Measurement by radioimmunoassay of the mitochondrial uncoupling protein from brown adipose tissue of obese (ob/ob) mice and Zucker (fa/fa) rats at different ages

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The concentration of the 'uncoupling protein' in brown adipose tissue mitochondria has been measured in lean and obese (ob/ob) mice and Zucker (fa/fa) rats at different ages using a specific radioimmunoassay. During the suckling period the concentration of the protein was similar in normal and mutant animals of both types, despite the decrease in mitochondrial GDP binding observed in the obese. The concentration of uncoupling protein was, however, decreased in adult ob/ob mice and adult Zucker rats compared with their respective lean siblings, in parallel with the decrease in GDP binding. It is concluded that there is a 'masked', or inactive, form of uncoupling protein in young ob/ob mice and fa/fa rats.

Brown adipose tissue *Uncoupling protein* *Obese mouse* *Obese rat* *GDP binding*

1. INTRODUCTION

The thermogenic function of brown adipose tissue (BAT) mitochondria is related to the presence of a 32-kDa 'uncoupling' protein in the inner mitochondrial membrane, which allows the dissipation of the proton gradient to be uncoupled from the synthesis of ATP via a proton conductance pathway [1,2]. The activity of this pathway has been measured principally by the specific binding of purine nucleotide ligands to the uncoupling protein [2]. However, the recent development of immunoassays [3,4] has allowed the uncoupling protein content of mitochondria to be quantitated directly. In rodents, BAT is thought to be the major site of both cold-induced and diet-induced thermogenesis [5–8]. These forms of thermogenesis involve tissue hypertrophy, mitochondrial proliferation and an increase in the mitochondrial concentration of the uncoupling protein [2,9–11].

The obesity of both the genetically obese (ob/ob) mouse and the Zucker (fa/fa) rat, which will develop in the absence of hyperphagia, is currently considered to result principally from a decrease in thermogenesis in BAT (review [12]). To date, studies on BAT in these mutants have mainly used the specific binding of [^{3}H]GDP to mitochondria to assess thermogenic activity, although in the case of the ob/ob mouse mitochondrial respiration, membrane potential and Ca^{2+} transport have also been measured [13,14]. Binding studies employing Scatchard analysis have indicated that the number of GDP binding sites in BAT mitochondria of ob/ob mice and Zucker rats fed ad libitum is decreased at room temperature [15,16]. Such studies have also indicated that the number of binding sites is decreased during the suckling period, and well before the development of overt obesity [13,17]. A decrease in the number

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of binding sites would suggest that the obese mutants have a decreased concentration of uncoupling protein, although a 'masked' form of the protein has been suggested from studies where acute changes in GDP binding have been demonstrated [18,19].

Preliminary results have indicated that the concentration of uncoupling protein is decreased in BAT mitochondria of adult ob/ob mice [20], but we show here in a study on both the ob/ob mutant and the Zucker fa/fa rat that this is a secondary change. In very young obese mutants the concentration of uncoupling protein is normal, despite the decreased level of GDP binding.

2. MATERIALS AND METHODS

2.1. *Animals*

Male lean (ob/+ or +/+) and obese (ob/ob) mice were obtained from a colony with the 'ob' gene on the 'Aston' background [13]. Litters were weaned at 21 days of age onto a commercial low fat/high carbohydrate diet (LAD-1, K and K Greef Chemicals, Croydon, England) which was fed ad libitum. The animals were housed in plastic cages in a room at 22 ± 2°C with a 12 h light/12 h dark cycle (light period from 07.00 h). Lean and obese mice were taken at 14 days, 26 days (just before development of hyperphagia) and 10 weeks of age. At the two older ages obese mice were identified visually, but the youngest animals were identified at 13 days of age by a cold stress test [13] and returned to the nest for use the following day.

