Myocardial Iron Metabolism in the Regulation of Cardiovascular Diseases in Rats

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Key Words

Iron • Diabetes • Myocardial infarction • Divalent metal transporter 1 • Transferrin receptor • Metal transporter protein 1

Abstract

The iron homeostasis plays an important role in cardiac function. To understand how it acts in diabetic and ischemic myocardial injury, we studied the myocardial iron metabolism in diabetic and myocardial ischemic rats. Diabetic rats were induced by intraperitoneal injection of streptozocin (STZ) after intragastric administration of a high-fat diet while the ischemic rat hearts were subjected to coronary artery ligation for 0.5, 1, 6, 12 or 24 h, respectively. In STZ-induced diabetic rats, the contents of serum and myocardial iron were found elevated obviously accompany with the decrease of hepatic iron determined by the flame emission atomic absorption spectroscopy. The levels of superoxide dismutase (SOD), malonaldehyde (MDA) and serum ferritin were increased in diabetic rats. Moreover, protein level of divalent metal transporter 1 (DMT1) was decreased while that for transferrin receptor (TfR) and metal transporter protein 1 (MTP1) was increased. In contrast, no alteration of iron concentration was

observed in the ischemic rats. The expression of DMT1, TfR and MTP1 has not changed after infarction. The findings suggested that diabetes mellitus (DM) induced the iron overload in the myocardium, at least in part by up-regulation of TfR. Meanwhile, down-regulation of DMT1 and up-regulation of MTP1 were induced to alleviate the excessive iron in the myocardium. However, myocardial infarction (MI) has not broken the balance of myocardial iron. In conclusion, the iron homeostasis reacts differently in DM and MI.

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Introduction

Iron, a transition metal, is an essential trace element for many biochemical, metabolic and biological activities [1]. It plays an important pathophysiological role in cardiovascular diseases such as iron overload cardiomyopathy and cardiac fibrosis [1-4]. Meanwhile, it has been reported that increased iron levels in serum and specific tissues may increase type II diabetes risks through several potential mechanisms in clinical studies [5, 6].

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Thus, more and more researchers focus on the relationship between diabetes mellitus (DM) and iron metabolism. However, the iron metabolism in the myocardium is still unclear. In addition, the disorder of systemic iron was reported in patients suffered from myocardial infarction [7]. Nevertheless, the relationship between MI and myocardial iron metabolism were not fully understood. Therefore, to increase our understanding of the underlying molecular mechanisms of myocardial damage, we focused on the myocardial iron regulation between DM and MI.

The generation of reactive oxygen species (ROS) by metal oxidants such as iron represents a potential damage mechanism in diabetic cardiomyopathy [8-10]. The alteration of iron homeostasis has been reported in diabetics and animal models [5, 6]. The management of the uptake and release of dietary iron is primarily by three key transporters, which comprise the proton-dependent divalent metal transporter (DMT1) [11-13], transferrin receptor (TfR) [12, 14, 15] and metal transporter protein 1 (MTP1) [16]. Among them, DMT1 and TfR import the iron into the cell by different mechanisms. In contrast, MTP1 exports ferrous iron (Fe^{2+}) into the plasma whereupon the iron is oxidized [17]. It has also been documented that iron is an important catalyst in free radical reaction and excess iron in myocardium can be potentially harmful via the generation of ROS [6]. Some studies also reported that L-type Ca^{2+} channel (LTCC) is another important pathway of uptaking Fe^{2+} into cardiomyocytes [18, 19].

Based on these findings, DMT1, TfR and MTP1 play a pivotal role together in myocardial iron homeostasis. Nevertheless, the relationships between the changes of their expression and myocardial iron especially in DM and MI conditions have not completely elucidated. Here, our study was designed to investigate the myocardial iron metabolism between diabetic and myocardial ischemic rats and further explain their exact mechanisms.

Materials and Methods

Experimental animals

Male wistar rats (the Animal Center of the Second Affiliated Hospital of Harbin Medical University, China) were used in this study. Rats were housed in a temperature ($23 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$) room with 12 h dark/light cycle and allowed food and water unlimited according to the policy of Good Laboratory Practice (GLP). Use of animals was in accordance with the regulations of the ethic committees of Harbin Medical University.

