Mitochondrial Respiratory Impairment in Streptozotocin-Induced Diabetic Rat Heart

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The purpose of this study was to examine mitochondrial respiratory impairment in the diabetic heart. Diabetes mellitus was induced in male Wistar rats by intravenous injection of streptozotocin (STZ) for 2 to 16 weeks (Group D). In some of the diabetic rats, insulin was injected for 2 or 3 weeks prior to sacrifice (Group I). Fasting blood glucose was markedly elevated to greater than 300 mg/dl in Group D and was similar to normal glucose levels in Group I. At 2 weeks after STZ injection, state 3 was only 59.1% of that in the control. Complex I and complex V activities were also significantly reduced to 43.4% and 71.7% of those in the control, respectively. These reductions recovered with insulin treatment. This phenomenon persisted for 16 weeks. Morphological studies revealed swelling of the mitochondria and an increase in lipid droplets in diabetic cardiomyocytes, and these were also improved with insulin treatment. We conclude that in the diabetic heart, disturbance of energy production in cardiac mitochondria is generated by the impairment of oxidative phosphorylation due to depression of complex I and complex V. These changes may contribute the cardiac dysfunction that is a complication of diabetes mellitus.

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Due to the recent increase in the number of patients with diabetes mellitus, myocardial involvement in diabetic patients has received much attention. In clinical practice, congestive heart failure is often found as a complication in diabetes mellitus, especially in cases with poor control. This is characterized by diastolic dysfunction. Furthermore in non-insulin dependent (type 2) diabetics, left ventricular function improves with correction of hyperglycemia. The cause of myocardial involvement in diabetes mellitus has been considered to be ischemia due to micro-angiopathy, and direct impairment of myocardial metabolism by hyperglycemia and insufficient insulin due to diabetes mellitus itself. We previously reported depressed Ca²⁺⁺-stimulated ATPase activity in sarcoplasmic reticulum (SR) changes in phospholipids and their fatty acid compositions in cardiac SR and reduced ATPase and acid phosphatase activities in diabetic myocardial cells. On the other hand, we have also reported suppression of both the Ca²⁺⁺-ATPase protein and mitochondrial respiratory chain in ischemic myocardium. This evidence suggests that both the energy-producing and -consuming systems are involved in both ischemic and diabetic myocardium. This study focused on energy production

Key words:
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by mitochondria in the diabetic heart from the acute to chronic stages in streptozotocin-induced diabetic rats, with special reference to the reversibility of mitochondrial impairment with insulin treatment.

MATERIALS AND METHODS

I) Experimental Protocol

The experimental protocol is shown in Fig 1. Three hundred 7-week-old male Wistar rats with a body weight of 170 g to 200 g were used. To induce diabetes mellitus, 65 mg/kg body weight of streptozotocin (STZ) (Sigma®) was dissolved in a citrate buffer (pH 4.5) and given in a single intravenous injection via the tail vein. The animals were maintained on normal rat chow and water ad libitum. The rats were separated into 5 groups at 2, 4, 8, 12 and 16 weeks following intravenous injection of STZ or citrate buffer alone. Those with fasting blood glucose levels above 300 mg/dl at the time of sacrifice were classified as diabetic (Group D). Some of the rats in Group D, for 2 or 3 weeks prior to sacrifice, blood glucose levels were maintained at physiological levels with subcutaneous injections of 2 to 10 units of lente-type insulin (Novolente MC®) (Group I). In Group 2-I, insulin treatment was started 2 days after STZ injection. Age-matched rats which were injected with citrate buffer alone were used as the control group (Group C). Under ether anesthesia, the beating heart was extracted and the following biochemical and ultrastructural analyses were performed.

