The influence of GDP on Ca^{2+} uptake by mitochondria of brown adipose tissue from lean and genetically obese (*ob/ob*) mice

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The specific binding capacity for purine nucleotides in brown-adipose-tissue mitochondria is thought to indicate the capacity of the proton-conductance pathway which leads to uncoupled respiration. This functional relationship was investigated in studies measuring initial Ca^{2+} -uptake rates and membrane potential in the presence or absence of GDP in brown-adipose-tissue mitochondria with different GDP-binding capacities. The mitochondria from pre-obese and obese *ob/ob* mice were less able than those from lean control mice to dissipate membrane potential in the absence of GDP. Mitochondria from the obese animals also maintained a higher Ca^{2+} -uptake rate without GDP in comparison with the rate found with mitochondria from the lean mice. The GDP-dependence of Ca^{2+} uptake was greater in brown-adipose-tissue mitochondria from cold-adapted animals than in those from animals kept at 22°C or at thermoneutrality (33°C). It is concluded that Ca^{2+} -uptake rate and membrane-potential values are depressed in the absence of GDP and indicate indirectly the influence of purine nucleotides on maintaining the proton electrochemical gradient in brown-adipose-tissue mitochondria. It is also apparent that the lower GDP-binding capacity in mitochondria from *ob/ob* mice is related to a decreased ability to dissipate the proton electrochemical gradient.

An alternative to the usual pathway directing substrate energy into ATP formation is found in brown-adipose-tissue mitochondria. Here an uncoupling mechanism can diminish the proton electrochemical gradient ($\Delta\bar{\mu}_{\text{H}^+}$) across the mitochondrial membrane, thus allowing more of the energy from substrate oxidation to be released as heat. This thermogenic process appears to be regulated by varying the amount and activity of a proton-conducting protein in the mitochondrial inner membrane (Heaton *et al.*, 1978). Proton conductance can be suppressed *in vitro*, and therefore respiration becomes fully coupled, by the binding of purine nucleotides (e.g. GDP) to this specific membrane protein (Nicholls, 1976). In genetically obese (*ob/ob* and *db/db*) mice the specific binding capacity for purine nucleotides is decreased compared with that in lean animals (Himms-Hagen & Desautels, 1978; Goodbody & Trayhurn, 1981). This relates well to the impaired ability for non-shivering thermogenesis

shown by the mutant mice (Trayhurn & James, 1978; Trayhurn, 1979).

From this information it has been postulated that *ob/ob* mice have a lower energy expenditure on non-shivering thermogenesis because of a decreased ability to dissipate $\Delta\bar{\mu}_{\text{H}^+}$ in brown-adipose-tissue mitochondria. However, no functional tests have demonstrated directly whether these mitochondria from *ob/ob* mice are less able than those from lean mice to vary $\Delta\bar{\mu}_{\text{H}^+}$. Because mitochondrial Ca^{2+} transport was being assessed in tissues from *ob/ob* mice (Fraser & Trayhurn, 1983), the opportunity was taken in the present studies to test the uncoupling capacity of brown-adipose-tissue mitochondria. The rate of uptake of Ca^{2+} by mitochondria is directly related to the membrane-potential ($\Delta\psi$) component of $\Delta\bar{\mu}_{\text{H}^+}$ (Nicholls & Åkerman, 1982) and can thus act as a qualitative index of $\Delta\bar{\mu}_{\text{H}^+}$. By measuring both Ca^{2+} -influx rates as well as $\Delta\psi$ in the presence and absence of added purine nucleotide (GDP), any functional differences between the genotypes in the control of mitochondrial thermogenesis can be determined.

Abbreviation used: TPMP⁺, methyltriphenylphosphonium.

A preliminary outline of part of this work has been presented previously (Fraser & Trayhurn, 1981).

Methods

Animals

Female 'Aston' *ob/ob* and lean mice were reared as described in the preceding paper (Fraser & Trayhurn, 1983). These were used as mature animals at 2–3 months of age or at a pre-obese stage, 14 days after birth. Female golden hamsters (*Mesocricetus auratus*) of an outbred MB strain were obtained from Intersimian Ltd., Abingdon, Oxon, U.K., and were acclimated at 5°C or 30°C for 3–4 weeks.

