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STUDIES ON THE ACTIVITY OF BROWN ADIPOSE TISSUE IN SUCKLING, PRE-OBESE, *ob/ob* MICE

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The properties and activity of brown adipose tissue have been investigated in suckling, pre-obese, *ob/ob* mice in order to determine whether decreased thermogenesis in the tissue precedes the development of obesity in this mutant. At 14 days of age there was no difference between the *ob/ob* and normal animals in the total amount of interscapular brown adipose tissue, and the DNA content, protein content, and cytochrome oxidase activity of the tissue were similar in the two groups of mice. Respiration rates of brown adipose tissue mitochondria in the presence of albumin were, however, greater in the normal than the *ob/ob* animals, although after the addition of GDP to recouple the mitochondria there was no difference between the two groups. The mitochondrial membrane potential, measured with [^{3}H]methyltriphenylphosphonium, was less affected by exogenous GDP in *ob/ob* mice than in normal animals. GDP binding to brown adipose tissue mitochondria, an index of the proton conductance pathway, was much greater in normal than in *ob/ob* mice at both 10 and 14 days of age; the decreased GDP binding in the mutant animals was found to result from a reduction in the number of binding sites. It is concluded that brown adipose tissue mitochondria of pre-obese *ob/ob* mice are more tightly coupled than those of normal siblings, and that the activity of the 'thermogenic' proton conductance pathway is lower in the mutant animals. A decrease in thermogenesis in brown adipose tissue is therefore an early event in the development of the *ob/ob* mouse and precedes the appearance of obesity.

Introduction

The genetically obese (*ob/ob*) mouse is very widely used in studies on the metabolic basis of body weight regulation and the factors which are involved in the aetiology of obesity [1]. A primary factor in the development of the obese state in the *ob/ob* mutant is a high 'metabolic efficiency', which appears to result principally from a decrease in whole-body energy expenditure on thermoregulatory non-shivering thermogenesis [2–4]. Recent measurements of regional blood flow and tissue oxygen consumption in vivo have indicated that the main site of non-shivering thermogenesis in adult rodents, as in the new-born, is brown adipose tissue [5–8]. Such measurements have also suggested that differences in the activity of brown adipose tissue can largely account for the difference in energy expenditure on non-shivering thermogenesis between obese and lean mice [7].

The principal mechanism for thermogenesis in brown adipose tissue is through the operation of a proton conductance pathway across the inner membrane of the mitochondria, and this results in mitochondrial uncoupling [9]. The proton conductance pathway can be blocked by purine nucleotides, at least in vitro, which recouple the mitochondria [9,10]. The pathway is associated with a specific membrane protein of molecular

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Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

weight 32000, to which purine nucleotides bind [11]; the extent to which purine nucleotides bind to brown adipose tissue mitochondria reflects the activity of the proton conductance pathway in non-hibernating species [12–16].

Purine nucleotide binding experiments have suggested that under normal environmental conditions the proton conductance pathway is less active in brown adipose tissue of adult obese (*ob/ob*) mice than in lean mice [17]. Binding experiments have also suggested that the acute response to cold in the brown adipose tissue of the obese mice is impaired [17], although the chronic response seems to be normal [18]. No studies have been made, however, on the thermogenic activity of brown adipose tissue in *ob/ob* mice before obesity has been established, i.e., before 4 weeks of age. Such studies are important in order to determine whether decreased thermogenesis in brown adipose tissue is a primary factor in the development of obesity in the *ob/ob* mouse, or whether it is simply secondary to the obese state. In a different mutant, the diabetic-obese (*db/db*) mouse, the purine nucleotide-binding capacity has, however, been shown to be decreased as early as at 14 days of age [19].

In the present study we have examined the properties and activity of brown adipose tissue from suckling, pre-obese, *ob/ob* mice and report the results of measurements of mitochondrial purine nucleotide (GDP) binding, respiration rate and membrane potential.

