GDP binding to brown-adipose-tissue mitochondria of diabetic–obese (*db/db*) mice

Decreased binding in both the obese and pre-obese states

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GDP binding to brown-adipose-tissue mitochondria from adult diabetic–obese (*db/db*) mice was significantly less than with lean siblings. Binding was also decreased in the mutant mice before obesity had begun to develop. Decreased GDP binding was found to result from a decrease in the number of binding sites.

Genetically obese animals have been widely used to investigate the fundamental mechanisms involved in the regulation of body weight. Studies on the most commonly used mutant, the obese (*ob/ob*) mouse, have indicated that a primary factor leading to the development of obesity is a decrease in whole-body energy expenditure on the non-shivering thermogenesis required to maintain homoeothermy (Trayhurn & James, 1978; Thurlby & Trayhurn, 1979). The main organ responsible for non-shivering thermogenesis in mature rodents, as in many newborn mammals and in hibernators, is brown adipose tissue (Foster & Frydman, 1978, 1979), where the mechanism for the generation of heat is through a proton-conductance pathway across the mitochondrial inner membrane (Nicholls, 1979). This proton-conductance pathway can be inhibited, and the mitochondria consequently recoupled, by purine nucleotides (e.g. GDP and ADP), which bind to a specific protein of mol.wt. 32 000 (Nicholls, 1976; Heaton *et al.*, 1978). In non-hibernating species, the extent to which purine nucleotides bind to brown-adipose-tissue mitochondria appears to be closely correlated with the 'thermogenic' activity of the tissue; thus binding is high after either cold-adaptation or the administration of noradrenaline to rats (Desautels *et al.*, 1978; Desautels & Himms-Hagen, 1979; Sundin & Cannon, 1980), and during early development in both rats (Sundin & Cannon, 1980) and guinea pigs (Rafael & Heldt, 1976). Purine-nucleotide-binding studies have also been used to show that there is a decrease in brown-adipose-tissue thermogenesis in adult *ob/ob* mice (Himms-Hagen & Desautels, 1978), a finding that has been substantiated by measurements of regional

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blood flow and tissue oxygen utilization *in vivo* (Thurlby & Trayburn, 1980).

At present it is not clear whether decreased purine nucleotide binding to brown-adipose-tissue mitochondria of adult *ob/ob* mice is a secondary consequence of the changes (e.g. hormonal) induced by the obese state, or whether it reflects a very early change in the thermogenic activity of the tissue which is important to the development of obesity. It is also not clear whether decreased binding results from a decrease in the number of binding sites, or from a change in affinity. In the present paper we report purine-nucleotide (GDP) binding experiments on brown-adipose-tissue mitochondria of the diabetic–obese (*db/db*) mouse, an obese mutant for which there is also physiological evidence to indicate that obesity may be due primarily to a decrease in thermogenesis (Trayhurn, 1979; Trayhurn & Fuller, 1980). The results obtained show that GDP binding to brown-adipose-tissue mitochondria is decreased not only in adult obese mice, but also in 14-day-old animals before obesity has developed. Binding is shown to be decreased because of a decrease in the number of binding sites.

Experimental

Materials

[³H]GDP (37 MBq/ml) and [¹⁴C]sucrose (9.3 MBq/ml) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Ferrocytochrome *c* (from pig heart), GDP (sodium salt), atractyloside, bovine serum albumin (fraction V, essentially fatty acid-free) rotenone and *p*-nitrophenylhydrazine were obtained from Sigma, Poole,

Dorset, U.K. All other chemicals were from BDH, Poole, Dorset, U.K., and were of AnalaR grade when available.

Animals

The mice used were from a diabetic-obese (*db/db*) colony established from breeding stock obtained from the Jackson Laboratory, Bar Harbor, ME, U.S.A. (Trayhurn, 1979). The *db* mutation was on a C57BL/ks background in 'repulsion' to the misty coat gene (*m*). The colony was housed at $23 \pm 1^\circ\text{C}$, and was subject to a 12h-light/12h-dark cycle. Food (Spillers-Spratts Rodent Breeding Diet 1; Spratts Patent Ltd., Barking, Essex, U.K.) and water were available *ad libitum*.

For studies on mature animals the *db/db* mice were taken at 8–10 weeks of age; heterozygotes (*db/+*) were used as lean controls. Experiments were also performed on 14-day-old mice. At this age the diabetic-obese animals cannot be differentiated visually from their siblings. A 'cold-stress' test similar to that used previously for *ob/ob* mice (Trayhurn *et al.*, 1977) was therefore developed to identify the *db/db* and *db/+* genotypes. Essentially, this involved monitoring the body temperature of mice caged individually for up to 1h at room temperature; *db/db* mice were identified by their sharp fall in temperature. The test was performed when the mice were aged 13 days, and after being marked the litter was returned to the nest for use the following day.