Mitochondria preparations

Mitochondria were prepared from pooled brown adipose tissue in the interscapular, subscapular, cervical and axillary regions by the method of Cannon & Lindberg (1979), as described previously (Fraser & Trayhurn, 1983).

Ca²⁺-uptake measurements

The initial rate of Ca²⁺ uptake by respiring mitochondria at 20°C was measured by using 200 μ M-⁴⁵Ca²⁺ as in the preceding paper (Fraser & Trayhurn, 1983). The Na⁺-free incubation medium contained 100 mM-sucrose/2 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/Tris (pH 7.2), bovine serum albumin (fatty acid-free) (2 mg/ml), 1 mM-KH₂PO₄, 5 mM-L- α -glycerophosphate [di(monocyclohexylamine) salt], 2.4 μ M-rotenone and, where appropriate, 1 mM-GDP (Tris salt). Mitochondria were present at a concentration of 1–2 mg of protein/ml.

Some of the control values with added GDP that were reported in the preceding paper (Fraser & Trayhurn, 1983) were the positive controls for the studies without GDP described below.

Mitochondrial membrane potential

The distribution of [³H]TPMP⁺ ions (New England Nuclear Chemicals G.m.b.H., Dreieich, Germany) across the mitochondrial inner membrane was used to determine $\Delta\psi$ (Scott & Nicholls 1980; Nicholls & Brand, 1980). Details of the procedure were as in the preceding paper (Fraser & Trayhurn, 1983).

Materials

Organic reagents were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K., except where otherwise indicated.

Results

Influence of GDP on Ca²⁺-uptake rate

The addition of GDP to brown-adipose-tissue mitochondria had a pronounced effect on the initial rate of Ca²⁺ uptake. For mitochondria from lean mice, the presence of GDP stimulated the absolute Ca²⁺-uptake rate by about 35% (Table 1). However, in the absence of GDP the radioisotopic method of detecting Ca²⁺-uptake revealed an apparent lag of 25–30 s before the onset of Ca²⁺ transport (Fig. 1a). Hence measurement from the moment of adding Ca²⁺ to the respiring mitochondria gives a considerably greater effect of GDP than does a simple comparison of the absolute uptake rates.

With mitochondria from *ob/ob* mice, addition of GDP also stimulated the initial rate of Ca²⁺ uptake, this time by about 25% (Table 1). Nevertheless the pattern of uptake was noticeably different from that of mitochondria from lean mice (Fig. 1b). Not only was there no 'lag' in the onset of Ca²⁺ uptake in the absence of GDP, but the absolute rate of uptake without GDP was in fact greater than that for mitochondria from lean mice in the presence of GDP. It is therefore apparent that, although GDP enhances the rate of Ca²⁺ accumulation by brown-adipose-tissue mitochondria from *ob/ob* mice, its effect is much less than with these mitochondria from lean mice.

Influence of GDP on membrane potential

An independent assessment of the effect of GDP on the electrophoretic driving force for Ca²⁺ uptake was made by measuring $\Delta\psi$ across the mito-

Table 1. Influence of GDP on initial Ca²⁺-uptake rates with brown-adipose-tissue mitochondria of mature and 14-day-old lean and *ob/ob* mice

The initial Ca²⁺-uptake rates were measured in respiring mitochondria at 20°C as described in the Methods section. Initial extramitochondrial Ca²⁺ concentration was 200 μ M. Mean values $\pm$ S.E.M. are given, with the numbers of experiments indicated in parentheses. The statistical significance of differences between preparations with and without 1 mM added GDP was estimated by Student's *t* test for unpaired data (**P* < 0.05); †*P* < 0.05 by paired *t* test.