Zucker (fa/fa) rats were bred from a colony maintained at the University of Southampton. Litters were weaned at 21 days of age, and fed a commercial low fat/high carbohydrate diet (Purina rat chow, Christopher Hill, Poole, England). Rats were housed in wire mesh cages in a room at 23 ± 2°C with a 14 h light/10 h dark cycle (light period from 06.00 h). Obese Zucker (fa/fa) rats and their lean (fa/+ or +/+) siblings were taken at 10 days, 21 days (immediately prior to weaning), 5 weeks and 12 weeks of age. Obese animals were identified at the two younger ages by their decreased rectal temperature together with their increase in inguinal fat pad weight [21].

2.2. *GDP binding assay*

Mitochondria were prepared from interscapular BAT as in [13,16]. GDP binding was measured by incubating the freshly prepared mitochondria at room temperature with 10 μM [^{3}H]GDP as described for ob/ob mice [13] and Zucker rats [16]. [^{3}H]GDP and [^{14}C]sucrose were obtained from Amersham International (Amersham, England).

The protein content of rat mitochondria was assayed as in [22], while a modified Lowry assay was used with the mouse mitochondria [23].

2.3. *Radioimmunoassay of uncoupling protein*

The concentration of uncoupling protein in mitochondrial preparations was determined by solid-phase radioimmunoassay [3]. Mouse protein standards were used with mitochondria from mice, and rat standards with rat mitochondria. Protein standards were prepared from BAT of cold-acclimated (4°C for 3 weeks) animals as in [24,25].

The statistical significance of differences between groups was assessed by Student's t-test.

3. RESULTS

3.1. *ob/ob mice*

There was no difference in body weight between lean and ob/ob mice at either 14 or 26 days of age, but at 10 weeks of age the obese weighed more than twice as much as the lean (table 1). At all 3 ages the amount of interscapular BAT in the obese mice was greater than that in the lean. Previous reports on ob/ob mice on the Aston background have, however, suggested that there is no hypertrophy of the tissue at 14 days of age [13,26]. Mitochondrial GDP binding was significantly lower in the ob/ob animals than in the lean at each of the ages studied, and this is in agreement with previous work [13,27]. The GDP binding values were highest for both groups of mice during the suckling period.

There was no significant difference between ob/ob and normal mice in the concentration of uncoupling protein in mitochondria at either 14 or 26 days of age (table 1). Only in the 10-week-old animals was a difference observed, the concentration in the obese being half that of the lean. Although the level of GDP binding fell with age there was no clear effect of age on the concentration of uncoupling protein.

Table 1

Concentration of uncoupling protein in brown adipose tissue mitochondria of lean and obese (ob/ob) mice at different ages

	14 days		26 days		10 weeks	
	Lean	Obese	Lean	Obese	Lean	Obese
Body wt (g)	8.8± 0.2 (10)	9.1± 0.3 (10)	17.2± 0.5 (11)	18.2± 0.8 (11)	32.3± 0.9 (9)	75.1± 3.0[c] (9)
Interscapular brown adipose tissue wt (mg)	76.9± 4.0 (10)	100.6± 9.9[a] (10)	137.6±13.1 (11)	377.0±23.6[c] (11)	159.3±11.0 (9)	672.6±55.3[c] (9)
GDP binding (pmol/mg mitochondrial protein)	572 ±49 (8)	292 ±38[b] (8)	367 ±36 (7)	251 ±20[b] (7)	258 ±26 (9)	173 ±25[a] (9)
Uncoupling protein (μg/mg mitochondrial protein)	29.7± 2.1 (10)	28.2± 2.4 (10)	37.6± 2.7 (11)	41.6± 3.2 (11)	38.8± 3.5 (9)	20.1± 2.4[c] (9)

Brown adipose tissue mitochondria were prepared from lean and obese mice at different ages and assayed as described in the text. The results are given as mean values ± SE with the number of animals shown in parentheses. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ compared with lean mice

3.2. *Zucker rats*

Table 2 gives the results obtained at different ages for the Zucker rat, and these showed a generally similar pattern to the ob/ob mouse. Differences between normal and fa/fa rats in body weight and the amount of interscapular BAT were not observed at either 10 days or 21 days of age, but at 5 and 12 weeks both parameters were considerably elevated in the obese. The absence of any significant increase in the weight of BAT in the fa/fa rat during the suckling period is in contrast to the results reported for Zucker rats in [17].

