Changes in the expression of cardiac mitofusin-2 in different stages of diabetes in rats

QIN GAO[1,2], XIAO-MEI WANG[1], HONG-WEI YE[1,2], YING YU[1,2], PIN-FANG KANG[3], HONG-JU WANG[3], SU-DONG GUAN[1] and ZHENG-HONG LI[1]

[1]Department of Physiology and [2]Anhui Key Laboratory of Tissue Transplantation, Bengbu Medical College, Bengbu 233030; [3]Department of Cardiovascular Disease, The First Affiliated Hospital of Bengbu Medical College, Bengbu 233004, P.R. China

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Abstract. The aim of this study was to investigate the role of mitofusin-2 (Mfn2) in different stages of diabetes in rats and to analyze the related mechanism(s). A diabetic model in SD rats was induced by a single intraperitoneal injection of 55 mg/kg streptozoticin (STZ). The hearts were isolated from diabetes mellitus (DM) rats at the fourth week (DM4W), eighth week (DM8W) and twelfth week (DM12W) and fasting blood glucose (FBG) levels and the ratio of heart weight to body weight (HW/BW) were measured. Malondialdehyde (MDA) content, superoxide dismutase (SOD) and caspase 3 activities were measured. The expression of Mfn2 of the left anterior myocardium at the mRNA level was detected using RT-PCR. In contrast to the normal group, in the DM4W, DM8W and DM12W groups, there was a significant increase in the FBG levels, but no difference among the DM4W, DM8W and DM12W groups. The HW/BW ratio as well as the MDA content were increased, while SOD activity was reduced. Caspase-3 activity was increased, while the expression of Mfn-2 mRNA levels was reduced. In addition, with the development of diabetic cardiomyopathy, the contents of MDA and caspase 3 were increased, whereas SOD activity and Mfn-2 mRNA levels were further reduced. In conclusion, our results indicated that with the development of diabetes, the expression of cardiac Mfn2 has showed a decrease, which may be associated with the decrease of antioxidant ability and progression of apoptosis.

Introduction

Diabetes mellitus (DM) induces various cardiovascular complications in the diabetic population, which has become the major cause of morbidity and mortality (1). Diabetic cardiomyopathy is a distinct clinical entity inducing functional, biochemical and morphological abnormalities in the heart, ultimately leading to heart failure (2). However, the mechanisms leading to cardiac changes are not fully comprehended.

Diabetic cardiomyopathy is accompanied by mitochondrial injury (3,4). Mitochondria undergo frequent fusion and fission, and the balance of these opposing processes regulates mitochondrial morphology (5). Mitochondrial fusion serves to keep up a tubular mitochondrial network and to optimize mitochondrial function, which is regulated by large GTPases, such as mitofusin-1 and -2 (Mfn1 and Mfn2). Previous studies have reported that Mfn2 polymorphic genes were present in type 1 and 2 diabetes patients. Type 2 diabetes downregulates the expression of Mfn2 mRNA in skeletal muscle (6). The overexpression of Mfn2 in diabetic rats was reported to have the potential to protect the kidney by inhibiting the activation of p38 and the accumulation of ROS; to prevent mitochondrial dysfunction and reduce the synthesis of collagen IV (7). Of all cell types and tissues, Mfn2 is predominantly expressed in the heart (8), at the same time its functional role in the cardiac myocyte is poorly understood, and there are no data regarding the changes of Mfn2 in the DM heart. Thus, we wanted to examine whether Mfn2 changes in diabetic cardiomyopathy and whether this change of cardiac Mfn2 is correlated with the pathological process of DM, since it may provide a novel strategy for the treatment of DM.

Excessive oxidative stress has been associated with the pathology and complications of diabetes. Hyperglycemia causes oxidative stress and cell death. Oxidative stress may induce mitochondrial fragmentation, which is correlated with mitochondrial fusion and fission. In the case that cardiac Mfn2 changes with the development of DM, it is of importance to examine whether there is any association with oxidative stress injury.

Apoptosis occurs in diabetic cardiomyopathy. Caspases are a large protein family of cysteine proteases that have been specifically linked with cell death (apoptosis). Among them, caspase 3 is a frequently activated death protease, catalyzing the specific cleavage of many key cellular proteins. Thus, we measured the changes of caspase 3 during the development of DM, and aimed to analyze the role of Mfn2 in the process.

