Oxidation and Phosphorylation in Liver Mitochondria from Alloxan and Streptozotocin Diabetic Rats[1] (35712)

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Gluconeogenesis, which is greatly stimulated in diabetes, requires a close interplay between mitochondria and cytosol. In rat liver, amino acid precursors of glucose undergo their initial metabolic transformations in the mitochondria. The metabolites which are produced diffuse into the cytosol and are converted to glucose. The cytosolic pathway is an anabolic sequence of reactions which requires energy as ATP. For example, the conversion of two pyruvate molecules to glucose requires the consumption of 6 ATP's. Since mitochondrial oxidative phosphorylation is the primary source of cellular ATP, one would not expect uncoupling to play a role in the etiology of diabetic hyperglycemia. However, the literature concerning the respiratory activities of diabetic mammalian liver mitochondria is controversial. By using manometric techniques, Vester and Stadie (1), Hall *et al.* (2), and Milanov (3) have reported decreased ATP synthesis, oxygen uptake and P:O ratio. Beyer and Shamoian (4) did not show an effect on P:O but demonstrated elevated rates of oxygen uptake. Other studies, however, have not reported significant differences between normal and diabetic mitochondria (5–7).

Recent reports in which oxygen uptake was measured polarographically are also conflicting. Boveris, *et al.* (8) have reported decreased oxygen uptake in diabetic mitochondria but no effect on the P:O ratio. On the other hand, Matsubara and Toshihiro (9–11) have shown decreased oxygen uptake and P:O ratio.

In the present study, we have investigated the respiratory activities of liver mitochondria from rats with chronic diabetes which was induced either by alloxan or streptozotocin. In addition, we have evaluated the possibility that the respiratory activities of the diabetic mitochondria were directly affected by alloxan.

Methods. Animals. Male Sprague-Dawley rats, weighing approximately 150 g, were fasted for 48 hr and made diabetic by administration of either alloxan or streptozotocin. Alloxan was prepared in distilled water, without adjusting pH, at a concentration of 100 mg/ml. The rats received two subcutaneous injections of this solution (100 mg/kg) 3 hr apart. Streptozotocin was prepared in 0.05 *M* citrate buffer, pH 4.5, at a concentration of 10 mg/ml. The rats received one intravenous injection of this solution (75 mg/kg). After the injections, Purina Lab Chow was fed *ad libitum* until the animals were killed 30 days later. No attempt was made to increase rat survival by the administration of insulin. Mortality was quite high; 65% of the alloxan diabetic and 10% of the streptozotocin diabetic rats died during the interim period. Of the remaining diabetic animals, only those with blood glucose levels above 300 mg % were used for the experiments in this report.

Respiratory activities. Mitochondria were isolated by the method of Schneider (12), as modified by Johnson and Lardy (13), and suspended in 0.25 *M* sucrose at a concentration of 30 mg protein/ml. The suspension was kept on ice and used within 2 hr. Respiration was measured polarographically with a Clark type oxygen assembly coupled to a polygraph (Gilson Medical Electronics). The 1.9-ml reaction mixtures contained 26.3 m*M* Tris–HCl, 1.1 m*M* EDTA, 15.8 m*M* KCl, 5.3 m*M* $MgCl_2$, 15.8 m*M* Pi, and 15.8 m*M* α-oxoglutarate or 10.6 m*M* pyruvate + 1.1

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TABLE I. Effects of Chronic Streptozotocin Diabetes on Liver Mitochondrial Respiratory Activities.

Rat treatment	No. rats	Substrate (m*M*)	Parameters studied[a]			
			Respiratory rate (μmoles O_2/min/g protein)			
			State 3	State 4	RC	P:O
None	5	α-oxoglutarate (15.8)	40.9 ± 1.1	8.06 ± 0.70	5.08 ± 0.17	2.64 ± 0.05
Diabetic	5	α-oxoglutarate (15.8)	54.0 ± 2.4	7.96 ± 0.41	6.80 ± 0.25	2.73 ± 0.05
p[b]			< 0.005	NS	< 0.001	NS
None	5	Pyruvate (10.6) Malate (1.1)	27.5 ± 1.5	7.79 ± 0.48	3.65 ± 0.22	2.54 ± 0.06
Diabetic	5	Pyruvate (10.6) Malate (1.1)	36.0 ± 4.8	8.67 ± 0.80	4.00 ± 0.54	2.67 ± 0.06
p			NS	NS	NS	NS

[a] The data are expressed as mean ± standard error of the mean.

