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MITOCHONDRIAL PROTEIN PHOSPHORYLATION AND CARDIOMYOPATHY IN GENETICALLY DIABETIC MICE: THE EFFECT OF ESTRONE TREATMENT

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The alpha-subunit of pyruvate dehydrogenase and succinyl-CoA synthetase are phosphorylated after incubation of cardiac mitochondria from genetically diabetic mice with [γ-^{32}P]ATP. There is significantly increased incorporation of ^{32}P into pyruvate dehydrogenase from diabetic mice when compared to controls. The enhanced rate of pyruvate dehydrogenase phosphorylation correlates well with the previously reported defective oxidative metabolism and decreased activity of this enzyme from diabetic mice. The relationship between abnormal mitochondrial function and development of cardiomyopathy in the diabetic mice has been studied further by *in vivo* estrone treatment. The results indicate that ultrastructural alterations of myocardium are closely associated with the defective pyruvate oxidation (via phosphorylation of pyruvate dehydrogenase) and both processes can be prevented by 7-12 weeks estrone treatment.

The diabetic syndrome (non insulin-dependent type) in C57BL/KsJ db/db mice (1) is accompanied by pathologic lesions of the heart (2). The development of cardiomyopathy in this strain of mice is characterized by ultrastructural changes as well as defective oxidative metabolism in cardiac mitochondria (3). There is a 30% decrease in state 3 oxidation rate in isolated cardiac mitochondria from diabetic mice when pyruvate + malate is used as substrate. It was recently shown (4) that this defective pyruvate oxidation may be due to inactivation of pyruvate dehydrogenase. Since the mammalian pyruvate dehydrogenase complex is inactivated by phosphorylation and reactivated by dephosphorylation (5-7), it was suggested (4) that higher levels of phosphorylation might be responsible for the inactivation of pyruvate dehydrogenase in mitochondria of diabetic mice. However, direct evidence showing increased incorporation of phosphate into the enzyme complex from diabetic mice was lacking. In the present study, we have carried out *in vitro*

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protein phosphorylation using cardiac mitochondria isolated from both diabetic and control mice. Since it has been shown (8) that estrone, a dehydroepiandrosterone metabolite, is effective in maintaining islet integrity and preventing the development of severe diabetes, the in vivo treatment of estrone in diabetic mice was also carried out to see if cardiomyopathy as well as inactivation of pyruvate dehydrogenase could be prevented.

Materials and Methods

Female, diabetic mice (C57BL/KsJ db/db) (Jackson laboratories, Bar Harbor, Maine) and their lean littermate controls were used. [γ-^{32}P]ATP was purchased from ICN Pharmaceuticals (Irvine, CA).

Estrone treatment Mice, 5 weeks of age were treated with estrone according to Coleman et al (8). Briefly, db/db mice and their controls were each divided into two groups of 25 animals. One group from control and diabetic mice was fed chow alone (old Guildford diet 96) and the other group was fed powdered chow into which estrone (0.005 g per 100 g of chow, Sigma Co.) had been incorporated. The treatment was carried out for 7-12 weeks. Mice were then sacrificed between 12-17 weeks of age. A ring of cardiac muscle was cut for ultrastructural observation and the remaining tissue pooled and used for biochemical studies.

Preparation of mitochondria Heart mitochondria were isolated using the nagarse method reported previously (3). Two to three hearts from animals of each group were used for the preparations.

Oxidative phosphorylation Using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio), oxygen consumption was monitored in a final volume of 1.5 ml at 30°C. The reaction mixture consisted of 0.225 M mannitol, 0.07 M sucrose, 1 mM EGTA, 0.01 M potassium phosphate pH 7.5, and substrate, each at the following concentration: 50 µM palmitylcarnitine + 2.5 mM malate; 5 mM pyruvate + 2.5 mM malate; or 5 mM succinate + 2.5 µM rotenone. State 3 (ADP-stimulated) respiration was initiated by addition of ADP (273 µM) and was followed by state 4 (ADP-limiting) respiration (3).

In vitro phosphorylation of mitochondrial proteins: Phosphorylation of mitochondrial proteins was performed as described by Yang and Smith (9). Reaction mixture consisted of the following in a final volume of 100 µl: 50 mM Tris. glycine pH 8.2, 10 mM $MgCl_2$, 5 µM [γ-^{32}P]ATP (5 µc) and 200 µg mitochondrial protein. The samples were preincubated at 30°C for 1 min prior to addition of the radioactive substrate. After initiation of the reaction by addition of substrate, samples were incubated for 30s at 30°C and quenched by the addition of 25 µl of SDS stop solution that contained 0.5M Tris-HCl pH 6.8, 10% SDS, 10% 2-mercaptoethanol, and 50% glycerol.