Mitochondrial preparation	Ca ²⁺ -uptake rate (nmol/min per mg of mitochondrial protein)	
	Without GDP	With 1 mM-GDP
Mature lean mice	36.9 $\pm$ 4.0 (8)	53.9 $\pm$ 5.7* (8)
Mature <i>ob/ob</i> mice	70.8 $\pm$ 5.6 (8)	98.4 $\pm$ 12.5† (8)
14-day-old lean mice	17.9 $\pm$ 6.6 (4)	93.4 $\pm$ 27.3* (4)
14-day-old <i>ob/ob</i> mice	51.6 $\pm$ 10.9 (4)	87.0 $\pm$ 7.3* (4)

Fig. 1. Uptake of Ca²⁺ by isolated mitochondria from brown adipose tissue of lean (a) and ob/ob (b) mice in the absence (○) and in the presence (●) of 1 mM-GDP
The curves are plotted from mean values ± S.E.M. (bars) for nine groups of lean and nine groups of ob/ob mice. For experimental details see the text.

chondrial membrane (Table 2). As reported in the preceding paper (Fraser & Trayhurn, 1983), there was no difference in Δψ between mitochondria from brown adipose tissue of lean and ob/ob mice in the presence of GDP. Yet when GDP was absent, mean values for mitochondrial Δψ were lower by 31.8 mV for the lean animals, but by only 20 mV for the ob/ob mice. Thus, both rates of Ca²⁺ uptake as well as direct measurement of Δψ indicate that brown-adipose-tissue mitochondria from ob/ob mice have less ability than those from lean mice to dissipate the membrane electrochemical potential. Furthermore, these results also confirm that the specific binding capacity for GDP (Himms-Hagen & Desautels, 1978) is functionally related to the capability for diminishing Δψ.

Ca²⁺ uptake and Δψ in mitochondria from pre-obese ob/ob mice

As described previously (Fraser & Trayhurn, 1983), brown-adipose-tissue mitochondria from ob/ob mice at 2–3 months of age have a higher capacity for transporting Ca²⁺ in the presence of GDP than do these mitochondria from lean mice. However, this difference was not apparent in pre-obese mice at 14 days of age. Despite this, mitochondrial Ca²⁺ uptake is clearly less affected by the absence of GDP from preparations from 14-day-old ob/ob mice than in those from their lean littermates. Ca²⁺-uptake rates

Table 2. Influence of GDP on Δψ in respiring brown-adipose-tissue mitochondria of mature and 14-day-old lean and ob/ob mice

Δψ was determined for mitochondria respiring at 20°C as described in the Methods section. For the mature mice, mean values are given ± S.E.M., with the numbers of experiments indicated in parentheses (*P < 0.01, **P < 0.001, compared with values without GDP; †P < 0.01, compared with values for lean mice without GDP). Mean values for two experiments are given for 14-day-old mice, with the individual values in parentheses.

Mitochondrial preparation	Mitochondrial Δψ (mV)	
	Without GDP	With 1 mM-GDP
Mature lean mice	102.3 ± 3.3 (6)	134.1 ± 4.4** (4)
Mature ob/ob mice	115.6 ± 2.3† (6)	135.6 ± 4.3* (4)
14-day-old lean mice	94.9 (90.5, 99.2)	149.1 (146.3, 151.9)
14-day-old ob/ob mice	108.6 (105.1, 112.0)	146.9 (143.0, 150.8)

by mitochondria from ob/ob and lean mice fell by 41% and 81% respectively without added GDP (Table 1). Similarly, in two experiments the mean Δψ was lower by 38.3 and 54.2 mV for ob/ob and lean mice respectively in the absence of GDP (Table

2). Thus, in agreement with GDP-binding studies (Goodbody & Trayhurn, 1982) which indicate decreased capacity of the proton-conductance pathway in the pre-obese *ob/ob* mice, the present results indicate a decreased ability to lower $\Delta\psi$ when GDP-binding sites on these mitochondria are unoccupied.