At each age the specific binding of GDP to brown adipose tissue mitochondria was significantly lower in the fa/fa rats than in the normal animals (table 2), the difference between genotypes being greatest for the adults, confirming previous observations [21]. As with the results on the ob/ob mouse there was no significant difference between normal and fa/fa rats in the concentration of uncoupling protein during the suckling period, immediately prior to weaning (21 days), or in the young (5 weeks) rats. However, the concentration of the protein was significantly

Table 2

Concentration of uncoupling protein in brown adipose tissue mitochondria of lean and obese Zucker (fa/fa) rats at different ages

	10 days		21 days		5 weeks		12 weeks	
	Lean	Obese	Lean	Obese	Lean	Obese	Lean	Obese
Body wt (g)	22.0±2.6 (6)	25.0±2.0 (6)	56.2±4.0 (6)	62.4±5.0 (6)	118.0±7.0 (6)	140±19.0 (6)	240±10.2 (5)	320±15.2[c] (5)
Interscapular brown adipose tissue wt (g)	0.19±0.01 (6)	0.18±0.02 (6)	0.20±0.02 (6)	0.25±0.02 (6)	0.32±0.02 (6)	0.50±0.02[c] (6)	0.37±0.03 (5)	2.76±0.10[c] (5)
GDP binding (pmol/mg mitochondrial protein)	282±14 (6)	210±9[b] (6)	264±12 (6)	186±10[c] (6)	230±15 (6)	146±15[b] (6)	220±14 (5)	120±11[c] (5)
Uncoupling protein (μg/mg mitochondrial protein)	11.0±0.7 (6)	10.7±0.5 (6)	11.9±0.3 (5)	9.7±0.9 (6)	8.0±0.4 (5)	8.6±1.2 (5)	13.9±1.7 (5)	8.1±0.6[a] (5)

Brown adipose tissue mitochondria were prepared from lean and obese Zucker rats at different ages and assayed as described in the text. The results are given as mean values ± SE with the number of animals shown in parentheses. [a] $p < 0.05$; [b] $p < 0.01$; [c] $p < 0.001$ compared with lean rats

lower in the obese than the lean at 12 weeks of age. The amount of uncoupling protein was not noticeably altered by age, but the GDP binding values fell slightly with increasing age.

3.3. *Effect of cold acclimation on ob/ob mice and Zucker rats*

When 4-week-old Zucker rats were cold-acclimated (4 ± 1°C) for 1 week they showed a normal increase in both GDP binding and in the concentration of uncoupling protein. The binding values were 530 ± 95 and 585 ± 110 pmol GDP/mg mitochondrial protein for lean and obese rats, respectively; the concentration of uncoupling protein was 53 ± 2 μg/mg mitochondrial protein in the lean and 57 ± 2 μg/mg mitochondrial protein in the obese fa/fa rats (mean values ± SE, $n = 2$). These results indicate that there is not an immunologically reactive but functionally abnormal form of uncoupling protein in the Zucker rat.

Adult obese mice (aged 7 weeks) also responded to moderate cold (13 ± 1°C) with a marked increase in the amount of uncoupling protein. After 2 weeks at 13°C the ob/ob mice had a concentration of uncoupling protein of 119 ± 20 μg/mg mitochondrial protein (mean values ± SE, $n = 5$), which is even higher than that reported previously for lean mice at 4°C [9].