Materials and methods

Animals. Thirty-six male Sprague-Dawley rats (200-250 g) were purchased from the Animal Center of the Bengbu Medical

Correspondence to: Professor Zheng-Hong Li, Department of Physiology, Bengbu Medical College, 2600 Dong Hai Ave., Bengbu 233030, P.R. China
E-mail: bbmclzh@126.com

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College. The rats were fed normal chow and had free access to water. They were kept at a constant temperature of (21±1˚C) with a fixed 12-h light/dark cycle. All animal procedures were conducted in accordance with the United States National Institutes of Health Guide and were approved by the Animal Use and Care Committee of the Bengbu Medical College.

Chemicals and reagents. Streptozotocin (STZ) was purchased from Sigma (St. Louis, MO, USA). Malondialdehyde (MDA) and superoxide dismutase (SOD) kits were purchased from the Nanjing Jiancheng Bioengineering Institute (China). The caspase 3 kit was purchased from Shanghai Genmed Scientifics Inc., (China). The primers for Mfn-2 were 5'-CTCAGGAGCAGCGGGTTTATTGTCT-3' and 5'-TGTCGAGGGACCAGCATGTCTATCT-3', the amplified fragment length was 412 bp, while the primers for β-actin were 5'-GATGGTGGGTATGGGTCAGAAGGAC-3' and 5'-GCTCATTGCCGATAGTGATGACT-3', the amplified fragment length was 630 bp. Any other chemicals used were of the highest purity available.

Induction of diabetes and experimental protocol. Diabetes was induced in overnight-fasted rats by administering a single intraperitoneal (i.p.) injection of 55 mg/kg STZ freshly dissolved in 0.1 mol/l sodium citrate buffer (pH 4.5). The control group was injected with a similar volume of sodium citrate buffer alone. Rats with a fasting blood glucose (FBG) level >16.7 mmol/l became diabetic 72 h subsequent to injection. Animals were randomly divided into control groups of 4, 8 and 12W, corresponding to diabetes at the fourth (DM4W), the eighth (DM8W) and the twelfth week (DM12W) groups, respectively (n=6).

Detection of fasting blood glucose (FBG), body weight (BW) and heart weight (HW). FBG level and BW were measured every 4 weeks subsequent to injections of STZ. The ratio of heart weight to body weight (HW/BW) was determined to indicate the degree of cardiac hypertrophy.

Detection of MDA content and SOD activity. At the end of the experimental period, 0.1 g heart tissue was homogenized in ice-cold PBS. MDA content and SOD activity were measured by commercially available kits, according to the manufacturer's instructions.

Detection of Mfn2 mRNA by RT-PCR. RT-PCR was used to detect Mfn2 mRNA expression in the heart. Briefly, total RNA was extracted with TRIzol, according to the manufacturer's instructions. Total RNA (2 μg)were reverse transcribed to cDNA, and PCR was performed following the routine method. PCR products were analyzed on 1% agarose gel. Densitometry result for Mfn2 gene was compared with the corresponding β-actin levels to account for loading differences.

Detection of caspase-3 activity. Caspase-3 activity was measured by commercially available kits, according to the manufacturer's instructions.

Statistical analysis. Values were expressed as the mean ± SEM. Statistical comparisons were carried out by one-way variance analysis and the Newman-Keuls test. P<0.05 were considered to indicate a statistically significant difference.

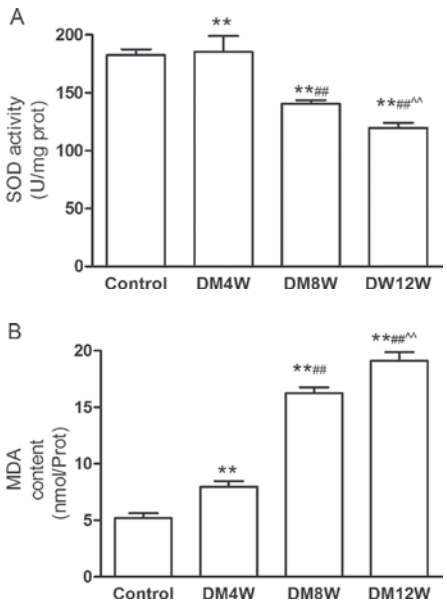

Figure 1. Changes of (A) cardiac SOD activity and (B) MDA content in diabetic rats. **P<0.01 compared with control; ##p<0.01 compared with DM4W; ^^p<0.01 compared with DM8W.

