Differentiation of rapid and slower-acting effects of insulin on mitochondrial processes in brown adipose tissue from streptozotocin-diabetic rats

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Insulin treatment of streptozotocin-diabetic rats restores the depressed palmitoyl-group oxidation observed in brown-adipose-tissue mitochondria from diabetic rats. A relatively rapid effect of insulin (5 h) to increase carnitine-dependent oxidation of palmitoyl-CoA and to increase overt carnitine palmitoyltransferase activity is differentiated from a slower effect of the hormone (1 day) to increase palmitoylcarnitine oxidation.

INTRODUCTION

The thermogenic response of brown adipose tissue is decreased in streptozotocin-diabetes (Rothwell & Stock, 1981; Seydoux *et al.*, 1983). Several possible lesions are implicated. These include a decrease in sympathetic-nerve activity (Rothwell *et al.*, 1983), a decrease in blood flow (Jamal & Saggerson, 1988a), a decrease in the abundance or function of the mitochondrial GDP-binding protein (Seydoux *et al.*, 1984; Jamal & Saggerson, 1988a), decreased mitochondrial carnitine-dependent oxidation of long-chain fatty acids (Seydoux *et al.*, 1983), owing to a decrease in the activity of CPT₁ (overt carnitine palmitoyltransferase) (Jamal & Saggerson, 1988b), and a generalized lesion in the respiratory chain, associated with decreased abundance of cytochromes *b* and (*a*+*a*₃) (Jamal & Saggerson, 1988b). Insulin has been implicated in the restoration of a normal thermogenic response on refeeding of starved rats (Rothwell *et al.*, 1983) and has been shown to increase blood flow rapidly through interscapular brown fat after administration to streptozotocin-diabetic rats (Jamal & Saggerson, 1988a). It was therefore decided to investigate the role of insulin to promote recovery of normal mitochondrial functions in brown adipose tissue of diabetic animals. In the present study we provide evidence for both rapid and longer-term effects of insulin in this respect.

MATERIALS AND METHODS

Chemicals

These were obtained as described by Jamal & Saggerson (1988b).

Animals

These were male Wistar rats bred in the animal colony at University College London. Animals were selected at body weight 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]. Two days after streptozotocin was chosen for continuity with Jamal & Saggerson (1988a,b), where some or all of the measurements were made with isolated brown adipocytes. In these previous studies it was found that recovery of adipocytes declined drastically 3–4 days after streptozotocin treatment. We were therefore constrained to using the animals during a period when they may show some inappetence, which in turn may contribute to the apparent effect of diabetes. Protamine zinc bovine insulin (Weddel Pharmaceuticals, London E.C.1, U.K.) was administered subcutaneously at 20 units/kg body wt. dispersed in 0.2 ml of 0.15 M-NaCl. 'One-day insulin-treated' diabetic rats received an insulin injection at approx. 10:00 h 2 days after streptozotocin, followed by a second injection at 10:00 h 2 days after streptozotocin, followed by a second injection at 10:00 h on the following day. These rats were then killed after a further 2 h. 'Short-term insulin-treated' diabetic rats received an insulin injection at approx. 10:00 h 2 days after streptozotocin. A further injection was administered at approx. 12:00 h, and the rats were killed after a further 3 h. All insulin-treated animals showed a negative 'Clinistix' test for urinary glucose.

Isolation of mitochondria from interscapular brown fat

Rats were killed by cervical dislocation. Interscapular brown adipose tissue from five animals was removed, pooled, and chilled to 0–4 °C in 0.25 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4), 1 mM-EGTA and fatty acid-poor albumin (10 mg/ml). Homogenates were prepared at 0–4 °C in the same medium, and mitochondria were isolated and purified by using Percoll (Armstrong & Halestrap, 1984) as described by Jamal & Saggerson (1988b), except that the centrifugation through Percoll was at 20000 *g*_{av} for 15 min. Protein contents of mitochondrial preparations were determined by the method of Lowry *et al.* (1951), with bovine albumin as standard.

Measurement of mitochondrial respiration

Approx. 0.75 mg of mitochondrial protein was incubated at 30 °C in a final volume of 2.0 ml. The conditions were as described by Harper & Saggerson (1975) and

Abbreviation used: CPT₁, overt form of carnitine palmitoyltransferase (EC 2.3.1.21).

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Table 1. Rates of palmitoyl-group respiration by brown-adipose-tissue mitochondria from normal, diabetic and insulin-treated diabetic rats

Mitochondria were incubated in 0.13 M-KCl/2 mM-EGTA/2 mM-MgCl₂/20 mM-Tris/HCl (pH 7.4)/2 mM 7.4/2 mM-potassium phosphate/1 mM-ADP/10 μ M-GDP containing fatty acid-poor albumin (20 mg/ml), 2.5 mM-potassium malonate and 10 μ M-potassium fluorocitrate. Other additions were: L-malate (2.5 mM); palmitoylcarnitine (50 μ M); palmitoyl-CoA (50 μ M); L-carnitine (1 mM). The values are means $\pm$ S.E.M. for the numbers of experiments shown in parentheses. Significant difference from the control ($P < 0.01$) is indicated by ^a. Significant difference from the diabetic ($P < 0.01$) is indicated by ^b.