4. DISCUSSION

Our results demonstrate that in both the ob/ob mouse and the Zucker fa/fa rat there is a normal concentration of uncoupling protein in BAT mitochondria during the suckling period and after weaning. Both mutants also show the normal response to cold acclimation of parallel increases in mitochondrial GDP binding and in the concentration of the uncoupling protein. The normal capacity of young obese Zucker rats for non-shivering thermogenesis has been demonstrated [28,29]. Similarly, ob/ob mice are able to respond to mild but not severe cold environments [30]. Only in adult obese mutants is the concentration of the uncoupling protein lower than in lean siblings. Since the thermogenic activity of BAT is impaired from an early age in the obese mutants [13,17,21] despite the presence of normal amounts of the uncoupling protein, these results suggest that the lack of protein is not a primary expression of the gene defect but is secondary to the obese state and its associated metabolic and endocrine changes [31].

In the adult animals the decrease in uncoupling protein is paralleled by the decrease in the level of GDP binding, but in the young mutants there is reduced GDP binding in spite of the presence of a normal concentration of the protein. There is therefore a dissociation in young ob/ob mice and Zucker rats between the level of binding and the amount of protein, which is similar to that which appears to occur in normal animals on acute cold exposure or following noradrenaline infusion [18,19]. A masked, or inactive, form of uncoupling protein has been postulated to exist [18,19] and our results are consistent with this idea. This concept is further supported by the demonstration that mitochondrial GDP binding of both lean and obese rats is elevated to the same level by noradrenaline administration, the response being maximal within 40 min [21,32].

Although Scatchard analysis of GDP binding data was not performed here, previous work on suckling ob/ob mice [13] and young Zucker rats [16,17] has shown that the decreased level of GDP binding in both mutants is due to a reduction in the number of binding sites and not to any change in affinity. In addition, studies on mitochondrial respiration, membrane potential and Ca^{2+} transport have all indicated that the activity of the proton conductance pathway is lower in suckling ob/ob mice than in lean animals [13,14]. Thus it is unlikely that the decrease in GDP binding observed in young obese mutants is anything other than a reflection of a reduction in mitochondrial proton conductance. GDP binding is therefore more an index of the activity of the proton conductance pathway than a measure of the amount of uncoupling protein per se, although clearly the amount of the protein must set the upper limit to the amount of GDP that can be specifically bound.

The principal reason for the decreased thermogenesis in BAT of Zucker fa/fa rats and ob/ob mice is considered to be a low activity of the sympathetic innervation to the tissue [33–37]. Our results indicate that in the young obese mutants the amount of uncoupling protein is not the limiting factor for BAT thermogenesis. However, under stimulation from the sympathetic nervous system may well be the reason for both this initial 'masking' of GDP binding sites and for the secondary

decline in the concentration of uncoupling protein with age. This hypothesis is supported by the recent demonstration that chronic noradrenaline infusions will initiate all the changes in BAT that are normally associated with cold acclimation and dietary-induced thermogenesis, i.e., it stimulates tissue growth, mitochondrial proliferation, and mitochondrial GDP binding and it increases the concentration of the uncoupling protein [38].

The reduction in uncoupling protein with age occurs despite the hyperphagia which develops in both the ob/ob mouse and fa/fa rat after weaning. Thus neither mutant exhibits the increase in concentration of uncoupling protein observed after overfeeding with a cafeteria diet [10,11]. Finally, it should be noted that the decrease in the concentration of uncoupling protein in mitochondria in the older obese mutants does not give a true reflection of the absolute reduction in thermogenic capacity since marker enzyme studies have shown that total mitochondrial content of BAT is also reduced in these mutants at this age [15,21], whereas mitochondrial content as well as the concentration of uncoupling protein are normal in the suckling and young obese mutants [13,16,17].

REFERENCES

[1] Nicholls, D.G. (1979) Biochim. Biophys. Acta 549, 1–29.

[2] Nicholls, D.G. and Locke, R.M. (1984) Physiol. Rev. 64, 1–64.

[3] Lean, M.E.J., Branch, W.J., James, W.P.T., Jennings, G. and Ashwell, M. (1983) Biosci. Rep. 3, 61–71.

[4] Cannon, B., Hedin, A. and Nedergaard, J. (1982) FEBS Lett. 150, 129–132.