[b] Probability of significant difference between means of control and experimental values by Student t test. NS indicates "not significant" ($p > 0.05$).

m*M* malate. After addition of 0.2 ml of mitochondrial suspension, sequential additions of ADP (0.194 m*M*) were made. Calculations of respiratory control ratio, P:O ratio, and oxygen uptake in states 3 and 4 were by the method of Chance and Williams (14). All experiments were at 21° and the concentration of O_2 in the media assumed to be 0.25 μmoles/ml.

Chemicals. Chemicals were of the highest commercially available grade. ADP was obtained from Nutritional Biochemicals Corp.; ATP, malic acid, α-oxoglutaric acid, pyruvic acid, tris (hydroxymethyl) aminomethane (Tris), Sigma Chemical Co.; KCl, sucrose, $MgCl_2$, KH_2PO_4, K_2HPO_4, ethylenediaminetetraacetic acid (EDTA), Mallinckrodt Chemical Co.; KOH, Fisher Chemical Co.; alloxan, CalBiochem; and streptozotocin, Upjohn Co.

Results and Discussion. Tables I and II summarize our results obtained with the

TABLE II. Effects of Chronic Alloxan Diabetes on Liver Mitochondrial Respiratory Activities.

Rat treatment	No. rats	Substrate (m*M*)	Parameters studied[a]			
			Respiratory rate (μmole O_2/min/g protein)			
			State 3	State 4	RC	P:O
None	6	α-oxoglutarate (15.8)	39.2 ± 2.2	7.87 ± 0.76	4.99 ± 0.12	2.63 ± 0.08
Diabetic	6	α-oxoglutarate (15.8)	55.1 ± 3.0	8.44 ± 0.47	6.55 ± 0.43	2.52 ± 0.04
p[b]			< 0.005	NS	< 0.01	NS
None	7	Pyruvate (10.6) Malate (1.1)	25.6 ± 1.2	8.00 ± 0.42	3.16 ± 0.15	2.57 ± 0.07
Diabetic	6	Pyruvate (10.6) Malate (1.1)	35.3 ± 1.0	8.50 ± 0.46	4.33 ± 0.14	2.68 ± 0.12
p			< 0.001	NS	< 0.001	NS

[a] The data are expressed as mean ± standard error of the mean.

[b] Probability of significant difference between means of control of experimental values by Student t test. NS indicates "not significant" ($p > 0.05$).

streptozotocin and alloxan diabetic mitochondria. In both diabetic conditions, with α-oxoglutarate and pyruvate + malate as substrates, oxygen uptake in state 3 was increased. Since this finding was not accompanied by increased state 4 respiration, the respiratory control ratio was also increased. However, the P:O ratios which represent the efficiency of oxidative phosphorylation were not altered.

The lack of effect on P:O ratio indicates that uncoupling of oxidative phosphorylation is not a primary lesion in the biochemistry of chronic diabetes. The previously demonstrated uncoupling of diabetic mitochondria (1–3, 9–11) must have been related to factors not directly correlated with hyperglycemia since all of the diabetic rats in our studies had blood glucose levels above 300 mg %. High levels of fatty acids in liver (9–11) and the inherent toxic effects of alloxan (10) have been suggested as possible uncoupling factors. Indeed, a direct correlation between free fatty acid level and uncoupling of rat liver mitochondria has been shown for acute diabetes caused by pancreatectomy and alloxan (10). However, it is not known whether or not the uncoupling effects of fatty acids occurred *in vivo* or were caused by the mitochondrial isolation procedure which disrupted cellular organization and permitted compartmentalized fatty acids to contact the mitochondria. In any case, mitochondria were not uncoupled as a consequence of chronic diabetes (Tables I and II).

