Changes induced by fasting and dietetic obesity in thermogenic parameters of rat brown adipose tissue mitochondrial subpopulations

Juan C. MATAMALA*, Magdalena GIANOTTI*, Jordi PERICÁS, Santiago QUEVEDO, Pilar ROCA, Andreu PALOU† and Francisco J. GARCÍA-PALMER

Laboratori de Biologia Molecular, Nutrició i Biotecnologia. Departament de Biologia Fonamental i Ciències de la Salut. Universitat de les Illes Balears. Crta. Valldemossa, km 7,5; 07071 Palma de Mallorca, Spain.

The effects of starvation on the thermogenic parameters of three different mitochondrial subpopulations in brown adipose tissue (BAT) of both post-cafeteria obese and lean rats were investigated. Tissue from different BAT depots from fed and 24 h starved rats were collected, pooled and three mitochondrial subpopulations were isolated by differential centrifugation; the M1 fraction (1000 ***g***), the M3 fraction (3000 ***g***) and the M15 fraction (15000 ***g***). Thermogenic parameters were measured in the three mitochondrial subtypes, and uncoupling protein (UCP) mRNA was determined in BAT. The results showed that starvation induced a decrease in mitochondrial turnover in BAT from both lean and obese rats. Moreover, a selective net loss of UCP from the lightest mitochondrial fraction (M15) in lean rats, with a concomitant reduction of UCP mRNA was observed. The reductions did not occur in obese rats and, as a result, a change in UCP distribution between the mitochondrial subpopulations was produced, with an increase in the M1 mitochondrial subtype. The lack of response of UCP to starvation observed in BAT of obese rats compared with the decrease seen in lean animals, is a consequence of a different mitochondrial subpopulation composition and/or a different response of a particular subpopulation to starvation.

INTRODUCTION

Brown adipose tissue (BAT) plays an important role in the regulation of energy balance because it is a major site of both non-shivering and diet-induced thermogenesis in small mammals [1,2] and perhaps also in humans [3]. Diet-induced thermogenesis results mainly from sympathetic stimulation of BAT [4]. Fasting leads to a marked decrease in non-shivering thermogenesis, which has been related to a decrease in GDP-binding and uncoupling protein (UCP) [5,6], and in UCP mRNA levels [7–9]. However, hypertrophied BAT of cafeteria-obese rats shows a significant decrease in GDP binding, and no changes in the other thermogenic parameters (although GDP binding does not necessarily correlate with UCP content because the UCP could be masked [5,6]). Therefore whatever the fasting-related atrophy mechanism, it seems that it is not fully operative in these rats and would be responsible for and/or contribute to the obesity status.

In order to assess the lack of thermogenic response to fasting in obese rats we have proposed that there are different UCP pools in mitochondria. There are several reasons for this fact. First, results of previous investigations by our group have shown that in transiently adrenergically-stimulated cultured brown adipocytes, the induced UCP is rapidly and specifically degraded after removal of noradrenaline (NA). On the other hand, in chronically adrenergically-stimulated cells the half-life of UCP is much longer and the degradation is non-specific [10]. These data suggest the existence of two different functional pools of UCP, a rapidly degradable pool of newly synthesized protein and a more stable pool which would be protected against specific degradative systems [10]. Secondly, it has been shown that mitochondria can be fractionated into different subpopulations, called heavy, medium and light mitochondria [11,12]. In liver, cold exposure acts differentially on the three mitochondrial fractions, having a more marked effect on the light fraction [11,12]. Under these conditions, in the medium and light fractions oxidative phosphorylation is somewhat uncoupled, whereas the heavy fraction remains coupled and ATP is synthesized [11–13]. These mitochondrial subpopulations have been fully characterized also in mouse BAT [14]. Thirdly, during cold acclimatization, the thermogenic parameters of the three mitochondrial subpopulations in rat BAT change in a different way [15]. The timing of the appearance of new UCP in the different mitochondrial subtypes varies, beginning in the lightest and continuing in the heaviest fractions. Only the UCP incorporated into the heavy mitochondrial subtypes is able to unmask GDP-binding sites, which implies that the size or maturation of mitochondria could be related to the ability to mask or unmask the GDP-binding sites of UCP [15]. Finally, the net loss of UCP upon removal of NA from murine BAT cell cultures affects mainly the lightest mitochondrial pools, with comparatively little effect on the heavy mitochondria [14]. Similar results have been obtained in a parallel situation *in vivo* (starvation of mice) [14].