Methods

Mice were killed by cervical dislocation, and brown adipose tissue was rapidly removed from the interscapular, subscapular and dorsocervical sites and dissected free of any adhering connective tissue or white adipose tissue. The protein content of interscapular brown adipose tissue and of mitochondrial preparations was measured by a modified Lowry assay (Schacterle & Pollack, 1973). Cytochrome oxidase activity was measured spectrophotometrically (Yonetani & Ray, 1965) and the DNA content was determined by a method

developed for white adipose tissue (Curtis-Prior *et al.*, 1975).

Mitochondria were prepared from brown adipose tissue obtained from up to four animals per group, as described by Cannon & Lindberg (1979). The purine-nucleotide-binding assay was performed at room temperature (20°C) by using $10\mu\text{M}$ -[^3H]-GDP, essentially as described by Nicholls (1976) with the modifications of Desautels *et al.* (1978), except that 'competition' with $100\mu\text{M}$ -ADP was generally omitted (see the Results section). Radioactivity was measured in a Packard Tri-Carb 2650 liquid scintillation counter, with a NCS-tissue-solubilizer/toluene-based scintillation solution.

Student's unpaired '*t*' test was used to determine the statistical significance of differences between lean and diabetic-obese animals.

Results

Most studies on brown adipose tissue have used material from the easily accessible interscapular site. Table 1 shows that there was more than twice as much interscapular brown adipose tissue in the diabetic-obese animals as in the lean controls. This hypertrophy, which also occurs in the *ob/ob* mutant (Himms-Hagen & Desautels, 1978), is the result of an increased amount of triacylglycerol. The DNA content of the interscapular fat-pads was similar in both diabetic-obese and lean mice, which indicates that the increased triacylglycerol in the tissue of the *db/db* mutants must be accommodated by a marked increase in brown-adipocyte size rather than by any increase in cell number. Both the total protein content and the cytochrome oxidase activity were significantly lower in the diabetic-obese animals than in the controls.

The purine-nucleotide-binding experiments were performed on mitochondria obtained by pooling brown adipose tissue from the interscapular, subscapular and dorsocervical sites. This procedure was adopted to minimize the number of animals needed to obtain sufficient material. In preliminary experiments no difference was noted in the amount of binding to mitochondria isolated from the interscapular site alone or from the pooled sites. The time

Table 1. *Properties of interscapular brown adipose tissue from adult and diabetic-obese (db/db) mice*
For details see the Experimental section. The results, which refer to the interscapular pads, are mean values $\pm$ S.E.M. for six animals in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with lean mice.

	Lean	Diabetic-obese
Body wt. of mice (g)	23 ± 2	$33 \pm 3^*$
Brown-adipose-tissue wt. (mg)	91.8 ± 5.0	$227.6 \pm 14.8^{***}$
DNA content of brown adipose tissue (μg)	167.7 ± 17.3	155.9 ± 13.9
Protein content of brown adipose tissue (mg)	8.8 ± 0.6	$5.6 \pm 0.4^{**}$
Cytochrome oxidase activity of brown adipose tissue (μmol of cytochrome <i>c</i> oxidized/min)	24.5 ± 0.4	$12.0 \pm 1.3^{***}$

Table 2. GDP binding to brown-adipose-tissue mitochondria of adult and 14-day-old lean and diabetic-obese (*db/db*) mice

Mitochondria were incubated with $10\mu\text{M}$ - $[^3\text{H}]\text{GDP}$ for 7 min at 20°C , pH 7.1. For details see the Experimental section. The specific activity of cytochrome oxidase was similar in the mitochondrial preparations of lean and diabetic-obese animals, indicating that differences in GDP binding between the two genotypes were not due to any difference in the purity of the preparations. The results are mean values $\pm$ S.E.M. for the numbers of groups of mice shown in parentheses. *** $P < 0.001$ compared with lean mice.

	GDP binding (nmol/mg of mitochondrial protein)	
	Lean	Diabetic-obese
Adult	0.257 ± 0.009 (9)	0.151 ± 0.010 (9)***
14-day-old	0.370 ± 0.024 (5)	0.208 ± 0.017 (5)***

course of the binding of $[^3\text{H}]\text{GDP}$ to brown-adipose-tissue mitochondria was the same in both the diabetic-obese and the lean mice. Binding was rapid and essentially maximal after 7 min, and this incubation time was therefore employed in subsequent experiments. The only difference observed between the normal and diabetic-obese animals was in the maximum binding (Table 2), which in the mutants was only 58% of that in the control mice; this suggests that brown-adipose-tissue thermogenesis is significantly decreased in adult diabetic-obese mice, as in the *ob/ob* mutant. It is important to note that decreased GDP binding in the diabetic-obese animals is unlikely to be due to the presence of mitochondria from contaminating tissue, since mitochondria from white adipose tissue (which is the most likely contaminant of brown fat in obese animals) are not isolated by the procedure used to prepare brown-adipose-tissue mitochondria (Cannon & Lindberg, 1979).

