Effects of hypothyroidism and hyperthyroidism on GDP binding to brown-adipocyte mitochondria from rats

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1. Rats were made hypothyroid by giving them a low-iodine diet with propylthiouracil for 4 weeks, or were made hyperthyroid by injection with tri-iodothyronine (T_3) over a 3-day period. 2. Brown adipocytes were isolated from the interscapular depots of these animals or from their euthyroid controls, followed by isolation of mitochondria from the cells. 3. Relative to cell DNA content, hypothyroidism decreased the maximum binding (B_{max}) of [3H]GDP to mitochondria by 50%. T_3 treatment increased binding by 37%. 4. These findings, which are discussed in relation to previously observed changes in brown adipose tissue after alteration of thyroid status, suggest that mitochondrial uncoupling for thermogenesis is less or more effective in hypothyroidism or hyperthyroidism respectively.

INTRODUCTION

The thermogenic response of brown adipose tissue is impaired in several physiological states. These include starvation (Rothwell *et al.*, 1984), diabetes (Rothwell & Stock, 1981; Seydoux *et al.*, 1983), lactation (Trayhurn *et al.*, 1982) and hypothyroidism (Mory *et al.*, 1981; Seydoux *et al.*, 1982). At the cellular and subcellular levels various changes have been noted which could contribute to this impairment of thermogenic function. Associated with starvation and lactation there is decreased function and/or abundance of the mitochondrial uncoupling protein 'thermogenin' (Rothwell *et al.*, 1983, 1984; Villarroya *et al.*, 1986; Trayhurn & Jennings, 1987). Brown adipocytes from diabetic rats exhibit decreased maximum rates of noradrenaline-stimulated O_2 uptake. This is accompanied by decreased GDP binding to mitochondria isolated from these cells (Jamal & Saggerson, 1988a), decreased mitochondrial respiration of various substrates [in particular fatty acids (Jamal & Saggerson, 1988b)] and decreased activity of overt carnitine palmitoyltransferase (Jamal & Saggerson, 1988b; Gualberto & Saggerson, 1989), an enzyme thought to be important in the regulation of fatty acid oxidation. Activation of lipolysis by noradrenaline, however, is not impaired in diabetes (Jamal & Saggerson, 1988a). Therefore in this state the thermogenic process at the level of the isolated adipocyte does not appear to be limited by supply of fatty acid substrate, but may be limited by access of this substrate to the mitochondria and by the availability of thermogenin.

In hypothyroidism also, the maximum rate of noradrenaline-stimulated O_2 uptake by isolated brown adipocytes is decreased. However, in this state this change is also accompanied by a decrease in the sensitivity to and maximum response of lipolysis to noradrenaline (Sundin *et al.*, 1984; Woodward & Saggerson, 1986). Also by contrast with the diabetic state, hypothyroidism does not result in any decrease in the activity of overt carnitine palmitoyltransferase in brown-adipose-tissue mitochondria (Saggerson & Carpenter, 1986). Drawing

parallels with starvation, lactation or diabetes, one might expect a decrease in the abundance and/or function of thermogenin in the hypothyroid state, and possibly the opposite in hyperthyroidism. Surprisingly, most previous studies of GDP binding to mitochondria isolated from whole brown fat do not support this expectation. Thyroidectomy or methimazole-induced hypothyroidism had no effect, or slightly increased GDP binding (Mory *et al.*, 1981; Triandafillou *et al.*, 1982), whereas chronic thyroxine treatment decreased this parameter (Sundin, 1981; Triandafillou *et al.*, 1982). It was only in the study of Bianco & Silva (1987), using an immunoassay procedure, that a decrease in thermogenin abundance was noted after thyroidectomy.

These findings raised the question of the cause of the decreased noradrenaline activation of O_2 uptake in hypothyroidism. It might be inferred that this is solely due to diminished fatty acid supply from lipolysis and does not result from any changes at the mitochondrial level. However, Sundin *et al.* (1984) demonstrated that the ability of fatty acids to stimulate respiration by brown adipocytes is decreased in hypothyroidism and increased in hyperthyroidism. In view of this, we decided to re-investigate the effect of these two states on GDP binding to mitochondria that were isolated solely from the adipocyte component of brown adipose tissue.