Effect of environmental temperature on GDP-dependent Ca^{2+} -uptake rates

Cold-adaptation of rats results in an increased capacity for non-shivering thermogenesis and an increase in the specific binding capacity for purine nucleotides in brown-adipose-tissue mitochondria (Desautels *et al.*, 1978; Sundin & Cannon, 1980). This suggests that adaptation to cold increases the concentration of proton-conductance channels across the mitochondrial inner membrane. When lean mice were acclimated at 33°C or at 5°C for 3–4 weeks, there was a 3-fold increase in the initial rate of Ca^{2+} -uptake by mitochondria of brown adipose tissue from cold-adapted compared with warm-adapted mice, as previously described (Fraser & Trayhurn, 1983). In the absence of GDP, mitochondria from mice adapted to 5°C were only able to take up Ca^{2+} sluggishly, so that on addition of GDP the Ca^{2+} -influx rate increased 7.8-fold. In contrast, mitochondria from mice adapted to thermoneutrality (33°C) were little affected by whether GDP was present or absent (Table 3). Indeed, in the two experiments performed, the presence of GDP inexplicably caused the Ca^{2+} -influx rate to fall.

Again, when *ob/ob* mice were cold-adapted at 12°C the addition of GDP caused a 5.6-fold increase in Ca^{2+} -uptake rate (Table 3), compared with only a 1.3-fold increase when the animals were maintained at 22°C (Table 1). Lean mice at 12°C showed a smaller effect of cold, giving a 3.9-fold increase in Ca^{2+} -uptake rate when GDP was added, compared with a 1.3-fold increase for animals at 22°C. Hence, for both lean and *ob/ob* mice, cold-adaptation increased the dependence on GDP of mitochondrial Ca^{2+} uptake. It is therefore concluded that, although in general brown-adipose-tissue mitochondria from *ob/ob* mice are less able than those from lean mice to discharge $\Delta\psi$ in the absence of GDP, both genotypes under the stimulus of cooling are nevertheless able to increase the capability for depressing $\Delta\psi$, presumably by enhancing the activity of the proton-conductance pathway. These results parallel those obtained in GDP-binding studies (Hogan & Himms-Hagen, 1980).

Similar effects of cold-adaptation on GDP-dependent Ca^{2+} uptake were found with brown-adipose-tissue mitochondria from hamsters, but the response was even more marked than in mice. In hamsters kept at thermoneutrality (30°C), GDP

Table 3. *Influence of GDP on initial Ca^{2+} -uptake rates with brown-adipose-tissue mitochondria from cold- and warm-adapted mice and hamsters*

Lean mice were acclimated at 33°, 12° or 5°C, and *ob/ob* mice were acclimated at 12°C, all for 2–4-week periods. Hamsters were acclimated at either 30°C or at 5°C for 3 weeks. Ca^{2+} -uptake rates were measured with brown-adipose-tissue mitochondria at 20°C as described in the Methods section. Mean values $\pm$ S.E.M. are given with the numbers of experiments indicated in parentheses. Mean values from two experiments each are given for mice at 33°C and hamsters at 30° and 5°C, with the individual values in parentheses.

Mitochondrial preparation	Ca^{2+} -uptake rate (nmol/min per mg of mitochondrial protein)	
	Without GDP	With 1 mM-GDP
Lean mice at 33°C	155.9 (178.9, 132.9)	69.8 (60.2, 79.4)
Lean mice at 12°C	24.8 $\pm$ 9.4 (3)	96.1 $\pm$ 14.3 (3)
Lean mice at 5°C	27.6 $\pm$ 3.0 (3)	215.4 $\pm$ 67.8 (3)
<i>ob/ob</i> mice at 12°C	47.8 $\pm$ 4.7 (3)	269.8 $\pm$ 64.5 (3)
Hamsters at 30°C	176.5 (178.4, 174.5)	447.9 (630.7, 265.1)
Hamsters at 5°C	28.8 (32.9, 24.6)	981.9 (878.8, 1085.0)

stimulated the Ca^{2+} -uptake rate by 2.5-fold. However, in hamsters adapted at 5°C the Ca^{2+} -uptake rate was stimulated by 34-fold in the presence of GDP (Table 3); thus the mitochondria from cold-acclimated hamsters are much more 'uncoupled' than those from warm-acclimated hamsters. Much higher rates of Ca^{2+} uptake in the presence of GDP were obtained in the hamsters than in the mice, and the rates were particularly high in the hamsters acclimated at 5°C.

Overall, the results obtained on Ca^{2+} transport in hamsters are not consistent with the view that in this hibernating species brown adipose tissue is always partially primed for thermogenesis irrespective of the environmental temperature (Nicholls, 1979).