Materials and Methods

Chemicals. [8-^{3}H]GDP (spec. act. 10–15 Ci/mmol), [U-^{14}C]sucrose (spec. act. 15 or 350 mCi/mmol) and ^3H_2O (spec. act. 0.9 mCi/mmol) were obtained from the Radiochemical Centre, Amersham, U.K., and [^{3}H]methyltriphenylphosphonium bromide (spec. act. 36 Ci/mmol) was from New England Nuclear Chemicals GmbH, Dreieich, F.R.G. GDP (sodium salt), rotenone, ferrocytochrome *c* (from horse heart), *p*-nitrophenylhydrazine, α-glycerophosphate (sodium salt), bovine serum albumin (fraction V, essentially fatty acid-free) glycylglyine and FCCP were each obtained from Sigma, Poole, U.K. Triton X-100 (scintillation grade) was obtained from Koch-Light Laboratories (Colnbrook, U.K.) and toluene was from May and Baker (Dagenham, U.K.). All other chemicals were purchased from BDH, Poole, U.K., and were AnalaR grade when available.

Animals. The animals used in this study were from a colony of *ob/ob* mice of the mixed background Aston variety [20]. The colony was housed in plastic cages in an animal room maintained at a temperature of 22 ± 2°C, with a 12 h light/12 h dark cycle (light period from 07.00 h). All the mice were obtained from parents heterozygous for the '*ob*' gene, the parental genotype having been established by previous breeding trials. The mice were used when aged 14 days, or younger. Since obese individuals cannot be distinguished visually from their siblings until they are approx. 4 weeks of age, a 'cold stress' test was used to differentiate those bearing the *ob/ob* genotype from those with a normal lean genotype (*ob/+* and *+/+*) [21]. The cold stress test involved monitoring the rectal temperature of mice caged individually at room temperature (20–22°C) for up to 60 min. Under these conditions mice with the *ob/ob* genotype show a marked fall in body temperature. Normal and *ob/ob* animals identified by the test were either used immediately or were marked and returned to the nest for use the following day.

General methods. The mice were killed by cervical dislocation, and brown adipose tissue was removed from the interscapular site and trimmed free of any white adipose tissue and connective tissue. In some experiments the subscapular and dorsocervical brown adipose tissue was also removed (see below). The DNA content of brown adipose tissue was determined by the method of Curtis-Prior et al. [22]. The total protein of brown adipose tissue was measured by a modification [23] of the procedure of Lowry et al. [43]. The same assay was also used for the determination of mitochondrial protein, except in the measurements of membrane potential where the biuret method was employed [24]. Cytochrome oxidase activity was assayed spectrophotometrically [25].

Mitochondrial studies. Mitochondria were prepared as described by Cannon and Lindberg [26], from brown adipose tissue pooled from the interscapular, subscapular and dorsocervical sites. The binding of purine nucleotides was measured by incubating the mitochondria with 10 μM [^{3}H]GDP

at pH 7.1 for 7 min at room temperature (20°C), essentially by the method of Nicholls [10], with the modifications described previously [19].

Mitochondrial respiration was measured at 24°C using a Yellow Springs YSI Model 53 oxygen electrode (Yellow Springs Instrument Co., OH, U.S.A.) as described by Nicholls [27]. The mitochondria were incubated in a medium (pH 7.1) containing 100 mM sucrose, 10 mM glycylglycine, and 5 μM rotenone together with 10 mM α-glycerophosphate (as substrate).

Membrane potential was measured by incubating the mitochondria in the same medium as that used for the respiration studies, but with the addition of bovine serum albumin (2.5 mg/ml), tetraphenylboron (3 μM), and [^{3}H]methyltriphenylphosphonium (1 μM) [28]. The incubations were carried out for 2.5 min at 24°C, and [^{14}C]sucrose was then added. After 30 and 60 s, samples of the incubation mixture were taken and filtered through a 0.65 μm cellulose acetate filter (Sartorius Instruments Ltd., Surrey, U.K.) under vacuum. ^{14}C and ^{3}H radioactivity was then measured in both the filter and the filtrate [28].

The volume of the mitochondrial matrix was determined by incubating the mitochondria in the presence of ^3H_2O and [^{14}C]sucrose, exactly as for the measurement of membrane potential except that tetraphenylboron and methyltriphenylphosphonium bromide were excluded. Matrix volume was then calculated by comparing the water-permeable and sucrose-permeable spaces [29].

