Changes in brown-adipose-tissue mitochondrial processes in streptozotocin-diabetes

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Diabetic rats were used as a source of brown-adipose-tissue mitochondria 2 days after a single subcutaneous injection of streptozotocin (100 mg/kg). Diabetes caused an 80 % decrease in carnitine-dependent oxidation of palmitoyl-CoA and a 50–60 % decrease in overt carnitine palmitoyltransferase activity. An additional lesion in brown-adipose-tissue mitochondrial oxidative capacity was also indicated, since diabetes increased by 30–50 % the rate of oxidation under uncoupled conditions of several respiratory substrates (i.e. malate + palmitoylcarnitine, malate + pyruvate, succinate, *NNN'*-tetramethyl-*p*-phenylenediamine + ascorbate). This decrease in mitochondrial function was accompanied by an approx. 30 % decrease in the abundance of cytochromes ($a + a_3$) and total cytochromes b .

INTRODUCTION

The role of brown adipose tissue in thermogenesis appears to be impaired in insulin-dependent diabetes. In accord with this, basal and noradrenaline-stimulated heat production by rat interscapular brown fat is decreased in streptozotocin-diabetic rats (Seydoux *et al.*, 1983), and diabetic rats do not exhibit an increase in metabolic rate after consuming a palatable high-energy (cafeteria) diet (Rothwell & Stock, 1981). There are several possible sites for this diabetes-induced lesion. It is likely that sympathetic-nerve activity in brown adipose tissue is decreased in diabetes (Rothwell *et al.*, 1983), together with a decrease in blood flow through the tissue (Jamal & Saggerson, 1988). Within the brown adipocyte itself it is likely that the abundance or function of the mitochondrial 32 kDa protein 'thermogenin' is decreased in diabetes, since GDP binding to mitochondria is decreased (Seydoux *et al.*, 1984; Jamal & Saggerson, 1988). In addition, a defect somewhere in the mitochondrial pathway of fatty acid β -oxidation is also inferred, since the carnitine-dependent conversion of [$1-^{14}\text{C}$]palmitoyl-CoA into acid-soluble products by brown-adipose-tissue homogenates is decreased in diabetes (Seydoux *et al.*, 1983) and increased after infusion of insulin (Seydoux *et al.*, 1984). This lesion in β -oxidation could in turn result from alterations in processes for transport of long-chain fatty acids into mitochondria or from changes in activities of oxidative enzymes. A more general alteration in respiration might also be inferred, since infusion of insulin significantly increases rat brown-adipose-tissue succinate dehydrogenase activity (Seydoux *et al.*, 1984). The present study with isolated mitochondria extends these observations and shows that diabetes causes both a decrease in overt carnitine palmitoyltransferase (CPT₁) activity and more generalized changes in the components of the respiratory chain.

MATERIALS AND METHODS

Chemicals

These were obtained and treated as described by Harper & Saggerson (1975) and Saggerson (1982). In addition, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was obtained from Boehringer Corp. (Lewes, East Sussex, U.K.).

Animals

These were male Sprague-Dawley rats bred in the animal colony at University College London. Animals were selected at body wt. 160–180 g and taken as diabetic 2 days after a single subcutaneous injection of streptozotocin (100 mg/kg) dissolved in 0.2 ml of 50 mM-sodium citrate buffer (pH 4.0) containing 0.15 M-NaCl, provided that they showed a strongly positive 'Clinistix' test for urinary glucose [$> 0.5\%$ (w/v) glucose]. Body-weight loss and hyperglycaemia were as reported by Saggerson & Carpenter (1987).

Isolation of brown adipocytes

This was as described by Woodward & Saggerson (1986), with the interscapular tissue as source.