SDS gel electrophoresis and autoradiography: Aliquots of the SDS quenched reaction mixture (containing 50 µg protein) were subjected to SDS gel electrophoresis according to Yang and Smith (9). Gels were stained overnight in a solution containing 0.1% Coomassie Blue, 25% isopropanol and 0.1% sodium carbonate. After drying, the labeled bands were detected by autoradiography using Kodak X-Omat XRP-1 film, the labeled bands of interest were then cut out and the radioactivity determined in a scintillation counter.

Electron Microscopy - Blocks of ventricular heart tissue extending from endocardial to epicardial surface were fixed in 4% glutaraldehyde- 3% paraformaldehyde and processed for electron microscopy as described previously (2).

Results and Discussion

In vitro phosphorylation of mitochondria: Yang and Smith (9) have shown that when mitochondria are incubated with [γ-^{32}P]ATP for 30s, only two proteins are phosphorylated. In the present study, two phosphoproteins with M.W. of 42,000 and 32,000 were detected from heart mitochondria of control or diabetic mice (Fig. 1A). The 42,000 dalton protein has been identified previously as the alpha-subunit of pyruvate dehydrogenase (PDH) and the 32,000 dalton protein as the autophosphorylated subunit of succinyl-CoA synthetase (SCS) (9). It is evident from Fig. 1A that there was increased incorporation of the labeled phosphate group into these two proteins with mitochondria preparations from hearts of diabetic mice as compared to controls. Fig. 1b shows the Coomassie Blue stain pattern of the same SDS-gel samples. There were approximately 14 major mitochondrial protein bands separated by electrophoresis with the 42,000 dalton radioactive band corresponding to Coomassie Blue stained band No. 8 and the 32,000 dalton radioactive band corresponding to a region between bands No.

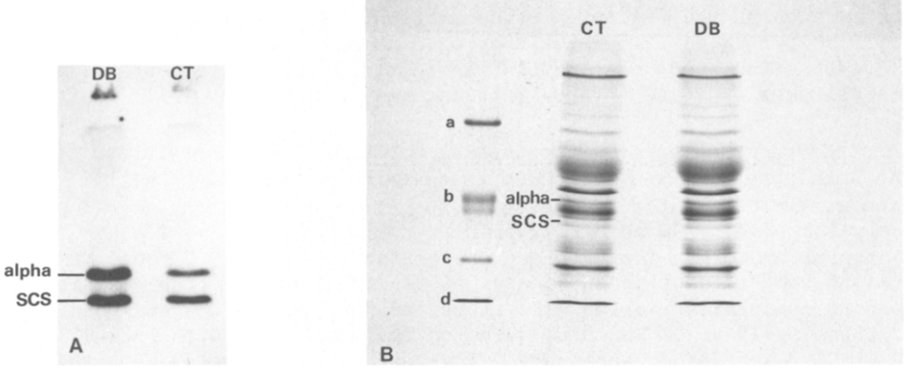

Fig 1. Mitochondrial protein phosphorylation. Cardiac mitochondria from genetically diabetic mice or lean-littermates are phosphorylated as described in Methods. SDS gel electrophoresis and autoradiography are performed. Fig 1A. autoradiogram. Fig 1B. Coomassie Blue stained gel. alpha: alpha-subunit of pyruvate dehydrogenase. SCS: Succinyl-CoA synthetase. Molecular weight standards are: phosphorylase a (a), bovine serum albumin (b), ovalbumin (c), and carbonic anhydrase (d). CT= control, DB= diabetic

10 and 11. Densitometric scans of the Coomassie Blue stained gel (data not shown) indicate no alterations in the individual bands between the diabetic and control groups; specifically, there was not an increase in the concentration of 42,000 dalton protein per mg of mitochondrial protein from diabetic mice as compared to controls. Furthermore, the increase in the extent of PDH phosphorylation in the preparation from diabetic mice is not due to a change in the ATPase activity, since increasing ATP concentrations or the inclusion of oligomycin (20 μg/ml) during incubation does not abolish this difference between samples of diabetic and control mice. Thus the increased radioactivity in the 42,000 dalton protein may be attributed to the enhanced pyruvate dehydrogenase kinase activity in mitochondria from diabetic mice. This result correlates well with the previous finding (4) where there was inactivation of pyruvate dehydrogenase from the mitochondria of the diabetic mice due to an increase in the proportion of phosphorylated enzyme (the inactive enzyme).