In the view of the studies on different mitochondrial subpopulations in both liver and BAT, together with the known role of BAT in diet-induced thermogenesis, and the differences in the response to fasting in BAT from obese animals, we devised the hypothesis that the lack of response in UCP levels observed in the BAT of obese rats compared with the decrease seen in lean animals [5,16,17] could be because the mitochondrial subpopulations in obese rat BAT are different and/or a different response

Abbreviations used: UCP, uncoupling protein or thermogenin; BAT, brown adipose tissue; NA, noradrenaline; COX, cytochrome *c* oxidase; IOD, integrated optical densities.

* These authors contributed equally to the work.

† To whom correspondence should be addressed.

of a particular subpopulation to starvation is produced. Thus, we decided to analyse the thermogenic parameters of the three mitochondrial fractions of rats made obese by dietary manipulation and to establish whether or not these changes were related to the processes involved in UCP expression and incorporation into the different mitochondrial subtypes.

MATERIALS AND METHODS

Animals

The female Wistar rats used were bred in our laboratory and were housed in cages in temperature (23 °C) and light (12 h light/12 h dark) controlled conditions. The four experimental groups used were as follows. Post-cafeteria obese rats ($n = 7$) were fed with cafeteria diet from day 10–100 of age, and then were fed standard diet until they were killed on day 150. The body weight of this group was 41 % greater than control rats when the cafeteria diet was stopped; after reversion to standard diet the body weight of the obese rats decreased and stabilized but remained 23 % heavier than controls on day 150, although the dietary daily intake was the same as that of the control rats, as described previously [16,18,19]. Control rats ($n = 6$) were age-matched and were fed standard rat diet until they were killed on day 150. Obese fasted rats ($n = 6$) and control fasted rats ($n = 6$) were fed the same diets as the obese and control groups, respectively, but both groups were fasted for 24 h before being killed.

Diets

Cafeteria diet was made up of the following foods: cookies with paté and sobrassada (typical Majorcan sausage), candies, bacon, biscuits, chocolate, peanuts, cheese, milk containing 20 % (w/v) sucrose and ensaimada (typical Majorcan cake). The composition of the cafeteria diet has been described previously [20], and was approx. 13.6 % lipid, 21.0 % carbohydrate, 9.0 % protein, 51.3 % water and 5.1 % others (by weight). The standard diet was purchased from Panlab, and was composed of 23.5 % protein, 48.9 % carbohydrate, 5.0 % lipid, 12 % water, 5.7 % ash and 4.0 % cellulose.

Isolation of samples

Animals were killed by decapitation at the start of the light cycle. A small piece of interscapular BAT (approx. 100 mg) was rapidly excised, frozen in liquid N_2 and stored at −70 °C for the determination of UCP mRNA. The rest of the interscapular BAT, cervical, axillar, perirenal and perioaortic BAT depots were excised and carefully dissected in order to avoid contamination with other tissues. The BAT samples were pooled, weighed and homogenized in 250 mM sucrose/1 mM Hepes/0.2 mM EDTA buffer, pH 7.0 in a Teflon/glass homogenizer operated by a power drill (Black and Decker) at 1600 r.p.m. for 3 strokes of 5–8 s duration, held in an ice bath. Aliquots of the homogenate were assayed for total protein content [21] and cytochrome *c* oxidase (COX) activity [22]. The mitochondrial subpopulations were isolated from the remaining homogenate.

Chemicals

[8-^{3}H]GDP and [U-^{14}C]sucrose were obtained from DuPont. GDP, Coomassie Blue, BSA, rotenone, ferrocytochrome *c*, Tween 20, buffers, goat anti-rabbit IgG conjugated to alkaline phosphatase, and *p*-nitrophenol phosphate were obtained from Sigma Chemicals. Other compounds were purchased from Boehringer, Merck and Panreac.