Additions	Rate of O ₂ consumption (nmol/min per mg of mitochondrial protein)			
	Control	Diabetic	Short-term insulin-treated diabetic	1-day insulin-treated diabetic
Malate (A)	54 $\pm$ 2 (4)	14 $\pm$ 1 (3)	45 $\pm$ 13 (3)	63 $\pm$ 13 (4)
Malate + palmitoylcarnitine (B)	400 $\pm$ 13 (4)	216 $\pm$ 16 (3)	228 $\pm$ 16 (3)	375 $\pm$ 16 (4)
B - A	346 $\pm$ 10 (4)	202 $\pm$ 13 (3) ^a	183 $\pm$ 9 (3) ^a	312 $\pm$ 13 (4) ^b
Malate	52 $\pm$ 2 (3)	16 $\pm$ 3 (3)	38 $\pm$ 7 (3)	69 $\pm$ 10 (4)
Malate + palmitoyl-CoA (C)	68 $\pm$ 3 (3)	29 $\pm$ 5 (3)	51 $\pm$ 7 (3)	91 $\pm$ 13 (4)
Malate + palmitoyl-CoA + L-carnitine (D)	290 $\pm$ 7 (3)	70 $\pm$ 6 (3)	216 $\pm$ 22 (3)	303 $\pm$ 22 (4)
D - C	225 $\pm$ 5 (3)	41 $\pm$ 7 (3) ^a	166 $\pm$ 10 (3) ^{ab}	212 $\pm$ 12 (4) ^b
B - A	1.6	4.9	1.1	1.5
D - C				

Jamal & Saggerson (1988b) for measurement of palmitoyl-group oxidation in the presence of malonate and fluorocitrate in the coupled state.

Measurement of CPT₁ activity

This was performed at 25 °C with 20–25 μ g of mitochondrial protein in a final volume of 1.0 ml by using a 5,5'-dithiobis-(2-nitrobenzoic acid)-linked assay (Saggerson, 1982) as described by Jamal & Saggerson (1988b).

Statistical methods

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

RESULTS AND DISCUSSION

The first two columns of Table 1 confirm the findings of Jamal & Saggerson (1988b) by demonstrating that palmitoyl-group oxidation by isolated mitochondria under conditions approximating to 'State 3' is decreased in the diabetic state. The decrease in carnitine-dependent oxidation of palmitoyl-CoA (82%) was more pronounced than the decrease in palmitoylcarnitine oxidation (42%), implicating CPT₁ as one of the lesions in diabetes. This is confirmed in Fig. 1, where, as also shown by Jamal & Saggerson (1988b), CPT₁ activity was significantly decreased by diabetes ($P < 0.01$ versus the control at all tested concentrations of palmitoyl-CoA). It should be noted that the activities shown in Table 1 and Fig. 1 are appreciably higher than those reported by Jamal & Saggerson (1988b). This is mainly attributed to the further purification of the mitochondria by using Percoll. It should, however, be noted that the present study uses a different rat strain (Wistar compared with Sprague-Dawley) and that Jamal & Saggerson (1988b) used mitochondria prepared from adipocytes for studies of respiration.

Treatment of diabetic rats over 1 day completely restored palmitoylcarnitine oxidation, carnitine-depen-

Fig. 1. CPT₁ activity in brown-adipose-tissue mitochondria from normal, diabetic and insulin-treated diabetic rats

Assays were performed in 150 mM-sucrose/60 mM-KCl/25 mM-Tris/HCl (pH 7.4)/1 mM-EGTA containing fatty acid-poor albumin (1.3 mg/ml) and 100 μ M-5,5'-dithiobis-(2-nitrobenzoic acid). Palmitoyl-CoA concentrations were as shown, and the reaction was initiated with 0.4 mM-L-carnitine. The values are means $\pm$ S.E.M. for three experiments in each case: $\circ$, control; $\blacksquare$, diabetic; $\square$, short-term insulin-treated diabetic; $\bullet$, 1-day insulin-treated diabetic. Both short-term and 1-day insulin treatments significantly increased CPT₁ activity relative to the untreated diabetic state ($P < 0.02$ at all tested palmitoyl-CoA concentrations).

dent oxidation of palmitoyl-CoA (Table 1) and CPT₁ activity (Fig. 1). When diabetic animals received the shorter-term (5 h) insulin treatment, no change in palmitoylcarnitine oxidation was observed whatsoever (Table

1). By contrast, the rate of carnitine-dependent palmitoyl-CoA oxidation was increased 4-fold to a rate which was 75% of the control rate (Table 1), and CPT₁ activity was significantly increased by 31%. The increases in CPT₁ activity after either period of insulin treatment were observed at all tested concentrations of palmitoyl-CoA (i.e. the $s_{0.5}$ for the acyl-CoA substrate was not appreciably altered from the mean value of 35 μ M, nor was the Hill coefficient appreciably different from 3.2 in any case).

The implications of these findings are that insulin appears to exert a relatively rapid effect on processes that are external to the mitochondrial inner membrane, namely CPT₁ and possibly the carnitine:acylcarnitine translocator. It is not clear at present why the percentage increase in carnitine-dependent oxidation of palmitoyl-CoA should so greatly exceed the percentage increase in CPT₁ activity on short-term insulin treatment. This might implicate the translocase as being another insulin-sensitive locus. By contrast, events associated with palmitoyl-group oxidation within the mitochondrial inner membrane appear to respond more slowly to insulin therapy. These processes could include the latent CPT and components of the respiratory chain.

Further work should be directed towards establishing

whether all or only some of these events are dependent on stimulation of synthesis of new proteins by insulin.

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