MATERIALS AND METHODS

Chemicals

These were obtained and treated as described by Woodward & Saggerson (1986) and Jamal & Saggerson (1988a).

Animals

These were male Sprague–Dawley rats bred in the animal colony at University College London. The rats were kept at 21 °C on a light/dark cycle of 13 h/11 h, with light from 06:00 to 19:00 h. They were fed *ad libitum* with Rat & Mouse No. 3 Breeding Diet (Special

Abbreviations used: T_3 , tri-iodothyronine; B_{max} , maximum binding at a given site.

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Diet Services, Witham, Essex, U.K.), which contained (by wt.) 21% digestible crude protein, 4% digestible crude oil and 39% starches and sugars. Hypothyroidism was induced by giving a low-iodine version of the Rat & Mouse No. 3 diet together with 0.01% (w/v) 6-n-propyl-2-thiouracil in the drinking water for 4 weeks as described by Chohan *et al.* (1984) and Woodward & Saggerson (1986). Hypothyroid animals and their euthyroid controls were used when aged 8 weeks. For studies on animals with elevated thyroid-hormone levels, animals were selected at 6 weeks of age (160–180 g body wt.) and injected subcutaneously (1 mg/kg body wt.) with T_3 dissolved in 10 mM-NaOH/0.03% (w/v) bovine serum albumin (Sugden *et al.*, 1983). T_3 injection was repeated 24 h and 48 h after the initial injection, and the rats were then killed 24 h after this final administration. Euthyroid controls for these animals were also selected at 160–180 g body wt. and were injected with 10 mM-NaOH/0.03% albumin, the same injection protocol being used as for the T_3 -treated rats. During the 3-day treatment period T_3 -treated rats showed negligible weight loss or gain, whereas their controls increased in weight by 10–15%.

Isolation of brown-adipocyte mitochondria

Brown adipocytes were isolated from the interscapular depots of several rats. This procedure, originally described by Fain *et al.* (1967) and elaborated by Nedergaard & Lindberg (1982), was as described by Woodward & Saggerson (1986). The freshly prepared cells were then homogenized in ice-cold 0.25 M-sucrose medium containing 10 mM-Tris/HCl (pH 7.4)/1 mM-EGTA/1 mM-dithiothreitol/fatty acid-poor albumin (1%, w/v) in a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle (six up-and-down strokes at 500 rev./min with a radial clearance of 0.19 mm). The centrifugation procedure to obtain mitochondria was identical with that used to obtain mitochondria from whole interscapular brown fat (Saggerson & Carpenter, 1982). The final mitochondrial pellet was resuspended in ice-cold 0.3 M-sucrose medium containing 10 mM-Tris/HCl (pH 7.4)/1 mM-EGTA. The protein concen-

tration was rapidly determined (Bradford, 1976), and most of the preparation was then used immediately for measurement of GDP binding. Other portions of the initial homogenate and of the final mitochondrial suspension were stored at -20°C before subsequent determinations (as appropriate) of DNA (Switzer & Summer, 1971), protein (Lowry *et al.*, 1951) and citrate synthase (EC 4.1.3.7) (measured in the presence of 0.1% Triton X-100) as described by Srere *et al.* (1963).

Specific binding of GDP to brown-adipocyte mitochondria

Total binding was measured by using [^3H]GDP and corrected by subtraction of the [^{14}C]sucrose space (Rial & Nicholls, 1983; Jamal & Saggerson, 1988a). 'Non-specific binding' was similarly measured at the same time in the presence of 1.0 mM unlabelled GDP and was subtracted from the total binding to give values for 'specific binding'.