Establishment of diabetic and high iron rat model

Twenty-four rats were randomly divided into three groups as follows: control group, DM group, high iron group. The procedures for establishment of diabetic model have been described in detail elsewhere [20]. Wistar rats (200–250 g) were intragastrically administered with fat emulsion (10 ml/d) for 15 d. Fat emulsion was prepared with 20 g lard, 1 g thyreostat, 5 g cholesterol, 1 g sodium glutamate, 5 g sucrose, 5 g saccharu, 20 ml tween-80, and 30ml propylene glycol, then added to 100 ml by distilled water. Fat emulsion was stored in a refrigerator at 4°C . DM was induced in the rats by intraperitoneal injection of 40 mg/kg each day streptozocin (STZ) (Sigma Chemicals) in a 0.1 M citrate buffer solution (pH 4.2) for 2 d. Blood samples were collected for measuring fasting blood glucose (FBG) level at 72 h after the last injection of STZ by Grace glucometer (Grace Medical, Inc. America) to ensure induction of DM (FBG ≥ 16.7 mM). The high iron was induced in the rats by injection with iron dextran injection (200 mg/kg each week) for 4 weeks.

Establishment of MI model

Eighteen male wistar rats (250–270 g) were randomly divided into six groups: control, MI for half an hour, MI for 1 h, 6 h, 12 h and 24 h, respectively. The animals were anaesthetized with chloral hydrate (300 mg/kg). A left thoracotomy was performed, and the left anterior descending coronary artery was ligated as described previously [21]. At given time during the experiments, remote tissues of ischemic areas in the left ventricles were quickly dissected for subsequent analysis. The control group of rats was handled in the same manner except the ligation of coronary artery.

Biochemical estimations of diabetic rats

Blood samples of DM rats were collected from tail vein to test FBG after fasting for 12 h. Left ventricles were homogenized and analyzed for superoxide dismutase (SOD), malonaldehyde (MDA) (Nanjing Jiancheng Bioengineering Institute, China). The serum ferritin was analyzed according to the manufacturer's instructions (Tianjin Hanyang Biologicals Technology Co., Ltd, China).

Determination of serum, myocardial and hepatic iron

Serum, myocardial and hepatic iron levels were determined by Flame Atomic Absorption Spectrophotometer (Shimadzu AA-6800, Japan) [22]. Tissue samples (0.1–0.2 g) were digested with a mixture of HNO_3 and H_2O_2 (2:1), then heated at $350\text{--}400^\circ\text{C}$. The final clear residue was adjusted to a final volume of 3.0 ml with 1.0 M HNO_3 .

Western Blot

The procedures were essentially the same as described previously [23, 24]. Proteins were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred moist to polyvinylidene difluoride membranes. Membranes were blocked by 5% nonfat dry milk in PBS and incubated overnight at 4°C . Membranes were washed with PBS containing 0.5% Tween 20 (PBS-T) and then incubated with primary antibody for 2 h, and incubated with secondary antibody for 1 h. The images were captured on the Odyssey Infrared Imaging System

Fig. 1. Fasting blood glucose (FBG) and body weight of the diabetic rats. (A) FBG, *** $P < 0.001$ vs. control, ++ $P < 0.01$, +++ $P < 0.001$ vs. DM. (B) Body weight, for the sake of clarity and clearer comparisons among different groups, only mean values ($n = 8$ in each group) are shown, (●), control; (+), DM rats; (□), high iron rats, * $P < 0.05$, *** $P < 0.001$ vs. control, +++ $P < 0.001$ vs. DM.

Fig. 2. The concentration of superoxide dismutase (SOD), malonaldehyde (MDA) and serum ferritin in diabetic rats and high iron rats. (A, B, C) The contents of SOD, MDA and serum ferritin were increased in the diabetic group. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. control, ++ $P < 0.01$, +++ $P < 0.001$ vs. DM.

(LI-COR Biosciences, Lincoln, NE, USA). Western blot bands were quantified using Odyssey v1.2 software by measuring the band intensity (area $\times$ OD) in each group and normalizing to the internal control.

Statistics and reagents

Polyclonal antibodies against DMT1, TfR and MTP1 were obtained from Alpha Diagnostic (San Antonio, TX, USA). The anti- β -actin antibody (polyclonal) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH; anti-GAPDH antibody) was provided by Kangcheng (Shanghai, China). All data were expressed as mean $\pm$ SEM and analyzed using SPSS13.0 software. Statistical comparisons among multiple groups were performed using analysis of variance (ANOVA). $P < 0.05$ was considered to be statistically significant.

Results

Fasting blood glucose (FBG) and body weight of the diabetic rats

Blood glucose levels in STZ-induced diabetic rats were significantly higher than that in the control group

Fig. 3. The levels of serum, myocardial and hepatic iron in diabetic rats and high rats. (A, B) The level of serum and myocardial iron were elevated in the diabetic group. (C) The level of hepatic iron in diabetic group was decreased. Data are expressed as mean $\pm$ SEM ($n = 8$ in each group). *** $P < 0.001$ vs. control, ++ $P < 0.01$, +++ $P < 0.001$ vs. DM.