Measurement of radioactivity. Radioactivity was measured in a Packard Tri-Carb 2650 liquid scintillation counter, with corrections being made for background counts and quench (using an external standard). An NCS tissue solubilizer/toluene-based scintillation solution was used in the purine nucleotide-binding experiments while a Triton-X-100/toluene-based scintillation solution was employed in the measurements of membrane potential.

Statistical analysis. Student's unpaired *t*-test was used to assess the statistical significance of differences between groups.

Results

General properties of brown adipose tissue

Table I shows the general properties of interscapular brown adipose tissue in suckling normal and *ob/ob* mice aged 14 days. At this age there was no difference in the body weight of the two groups of mice, nor was there any difference in the total amount of interscapular brown adipose tissue. The DNA content of the interscapular brown fat pads was not significantly different in the *ob/ob* and normal animals at 14 days of age, suggesting that there were similar numbers of brown adipocytes. The protein content and cytochrome oxidase activity of the brown adipose tissue were also not different, which indicates that the total oxidative capacity of the tissue was the same in both normal and pre-obese animals.

TABLE I

INTERSCAPULAR BROWN ADIPOSE TISSUE FROM NORMAL AND *ob/ob* MICE AGED 14 DAYS

For experimental details see the text. The results are expressed as mean values ± S.E. for six *ob/ob* and six normal mice.

	Normal	*ob/ob*
Body weight of mice (g)	9.8 ± 0.4	9.8 ± 0.3
Rectal temperature following 40 min 'cold' exposure (20–22°C)	33.8 ± 0.2	30.5 ± 0.7 [a]
Brown adipose tissue weight (mg)	49.8 ± 5.3	50.0 ± 3.7
DNA content of brown adipose tissue (μg)	202 ± 8	174 ± 16
Protein content of brown adipose tissue (mg)	6.0 ± 0.4	6.2 ± 0.4
Cytochrome oxidase activity of brown adipose tissue (μmol cytochrome *c* oxidised/min)	14.6 ± 1.0	13.1 ± 1.7

[a] $P < 0.001$, compared to the normal mice.

Mitochondrial respiration

Respiration rates were measured, using α-glycerophosphate as substrate, in mitochondria isolated from brown adipose tissue pooled from the interscapular, subscapular and dorsocervical sites. The results obtained with both *ob/ob* and normal animals are given in Table II. In the absence of albumin low respiration rates were found with both groups of mice. On adding albumin, which removes the inhibitory effects of endog-

TABLE II

RESPIRATION RATES OF MITOCHONDRIA FROM BROWN ADIPOSE TISSUE OF NORMAL AND *ob/ob* MICE AGED 14 DAYS—THE EFFECT OF GDP

Normal and *ob/ob* mice were identified at 14 days of age and used immediately. Respiration rates were measured at 24°C, pH 7.1, with α-glycerophosphate as substrate. Additions were made sequentially. For full experimental details see text. The results are expressed as mean values ± S.E. for four groups of normal and four groups of *ob/ob* mice (two animals were used to obtain the mitochondrial preparations for each group).

Additions	Mitochondrial respiration (nmol O_2/min per mg mitochondrial protein)	
	Normal	*ob/ob*
–	39.6 ± 5.8	24.8 ± 3.0
Albumin (2.5 mg/ml)	120.5 ± 6.9	85.2 ± 3.3 [a]
GDP (1 mM)	64.8 ± 5.7	64.8 ± 2.2
FCCP (5 μM)	110.9 ± 4.4	81.0 ± 4.6 [a]

[a] $P < 0.01$, compared to the normal mice.

enous fatty acids on the activity of α-glycerophosphate dehydrogenase [27,30], large increases in respiration rate were observed. Respiration after the addition of albumin was 41% higher in the normal mice than in the mutants. When GDP was then added to recouple the mitochondria, and thereby restore respiratory control, the respiration rates fell and the rates became identical in the two groups of mice. The difference in respiration rate before and after adding GDP was nearly 3-times higher in the mitochondria from the normal mice than from the *ob/ob*.