II) Biochemical Analyses

I) Extraction of Mitochondria:

All procedures were carried out at 4°C unless otherwise noted, and all of the reagents were reagent grade. Protein determination was made by a biuret method. Mitochondria were extracted from left ventricular tissue using the modified method of Sordahl and Stewart with alkaline protease (Nagarse®, Nagarse Co, Ltd)13. Briefly, 3 g of chopped ventricular tissue was homogenized with 40 ml of a mixture containing 0.25 mol/L sucrose, 0.01 mol/L glycoletherdiaminetetra-acetic acid (GEDTA) and 0.03 mol/L tris-HCl (pH 7.2) with a Polytron homogenizer (Brinkmann Instruments), and then centrifuged at 27,000 × G for 10 min. One mg of Nagarse per g of starting tissue was added to the pellet and suspended with a Vortex mixer and incubated at 4°C for 8 min. After
suspension in an isolated medium containing 0.18 mol/L KCl, 0.01 mol/L GEDTA, 0.5% (wt/vol) bovine serum albumin (BSA) and 0.03 mol/L tris-HCl (pH 7.2) with a loose-fitting Teflon pestle using glass homogenizers, centrifugation was performed at 300×G for 5 min. The supernatant was centrifuged twice at 12,000×G for 10 min. The mitochondrial pellets were suspended in a solution of 0.18 mol/L KCl, 0.5% BSA and 0.03 mol/L tris-HCl (pH 7.2), and the amount of mitochondrial protein was adjusted to a final concentration of 5 mg/ml. Some of the mitochondria were sonicated with a sonifier (Cell Disruptor 200, Branson Co, Ltd, Switzerland) for 60 sec, and sub-mitochondrial particles were prepared.

2) Measurement of Oxidative Phosphorylation:
The activity of oxidative phosphorylation in mitochondria was measured by the method of Hagihara\textsuperscript{14} using oxygen electrodes (Oxygen Consumption Recorder PO-100A, Yunagimoto Mfg. Co, Ltd, Tokyo). Two ml of reaction medium consisted of 0.01 mol/L KCl, 0.0001 mol/L ethylenediaminetetraacetic acid (EDTA), 0.002 mol/L MgCl\textsubscript{2}, 0.25 mol/L sucrose, 0.02 mol/L potassium phosphate buffer (pH 7.2) and 0.75 mg of mitochondrial protein. The oxygen saturation was estimated to be 245 nmol/ml at 25°C. Succinate or glutamate plus malate was used as the substrate with active oxygen consumption initiated by the addition of 0.025 ml 50 mmol/L ADP. The rate of oxygen consumption in state 3 was determined by a polarographic record using the method of Chance and Williams\textsuperscript{15}

3) Measurement of NADH-Coenzyme Q (CoQ) Reductase (Complex I) Activity:
Complex I activity was measured by the modified method of Hatefi and Rieske\textsuperscript{16} using a spectrophotometer to determine the initial rate of oxidation of NADH at 340 nm. The reaction was started by adding 0.4 mg of sub-mitochondrial particles to a reaction medium containing 0.08 mol/L tris-HCl (pH 8.0), 0.003 mol/L NaN\textsubscript{3}, 0.04 mmol/L CoQ\textsubscript{3} and 0.03 mmol/L ADP at 37°C.

4) Measurement of Succinate Dehydrogenase-CoQ Reductase

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(Complex II) Activity:
Complex II activity was measured according the modified method of Ziegler and Rieske\textsuperscript{17} by measuring the rate of reduction of 2,6-dichloroindophenol sodium (DCIP) at 500 nm with a spectrophotometer. The reaction was initiated by adding 0.05 mg of submitochondrial particles to a reaction solution containing 0.02 mol/L sodium succinate, 0.01 mmol/L EDTA, 0.01% triton X-100, 0.1 mol/L potassium phosphate (pH 7.0), 0.0017% DCIP, and 0.04 mmol/L CoQ\textsubscript{3} at 37°C.

5) Measurement of Cytochrome Oxidase (Complex IV) Activity:
Complex IV activity was measured according to the modified method of Orii and Okunuki\textsuperscript{18} by adding 0.05 mg of submitochondrial particles to a reaction solution containing 0.0045 mol/L cytochrome C (reduced form) and 0.15 mol/L sodium phosphate buffer (pH 5.0). The change in the concentration of reduced cytochrome C was measured at 500 nm and 37°C.

