +Nrf2 group, the damage degree of myocardial cells in 5 mice was significantly improved, and the inflammatory cells in the interstitium were also significantly reduced.(Figure 2)

Effects of Mst1-Nrf2 on autophagosomes in diabetic cardiomyopathy mice

The morphology and number of autophagosome in cardiomyocytes were observed under electron microscope. The number of autophagosomes in myocardial cells of mice in wild group was very low. However, the number of cardiomyocyte autophagosomes in Mst1 knockout group was significantly increased, and the number of cardiomyocyte autophagosomes in Mst1 knockout +Nrf2 group was the largest among the 6 groups. Compared with the wild group, the number of cardiomyocyte autophagosomes in DCM group was significantly reduced or not. Compared with DCM, the number of autophagosomes in the DCM+Mst1 knockout group was increased, and the number of autophagosomes in the DCM+Mst1 knockout +Nrf2 group was significantly increased.(Figure 3)

Effects of Mst1-Nrf2 on autophagy and apoptosis-related proteins in myocardial tissue with each group.

To explore the effects of Mst1-Nrf2 on down-regulation of apoptosis protein and up-regulation of autophagy protein, the results showed that Mst1-Nrf2 could down-regulate the expression of apoptosis-related proteins and up-regulate the expression of autophagy related proteins in STZ-induced diabetic mice. $P < 0.01$ The difference was statistically significant. (Figure 4)

Effects of Mst1-Nrf2 on Keap1-Nrf2 pathway-related proteins in myocardial tissue with each group.

To explore the effects of Mst1-Nrf2 on *Keap1-Nrf2 pathway-related proteins in myocardial tissue with each group*, the results showed that Mst1-Nrf2 could up-regulate the expression of Nrf2 and p62 proteins in STZ-induced diabetic mice. $P < 0.01$ The difference was statistically significant. (Figure 5)

Discussion

This study proved that Mst1-Nrf2 could significantly reduce the progression and apoptosis of diabetic cardiomyopathy, down-regulate the Keap1-Nrf2 pathway, and increase autophagy. It is well known that in diabetic cardiomyopathy, hyperglycemia and fluctuation of blood glucose level lead to increased apoptosis, which can induce acute oxidative stress of myocardial cells, lead to cardiomyopathy changes, and ultimately lead to myocardial systolic and diastolic dysfunction^[21,22,23]. This study confirmed that Mst1-Nrf2 can effectively relieve myocardial fibrosis and apoptosis caused by diabetic cardiomyopathy^[24]. Apoptosis and autophagy are key factors in cardiovascular complications of diabetes mellitus. Therefore, a thorough understanding of the relationship between apoptosis and autophagy in the pathogenesis of diabetic cardiomyopathy is of great significance in the early prevention and treatment of diabetic cardiomyopathy^[25,26].

Mst1 is widely expressed in all tissues of human body, and it has a regulatory effect on apoptosis. Mst1 is a protein kinase activated during apoptosis. Upregulation of Mst1 results in decreased autophagy level and increased protein aggregation. Overexpression of Mst1 increased myocardial apoptosis in vivo and in vitro. Enhanced apoptosis by bcl-2 interaction with Bax to form an active conformation through Bax^[27]. We considered whether Mst1 interferes with the interaction between bcl-2 and Bax, and Beclin1 and Bcl-2 in addition to promoting the interaction^[28]. As expected, Mst1 knockout enhanced the interaction between Bcl-2 and Bax in diabetic mice. Therefore, Mst1 induces the separation of Bcl-2 from Bax, enhances apoptosis and promotes the development of DCM^[29,30].

In physiological state, Keap1 acts as a negative regulator of Nrf2 and maintains intracellular Nrf2 levels by mediating Nrf2 ubiquitination. When cardiomyocytes are in stress state such as high glucose environment, autophagy level is up-regulated after Mst1 knockout, and p62 protein can induce autophagosome to engulf Keap1, resulting in Nrf2 dissociation and down-regulation of Nrf2 ubiquitination level. The direct interaction between Keap1 and p62 leads to a decrease in Nrf2 ubiquitination and an increase in Nrf2 stability. Mst1 gene knockout enhances autophagy and removes inhibition of Keap1. Abnormal accumulation of Nrf2 depends on p62 and Keap1 functions. This mechanism is known as the atypical mechanism of Nrf2 activation. This atypical Nrf2 activation

mechanism is caused by autophagy deregulation, which provides a new idea for studying the regulation of Mst1-Nrf2 pathway^[31,32].

Conclusions

This study used Mst1 gene knockout mice and Keap1-Nrf2 pathway inhibitor as interference factor to evaluate the protective effect of Mst1-Nrf2 in diabetic cardiomyopathy animal model. In Mst1-Nrf2 pretreated mice, Nrf2 protein expression was up-regulated and correlated with Keap1-Nrf2 induction, resulting in increased stability of Nrf2^[33]. Mst1-Nrf2 can up-regulate the level of autophagy-related proteins, down-regulate the level of apoptosis-related proteins and promote the formation of autophagosomes in cardiomyocytes. Mst1-Nrf2 can also effectively relieve myocardial fibrosis induced by diabetic cardiomyopathy. These studies suggest that Mst1-Nrf2 plays a role in the protective mechanism of diabetic cardiomyopathy^[34].

Declarations

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

JW contributed to data analysis and paper writing, NW, LLW, LJ and WX : Writing - Review & Editing, Supervision, Validation, Data Curation and GWL contributed to the study design and paper writing.

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NOMENCLATURE

Mst1, stk4 gene. Nrf2, Kelch-like ECH-associated protein-1/nuclear factor E2. Keap1, Cytoplasmic junction protein. STZ, streptozocin.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study by the GraphPad Prism 5.

Ethics approval and consent to participate

Approval no. QMU-AECC-2021-205.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Changes of body weight and diabetes-related indexes in each group. (A) Changes of body weight in each group. (B) Changes of blood glucose in each group. (C) Changes of TG in each group. (D) Changes of T-CHO in each group. (E) Changes of HDL in each group. Body weight : DCM group is $P<0.05$ vs. control group. $P<0.01$ vs. DCM group. Other indicators: $P<0.01$ vs. control group. $P<0.01$ vs. DCM group.

Figure 2

Effect of myocardial fibrosis in each group. (magnification, x100)

Figure 3

Effect of myocardial autophagy in each group. (magnification, x400)

Figure 4

Effects of autophagy and apoptosis-related proteins on myocardial tissue in each group. (A) WB (B) Bax (C) Bcl-2 (D) WB (E) LC3 (F) Beclin1 $P<0.01$ DCM group vs. control group. $P<0.01$ DCM+Mst1 group or DCM+Mst1+Nrf2 group vs. DCM group. $P<0.01$ DCM+Mst1+Nrf2 group vs. DCM+Mst1 group.

Figure 5

Effects of Keap1-Nrf2 pathway-related proteins on myocardial tissue in each group. (A) WB (B) Nrf2 (C) p62 (D) WB (E) Mst1 $P<0.01$ DCM group vs. control group. $P<0.01$ DCM+Mst1 group or DCM+Mst1+Nrf2 group vs. DCM group. $P<0.01$ DCM+Mst1+Nrf2 group vs. DCM+Mst1 group.