Separation of mitochondrial subpopulations from BAT

The homogenate was filtered through a layer of gauze and centrifuged at 15000 ***g*** for 15 min at 4 °C. The fat layer and supernatant were discarded, and the pellet was resuspended in homogenizing buffer to the original volume. Nuclei and cell debris were removed by centrifugation at 500 ***g*** for 10 min at 4 °C. The resulting supernatant was subjected to three sequential centrifugation steps for 10 min, at 1000 ***g***, 3000 ***g*** and 15000***g*** respectively, giving the heavy (M1), medium (M3) and light (M15) mitochondrial fractions. The pellets were resuspended in a minimal volume of the homogenizing buffer. Mitochondrial protein was measured by the method of Bradford [21] using BSA as standard, and COX activity was measured using a spectrophotometric method [22]

GDP binding

Specific GDP binding of the three mitochondrial fractions was measured as previously described [23] with slight modifications. The mitochondrial pellets were resuspended in the homogenizing buffer and, briefly, 100 μl portions (about 2 mg of protein) were incubated at 30 °C for 10 min in 2 ml of incubation buffer (20 mM Tes, pH 7.1) containing 100 mM [U-^{14}C]sucrose (6 kBq/ml), 10 μM [8-^{3}H]GDP (ammonium salt) (10 kBq/ml), 1 mM EDTA and 5 μM rotenone. Non-specific binding was assessed by the addition of unlabelled GDP (25 μl of 10 mM GDP) and incubation for 10 min at 30 °C. [U-^{14}C]Sucrose was used to quantify the space between mitochondria. Specific and non-specific GDP binding was estimated after centrifugation of 0.4 ml mitochondrial samples in incubation medium (Microfuge). The radioactivity in the pellets and supernatants was measured (Beckman LS-3800) using a dual-label program for ^{3}H and ^{14}C.

Determination of UCP levels by ELISA

Plates (96-well, Nunc) were coated with 130 ng of purified UCP and incubated for 3 h at 37 °C. The plates were then recoated with 100 μl of 1 % (w/v) BSA in 0.9 % NaCl/0.05 % Tween 20 (by vol.) and incubated for 3 h at 37 °C. The wells were then washed with 3 × 400 μl of 0.9 % NaCl/0.05 % Tween 20. Purified UCP (used as standard) and diluted mitochondrial samples (0.1–1μg of mitochondrial protein) were incubated with rabbit antiserum (1:2000 final dilution) at 37 °C. The plates were incubated at room temperature for 2 h; the wells were then rinsed three times with 0.9 % NaCl/0.05 % Tween 20. For development, 100 μl of goat anti-(rabbit) IgG conjugated with alkaline phosphatase (diluted 1000-fold in PBS/0.05 % Tween 20) was added to each well and the plates were incubated overnight at 4 °C and then washed three times with 0.9 % NaCl/0.05 % Tween 20 (by vol.). A 100 μl volume of *p*-nitrophenyl phosphate (1 mg/ml dissolved in triethanolamine buffer, pH 9.8, containing 1 mM $MgCl_2$) was then added to each well and the plates were incubated at room temperature. The absorbance at 405 nm of each well was measured (MIOS spectrophotometer, Merck).

Isolation of tissue total RNA

The isolation of total RNA was carried out using guanidinium hydrochloride [24] with some modifications. The purified total RNA was dissolved in diethylpyrocarbonate-treated water and was quantified by measuring the absorbance at 260 nm.

Preparation of the UCP-cDNA digoxigenin-11-dUTP-labelled probe

Total RNA (131 ng) from a sample of BAT was retrotranscribed and amplified following the protocol supplied with the GeneAmp

Table 1 Effects of obesity and 24 hour fasting on rat BAT general parameters

The data represent the mean ± S.E.M. (control, control fasted, obese fasted, $n = 6$; obese, $n = 7$) and were analysed by a two- and one-way ANOVA as described in the Materials and methods section. Significant differences ($P < 0.05$): *fed versus fasted; †obese versus control. ANOVA significances ($P < 0.05$): D, effect of diet, S, effect of fasting, D × S, interaction of diet and fasting, NS, not significant.