Isolation of mitochondria

For measurement of respiration or CPT₁ activity, these were isolated from adipocytes and whole tissue respectively by procedures described by Harper & Saggerson (1975) and Saggerson (1982). Homogenates were prepared at 0–4 °C in 0.25 M-sucrose medium containing 10 mM-Tris/HCl (pH 7.4), 1 mM-EGTA and fatty acid-poor albumin (10 mg/ml). These were initially centrifuged at 3000 g_{av} for 1 min, and the resulting supernatant was then centrifuged at 20000 g_{av} for 1 min. The resulting mitochondrial pellet was washed once by

Abbreviations used: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; CPT₁ and CPT₂, the overt and latent forms respectively of carnitine palmitoyltransferase (EC 2.3.1.21); IC₅₀, concentration of inhibitor causing 50 % inhibition; NbS₂, 5,5'-dithiobis-(2-nitrobenzoic acid); TMPD, *NNN'*-tetramethyl-*p*-phenylenediamine.

resuspension in 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4)/1 mM-EGTA, resedimented at 20000 g_{av} for 1 min and finally suspended in 0.3 M-sucrose/10 mM-Tris/HCl (pH 7.4)/1 mM-EGTA. For measurements of cytochrome contents, the isolation procedure was modified and the mitochondria were purified by using Percoll (Armstrong & Halestrap, 1984). Mitochondria were obtained from interscapular adipose tissues as described above, except that the initial supernatant after the 5000 g_{av} centrifugation was centrifuged once more at 5000 g_{av} for 1 min before sedimentation of the mitochondria at 20000 g_{av} for 1 min and washing as described above. The washed mitochondria were then suspended in 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4), layered on to Percoll (17.5%, w/v, in the same medium) and centrifuged at 9500 g_{av} for 15 min. The pellet obtained was washed by suspension in 0.25 M-sucrose/10 mM-Tris/HCl (pH 7.4), centrifuged at 20000 g_{av} for 5 min and finally resuspended in the same medium.

Measurement of mitochondrial respiration

O₂ uptake was measured polarographically with an oxygen electrode (Rank Bros., Bottisham, Cambridge, U.K.). The electrode was calibrated by addition of spectrophotometrically standardized NADH to media containing phenazine methosulphate (Harper & Saggerson, 1975).

Samples (0.1 ml) of mitochondrial preparations were incubated at 30 °C in final volumes of 2.0 ml in the electrode chamber in 0.13 M-KCl/2 mM-EGTA/2 mM-MgCl₂/20 mM-Tris/HCl (pH 7.4)/2 mM-potassium phosphate/1 mM-ADP/10 μ M-GDP containing fatty acid-poor albumin (20 mg/ml). When palmitoyl-group oxidation was measured, this medium also contained 2.5 mM-potassium malonate and 10 μ M-potassium fluorocitrate (Harper & Saggerson, 1975). Other additions to this medium (see Table 1) were succinate, 1.5 mM; L-malate, 2.5 mM; pyruvate, 1 mM; palmitoylcarnitine, 50 μ M; palmitoyl-CoA, 50 μ M; carnitine, 1 mM; TMPD, 0.1 mM; sodium ascorbate, 3 mM; CCCP, 20 μ M; rotenone, 2.5 μ M. Preliminary experiments (results not shown) established that maximal effects were obtained with these concentrations of palmitoylcarnitine, palmitoyl-CoA, carnitine and CCCP.

Measurement of overt carnitine palmitoyltransferase (CPT₁) activity

Freshly isolated mitochondria (300–400 μ g of protein) were used to assay CPT₁ at 25 °C radiochemically (Saggerson & Carpenter, 1986) or spectrophotometrically in an NbS₂-linked assay (Saggerson, 1982). All assays were in 1.0 ml volumes containing 150 mM-sucrose, 60 mM-KCl, 25 mM-Tris/HCl (pH 7.4), 1 mM-EGTA and fatty acid-poor albumin (1.3 mg/ml). Palmitoyl-CoA concentrations are indicated in Table 2. In addition the spectrophotometric assay contained 0.1 mM-NbS₂, whereas 1 mM-dithiothreitol was present in the radiochemical assay. Both assays were initiated by the addition of 0.4 mM-L-carnitine, with 0.5 μ Ci of L-[Me-³H]carnitine also present in the radiochemical assay.