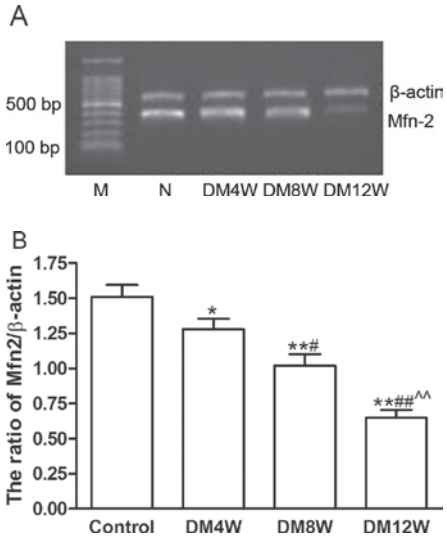

Figure 2. Expression of (A) cardiac Mfn2 mRNA and (B) result analysis in diabetic rats. P<0.05, **p<0.01 compared with control; #p<0.05; ##p<0.01 compared with DM4W; ^^p<0.01 compared with DM8W.

Results

Changes of FBG, BW, HW and the HW/BW-ratio. The changes in FBG, BW, HW and the HW/BW-ratio at different stages of diabetes are shown in Table I. In contrast to the control group, the FBG in DM groups was increased significantly, and there were no differences at different stages of DM. Compared with the control animals, BW significantly decreased at 4, 8 and 12W after STZ injection, and there were statistically significant differences in the different stages of DM. HW/BW,

Table I. Changes of fasting blood glucose, heart weight, body weight and heart weight/body weight in the different groups.

Fasting blood glucose (mmol/l)	4W	8W	12W
Control	6.05±1.02	5.88±1.14	5.75±0.63
DM	25.30±2.99[a]	25.65±3.19[a]	27.88±4.25[a]
Body weight (g)	4W	8W	12W
Control	382.67±5.95	440.53±3.84[b]	525.00±5.36[b,c]
DM	235.58±6.43[a]	185.46±3.85[a,d]	147.50±5.42[a,d,e]
Heart weight (mg)	4W	8W	12W
Control	1392.66±35.76	1528.63±43.25[b]	1861.7±181[b,c]
DM	925.45±22.6[a]	830.34±37.93[a]	784.68±18.75[a]
HW/BW ($mgxg^{-1}$)	4W	8W	12W
Control	3.64±0.04	3.47±0.07	3.54±0.10
DM	3.93±0.21	4.48±0.11[a,d]	5.32±0.06[a,d,e]

[a]P<0.01 compared with control; [b]p<0.01 compared with control 4W; [c]p<0.01 compared with control 8W; [d]p<0.01 compared with DM4W; [e]p<0.01 compared with DM8W.

however, was significantly increased in different DM stages corresponding to the control groups (Table I).

Changes of MDA content and SOD activity in heart tissue. There were no statistically significant differences of MDA content and SOD activity in the 4, 8 and 12W control groups (data not shown). In contrast to the control group, MDA content was increased, while SOD activity was decreased with the development of DM (Fig. 1). This suggested that with the development of DM, oxide stress injury was increased.

Change of Mfn2 mRNA level in heart. In contrast to control groups, the Mfn2 mRNA expression level in the heart decreased in the different stages of DM and was further decreased with the development of DM (Fig. 2).

Change of caspase-3 activity in heart tissue. In contrast to the control group, caspase-3 activity increased with the development of DM (Fig. 3). It suggested that with the development of DM apoptosis was aggravated.