Addition of the uncoupler FCCP resulted in an increase in respiration rate with both normal and *ob/ob* mitochondria, to values close to those obtained before the addition of GDP.

Purine nucleotide binding

The purine nucleotide-binding assay was performed principally on 14-day-old animals, using [^{3}H]GDP, and the effects of 'cold exposure' were also investigated. This was done by comparing the results obtained in mice identified on the previous day (day 13) to those obtained when the brown adipose tissue was removed immediately after the 'cold stress' test.

Table III shows that GDP binding was much greater in the mitochondria of the normal than the *ob/ob* mice. This was the case whether the identification of genotype was made immediately before the assay or on the preceding day. There was a clear response to the effects of cold exposure, both groups of mice showing an increase in GDP binding. Although the proportional increase in binding was greater in the *ob/ob* mice, the absolute increase was the same in the two groups.

Some exploratory GDP-binding experiments were also performed on 10-day-old animals whose genotype had been identified on the preceding day (Table III). In the lean mice binding was significantly higher at this age than in the 14-day-old animals. The large difference in binding between the normal and the *ob/ob* observed in the 14-day-old animals was also clearly apparent at 10 days of age. Thus, even within the first 2 weeks of life the proton conductance pathway in brown adipose tissue appears less active in *ob/ob* mice than normal animals.

In order to determine whether the lower GDP binding of *ob/ob* mice was due to a decrease in the number of binding sites or to a decrease in affinity, a Scatchard analysis was performed. This was done using 14-day-old mice identified when aged 13 days. The Scatchard plots which were obtained are shown in Fig. 1. Linear plots ($r = 0.99$) were observed with the *ob/ob* as well as the normal mice, indicating that in both groups there is only one type of binding site. The maximum number of binding sites was much greater in the normal mice than in the *ob/ob* animals; values of 910 and 364 pmol GDP/mg mitochondrial protein were obtained for normal and *ob/ob* animals, respectively. The apparent dissociation constant (K_d) of 2.0 μM was the same for both groups of mice. This value is very similar to that obtained in adult *ob/ob* mice (Goodbody, A.E., unpublished data) and to that found previously with lean and diabetic-obese (*db/db*) mice of the C57BL/Ks strain [19].

Membrane potential

Membrane potential ($\Delta\psi$) was measured using [^{3}H]methyltriphenylphosphonium [28], a lipophilic cation which distributes across membranes according to a Nernst equilibrium [31]. The measure-

TABLE III

PURINE NUCLEOTIDE (GDP) BINDING TO MITOCHONDRIA FROM BROWN ADIPOSE TISSUE OF NORMAL AND *ob/ob* MICE AGED 10 AND 14 DAYS

Normal and *ob/ob* mice were identified either on the day before the experiment or immediately after the test for the identification of genotype. Mitochondria were incubated for 7 min at 20°C in a medium (pH 7.1) containing 10 μM [^{3}H]GDP. For full experimental details see text. The results are expressed as mean values ± S.E. with the number of individual animals in parentheses.

Age at time of assay (days)	Age at time of identification (days)	GDP bound (pmol GDP/mg mitochondrial protein)	
		Normal	*ob/ob*
14	13	527.4 ± 19.7(5)	199.9 ± 21.3(5) [b]
14	14 [a]	680.5 ± 24.3(5) [c]	353.8 ± 12.1(4) [b,c]
10	9	762.5 ± 33.3(3) [c]	250.0 ± 20.6(2) [b]

[a] These results represent the acute effect of cold.
[b] $P < 0.001$, compared to the normal mice.
[c] $P < 0.001$, compared to the 14-day-old mice of the same genotype identified on day 13.

ments were made on mitochondria isolated from 14-day-old animals tested and identified at 13 days of age; the results obtained are shown in Table IV. In the freshly isolated mitochondria incubated without any exogenous purine nucleotide, the membrane potential of the *ob/ob* animals was 12 mV greater than with the normal mice. In the presence of GDP a higher membrane potential was obtained with both groups, but in this case $\Delta\psi$ in the mitochondria of the normal mice was 9 mV higher than in the mutant animals. The net effect of GDP was to cause a much greater rise in membrane potential in the normal animals. In the presence of FCCP (with GDP) membrane potential was rather lower than in the mitochondria incubated in the absence of GDP.