6) Measurement of Dinitrophenol (DNP)-Stimulated ATPase Activity:
The activity of mitochondrial DNP-stimulated ATPase activity, which is involved in the final step of ATP production, was measured by the modified method of Pullman et al\textsuperscript{19} One ml of reaction solution containing 0.21 mol/L sucrose, 0.02 mol/L KCl, 0.02 mol/L tris-malate (pH 7.2) and 5 mg mitochondria was used. The reaction was initiated by adding 0.001 mol/L DNP and 0.03 mol/L ATP. Following incubation for 10 min at 25°C, the reaction was stopped by adding 2 ml of 20% trichloroacetic acid. The amount of free inorganic phosphate derived from ATP was measured by the method of Fiske and SubbaRow\textsuperscript{20}

III) Ultrastructural Observation
Chopped heart muscle was fixed with 2.5% glutaraldehyde and 0.1 mol/L Nacacodylate (pH 7.4) for 2 h at 0°C. The sample was rinsed with 0.1 mol/L cacodylate and sucrose, fixed in 1% OSO\textsubscript{4}, dehydrated in ethanol and embedded in Epon 812. The blocks were sliced with an ultramicrotome (Sorvall MT-2) and observed with an electron microscope (Hitachi H-7000).
Statistics:
Unless specifically noted, the results are expressed as the mean ± SD. The experimental values were statistically analyzed with an unpaired Student's t-test. Differences were considered significant at p < 0.05.

RESULTS

Blood glucose levels: Induction of diabetes mellitus was confirmed by an elevation of the blood glucose concentration. The fasting blood glucose levels for each group at the time of sacrifice are shown in Fig 2. Group C showed normal blood glucose levels at 2 and 16 weeks (Groups 2-C and 16-C) of 105.0 ± 9.9 mg/dl and 111.4 ± 36.0 mg/dl, respectively. Group D showed a markedly elevated blood glucose level at 2 weeks Group 2-D of 416.6 ± 42.1 mg/dl, with sustained elevation of blood glucose until 16 weeks. Group I showed blood glucose levels of 131.2 ± 20.8 mg/dl and 125.3 ± 40.5 mg/dl at 2 and 16 weeks (Groups 2-I and 16-I), respectively, which were not significantly different from the values in the control.

Body weight and heart/body weight ratio:

Fig 2. Blood glucose level.
C: age-matched controls; D: STZ-induced diabetic rats; I: insulin-treated diabetic rats. Values are expressed as the mean ± SD. **p < 0.001 vs C, ***p < 0.001 vs I

Fig 3. Body weight and heart/body weight ratio in diabetic rats. Fig 3-a: Body weight; Fig 3-b: Heart/body weight ratio.
Dotted, hatched and solid bars indicate normal (C), diabetic (D) insulin-treated (I) groups, respectively. Values are expressed as the mean ± SD.
*p < 0.05, **p < 0.01, ***p < 0.001 vs C, *p < 0.05, ***p < 0.001 vs D
Fig 4. Oxygen consumption rate in state 3 of cardiac mitochondria in diabetic rats. Succinate was used as a substrate. Dotted, hatched and solid bars indicate Groups C, D and I respectively. Values are expressed as the mean ± SD.

* p < 0.01, ** p < 0.001 vs C

Fig 5. Oxygen consumption rate in state 3 of cardiac mitochondria in diabetic rats. Glutamate plus malate was used as a substrate. Dotted, hatched and solid bars indicate Groups C, D and I respectively. Values are expressed as the mean ± SD.

* p < 0.05, ** p < 0.01, *** p < 0.001 vs C

As reported previously, the body weight in Group C increased steadily with age. However, the increase in Group D was inhibited at 2 weeks after the intravenous injection of STZ. Body weight in Group I was significantly greater than that of Group D from weeks 4 to 16. A significant difference was also found between Groups I and C from weeks 4 to 16. The weight of the heart in Group D was greater than those in Groups C and I. Therefore, the heart/body weight ratio was significantly greater in Group D than in Groups C and I from weeks 2 to 16 (Fig 3).