Fig. 4. The expression of iron transporters in diabetic rats. (A, C, E) Western blot results for TfR, DMT1 and MTP1 expression in the left ventricles. (A, B, E and F) DM and high iron enhanced the expression of TfR and MTP1 in the myocardium and reduced the expression of DMT1 (C, D). Values are given normalized to band intensity of β -actin (anti- β -actin antibody) used as internal control. All values are expressed as mean $\pm$ SEM (n = 5 in each group). ** P <0.01 vs. control.

(P <0.001, Fig. 1A), but no significant differences were found between the high iron and control groups (P >0.05, Fig. 1A). In addition, the body weight increased gradually in the control and high iron groups, whereas it decreased after injection of STZ in diabetic group (Fig. 1B).

The concentration of SOD, MDA and serum ferritin in diabetic rats

The levels of SOD and MDA in the diabetic group were significantly elevated compared with the control group (P <0.001, Fig. 2A, B). For example, the content of SOD in the control group was 2.51 ± 0.15 , while in the DM group it reached 4.66 ± 0.14 (Fig. 2A). Similar results were obtained in Fig. 2B. The serum ferritin contents in the diabetic and high iron groups were higher than that in the control group (P <0.001, Fig. 2C). Moreover, the levels of SOD, MDA in high iron group were lower than diabetic group (Fig. 2A, B) while the level of serum ferritin was similar in these two groups (Fig. 2C).

Levels of iron in serum, myocardial and hepatic in diabetic rats

It was observed that the iron contents in both serum and myocardial were higher in diabetic and high iron

groups compared with the control group (P <0.001, Fig. 3A, B). For example, the iron level in serum in DM group was 2.4 fold of control group, the content of myocardial iron in DM group was 40% more than that in control group (Fig. 3A, B). It suggested that the iron overload in myocardium was induced by DM (Fig. 3A, B). In contrast, the amount of hepatic iron in the diabetic was markedly lower than that in the control group, while was higher in the high iron group compared with that in the control group (P <0.001, Fig. 3C).

The protein expression of TfR, DMT1 and MTP1 in diabetic rats

Western blotting analysis was performed to verify the presence of TfR, DMT1 and MTP1 proteins in the heart and determine the effect of diabetes on their expression in the myocardium. The protein expressions of TfR and MTP1 in left ventricles were significantly increased in the diabetic and high iron groups compared with that in the control group (P <0.01, Fig. 4A, B, E, and F). Meanwhile, the expression of DMT1 in the diabetic and high iron groups were obviously decreased compared with that in the control group (P <0.01, Fig. 4C, D). However, the protein expressions of TfR, DMT1 and

MTP1 were not statistically different between diabetic and high iron groups.

Reactive Oxygen Species in the heart and ferritin in serum in MI rats

The SOD level was increased significantly after myocardial ischemia for 6 h (Fig. 5A). The MDA level was obviously elevated after myocardial infarction compared with that in the control group (Fig. 5B). These results indicated that the oxidative injury was induced by MI. However, the serum ferritin in these groups was not significantly different ($P>0.05$, Fig. 5C).

Iron levels in serum, myocardial and hepatic in MI rats

The iron contents of serum, myocardial and hepatic were not significantly different in the ischemic model for different time compared with that in the control group (Fig. 6).

The protein expression of TfR, DMT1 and MTP1 in MI rats

The protein expressions of TfR, DMT1 and MTP1 in the ischemic myocardium were further detected by western blot technique after testing the content of iron. Consistent with the result of iron concentration, none of those three proteins was changed in MI rats (Fig. 7).

Discussion

The main finding of this study is that the myocardial iron metabolism was different between DM and MI rats. This notion was strongly supported by the following evidences: (1) the overload of myocardial iron was observed in the STZ-induced diabetic model which was characterized by the increased iron levels in serum and myocardium and the decrease of hepatic iron; (2) no redundant iron was found in the serum, myocardium or liver after MI; (3) the protein levels of important iron transporters of myocardium were changed in DM whereas none was altered in MI.