It should be noted that the true extent of the potential change induced by GDP has been substantially understated in the present study, since only the membrane potential component of the proton electrochemical potential has been mea-

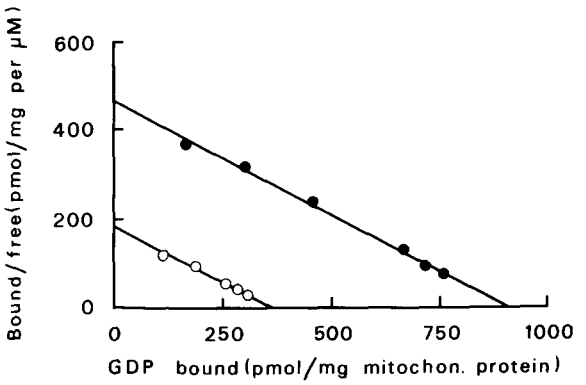

Fig. 1. Scatchard plot of GDP binding to brown adipose tissue mitochondria from normal (●) and *ob/ob* (○) mice aged 14 days. The mice were identified at 13 days of age and used the following day. Mitochondria were prepared from brown adipose tissue pooled from three normal and three *ob/ob* animals. The incubations with [^{3}H]GDP were carried out at 20°C, pH 7.1, for 7 min. For other experimental details see text.

TABLE IV

MEMBRANE POTENTIAL IN MITOCHONDRIA FROM BROWN ADIPOSE TISSUE OF NORMAL AND *ob/ob* MICE AGED 14 DAYS

Normal and *ob/ob* mice were identified at 13 days of age, and used the following day. Mitochondria were incubated at 24°C in a medium (pH 7.1) containing [^{3}H]methyltriphenylphosphonium. For full experimental details see text. The results are expressed as mean values ± S.E. for three groups of normal and three groups of *ob/ob* mice (two animals were used to obtain the mitochondrial preparations for each group). The results with FCCP were obtained in the presence of GDP.

	Membrane potential (mV)			
	−GDP	+GDP (1 mM)	Δ with GDP	+FCCP (5 μM)
Normal	111 ± 7	157 ± 5	47 ± 3	95 ± 2
ob/ob	123 ± 8	148 ± 6	25 ± 7 [a]	99 ± 4

[a] $P < 0.05$, compared to the normal mice.

sured. Parallel measurements of $\Delta\psi$ and ΔpH are required to determine the proton electrochemical potential.

Discussion

Obesity is not detectable visually in genetically obese (*ob/ob*) mice until 1 week after weaning when the animals are aged 4 weeks. At this stage the total body lipid content of the mutant animals is approx. 3-times that of lean siblings [20,32]. Previous studies on the activity of brown adipose tissue in *ob/ob* mice have been conducted on animals rather older than 4 weeks [7,17,18]. Consequently, it is not known whether the decreased thermogenesis found in brown adipose tissue of *ob/ob* mice studied when substantially obese is simply secondary to the changes, e.g., endocrinological, brought about by the obese state, or whether it occurs early in development and may therefore be related to the primary genetic defect.

In the present experiments the activity of brown adipose tissue has been examined primarily in mice aged 14 days. At this stage the total body lipid content of Aston variety animals bearing the *ob/ob* genotype is only slightly greater than that of normal littermates [20]. The results of the present study indicate that there are no gross structural changes in brown adipose tissue of the *ob/ob* mutant during the first 2 weeks of life. In particular, there is no change in the apparent number of brown adipocytes, and the absence of any weight increase in the interscapular pads indicates that there is no major excess accumulation of triacylglycerol (Table I). In adult *ob/ob* mice, however, the triacylglycerol content of interscapular brown adipose tissue is much higher than that of lean animals [17].