Parameter	Control fed	Control fasted	Obese fed	Obese fasted	ANOVA
Body weight (g)	325 ± 3	304 ± 1*	424 ± 13†	421 ± 13†	D, D × S
BAT weight (mg)	900 ± 90	617 ± 50*	1540 ± 120†	1420 ± 110†	D, D × S
Protein content (mg/g of tissue)	60.0 ± 1.7	59.4 ± 4.0	52.4 ± 1.9†	57.0 ± 4.1	NS
COX activity (units/g of tissue)	53.1 ± 5.4	59.0 ± 4.8	44.0 ± 4.4	47.6 ± 5.2	NS

RNA PCR kit (Perkin Elmer-Cetus), except that a mixture of dTTP/digoxigenin-dUTP (0.65:0.35) was used instead of dTTP. The sequences of primers used in this method were as follows: upstream primer 3′-GGAAAGGTGTCACAACTGTTCG-5′ and downstream primer 3′-CGACCCTCTGGTGCTTATT-TTG-5′, resulting in an amplification of the 496 bp fragment containing exons 4, 5, 6, part of exon 3, and part of the 3′-terminal extreme after exon 6. The amplification reactions were carried out in 35 cycles; each cycle comprised a denaturing step (95 °C for 1 min) followed by a primer annealing and DNA polymerization step (60 °C for 1 min). The samples were then heated at 60 °C for 7 min in order to complete the polymerization process. The digoxigenin-labelled DNA probe was purified by extraction with phenol/chloroform/isopentyl alcohol (25:24:1, by vol.), precipitated with ethanol/10 M ammonium acetate, redissolved in water and quantified by measuring the absorbance at 260 nm [25].

Preparation of the β-actin-cDNA digoxigenin-11-dUTP-labelled probe

About 1 μg of total RNA from a sample of BAT was retro-transcribed and amplified following the protocol supplied with the β-actin primer set for RT-PCR kit (Stratagene) except that a mixture of dTTP/digoxigenin-dUTP (0.65:0.35) was used instead of dTTP. The sequences of primers used in this method were as follows: sense primer 206-TGTGATGGTGGGAATGGG-TCAG-227 and antisense primer 698-TTTGATGTCACGCAC-GATTTCC-714, resulting in an amplification of the 514 bp fragment. The amplification reaction was as follows: 5 min denaturation at 94 °C, 5 min annealing at 60 °C, followed by 35 cycles of 1.5 min at 72 °C, 45 s at 94 °C and 45 s at 60 °C, with a final extension of 10 min at 72 °C. The digoxigenin-labelled DNA probe was purified by extraction in phenol/chloroform/isopentyl alcohol (25:24:1, by vol.), precipitated with ethanol/10 M ammonium acetate, redissolved in water and quantified by measuring the absorbance at 260 nm [25].

Dot–blot determination of UCP mRNA and β-actin mRNA

Aliquots (5 μg) of total RNA from BAT samples were denatured in an ice-cold 10 mM NaOH containing 1 mM EDTA before application to a positively charged nylon membrane (Boehringer Mannheim). Alkali transfer was performed in a Bio-Dot® SF microfiltration apparatus (Bio-Rad) according to the protocol supplied by the manufacturer. After transfer, the membrane was washed in 2 × 0.15 M NaCl/0.015 M sodium citrate containing 0.1 % (w/v) SDS. Prehybridization (1 h) and hybridization (16 h) with UCP-cDNA digoxigenin-11-dUTP-labelled probe and immunological chemiluminiscent detection were carried out as described [26], using a Boehringer kit. Both procedures were performed at 65 °C in the absence of formamide and the washing buffer was the same as that used in Church and Gylbert hybridizations [26]. The concentration of the labelled probe in the hybridization solution was 16 ng/ml.

After immunological detection, the membrane was exposed to a photographic film (Kodak X-OMAT), which was later analysed by scanner photodensitometry. The integrated optical densities (IOD) of the bands were quantified using the Bioimage program (Millipore).