There is a growing body of evidence that increased oxidative stress and the generation of ROS is one of the important mechanisms of diabetic and ischemic cardiomyopathy [25, 26]. In addition, it was indicated that the generation of ROS correlated with metal oxidants such as iron [27]. During our experiments, both DM and MI models induced the elevation of oxidative injury. Specifically, redistribution of iron and their transporters

Fig. 5. The concentration of SOD, MDA and serum ferritin in MI rats. (A) The content of SOD was increased from ischemic for 6 h. (B) The contents of MDA was increased in MI group. (C) The level of serum ferritin did not change in MI group. Data are expressed as mean $\pm$ SEM (n = 3 in each group). * $P<0.05$, ** $P<0.01$, *** $P<0.001$ vs. control.

Fig. 6. The levels of serum, myocardial and hepatic iron in MI rats. (A, B, C) None of the iron was altered in MI group. Data are expressed as mean $\pm$ SEM (n = 3 in each group).

explained the production of ROS in DM hearts. However, no relationship between the increase of ROS and iron transporters was found in MI hearts. Previous studies have indicated that the pH value of cardiomyocytes was decreased during ischemia, which induces the release of low molecular iron. Then, low molecular iron catalyzes

Fig. 7. The expression of iron transporters in MI rats. (A, C, E) Western blot results for TfR, DMT1 and MTP1 expression in the left ventricles. MI did not alter the expression of TfR, DMT1 and MTP1. Values are given normalized to band intensity of GAPDH (anti-GAPDH antibody) used as internal control. All values are expressed as mean $\pm$ SEM (n = 3 in each group).

the Fenton reactions which produces oxygen radicals to cause the increase of ROS. Meanwhile, the iron changed its bounding state to free state without altering its amount during this process [28]. Thus, MI may cause the increase of ROS by this pathway in the present study.

In our study, the high contents of iron in serum and myocardial were detected in the diabetic model (Fig. 3A, B). One of the possible mechanisms of iron overload was the up-regulation of TfR (Fig. 4A), by which excessive iron imported into the cardiomyocytes. After that, high concentration iron induced the down-regulation of DMT1 and up-regulation of MTP1 to alleviate the overload status (Fig. 4C, E). Therefore, our data proved that there may be a feedback protective mechanism in the iron regulation to sustain the function of the cardiomyocytes. Likewise, Ward et al demonstrated the down-regulation of DMT1 and up-regulation of TfR in the kidney of STZ-diabetes. In their studies, the expression of DMT1 in the renal, proximal tubules and collecting ducts was found significantly reduced and TfR expression increased in STZ-diabetic Sprague-Dawley rats [27]. At the same time, renal DMT1 expression was also decreased in wistar rats following 12 weeks of STZ-diabetes, which was fully restored by insulin-

replacement [27]. This increases the likelihood that this observation is a general phenomenon in diabetic rats. Such possible interactions between oxidative stress and the dynamic balance of DMT1 and TfR prompted the iron metabolism designed to attenuate the development of oxidative stress in experimental diabetes.

As we found, more iron existed in the myocardium of DM rats, where is the additional iron from? As shown in our data, the hepatic iron in the DM group was severely decreased compared with the control group (Fig. 3C) which is opposite to the contents in serum and myocardium (Fig. 3A, B). Systemic iron balance is regulated at the level of intestinal absorption, iron recycling and liver mobilization. Liver is an important metabolic organ, and the additional iron may at least partly from the release of hepatic.

Studies show that elevated iron mediated injury might play an important role in the development of a number of cardiovascular diseases such as heart ischemia-reperfusion injury, arrhythmia, heart failure and dilated cardiomyopathy [2, 29, 30]. Despite the connection between elevated cardiac free iron contents and cardiac ischemia has been well documented [31], the regulation of cardiac iron metabolism in DM and MI

remains poorly understood. Although the levels of SOD and MDA were elevated in both models to cause the oxidative injury (Fig. 2A, B, Fig. 5A, B), the iron status in MI was totally different from DM. For example, the serum and myocardial iron did not change in MI rats (Fig. 6A, B) whereas increased markedly in the diabetic group (Fig. 3A, B). In addition, the content of hepatic iron in MI apparently kept in the normal level (Fig. 6C). Consistent with the iron level, these iron transporters also sustained with their normal condition after ischemia for corresponding time (Fig. 7). As the main protein, ferritin can combine with the ferrous. Therefore, the elevated serum ferritin in DM rats confirmed the iron-overload status (Fig. 2C). On the contrary, the serum ferritin in MI rats has not altered in the ischemic condition (Fig. 5C).

Taken together, these findings in this study provided information about the iron homeostasis in these two different oxidative-injury diseases. The level of myocardial iron and the iron transporters reacts differently in these two models. According to our study, the iron transporters TfR, DMT1 and MTP1 may be potential therapeutic targets in the treatment of iron disorder.

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