Measurement of mitochondrial cytochrome contents

These were determined as described by Van Gelder (1978), with a Perkin-Elmer 356 dual-wavelength spectrophotometer. Mitochondria were dispersed at 1 mg/ml in 100 mM-Tris/HCl buffer (pH 8.0) containing Triton

X-100 (1%, v/v). The concentration of cytochromes ($a + a_3$) was determined from the $\Delta A_{605-630}$ on reduction with 5 mM-ascorbate + 50 μ M-TMPD, by using an absorbance coefficient of 37.4 mM⁻¹·cm⁻¹. The total amount of cytochromes b was determined from the $\Delta A_{563-577}$ for the dithionite-reduced preparation minus the TMPD + ascorbate-reduced preparation, by using an absorbance coefficient of 25.6 mM⁻¹·cm⁻¹.

Expression of results

Values are expressed relative to protein contents of mitochondrial preparations determined by the method of Lowry *et al.* (1951), with bovine albumin as standard. DNA contents of adipocyte preparations were measured fluorimetrically (Switzer & Summer, 1971). Citrate synthase activity was measured (Saggerson & Carpenter, 1986) both in adipocyte homogenates and in mitochondrial preparations and was used as a reference enzyme to calculate the amount of mitochondrial protein per quantity of cell DNA and thereby to relate respiratory rates to cell DNA.

Statistical significance was evaluated by Student's *t* test for unpaired samples.

RESULTS

In the experiments described below, we used diabetic rats only 48 h after administration of streptozotocin. It is possible that part of the reported changes are related to short-term alterations in food intake. Attempts to make similar measurements in chronic diabetes, e.g. 1–2 weeks after streptozotocin administration, were not successful because of extensive depletion of the tissue during this period. Associated with this, it also became impossible to isolate adipocytes in significant yield more than 4–5 days after streptozotocin.

To study palmitoyl-group oxidation, mitochondria isolated from brown adipocytes were incubated under State-3 conditions (albumin, GDP and ADP present) with the citric acid cycle blocked by fluorocitrate + malonate (Harper & Saggerson, 1975). Under these conditions respiration with malate alone was low (Table 1), as found previously with liver or white-adipocyte mitochondria under similar conditions (Harper & Saggerson, 1975). Subsequent addition of palmitoylcarnitine then substantially increased respiration. This palmitoylcarnitine-dependent respiration ($B - A$ in Table 1) was decreased by 40% in diabetes. It is unlikely that these State-3 respiratory rates were limited by palmitoylcarnitine transport into mitochondria or by CPT₂ activity, since uncoupling by CCCP doubled the rate of respiration. Under uncoupled conditions oxidation of malate + palmitoylcarnitine was still decreased by 37% in diabetes. Addition of palmitoyl-CoA to mitochondria caused little enhancement of respiration over the low basal rate seen with malate (plus malonate and fluorocitrate) alone, suggesting that the mitochondria were relatively intact. For mitochondria from normal rats, subsequent addition of L-carnitine then increased respiration. However, this carnitine-dependent palmitoyl-group oxidation ($D - C$ in Table 1) was only approx. 25% of the rate of palmitoylcarnitine-dependent oxidation, implying a rate-limiting role for CPT₁. Unlike palmitoylcarnitine oxidation (Table 1), respiration of palmitoyl-CoA + carnitine was not increased by the uncoupler CCCP (results not shown). This observation also supports the proposal that CPT₁ is rate-limiting.

Table 1. Rates of respiration of various substrates by brown-adipocyte mitochondria from normal and diabetic rats

Mitochondria were isolated from brown adipocytes. Concentrations of various substrates etc. are given in the Materials and methods section. The values are means $\pm$ S.E.M. for the numbers of separate preparations shown in parentheses; ^a $P < 0.05$, ^b $P < 0.02$, ^c $P < 0.01$, ^d $P < 0.001$ for effects of diabetes.