The membrane was dehybridized as described in the manufacturer's protocol (Boehringer Mannheim). Prehybridization and hybridization were repeated using the same conditions, except that a β-actin-cDNA digoxigenin-11-dUTP-labelled probe was used. The concentration of the probe in the hybridization solution was 50 ng/ml. Immunological detection and IOD were carried out as described above

Statistics

All data are presented as group mean values ± S.E.M. Differences between groups were assessed by one-way and two-way analysis of variance (ANOVA) and Student–Newman-Keuls *post hoc* comparisons. The analyses were performed with DBASE IV and SPSS-X packages on a VAX8820 computer.

RESULTS AND DISCUSSION

As shown in Table 1, BAT weight decreased with starvation in control but not in obese animals, as reported previously [5,16,17].

Table 2 shows the mitochondrial protein content and both specific and total COX activity of BAT tissue. The results in fed obese rats show a higher degree of individual variability, not only in protein levels, but also in the other parameters measured. The results indicate that the response of mitochondrial protein levels to starvation is quite different in the three mitochondrial fractions. The combined total mitochondrial protein content was significantly less in control rats, mainly due to the reduction in the M15 fraction. It has been noted that the morphological response of mitochondria to metabolic and environmental alterations such as starvation is their propensity to enlarge [27,28]. Our data are in accordance with mitochondrial enlargement in response to starvation (i.e., the reduced protein in the M15 fraction and the increase in M1 would be expected to lead to an increase in mitochondrial size).

Table 3 shows GDP binding and UCP levels. GDP-binding studies have been used widely as a method of assessing the thermogenic status of BAT mitochondria *in vitro*, but whether the binding studies are an indirect measure of the concentration

Table 2 Effects of obesity and 24 h fasting on the distribution of mitochondrial protein (total tissue) and specific and total COX activity among mitochondrial fractions

The data represent the mean ± S.E.M. (control, control fasted, obese fasted, $n = 6$; obese, $n = 7$) and were analysed by a two- and one-way ANOVA as described in the Materials and methods section. Significant differences ($P < 0.05$): * fed versus fasted; †obese versus control; ‡M15 versus M1; §M3 versus M1. ANOVA significances ($P < 0.05$): D, effect of diet; S, effect of fasting; F, effect of mitochondrial fractions; S × F, interaction of fasting and mitochondrial fractions; NS, not significant.

Parameter	Control fed	Control fasted	Obese fed	Obese fasted	ANOVA
Total mitochondrial protein (mg/tissue)					
M1	5.49 ± 0.84	5.73 ± 0.56	8.80 ± 1.87	9.75 ± 1.30†	D
M3	7.48 ± 0.98	5.72 ± 0.40	12.0 ± 1.7†	10.6 ± 1.4†	S
M15	9.48 ± 0.84‡	5.05 ± 0.43*	12.2 ± 1.8	9.43 ± 1.50†	
Total	22.5 ± 2.1	17.0 ± 1.3*	33.0 ± 4.9	29.8 ± 4.0†	
Specific COX activity (units/mg of mitochondrial protein)					
M1	1.72 ± 0.17	1.59 ± 0.11	1.88 ± 0.30	1.69 ± 0.15	F
M3	2.01 ± 0.12	2.33 ± 0.28§	1.99 ± 0.27	2.29 ± 0.26§	
M15	2.32 ± 0.26	2.74 ± 0.29‡	2.67 ± 0.24	2.49 ± 0.16‡	
Total COX activity (units/tissue)					
M1	9.98 ± 2.19	9.07 ± 0.83	17.0 ± 4.1	16.6 ± 3.1†	D
M3	15.3 ± 2.6	13.6 ± 2.1	24.3 ± 3.6†	23.9 ± 3.9†	S
M15	22.1 ± 3.4‡	15.2 ± 2.4	32.4 ± 2.7†‡	22.4 ± 2.4*†	F
Total	47.4 ± 6.8	37.9 ± 5.0	73.7 ± 9.0†	62.9 ± 8.0†	

Table 3 Effects of obesity and 24 h fasting on the distribution of specific and total GDP binding, and specific and total UCP content among mitochondrial fractions

The data represent the mean ± S.E.M. (control, control fasted, obese fasted, $n = 6$; obese, $n = 7$) and were analysed by a two- and one-way ANOVA as described in the Materials and methods section. Significant differences ($P < 0.05$): *fed versus fasted; †obese versus control; ‡M15 versus M1; §M3 versus M1. ANOVA significances ($P < 0.05$): D, effect of diet; S, effect of fasting; F, effect of mitochondrial fractions; D × S, interaction of diet and fasting; S × F, interaction of fasting and mitochondrial fractions; NS, not significant.