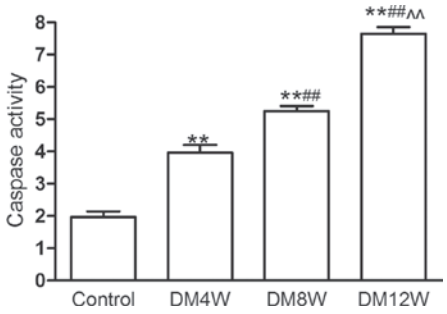

Figure 3. Changes of caspase-3 activity in diabetic rats. [**]P<0.01 compared with control; [##]p<0.01 compared with DM4W; [^^]p<0.01 compared with DM8W.

Discussion

In the present study, we determined that with the development of DM, cardiac oxidative stress and apoptosis were aggravated, the cardiac Mfn2 mRNA level decreased, suggesting that the downregulation of Mfn2 is likely to be correlated with oxidative stress injury and apoptosis in DM. This is the first study to report the correlation of DM and cardiac Mfn2 expressions.

STZ is a drug that causes sustained insulin deficiency and elevated serum glucose levels by selectively destroying pancreatic β-cells to induce DM. Investigators had observed that STZ-induced DM imitated the structural and cellular abnormalities of DM, including cardiac apoptosis, hypertrophy, fibrosis and cardiac inflammation. Consequently, in our experiment we selected STZ to induce DM. In the present study, FBG levels were demonstrated to be elevated in different stages of DM, confirming an occurring abnormality of the glucose metabolism in a STZ-induced diabetes model.

Evidence has shown reactive oxygen species (ROS) to be accumulated at the onset and throughout the development of diabetic cardiomyopathy (9,10). Free radical production was increased in DM patients, while hyperglycemia appeared to be the key factor in the generating of ROS, which lowers the concentrations of antioxidant enzymes. Oxidative stress plays a crucial role in complications of diabetes and induces the production of highly reactive oxygen radicals that are toxic to cells, while having been linked to protein glycation and/or glucose auto-oxidation. The present results have proven a

decreased SOD activity and an elevated MDA content in the diabetic heart, indicating that oxidative stress injury is aggravated with the development of diabetes. SOD scavenges super oxide radicals by converting them to H_2O_2 and oxygen. The decrease in SOD activity in diabetic rats may result from the inefficient scavenging of ROS, indicating that oxidative enzymes were inactivated, while deleterious effects occurred most likely due to accumulated super oxide radicals, or because the enzymes were glycosylated.

Mfn2 encodes a mitochondrial protein that is involved in maintaining the mitochondrial network and regulates mitochondrial metabolism and intracellular signaling. Elimination of Mfn2 in fibroblasts, L6E9 myotubes or Opa1 in mouse embryonic fibroblasts increased mitochondrial fission and decreased mitochondrial membrane potential, oxygen consumption, glucose and palmitate oxidation as well as respiratory complex activity (11-13). In recent years, Mfn2 has been reported to be correlated also with the pathological changes of some diseases related to oxidative stress, energy metabolism and mitochondrial apoptotic signaling. There have been different opinions regarding the role of Mfn2. The overexpression of Mfn2 alleviated high-glucose-induced glomerular mesangial cell proliferation and elevated apoptosis (14). Mfn2 gene can significantly promote apoptosis via Bax and may inhibit proliferation in hepatocellular carcinoma cells (15). Mfn2 silencing inhibited oxidative stress-induced apoptosis in H9c2 cells (16). Other authors, however, reported that Mfn2 deficiency exacerbated renal epithelial cell injury by promoting Bax-mediated mitochondrial outer membrane injury and apoptosis (17). The expression of Mfn2 was markedly downregulated in vascular smooth muscle cells in spontaneously hypertensive rats (SHR) (18). The overexpression of Mfn2 inhibited the proliferation of hyper-proliferative vascular smooth muscle cells *in vitro* and *in vivo* (19). In our experiment, we observed that with the development of diabetes, and the aggravation of oxidative stress injury in DM, caspase-3 activity increased, while Mfn2 mRNA expression was decreased, suggesting that the decrease of the Mfn2 expression may be crucial to diabetes pathophysiology, as well as being closely associated with oxidative stress injury and apoptosis in DM.

In conclusion, we found that with the development of diabetes, along with an aggravation of oxidative stress and apoptosis, Mfn2 expression was decreased, suggesting that Mfn2 might be an important regulator of diabetes. However, additional studies are required to confirm this hypothesis.

Acknowledgements

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