Additions	Control		Diabetic	
	(nmol/min per mg of mitochondrial protein)	(nmol/min per 100 μ g of adipocyte DNA)	(nmol/min per mg of mitochondrial protein)	(nmol/min per 100 μ g of adipocyte DNA)
Malate (A)	8 $\pm$ 1 (5)	41 $\pm$ 3 (5)	14 $\pm$ 2 (4)	55 $\pm$ 9 (4)
Malate + palmitoylcarnitine (B)	85 $\pm$ 3 (4)	444 $\pm$ 17 (4)	60 $\pm$ 5 (4) ^c	235 $\pm$ 20 (4) ^d
B - A	77 $\pm$ 3 (4)	403 $\pm$ 16 (4)	46 $\pm$ 6 (4) ^c	219 $\pm$ 4 (4) ^d
Malate + palmitoylcarnitine + CCCP	192 $\pm$ 11 (4)	1001 $\pm$ 56 (4)	121 $\pm$ 13 (4) ^c	472 $\pm$ 51 (4) ^d
Malate	12 $\pm$ 2 (4)	61 $\pm$ 11 (4)	15 $\pm$ 4 (4)	59 $\pm$ 14 (4)
Malate + palmitoyl-CoA (C)	17 $\pm$ 2 (4)	89 $\pm$ 9 (4)	16 $\pm$ 4 (4)	64 $\pm$ 17 (4)
Malate + palmitoyl-CoA + L-carnitine (D)	35 $\pm$ 5 (5)	183 $\pm$ 26 (5)	20 $\pm$ 3 (3) ^a	81 $\pm$ 13 (3) ^b
D - C	19 $\pm$ 5 (4)	106 $\pm$ 27 (4)	4 $\pm$ 2 (3) ^a	18 $\pm$ 9 (3) ^a
B - A	3.9	3.8	10.2	12.5
D - C				
Malate + pyruvate	47 $\pm$ 7 (4)	247 $\pm$ 37 (4)	33 $\pm$ 1 (4)	128 $\pm$ 3 (4) ^b
Malate + pyruvate + CCCP	104 $\pm$ 11 (4)	540 $\pm$ 59 (4)	58 $\pm$ 12 (4) ^a	227 $\pm$ 45 (4) ^c
Succinate + rotenone + CCCP	85 $\pm$ 7 (4)	499 $\pm$ 65 (4)	43 $\pm$ 12 (3) ^a	199 $\pm$ 52 (3) ^b
TMPD + ascorbate + rotenone + CCCP	108 $\pm$ 6 (4)	623 $\pm$ 48 (4)	71 $\pm$ 7 (3) ^b	326 $\pm$ 21 (3) ^c

Table 2. CPT₁ activities in brown-adipose-tissue mitochondria from normal and diabetic rats

Mitochondria were isolated from interscapular brown adipose tissues and CPT₁ activity was measured by the NbS₂-linked assay as described in the Materials and methods section. The values are means $\pm$ S.E.M. for five separate preparations; ^a $P < 0.05$, ^b $P < 0.02$, ^c $P < 0.01$ for effects of diabetes.

Palmitoyl-CoA concn. (μ M)	CPT ₁ activity (nmol/min per mg of mitochondrial protein)	
	Control	Diabetic
40	4.3 $\pm$ 0.5	2.1 $\pm$ 0.2 ^c
60	6.7 $\pm$ 1.1	3.0 $\pm$ 0.3 ^b
80	8.6 $\pm$ 2.1	3.5 $\pm$ 0.5 ^a

In diabetes, carnitine-dependent palmitoyl-group oxidation was decreased by 80 % relative to the control (Table 1), suggesting that diabetes decreases CPT₁ activity. This was confirmed by direct assay of CPT₁ in intact mitochondria freshly isolated from interscapular brown fat (Table 2). At three different palmitoyl-CoA concentrations it was found that CPT₁ activity was decreased by 50–60 %. This change may be contrasted with that seen in the liver in diabetes. Harano *et al.* (1972) observed a significant increase in CPT₁ activity in liver mitochondria from diabetic rats. Hepatic CPT₁ shows an additional adaptive response to diabetes, in that its sensitivity to the physiological regulator malonyl-CoA is decreased (Cook *et al.*, 1984). Using a radiochemical assay for CPT₁ [the NbS₂-linked assay could not be used, since NbS₂ interferes with the action of malonyl-CoA (Saggerson & Carpenter, 1982)], we ob-

