Evidence for decreased GDP binding to brown-adipose-tissue mitochondria of obese Zucker (*fa/fa*) rats in the very first days of life

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GDP binding to brown-adipose-tissue mitochondria of obese Zucker-rat (*fa/fa*) pups aged 2–14 days was significantly less than in lean control rats. Scatchard analysis in 10-day-old pups suggests that there was a large decrease in GDP-binding sites. However, a significant increase in fat content in brown adipose tissue of 2-day-old pre-obese pups raised the question of the sequential order and causal relationship between these two derangements.

A decreased energy expenditure has been well documented in obese Zucker rats; lower rates of oxygen consumption, demonstrated first in obese adults (Bray, 1969), have also been found in the mutants by 3 weeks of age (Kaplan, 1979), and even by 7 days of age, as reported by Planche *et al.* (1983). There is strong evidence to suggest that the decrease in energy expenditure could be achieved through an impaired thermoregulatory thermogenesis. Thus Zucker rats exhibit subnormal thermogenic responses to both cold (York *et al.*, 1972; Trayhurn *et al.*, 1976) and diet (Rothwell *et al.*, 1981). Moreover, rectal (Godbole *et al.*, 1978) and core temperatures (Planche *et al.*, 1983) have been shown to be lower in obese pups than in their lean littermates within the first weeks of life, strongly suggesting that impaired thermogenesis could play a primary role in the onset of this genetic obesity.

Brown adipose tissue is of major importance in the regulation of body temperature. It is responsible for the increase in heat production in both non-shivering thermogenesis after cold exposure (Foster & Frydman, 1978) and diet-induced thermogenesis (Rothwell *et al.*, 1981). The primary mechanism responsible for the thermogenesis in brown fat appears to be the mitochondrial proton-conductance pathway described by Nicholls (1976), which translocates protons across the mitochondrial inner membrane without coupling to ATP synthesis. This pathway, which is inhibited by purine nucleotides binding to a specific M_r 32000 protein, can be monitored by the specific binding of [3 H]GDP to brown-adipose-tissue mitochondria.

In two relevant models of genetic obesity, the *db/db* and *ob/ob* mice, a decrease in brown-adipose-

tissue thermogenic activity, as assessed by GDP binding, was observed by 2 weeks of age, before obesity has begun to develop (Goodbody & Trayhurn, 1981, 1982). In Zucker rats, a decreased specific GDP binding to brown-adipose-tissue mitochondria has also been demonstrated in both mature (Triandafilou & Himms-Hagen, 1983) and young (6 weeks old) animals (Holt & York, 1982). However, at this age considerable body-fat accretion, contributed to by the very high lipogenic activity present in liver and in white and brown adipose tissues (Bazin & Lavau, 1982; Lavau *et al.*, 1982), has already taken place in obese rats, decreasing heat losses and thereby the need for thermogenesis. Therefore the aim of the present work was to investigate whether an impairment of the mitochondrial proton-conductance pathway could be found in Zucker *fa/fa* rats early enough in life to be ascribed a primary role in the development of this obesity.

Materials and methods

Zucker rats are bred in our laboratory from pairs originally provided by the Harriet G. Bird Memorial Laboratory, Stowe, MA, U.S.A. The animals used in this study were obtained from obese (*fa/fa*) males and heterozygous lean (*Fa/fa*) females. Pups and their mothers were housed in a temperature-controlled room (22–23°C) on a fixed 12h-light (07:00–19:00h)/12h-dark cycle. The dams were fed on stock diet (UAR, 91360 Epinau sur Orge, France) *ad libitum*. The pups were used at 2, 5, 7, 10 and 14 days of age. Interscapular brown-adipose-tissue samples (30–80mg) were surgically removed under light diethyl ether anaesthesia, so that the pups could be kept alive for later (7 weeks of age)

genotype diagnosis. Care was taken to maintain the pups at 28–30°C during the surgical procedure. Interscapular brown-adipose-tissue biopsies were homogenized at 0°C in Tris (10 mM)/EDTA (2 mM)/sucrose (0.25 M) buffer, pH 7.2, and mitochondria were prepared by the method of Slinde *et al.* (1975). The protein content of the mitochondrial preparation was measured by the method of Lowry *et al.* (1951), and the activity of cytochrome oxidase was measured by a polarographic method (Schnaitman *et al.*, 1967).

Specific [^3H]GDP binding was assayed in duplicate at room temperature (20°C) by using the method of Nicholls (1976) as modified by Desautel *et al.* (1978) in the presence and absence of 100 μM -GDP. We consistently observed that the unspecific binding was negligible. Extramitochondrial water was routinely estimated by incubation in the presence of [^3H]inulin. After incubation for 3 min, which was a sufficient time to reach equilibrium, samples of the mitochondrial suspension were layered on 100 μl of oil of *d* 1.025 [(dibutyl phthalate (*d* 1.05)/dinonyl phthalate (*d* 0.98), 20:11, v/v] placed in the bottom of Beckman Microfuge tubes. During centrifugation (1 min) the mitochondria were sedimented through the oil, ensuring a good separation from the incubation medium. The bottom of the tube containing the pellet was cut off and transferred into a scintillation vial containing tissue solubilizer (BTS 450, Beckman). After addition of scintillation fluid (Ready-solv NA, Beckman), the radioactivity was measured. Scatchard analysis of [^3H]GDP binding was performed as described above, with [^3H]GDP concentrations ranging from 0.2 to 10 μM .