Mitochondrial Oxidative Phosphorylation:
The activities of oxidative phosphorylation are shown in Figs 4 and 5. When succinate was used as the substrate, a significantly reduced rate of oxygen consumption in state 3 was found in Group 2-D as compared to Group 2-C. A similar difference was found at 16 weeks after the onset of diabetes.
mellitus. The reduction in the rate of oxygen consumption in Group 2-D was improved significantly with insulin treatment from 59.1% to 89.5% (Group 2-I). This improvement was also observed in Groups 4-I to

16-I. Group I was significantly different from both Groups C and I at 12 and 16 weeks. Similar results were obtained when glutamate plus malate was used as the substrate.

Activities of Enzymes in the Mitochondrial Electron Transport System:
The activity of NADH-CoQ reductase (complex I) is shown in Fig 6. Complex I activity in Group 2-C was 0.076 ± 0.010 μmol NADH/mg protein per min. In Group 2-D, this value was significantly reduced to 0.033 ± 0.006 (p < 0.01). The activity in Group 2-I (0.081 ± 0.008) was similar to that in Group 2-C. At 4 to 16 weeks after the onset of diabetes mellitus, there was a significant improvement in activity with insulin treatment. In contrast, there was no significant difference in succinate dehydrogenase-CoQ reductase (complex II) activity between Groups 2-C, 2-D and 2-I groups; 0.33 ± 0.06, 0.29 ± 0.06 and 0.36 ± 0.05 μmol DCPIP/mg protein per min, respectively (Fig 7). Similarly, there were no significant differences between Groups
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Fig. 10. Electron micrographs of diabetic myocardium. A: normal myocardium in Group C. Fine structures are well preserved; B: diabetic myocardium in Group D. Swollen mitochondria and lipid droplets are observed; C: Insulin-treated myocardium. Mitochondrial swelling has improved and there are fewer lipid droplets. The bar indicates 1 μm. Mt: mitochondria; Mf: myofibrils; Lp: lipid droplets.

Activity is presented in Fig. 9. This activity in Group 2-C was 20.98 ± 1.36 μmol Pi/mg protein per h, which was significantly greater than that in Group 2-D (15.04 ± 3.01) (p < 0.01). The value in Group 2-I (20.72 ± 0.57) was not significantly different from that in Group 2-C. This tendency was also observed at 4 to 16 weeks.

Electron Microscopic Observation of Myocytes:
Myocytes in Group 8-D were examined by electron microscope, and compared with those in Groups 8-C and 8-I (Fig. 10). No remarkable changes were found in the myofibrils in Group 8-D. However, swelling of mitochondria, reduction in the density of the mitochondrial matrix and abnormal arrangement were noted. An increase in glycogen granules and lipid droplets were found near the mitochondria. Mitochondrial swelling in Group 8-I was only slight, while there was no improvement in the abnormal arrangement. Glycogen granules and lipid droplets also tended to be reduced.

DISCUSSION

Several etiological studies, such as the Framingham study, have shown that heart disease is a major complication of diabetes mellitus, with the mortality rate due to heart disease higher than that in non-diabetic groups. Rubler et al. reported a new type of cardiomyopathy associated with diabetic glomerulosclerosis. Hamby et al. were the first to propose the concept of diabetic cardiomyopathy due to pathological changes in small coronary vessels. On the other hand, from a biochemical perspective, a decrease in alpha and beta receptors, a reduction in actomyosin and myosin ATPase activity, and changes in myosin isozymes were all reported in diabetes. With regard to mitochondrial function, there have been reports of increases in long chain acylCoA and long chain acylcarnitine, a reduction in pyruvate dehydrogenase activity, a decrease in total NAD+/NADH content, an accumulation of beta hydroxy fatty acid in the metabolism of free fatty acids, and reductions in both Ca++ uptake and the membrane potential. However, there has been no detailed examination of the electron
transport system in diabetes.