The effect of estrone treatment on abnormal mitochondrial function and cardiomypathy

Coleman et al. have shown (8) that estrone is effective in maintaining islet cell integrity and preventing the development of severe diabetes while having little effect on the amount of food intake or the rate of weight gain. In order to study the relationship of pyruvate dehydrogenase inactivation with the development of cardiomyopathy in diabetic mice, the *in vivo* treatment of estrone in diabetic mice was carried out. Table 1 shows the effect of *in vivo* estrone treatment on incorporation of phosphate into the 42,000 dalton and 32,000 dalton mitochondrial proteins. To minimize variation, experiments were carried out in a paired manner using heart mitochondria from both diabetic and control mice. The incorporation of ^{32}P into radioactive bands (cpm per mg of mitochondrial protein) for either estrone treated or untreated diabetic mice were then expressed as a ratio relative to the respective lean-littermate controls. It can be seen from Table 1, that for untreated diabetic mice, the ^{32}P incorporation ratio for both pyruvated dehydrogenase and succinyl-CoA synthetase are greater than 1 (or approximately 30% higher than controls). Presumably the

Table 1. Effect of *in vivo* estrone treatment on mitochondrial protein phosphorylation

Mouse	^{32}P incorporation ratio *(R)	
	PDH	SCS
diabetic untreated	1.23	1.09
	1.40	1.48
	1.42	1.62
	1.28	1.22 (1.36 ± 0.23)
	1.24 (1.31 ± 0.12)	1.06
	1.35	1.75
	1.20	1.38
	1.15	1.38
	1.50	1.28
diabetic treated with estrone	1.19	1.0
	0.66	1.01
	0.71 (0.92 ± 0.20)	1.16 (1.11 ± 0.10)
	1.0	1.21
	0.94	1.05
	1.0	1.23
	P<0.001	P<0.05

* Heart mitochondria were isolated from diabetic mice and from their lean littermate controls. In vitro phosphorylation experiments were carried out side by side with these two preparations of mitochondria. After SDS-gel electrophoresis and autoradiography, ^{32}P radioactive bands were excised and counted. Results are expressed as ^{32}P incorporation ratio (R)

$$\text{Where } R = \frac{(\text{CPM/mg mitochondrial protein}) \text{ diabetic}}{(\text{CPM/mg mitochondrial protein}) \text{ control}}$$

Bracketed data are means and standard deviations for each group.

increased ^{32}P incorporation into PDH of heart mitochondria from diabetic mice is due to enhanced pyruvate dehydrogenase kinase activity. Treatment with estrone that began at 5 weeks of age (before the development of severe diabetes) and terminated at 17 weeks of age prevented this increase in ^{32}P incorporation so that the ratio remains at 0.92 for PDH and 1.11 for SCS. Statistical analysis by Student's t-test indicated that the differences between the treated vs, untreated group are highly significant for the phosphorylation of PDH ($P< 0.001$), but is only borderline for SCS ($P< 0.05$). It should be mentioned that estrone treatment did not affect the mitochondrial protein phosphorylation in the control mice (data not shown). While the effect of estrone treatment on PDH phosphorylation may be explained on the basis of the pyruvate dehydrogenase kinase activity, its effect on the autophosphorylation of succinyl-CoA synthetase is not clear.

Table 2. Effect of *in vivo* estrone treatment on state 3 pyruvate oxidation in isolated cardiac mitochondria*

Mouse	State 3 oxidation rate (% of control)	
Diabetic Untreated	55 73 63 52	(60 ± 9)
Diabetic Treated with estrone	73 100 86 82	(85 ± 11)
	P<0.01	

* Isolated heart mitochondria from diabetic mice with and without estrone treatment are compared with their respective control littermates in the oxidative phosphorylation studies. State 3 represents oxidation in the presence of ADP. Bracketed date are mean and standard deviations for each group. The Student's t-test was used and significance defined as $P \leq 0.05$.

To further demonstrate that estrone treatment was effective in the modification of cardiomyopathy, the oxidative phosphorylation studies were carried out. Table 2 shows the effect of *in vivo* estrone treatment on state 3 pyruvate oxidation in isolated mitochondria from matched pairs of diabetic and control animals. The state 3 oxidation rates (natom oxygen per mg mitochondria per min.) for either estrone treated or untreated diabetic mice are expressed as percentage relative to their respective controls. It can be seen from Table 2 that for untreated diabetic mice, the pyruvate oxidation rate is only 60% of that for control mice, representing a 40% depression. In contrast, estrone treatment causes a significant increase in this rate such that pyruvate oxidation in isolated mitochondria nearly returns to normal, representing 85% of the control value. It is noteworthy that this normalization in mitochondrial oxidative metabolism is preceded by the normalization of blood glucose level (data not shown).