[5] Foster, D.O. and Frydman, M.L. (1978) Can. J. Physiol. Pharmacol. 56, 110–122.

[6] Foster, D.O. and Frydman, M.L. (1979) Can. J. Physiol. Pharmacol. 57, 257–270.

[7] Thurlby, P.L. and Trayhurn, P. (1980) Pflügers Arch. 385, 193–201.

[8] Rothwell, N.J. and Stock, M.J. (1981) Pflügers Arch. 389, 237–242.

[9] Ashwell, M., Jennings, G., Richard, D., Stirling, D.M. and Trayhurn, P. (1983) FEBS Lett. 161, 108–112.

[10] Ashwell, M., Rothwell, N.J., Stock, M.J., Stirling, D. and Winter, P. (1984) Proc. Nutr. Soc. 43, in press.

[11] Nedergaard, J., Raasmaja, A. and Cannon, B. (1984) Biochem. Biophys. Res. Commun. 122, 1328–1336.

[12] Trayhurn, P. (1984) Clin. Endocrinol. Metab. 13, 451–474.

[13] Goodbody, A.E. and Trayhurn, P. (1982) Biochim. Biophys. Acta 680, 119–126.

[14] Trayhurn, P. and Fraser, D.R. (1983) Biochem. J. 214, 171–175.

[15] Goodbody, A.E. (1982) PhD Thesis, University of Cambridge.

[16] Holt, S. and York, D.A. (1982) Biochem. J. 208, 819–822.

[17] Bazin, R., Eteve, D. and Lavau, M. (1984) Biochem. J. 221, 241–245.

[18] Desautels, M., Zaror-Behrens, G. and Himms-Hagen, J. (1978) Can. J. Biochem. 56, 378–383.

[19] Desautels, M. and Himms-Hagen, J. (1979) Can. J. Biochem. 57, 968–976.

[20] Ashwell, M., Jennings, G. and Trayhurn, P. (1983) Biochem. Soc. Trans. 11, 727–728.

[21] York, D.A., Holt, S., Rothwell, N.J. and Stock, M.J. (1984) Am. J. Physiol. 246, E391–E396.

[22] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 267–275.

[23] Schacterle, G.R. and Pollack, R.L. (1973) Anal. Biochem. 51, 654–655.

[24] Lin, C.S. and Klingenberg, M. (1980) FEBS Lett. 113, 299–303.

[25] Lin, C.S. and Klingenberg, M. (1982) Biochemistry 21, 2950–2956.

[26] Mercer, S.W. and Trayhurn, P. (1983) Biochem. J. 212, 393–398.

[27] Himms-Hagen, J. and Desautels, M. (1978) Biochem. Biophys. Res. Commun. 83, 628–634.

[28] Holt, S., York, D.A. and Fitzsimmons, J.T. (1983) Biochem. J. 214, 215–223.

[29] Triandafillou, J. and Himms-Hagen, J. (1983) Am. J. Physiol. 244, E145–E150.

[30] Trayhurn, P. and James, W.P.T. (1978) Pflügers Arch. 373, 189–193.

[31] Bray, G.A. and York, D.A. (1979) Physiol. Rev. 59, 719–809.

[32] York, D.A., Holt, S.J. and Marchington, D. (1985) Int. J. Obesity, in press.

[33] Levin, B., Triscari, J. and Sullivan, A.C. (1983) Am. J. Physiol. 245, E87–E93.

[34] Levin, B., Triscari, J. and Sullivan, A.C. (1983) Am. J. Physiol. 245, R367–R371.

[35] Holt, S., Marchington, D. and York, D.A. (1984) Proc. Nutr. Soc. 43, 69A.

[36] Knehans, A.W. and Romsos, D.R. (1982) Am. J. Physiol. 242, E253–E261.

[37] Zaror-Behrens, G. and Himms-Hagen, J. (1983) Am. J. Physiol. 244, E361–E366.

[38] Mory, G., Bouillaud, F., Combes-George, M. and Ricquier, D. (1984) FEBS Lett. 166, 393–396.