At least two additional litters were used at each age for the determination of brown-adipose-tissue lipid content as described by Folch *et al.* (1957).

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

Results and discussion

The changes in mitochondrial protein, cytochrome oxidase activity and fat content of brown adipose tissue from 2 to 14 days of age in lean and obese pups are tabulated in Table 1. After a sharp 50% increase between days 2 and 5, the mitochondrial protein per g fat-free weight of tissue kept rising steadily, reaching by 14 days of age a value double that at 2 days of age; the developmental patterns were identical in the two groups of pups. Except at 7 and 14 days of age, where a slight difference between the two genotypes was observed, the fat-free brown adipose tissues of lean and obese pups were very similar in their mitochondrial protein content per gram. Cytochrome oxidase activity, a mitochondrial marker enzyme, underwent no significant changes over the time period investigated in any of the groups. The specific activity of this enzyme was strictly the same in both genotypes, in good agreement with the observations made in 40-day-old obese Zucker rats, where a normal succinate:cytochrome *c* oxidoreductase activity was reported (Holt & York, 1982). The fat content of brown adipose tissue was substantial in the very first days of life, amounting to 24% of the wet weight of tissue in lean rats (Table 1). Then, between 5 and 7 days of age, the lipid content fell dramatically to a low value, 11%. This was followed by a rapid fat accretion process, which brought the percentage of lipid back to 30% by day 14. Obese pups exhibited a profile of developmental changes in lipid content of brown adipose tissue strictly similar to that observed in their lean littermates, including the 2-

Table 1. *Properties of interscapular brown adipose tissue from lean (Fa/fa) and obese (fa/fa) Zucker pups aged 2–14 days*
For experimental details see the text. The values are means $\pm$ S.E.M. for the numbers of rats shown in parentheses:
* $P < 0.05$ and ** $P < 0.01$ versus values for lean rats.

Age (days)	Rats	Mitochondrial protein (mg/g fat-free wt. of tissue)	Cytochrome oxidase activity (μmol of O_2 /min per mg of protein)	Fat (%)
2	Lean	19.7 $\pm$ 1.13 (17)	1.9 $\pm$ 0.21 (13)	24.3 $\pm$ 1.10 (17)
	Obese	19.4 $\pm$ 1.35 (14)	1.7 $\pm$ 0.28 (9)	28.2 $\pm$ 1.84 (12)*
5	Lean	33.6 $\pm$ 2.4 (17)	2.35 $\pm$ 0.058 (12)	23.8 $\pm$ 1.57 (9)
	Obese	29.5 $\pm$ 3.5 (11)	2.26 $\pm$ 0.118 (8)	33.0 $\pm$ 1.56 (12)**
7	Lean	36.0 $\pm$ 1.12 (12)	2.45 $\pm$ 0.184 (8)	11.1 $\pm$ 0.76 (9)
	Obese	30.9 $\pm$ 1.16 (16)**	2.38 $\pm$ 0.142 (12)	14.9 $\pm$ 0.60 (10)**
10	Lean	38.8 $\pm$ 1.14 (15)	2.32 $\pm$ 0.041 (8)	18.6 $\pm$ 1.88 (7)
	Obese	36.3 $\pm$ 1.52 (17)	2.29 $\pm$ 0.099 (10)	29.2 $\pm$ 1.26 (9)**
14	Lean	42.4 $\pm$ 0.70 (13)	2.62 $\pm$ 0.234 (13)	29.0 $\pm$ 1.32 (4)
	Obese	46.7 $\pm$ 1.70 (7)*	2.21 $\pm$ 0.176 (8)	44.3 $\pm$ 1.19 (5)**

fold decrease between days 5 and 7. However, the brown-adipose-tissue lipid content was significantly (20%) higher in obese than in lean pups as early as 2 days of age, the difference between genotypes increasing with the age of the pups. This result shows that the large fat depot previously reported in brown adipose tissue of 2-week-old *fa/fa* pups (Bazin *et al.*, 1983) is already present within the first days of life.