Expression of results and statistical methods

Citrate synthase was assayed both in the initial homogenates and in the final mitochondrial preparations. This activity was then used as a reference enzyme to allow expression of the GDP binding relative to the DNA content measured in the initial homogenates (Jamal & Saggerson, 1988a). Values of n refer to numbers of separate mitochondrial preparations (each from the pooled brown adipose tissues of several rats). Results are shown as means $\pm$ S.E.M. Where S.E.M. values are not shown in Figures, these lie within the symbol. GDP-binding data were analysed by a non-linear weighted least-squares curve-fitting procedure, by using a search-type minimization (Colquhoun, 1971).

RESULTS AND DISCUSSION

[^3H]GDP binding to brown-adipocyte mitochondria could be satisfactorily fitted to a single site model. Fig. 1 and Table 1 show that hypothyroidism significantly decreased the B_{max} of this binding by approx. 50%

Fig. 1. Binding of [^3H]GDP to brown-adipocyte mitochondria from hypothyroid (■) and euthyroid (□) rats

Values of n are shown in Table 1 together with estimates of K_D and $B_{\text{max}} \pm \text{S.E.M.}$, which were computed from the values shown. The lines in the Scatchard plots are simply those that fit these computer-estimated parameters.

Table 1. Calculated parameters for binding of [³H]GDP to brown-adipocyte mitochondria

These were derived from the experiments shown in Figs. 1 and 2. ^a*P* < 0.02, ^b*P* < 0.01, ^c*P* < 0.001 versus the appropriate euthyroid control.

Fig.	Condition	<i>n</i>	GDP binding to mitochondria (<i>B</i> _{max.})		
			(pmol/mg of mitochondrial protein)	(pmol/100 μg of cell DNA)	<i>K</i> _D (μM)
1	Euthyroid (8 weeks)	7	588 ± 71	2013 ± 69	1.4 ± 0.1
	Hypothyroid	7	296 ± 24 ^b	1038 ± 32 ^c	2.1 ± 0.2 ^a
2	Euthyroid (6 weeks)	3	668 ± 43	1452 ± 107	2.4 ± 0.4
	Hyperthyroid	3	740 ± 33	1994 ± 71 ^a	1.7 ± 0.1

Fig. 2. Binding of [³H]GDP to brown-adipocyte mitochondria from hyperthyroid (●) and euthyroid (○) rats

Values of *n*, *K*_D and *B*_{max.} are given in Table 1.

irrespective of whether this was related to mitochondrial protein or to cell DNA. This decrease correlates extremely well with the 50% decrease in the maximum rate of noradrenaline-stimulated O₂ uptake observed by Woodward & Saggerson (1986) using similarly treated rats from the same animal colony. In the same study Woodward & Saggerson (1986) observed a 57% decrease in the maximum rate of lipolysis. Table 1 shows that hypothyroidism also caused a small, but significant, increase in the *K*_D for GDP. In general this value has not been found by others to be affected by changes in physiological state, and we do not understand the reason for this change.

Treatment with T₃ for 3 days, by contrast, significantly increased the *B*_{max.} for [³H]GDP binding relative to cell DNA by 37% (Fig. 2 and Table 1). This value was not, however, significantly increased relative to mitochondrial protein content, because the treatment with T₃ also increased the latter by 25% relative to cell DNA. It was noted that the 6- and 8-week-old euthyroid samples differed in that the cells from the younger rats had a lower *B*_{max.} (*P* < 0.01) and a higher *K*_D (*P* < 0.05) for

GDP binding. It is not known whether these changes reflect the age difference or the fact that the experiment shown in Fig. 2 was performed slightly later in the year than that shown in Fig. 1. It must be stressed that the hypothyroid and the hyperthyroid samples were prepared and measured at the same times as their appropriate controls.