The major change observed in brown adipose tissue of the pre-obese *ob/ob* mice lies in mitochondrial function. In the absence of exogenous GDP, respiration was lower than in mitochondria from brown adipose tissue of normal mice, and the membrane potential was higher (Tables II and IV). These differences were essentially abolished when the mitochondria were fully coupled by incubation with GDP; similar results have been obtained with adult *ob/ob* and lean mice (Goodbody, A.E., Fraser, D.R. and Trayhurn, P., unpublished data). Therefore, freshly isolated brown adipose tissue mitochondria from *ob/ob* mice appear to be more 'tightly coupled' than those from lean animals, and this is the case in both the obese and pre-obese states.

The activity of the proton conductance pathway in brown adipose tissue is now generally assessed by the purine nucleotide-binding assay [12–16]. In mature *ob/ob* mice decreased GDP binding, and therefore decreased thermogenesis, has been observed in animals maintained at normal environmental temperatures [17]. In the present study much lower GDP binding was found with *ob/ob* mice than with normal animals at both 14 and 10 days of age (Table III), and this was the result of a decrease in the number of binding sites (Fig. 1). Pre-weanling *ob/ob* mice responded to cold stress in the same way as the normal animals, by an increase in GDP binding. This contrasts with adult *ob/ob* mice who show no increase in GDP binding on acute cold exposure [17]; the hyperinsulinaemia of the adult obese animal [1] may be responsible for its failure to respond acutely to cold.

An attempt was also made to determine whether GDP binding was lower in *ob/ob* mice than in normal animals at an age even younger than 10 days (results not shown). Litters of 5- and 7-day-old mice were examined, but the test for identifying those animals with the *ob/ob* genotype cannot be used at this age. The GDP binding assay was therefore performed on brown adipose tissue mitochondria prepared from each individual mouse in the litter, with a view to determining whether there was a bimodal distribution in the binding data. Although a wide spread of values was obtained, ranging from 180 to 435 pmol GDP/mg mitochondrial protein, no clear bimodal distribution was observed from which a tentative assignment of normal and *ob/ob* genotypes could be made.

Decreased activity of possible thermogenic mechanisms other than the proton conductance pathway in brown adipose tissue has also been observed in *ob/ob* mice. Particular attention has been paid to Na^+ transport across the plasma membrane in tissues such as skeletal muscle and the liver. Reduced $(Na^+ + K^+)$-ATPase activity has been observed, either by direct measurement of enzyme activity or by [^{3}H]ouabain-binding

studies, in skeletal muscle, kidney and liver of mature *ob/ob* mice [33–36]. A decrease in the number of $\{Na^+ + K^+\}$-ATPase 'enzyme units' has also been found in skeletal muscle, though not the liver, of the *ob/ob* mutant compared to normal animals, as early as 14 days of age [37].

Whether a decrease in Na^+-pump activity in tissues such as skeletal muscle could make a quantitatively significant contribution to the low-energy expenditure on non-shivering thermogenesis of the *ob/ob* mouse has not been established. Estimates of the energetic cost of Na^+ transport vary from up to 50% of cellular energy expenditure [38,39] to a recent value of no more than 5% [40]. Recent studies have also indicated that skeletal muscle plays little or no direct role in non-shivering thermogenesis in small rodents [5,6,41], while measurements of regional blood flow and tissue oxygen utilization in vivo have shown that differences in brown adipose tissue account quantitatively for the difference in non-shivering thermogenesis between lean and obese mice [7]. It is probable, therefore, that any decrease in energy expenditure on Na^+ pumping in skeletal muscle and other tissues makes at most only a minor contribution to the overall decreased thermogenesis of the *ob/ob* mutant.

In conclusion, it is clear from the present results that reduced brown adipose tissue thermogenesis occurs early in the development of the *ob/ob* mutant, and is not a secondary factor of obesity. A similar conclusion has also been drawn with the *db/db* mutant [19], and this underlines the likely importance of brown adipose tissue in whole-body energy regulation in small rodents. Finally, it should also be noted from the present work that decreased thermogenesis in brown adipose tissue would account for both the cold-induced hypothermia [21] and low metabolic rate [42] of the suckling, pre-obese, *ob/ob* mutant.

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