In this study, the respiratory activity of myocardial mitochondria indicated a significant reduction in the rate of oxygen consumption in state 3 using either succinate or glutamate-malate as substrates beginning 2 weeks after the onset of diabetes mellitus. With insulin treatment, an improvement in the rate of oxygen consumption in state 3 was found at both 2 and 16 weeks. These results indicate that streptozotocin itself is not directly cardiotoxic, but mitochondrial oxygen consumption is reduced in the diabetic state. Pierce and Dhallan reported that mitochondria in rat myocardium at 8 weeks after the onset of diabetes mellitus showed a reduced rate of oxygen consumption in state 3 with glutamate as the substrate, as well as a reduction in Mg$^{2+}$-dependent ATPase activity, and that insulin treatment for 4 weeks returned the rate of oxygen consumption to normal levels, which is consistent with our results. Furthermore, in measuring the activities of enzymes in the electron transport system, a decrease in NADH-CoQ reductase (complex I) activity, which involves NAD, and in DNP-stimulated ATPase (complex V) activity, which is the final step in ATP production, were found. Treatment with insulin improved these deficiencies in the electron transport system at 16 weeks. It has been reported that oxygen consumption with glutamate plus malate as the substrate parallels the activities of complexes I and III while that with succinate as the substrate parallels that of complex III. Based on our results, which show reductions in both state 3 respiration with succinate as a substrate and DNP-ATPase activity, we speculate that complex III activity is impaired. In other words, complex I and V functions in the electron transport system are impaired, which in turn impairs ATP production. In contrast, complex II and IV functions are considered to protect against diabetic stress. Miller reported a relationship between the reduced content of ATP in the diabetic heart and impaired cardiac function. This finding also suggests impaired ATP production by mitochondria. It is difficult to determine which specific factors affect the reduction in mitochondrial function in the diabetic state. Although it is possible to rule out abnormalities of the major arteries in STZ diabetic myocardium, we cannot exclude impairment of the microvasculature. Ischemia reportedly reduces the rate of oxygen consumption in state 3 in mitochondria and impairs enzymes in the electron transport system (complexes I and V). In the mitochondria in diabetic hearts, it is possible that local myocardial perfusion may be reduced. However, a myriad of metabolic disturbances which accompany the diabetic state cannot be dismissed, including hypothyroidism, reduced intracellular pH, and oxygen free radicals, which may in turn depress the activity of enzymes in the electron transport system. Mitochondrial function is improved with the recovery of metabolism by insulin. In addition, it has been suggested that the differences in activity between complexes I and V and complexes II and IV which are found in diabetes are caused by structural changes and the different location of each enzyme in the mitochondrial inner membrane.

Electron microscopic examination of myocardial cells revealed that diabetic myocardium shows swollen mitochondria and an increase in lipid droplets. Seager et al reported swollen mitochondria, clearing of the mitochondrial matrix and marked increases in both lysosomes and lipid droplets. However, neither atherosclerosis plaque nor structural changes in the smooth muscles or endothelial cells in the small arteries arterioles or capillaries were seen in the myocardium of rats at 8 weeks after the onset of diabetes mellitus. Swelling of mitochondria suggests impaired energy production, and the increase in lipid droplets may be due to the fact that energy production by mitochondria depends on lipids rather than glucose. Both morphological changes and functional changes could be reversed with insulin treatment even in long-term diabetes. With continued study of the long-term changes in the STZ diabetic heart, it is expected that the process of reversible change with insulin treatment will become clear.

In conclusion, the results of our study suggest that in the mitochondria of diabetic myocardium, energy production is impaired soon after onset by the impairment of phosphorylation due to a reduction in the
activities of enzymes in the electron transport system (complex I and complex V), and these changes can be reversed by insulin treatment. These results may also contribute to the development of cardiac dysfunction as a complication of diabetes mellitus.

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