Discussion

The proton electrochemical potential across the inner membrane of mitochondria is dependent on both $\Delta\psi$ and the pH gradient (ΔpH) according to the relationship:

$$\frac{\Delta\bar{\mu}_{\text{H}^+}}{F} = \Delta\psi - 59 \Delta\text{pH}$$

where F is the Faraday (Nicholls, 1974). The decrease in $\Delta\bar{\mu}_{H^+}$ caused by the inward conductance of protons in brown-adipose-tissue mitochondria will be mainly a result of the fall in the pH gradient. However, under the experimental conditions reported here, $\Delta\psi$ is also mainly determined by proton extrusion linked to respiration. Therefore variation in the activity of the proton-conductance pathway will lead to variable values for $\Delta\psi$ as well as for ΔpH . Although changes in the membrane potential will be less marked than changes in the value of $-59 \Delta pH$, measurement of the membrane potential gives a qualitative estimate of changes in protonmotive force (Nicholls, 1974). Similarly, because $\Delta\psi$ is the main determinant of the electrochemical potential, which provides energy for Ca²⁺ accumulation (Nicholls & Åkerman, 1982), measurement of the rate of Ca²⁺ uptake by mitochondria is a further indirect assay for changes in $\Delta\bar{\mu}_{H^+}$.

The influence of GDP on Ca²⁺-uptake rate by brown-adipose-tissue mitochondria was greater with mitochondria from lean than from *ob/ob* mice. Moreover, in the absence of GDP, mitochondria from lean mice showed an apparent lag before the onset of Ca²⁺ uptake. If the low rate of Ca²⁺ uptake without GDP indicates that these mitochondria were substantially uncoupled, it is likely that the 'lag' was an artifact of the Ruthenium-Red-quench technique for measuring Ca²⁺ transport. The initial accumulation of ⁴⁵Ca²⁺ was probably being immediately released through the influx uniporter, the reversal of which is not inhibited by Ruthenium Red under conditions of low $\Delta\psi$ (Puskin *et al.*, 1976). With mitochondria from the *ob/ob* mice incubated without GDP, the absence of this uptake lag, the maintenance of a higher $\Delta\psi$ and the faster Ca²⁺-influx rate all indicate that the decreased GDP-binding capacity of these mitochondria is indeed associated with less ability, in comparison with lean mice, to dissipate the proton gradient.

A further argument in favour of the GDP-dependence of $\Delta\psi$ in brown-adipose-tissue mitochondria comes from the experiments on cold-adaptation. When either lean or *ob/ob* mice were acclimated to cold, with a consequent increase in GDP-binding capacity, the absence of GDP caused a marked decline in the Ca²⁺ uptake rate by these mitochondria. Thus with the availability of more GDP-binding sites there is indeed an increased capability for discharging $\Delta\psi$ by means of an increased inward flow of protons. Conversely, when GDP-binding capacity is low, as in mitochondria from *ob/ob* mice maintained at 22°C, the ability to decrease $\Delta\psi$ is limited and the mitochondria remain more tightly coupled when GDP is absent.

In summary, it is concluded that brown-adipose-tissue mitochondria from obese and pre-obese *ob/ob* mice are less able than these mitochondria from lean mice to decrease $\Delta\psi$ in the absence of GDP. Because the value of $\Delta\psi$ is in these experiments affected by changes in the pH gradient and indicates changing values of $\Delta\bar{\mu}_{H^+}$, the deduction can also be made that the mitochondria from *ob/ob* mice are less able than those from lean mice to dissipate $\Delta\bar{\mu}_{H^+}$. Although regulation of the proton-conductance pathway was here demonstrated with GDP *in vitro*, this purine nucleotide need not be the actual physiological regulating agent *in vivo*. Similarly, although the brown-adipose-tissue mitochondria from *ob/ob* mice have this functional impairment, which is well correlated with an impaired ability for non-shivering thermogenesis *in vivo*, this need not be the primary genetic defect in these animals. Factors regulating the number of proton-conducting units in the mitochondrial membrane are possibly more directly linked to the mutant gene.

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