In many cases a change in the *B*_{max.} for GDP binding is taken as indicating a change in the abundance of thermogenin (see, e.g., Nedergaard *et al.*, 1984; Nedergaard & Cannon, 1985; Sundin *et al.*, 1987). In other instances, however, the extent of GDP binding can be dissociated from the thermogenin content of brown-fat mitochondria (Desautels *et al.*, 1978; Gribskov *et al.*, 1986; Trayhurn *et al.*, 1987; Trayhurn & Jennings, 1987; Milner *et al.* 1988), particularly during acute changes in physiological state. In the present study we cannot exclude the possibility that some of the increase in GDP binding seen after only 3 days of T₃ treatment could represent an 'unmasking' phenomenon. However, since the extent of GDP binding has been shown to parallel the proton conductance of the mitochondria (Rial &

Fig. 3. Relationship between brown-adipocyte respiration and lipolysis in the euthyroid (□) and hypothyroid (■) states

The values, taken from Figs. 5(a) and 5(b) of Woodward & Saggerson (1986), are means of 4–5 separate experiments. These represent measurements made when cells were incubated with 5 mM-glucose, albumin (40 mg/ml), adenosine deaminase (1 unit/ml) and various concentrations of noradrenaline.

Nicholls, 1984), we feel confident in predicting decreased and increased capacity for mitochondrial proton flux in hypothyroidism and hyperthyroidism respectively.

It is unclear why our findings are at variance with those of others (Sundin, 1981; Mory *et al.*, 1981; Triandafillou *et al.*, 1982), i.e. whether this could relate to the treatments of the animals or to the analytical method employed. Mory *et al.* (1981) noted a > 20-fold decrease in plasma thyroxine concentration after thyroidectomy. Unfortunately Triandafillou *et al.* (1982) did not provide any values for thyroid-hormone concentrations. In our hands induction of hypothyroidism using propylthiouracil decreased plasma T_3 concentration from 1.06 nM to 0.17 nM and decreased thyroxine to undetectable levels (Chohan *et al.*, 1984). Very similar changes in plasma T_3 concentrations in hypothyroidism were reported by Bianco & Silva (1987). It may also be relevant that Sundin (1981) and Triandafillou *et al.* (1982) induced hyperthyroidism by treatment with thyroxine over 3 or 1 weeks respectively, whereas we treated with T_3 over only 3 days. With regard to analytical methods, previous measurements of GDP binding were made with mitochondria isolated from the whole heterogeneous mass of the brown adipose tissue (Sundin, 1981; Mory *et al.*, 1981; Triandafillou *et al.*, 1982). It would seem advisable, if possible, to only use mitochondria obtained from the adipocyte population. In addition, in previous studies, GDP binding was measured either under undefined conditions by equilibrium dialysis (Mory *et al.*, 1981) or only at one concentration of GDP (10 μM) (Sundin,

1981; Triandafillou *et al.*, 1982) without correction for the low-affinity 'non-specific' binding which is still apparent after correction for the sucrose space (Rial & Nicholls, 1983). A full binding analysis with appropriate correction for low-affinity binding is obviously desirable.

Finally, we wish to elaborate on our interpretation of previously published findings. Jamal & Saggerson (1988a) estimated that, in normal brown adipocytes stimulated with sub-maximally effective concentrations of noradrenaline, virtually all of the fatty acid released by lipolysis is likely to become oxidized. This is based on the estimation that 3 μmol of fatty acid/h (equivalent to 1 μmol of glycerol released/h) will support an increase in respiration of 1.15 μmol of O_2 /min (69 μmol of O_2 /h). In diabetes the relationship between O_2 uptake and lipolysis was changed such that only approx. 10% of the available fatty acid appeared to be oxidized (see Fig. 2 of Jamal & Saggerson, 1988a). Fig. 3 shows a similar treatment of data from Woodward & Saggerson (1986); the relationship between respiration and lipolysis is also changed in hypothyroidism, such that only 26% of the fatty acid liberated by lipolysis is oxidized at any of the observed rates of lipolysis ($r = 0.99$ for correlation of the two processes in the hypothyroid state). We can now suggest that this may, at least in part, be because of a decrease in thermogenin function and/or abundance. It is also pertinent that hypothyroidism approximately doubles the capability of brown adipocytes to esterify fatty acids (Baht & Saggerson, 1988). This may lead to the diversion of some of the lipolysis product toward re-esterification rather than oxidation.

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