The developmental changes in GDP binding to brown-adipose-tissue mitochondria from 2 to 14 days of age are depicted in Fig. 1. In lean pups, the GDP-binding capacity of brown-fat mitochondria, which averaged 0.16 nmol/mg of protein during the first days of life, increased suddenly to 0.3 nmol/mg of protein by 7 days of age and thereafter stabilized at this high value. In contrast, in obese pups the GDP-binding capacity at 10–14 days was not significantly higher than that at 2 days; however, we observed a peak value at 7 days of age. The

pattern of GDP binding observed in lean pups differs from that reported by Sundin & Cannon (1980) in Sprague–Dawley rats, where a peak value was observed by 3–4 days *post partum*, followed by a decrease in the second week of life. Our data suggest that activation of brown adipose tissue occurred in lean pups during the second week of life. This could be interpreted as a reflection of the increased need for regulatory heat production as pups are exposed to a lower environmental temperature (switch from nest to cage; it is the natural behaviour of the animals to move out of the nest after the first few days of life). The steady low GDP binding found in brown adipose tissue of obese animals during the same period could result from an impaired thermogenic response to cold; alternatively, behavioural differences or the development of body-fat stores that occurs in the mutants within the first 2 weeks of life (Zucker & Antoniades, 1972) might lessen their need for thermogenesis. Table 1 and Fig. 1 show that, regardless of the genotype of the pups, the burst of GDP binding that occurred at 7 days of age was concomitant with a depletion of the fat content of the tissue, indicating that an increased thermogenic activity had taken place fuelled by the fatty acids derived from endogenous lipid stores.

The finding in the present work was that the GDP-binding capacity, at 2 days of age and at each subsequent time point investigated, was significantly lower in obese than in lean pups, from a 30% decrease at 2 days of age to more than 50% at 14 days of age. To determine whether the large decrease in GDP binding observed in brown-adipose-tissue mitochondria of pups of the *fa/fa* genotype resulted from a change in the number of binding sites or from a change in affinity, we performed a Scatchard analysis. This was done at 10 days of age, when enough tissue could be dissected out for the study. Under our experimental conditions, where GDP binding was measured only over a limited range of ligand concentrations, owing to the minute amounts of tissue available, we obtained a linear Scatchard plot (Fig. 2) in the two groups of rats, indicating only one type of binding sites. The presence of two mitochondrial GDP-binding sites has been established in both hamster (Rial & Nicholls, 1983) and rat (Bryant *et al.*, 1983). The class of sites revealed under our conditions had binding characteristics (K_D around $1 \mu\text{M}$) close to those of the low-affinity sites described in rats and whose capacity was shown to be regulated by cold and diet (Bryant *et al.*, 1983). The apparent dissociation constant (K_D) was the same for lean and obese pups ($0.99 \pm 0.10 \mu\text{M}$ and $1.10 \pm 0.11 \mu\text{M}$ respectively), indicating that there was no change in the affinity for GDP in obese pups. However, the maximum

Fig. 1. Development of GDP binding to interscapular brown-adipose-tissue mitochondria of Zucker rats. For experimental details see the text. The values are means $\pm$ S.E.M.: ** $P < 0.01$ compared with values for lean pups at the same age. The numbers of lean pups (●) were 17, 18, 13, 15 and 13, and of obese pups (○), 14, 12, 16, 17 and 7, at 2, 5, 7, 10 and 14 days respectively.

Fig. 2. Scatchard plot of GDP binding to brown-adipose-tissue mitochondria from one lean (●) and one obese (○) 10-day-old Zucker pups

Details of the experimental procedure are given in the Materials and methods section.

binding was decreased from 270 pmol/mg in lean to 150 pmol/mg in obese pups ($P < 0.01$, $n = 9$). Therefore the large fall in the number of GDP-binding sites reported by Holt & York (1982) in the brown adipose tissue of obese rats aged 6 weeks was already present in the very first days of life. The present study does not elucidate the mechanism responsible for the decreased number of GDP-binding sites found in the mutants. Current experiments suggest that the decrease in GDP binding in 10-day-old *fa/fa* pups can be ascribed to a decrease in the amount of the M_r -32000 protein as observed by gel electrophoresis (R. Bazin, D. Ricquier & M. Lavau, unpublished work). The mechanism by which the concentration of the uncoupling protein in brown adipose tissue of obese pups would be depressed has yet to be investigated. A pre-synaptic decrease in sympathetic activity (Levin *et al.*, 1981) and a defect in the post-synaptic mechanism controlling thermogenesis in brown adipose tissue (Levin *et al.*, 1982) have been demonstrated in adult obese Zucker rats. Holt *et al.* (1983) have suggested that the decreased thermogenesis in obese Zucker rats could result from corticosterone inhibition of the sympathetic-nervous-system response to a dietary

signal. Moreover, the importance of noradrenaline in the control of the amount of the M_r -32000 protein has been shown (Mory *et al.*, 1984). Taken together, these results strongly suggest that an altered sympathetic activity could play a key role in the depressed thermogenic capacity of *fa/fa* pups.

In conclusion, the present work shows that a defective proton-conductance pathway, as monitored by GDP binding to mitochondria, was present in brown adipose tissue of *fa/fa* pups as early as 2 days of age, supporting the hypothesis that an impairment in the thermogenic capacity of brown adipose tissue could play a determinant role in the development of obesity in the Zucker rat. However, we were not able to demonstrate the presence of a decreased GDP binding independently of an increased fat content of brown adipose tissue. Therefore the question of the sequential order and causal relationship between these two derangements remains to be solved.

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