Alloxan affects mitochondria directly by causing inhibited O_2 uptake and uncoupling (15). Boveris, *et al.* (8) have suggested that these direct effects of alloxan may have caused the depressed respiratory activities and uncoupling described by Hall, *et al.* (2) and Haugaard and Haugaard (16) in mitochondria from alloxan diabetic rats. In order to investigate the direct action of alloxan in the absence of acute diabetes, rats were made chronically diabetic with alloxan as described under *Methods;* however, 60 hr before killing, the animals were reinjected (200 mg/kg). The results of this experiment, which are summarized in Table III, show that the second dose did not produce any reversal of the diabetic effects shown in Table II. Thus, it can be concluded that the mitochondrial respiratory processes had recovered from alloxan poisoning within 60 hr. Since Hall, *et al.* (2) and Haugaard and Haugaard (16) used their diabetic rats several weeks after injection, it is improbable that their results were caused by alloxan toxicity.

Stimulation of state 3 respiration (Tables

TABLE III. Effects of Alloxan Administration to Chronic Alloxan Diabetes Rats on Mitochondrial Respiratory Activities.

Rat treatment	No. rats	Substrate (m*M*)	Parameters studied[a]			
			Respiratory rate (μmoles O_2/min/g protein)			
			State 3	State 4	RC	P:O
None	4	α-oxoglutarate (15.8)	43.5 ± 2.7	9.09 ± 0.58	4.79 ± 0.27	2.54 ± 0.07
Diabetic	4	α-oxoglutarate (15.8)	67.6 ± 4.8	9.10 ± 0.66	7.47 ± 0.41	2.55 ± 0.06
p[b]			< 0.01	NS	< 0.005	NS
None	4	Pyruvate (10.6) Malate (1.1)	27.6 ± 1.4	8.44 ± 0.40	3.28 ± 0.17	2.56 ± 0.04
Diabetic	5	Pyruvate (10.6) Malate (1.1)	35.0 ± 1.6	8.64 ± 0.44	4.04 ± 0.28	2.58 ± 0.06
p			< 0.025	NS	NS	NS

[a] The data are expressed as mean ± standard error of the mean.

[b] Probability of significant difference between means of control and experimental values by Student t test. NS indicates "not significant" ($p > 0.05$).

I–III) was seen with both streptozotocin and alloxan diabetes. This finding was in agreement with the work of Beyer and Shamoian (4) for liver mitochondria from pancreatectomized dogs and with that of Ogura (17) for liver mitochondria from diabetic rats. Both of these studies showed increased respiratory activity with diabetic mitochondria. The reasons for these findings are not known and there is no indication as to whether or not they occurred *in vivo*. Possible explanations include increased mitochondrial permeability to substrates and increased enzymic capacity for oxidation. The latter possibility is supported by the finding of Boveris, *et al.* (8) that mitochondrial ubiquinone content is increased by pancreatectomy.

Summary. Respiratory activities were determined for liver mitochondria from rats with chronic alloxan and streptozotocin diabetes. Only those rats with blood glucose levels above 300 mg %, 30 days after injection of the diabetogenic substances were used. Both alloxan and streptozotocin diabetic mitochondria showed increased rates of respiration with α-oxoglutarate and L-malate + pyruvate as substrates but the P:O ratios were not altered. Administration of alloxan (200 mg/kg) to chronically diabetic rats 60 hr prior to killing did not cause further alterations in mitochondrial function which would have been indicative of direct mitochondrial poisoning by alloxan.

It can be concluded that altered efficiency of oxidative phosphorylation in the liver mitochondria is not a primary lesion in the biochemistry of chronic diabetes. Poisoning of liver mitochondria by the direct action of alloxan is not an acceptable explanation for the altered mitochondrial functions previously seen with diabetic liver mitochondria when the alloxan was administered subcutaneously more than 60 hr before rat sacrifice.

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