served, by contrast, that diabetes did not alter sensitivity of the brown-adipose-tissue enzyme to malonyl-CoA. When assayed with 80 μ M-palmitoyl-CoA the IC₅₀ for malonyl-CoA was 2.1 μ M in both the normal and the diabetic states (results not shown). In these experiments all of the measured CPT activity was inhibited by a high concentration of malonyl-CoA (10 μ M), which gave further assurance that the mitochondria were intact and that some malonyl-CoA-insensitive CPT₂ was not being measured in addition to CPT₁.

Table 1 shows that in the uncoupled state the oxidations of substrates other than palmitoylcarnitine were also decreased in diabetes, i.e. in the presence of CCCP rates of oxidation (per mg of mitochondrial protein) of malate + pyruvate, succinate and TMPD + ascorbate were decreased by 44 %, 49 % and 34 % respectively. All of these changes were also seen when citrate synthase was used as a reference enzyme (see the Materials and methods section), and values were expressed relative to adipocyte DNA. This form of expression eliminates the possibility that mitochondrial fractions isolated from cells of diabetic rats might contain more non-mitochondrial contaminating protein. However, it also encompasses the likelihood that the amount of mitochondrial protein per quantity of cell DNA is lower in diabetes (by approx. 23 %).

A general decrease in uncoupled respiratory capacity might be attributed to a co-ordinated decrease in the activities of several oxidative enzymes and/or of mitochondrial substrate translocators. It could also be attributed to a decrease in the function or abundance of respiratory-chain components. This possibility was tested in the experiment shown in Table 3, where mitochondria purified on Percoll to remove non-mitochondrial contaminating material were assayed for their cytochrome contents. Table 3 shows that the contents of cytochromes ($a + a_3$) and b were decreased by 29 % and 32 % respectively in diabetes.

Table 3. Cytochrome contents of brown-adipose-tissue mitochondria from normal and diabetic rats

Mitochondria were isolated from interscapular brown adipose tissues, purified by fractionation in Percoll, and cytochrome contents were measured as described in the Materials and methods section. The values are means $\pm$ S.E.M. for three separate preparations; ^a $P < 0.05$, ^b $P < 0.02$ for effects of diabetes.

Cytochrome	Mitochondrial cytochrome contents (nmol/mg of protein)	
	Control	Diabetic
($a + a_n$)	0.21 ± 0.01	0.15 ± 0.01^b
Total b	0.31 ± 0.01	0.21 ± 0.03^a

DISCUSSION

It is generally accepted that long-chain fatty acids are important substrates for brown-adipose-tissue thermogenesis. Seydoux *et al.* (1983) have indicated a lesion somewhere in the process of fatty acid β -oxidation in diabetes. In the present study we show that there are at least two lesions.

First, the entry of fatty acids into oxidative metabolism through CPT₁ is curtailed. It is not known if this reflects a change in the abundance of CPT₁ protein or modification of existing CPT₁. Sensitivity of CPT₁ to malonyl-CoA is not modified by diabetes in this tissue, but obviously the change observed here at the level of the isolated mitochondria will be overlaid by possible changes in malonyl-CoA concentration in diabetes. This possibility has not been investigated at present.

Second, general oxidative capability of the mitochondria is decreased, and this is accompanied by a decrease in the abundance of some cytochromes. This suggests that diabetes decreases the capacity both for thermogenin-mediated H⁺ influx (Seydoux *et al.*, 1984; Jamal & Saggerson, 1988) and for respiratory-chain-mediated H⁺ efflux.

It is therefore clear that several loci are involved in the impairment of the thermogenic function of brown adipose tissue in the diabetic state, and it would be of interest to investigate reversal of these changes by insulin injection or infusion.

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