Fig. 2 shows the ultrastructural features of cardiac tissue from estrone treated or untreated diabetic mice. It is evident that the ultrastructural lesions occurring in the myocardium of untreated diabetic mice such as the degeneration of mitochondria giving rise to residual bodies (2) are prevented by 12 weeks of estrone treatment (compare Fig. 2A and B). No myocardial

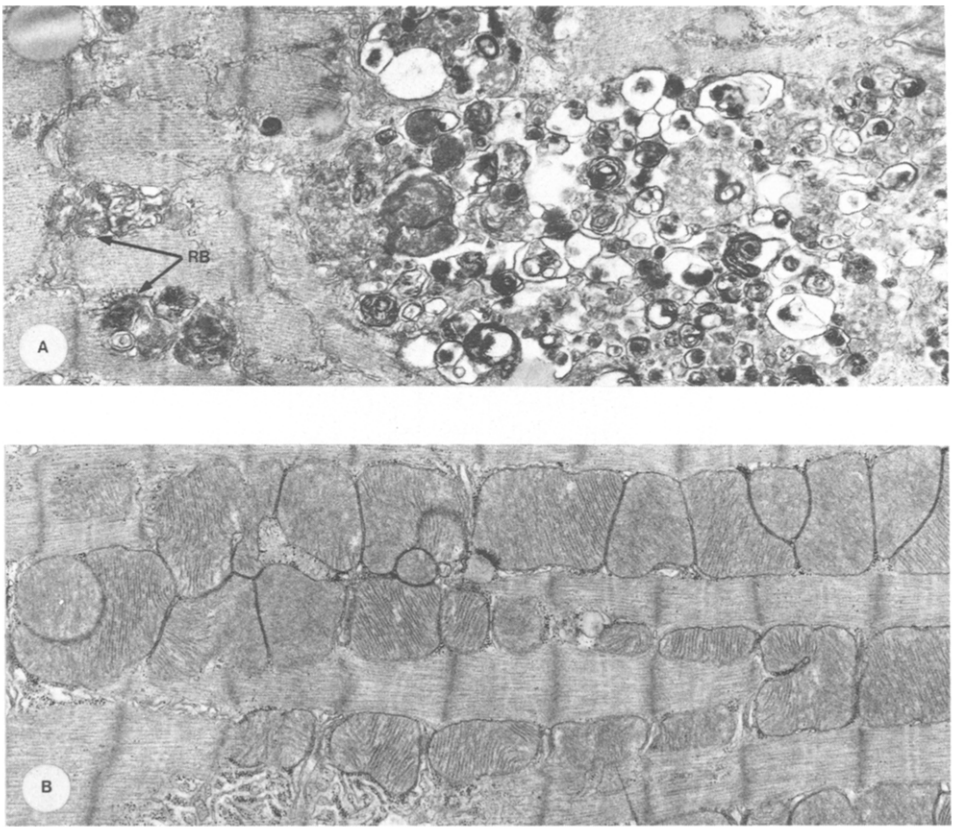

Fig 2. Electron micrographs of cardiac myocytes from 17 weeks old diabetic mice.
A. Ventricular myocyte from animal fed chow alone. Residual bodies (RB) are seen in interfibrillar spaces and coalesce to form extensive conglomerates of dense bodies and myelin figures.
B. Ventricular myocyte from a mouse receiving 12 weeks estrone treatment displays normal striated pattern and intermyofibrillar rows of mitochondria - x15,000.

alterations were observed by elecron microscopy in control mice throughout estrone treatment. Since estrone treatment did not affect the weight gain in diabetic mice (data not shown), the results from Fig. 2 suggest that cardiomyopathy and obesity in the diabetic mice are two unrelated events. This suggestion is also supported by ultrastructural observations in obese mice C57BL/6J ob/ob where lipid accumulation in the ventricular myocytes is not associated with degenerative changes of cardiac muscle (2). The present study suggests that abnormal mitochondrial function such as PDH phosphorylation may play an important role in the development of cardiomyopathy. Bazaes et al (10) have also reported higher phosphate content of muscle phosphofructokinase in

diabetic mice. These observations suggest that diabetes modifies the phosphorylation-dephosphorylation processes of muscle cells by increasing the rate of kinase reactions. In support of this, insulin was shown to inhibit endogenous protein phosphorylation in normal mouse diaphragm (11). It has been suggested that insulin stimulates the generation of intracellular mediators (12, 13). It remains to be seen whether the mediator(s) represent(s) an inhibitory signals(s) to many phosphorylation systems in the cell.

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