In order to distinguish between specific and non-specific binding, the GDP-binding assay was also performed in the presence of $100\mu\text{M}$ -ADP (+atractyloside), as described by Desautels *et al.* (1978). Although the 'specific' binding was lower than that shown in Table 2, the difference between the lean and diabetic-obese animals was maintained. However, in such competition studies a concentration of ADP higher than $100\mu\text{M}$ may be required, since after preincubation with $200\mu\text{M}$ -GDP (non-radioactive) negligible binding of $[^3\text{H}]\text{GDP}$ was observed, indicating that there is little or no non-specific binding of GDP to brown-adipose-tissue mitochondria from mice.

GDP-binding experiments were also performed on

Fig. 1. Scatchard plot of GDP binding to brown-adipose-tissue mitochondria from lean (●) and diabetic-obese (○) mice

Mitochondria obtained from adult animals were incubated at 20°C for 7 min, pH 7.1. For other details see the text.

pre-weanling mice aged 14 days. At this age obesity is not present, but an increase in the storage of triacylglycerol begins to occur at about this stage in development (Le Marchand-Brustel & Jeanrenaud, 1978). GDP binding was higher in both the lean and the diabetic-obese mice at 14 days of age than in their counterparts aged 8–10 weeks (Table 2), and this reflects the greater requirement for thermogenesis in very young animals. The difference between the two genotypes observed with mature animals was still apparent, however, with the binding in the diabetic-obese being only 56% of that in the lean.

A Scatchard analysis of GDP binding to brown-adipose-tissue mitochondria of adult mice was performed, and the plots obtained are shown in Fig. 1. The plots were linear ($r = 0.99$) for both lean and diabetic-obese animals, indicating that for each genotype only one type of binding site is present. The apparent dissociation constant (K_D) was the same for both groups of mice, $1.6\mu\text{M}$ at pH 7.1. This value is closer to that obtained in rats by Sundin & Cannon (1980) than to that reported by other authors for rats (see Sundin & Cannon, 1980) or hamsters (Nicholls, 1976). Although the affinity for GDP does not appear to differ between brown-adipose-tissue mitochondria of diabetic-obese and lean mice, there was a difference between the two groups in the maximum binding capacity. For lean mice the maximum binding was 0.27 nmol of GDP/mg of mitochondrial protein, whereas for diabetic-obese animals a value of 0.22 nmol of GDP/mg of mitochondrial protein was obtained.

Discussion

Since purine nucleotide binding to brown-adipose-tissue mitochondria closely reflects the thermogenic activity of the tissue in non-hibernating animals (Rafael & Heldt, 1976; Desautels *et al.*, 1978; Desautels & Himms-Hagen, 1979; Sundin & Cannon, 1980), the present results indicate that there is a marked decrease in brown-adipose-tissue thermogenesis in the diabetic-obese (*db/db*) mouse, as in the *ob/ob* mutant (Himms-Hagen & Desautels, 1978; Thurlby & Trayhurn, 1980). The present results also show that this decrease is not secondary to any of the changes induced by the obese state, which suggests that impaired thermogenesis in brown adipose tissue may be a primary factor in the development of obesity in diabetic-obese mice. Purine-nucleotide-binding studies have not been reported for 'pre-obese' *ob/ob* mice, but our preliminary results (A. E. Goodbody & P. Trayhurn, unpublished work) indicate that a decrease in brown-adipose-tissue thermogenesis also precedes the development of obesity in this mutant. The Scatchard plots (Fig. 1) demonstrate that, at least in the diabetic-obese strain, lower GDP binding is related to a fall in the number of binding sites rather than to any change in affinity; our preliminary results again indicate that this is also true for the *ob/ob* mouse. Thus in the obese mutants there is likely to be a decrease in the amount of the 32000-mol.wt. 'uncoupling' protein to which purine nucleotides bind (Nicholls, 1976; Heaton *et al.*, 1978).

In conclusion, the present results support the hypothesis that defective brown-adipose-tissue thermogenesis may be a general factor responsible for the development of obesity, an idea which is further supported by experiments on 'cafeteria' rats, where it has been demonstrated that large increases in voluntary food intake can be 'buffered' by increased diet-induced thermogenesis (Rothwell & Stock, 1979). This thermogenesis is associated with hypertrophy of interscapular brown adipose tissue and with an increase in GDP binding to brown-adipose-tissue mitochondria (Brooks *et al.*, 1980).

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