Parameter	Control fed	Control fasted	Obese fed	Obese fasted	ANOVA
Specific GDP-binding (pmol GDP/mg of mitochondrial protein)					
M1	396 ± 62	241 ± 15*	370 ± 43	290 ± 15†	S
M3	444 ± 71	194 ± 39*	393 ± 46	342 ± 26†	F
M15	274 ± 34	147 ± 36*	274 ± 31	236 ± 16†	D × S
Total GDP-binding (pmol GDP/tissue)					
M1	2258 ± 228	1441 ± 226*	3643 ± 1027	2649 ± 392†	D
M3	3589 ± 576	1108 ± 228*	5107 ± 1045	3696 ± 645†	S
M15	2370 ± 299	813 ± 225*	3733 ± 765	2019 ± 385†	F
Total	8217 ± 999	3363 ± 318*	12484 ± 2616	8411 ± 1041†	
Specific UCP content (μg UCP/mg of mitochondrial protein)					
M1	12.0 ± 1.9	12.9 ± 2.8	10.8 ± 1.9	23.5 ± 1.7*†	D
M3	15.4 ± 3.9	14.1 ± 2.4	23.6 ± 5.1§	21.7 ± 2.2†	
M15	16.6 ± 1.5	9.89 ± 2.20*	21.6 ± 2.9‡	21.4 ± 2.1†	
Total UCP content (μg UCP/tissue)					
M1	59.7 ± 6.1	73.8 ± 16.6	102 ± 41	242 ± 46*†	D
M3	103 ± 23	75.2 ± 7.8	262 ± 55†§	239 ± 46†	S × F
M15	141 ± 21‡	50.2 ± 10.9*	250 ± 40†‡	195 ± 27†	
Total	304 ± 36	188 ± 25*	590 ± 135†	709 ± 120†	

of UCP is not yet clear [2,29–38]. Fasting has been reported to depress the thermogenic capacity of BAT mitochondria, assessed by GDP binding in the rat [39] and UCP levels in mice [40], or, in hamsters, to reduce BAT thermogenic function exclusively by lowering mitochondrial protein concentrations without changing the UCP concentration or GDP binding [41]. Our previous studies on UCP response to fasting showed a selective loss of GDP binding and lowered UCP levels in control rats, whereas

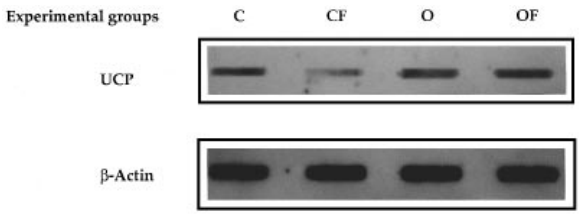

Figure 1 Dot–blot analysis of UCP mRNA

Total RNA (5 μg) isolated from BAT was hybridized with oligonucleotide DNA probes to UCP or β-actin. The experimental groups were: C, control; CF, fasted; O, obese; OF, obese fasted.

there was a fasting-induced decrease in GDP binding without any loss of UCP in both cafeteria and post-cafeteria obese rats [5,6].

The present data indicate that the decrease in UCP levels in lean rats can be ascribed to loss of specific and total UCP in the M15 mitochondrial fraction, with minor or no changes in the other fractions. The decrease was great enough to account for the diminished UCP of the pooled fractions. However, the response to starvation in lean rats was quite different from the response in obese rats where specific UCP is not decreased in the M15 fraction but is increased in the M1 fraction, although in fasted obese rats the UCP levels are similar in all three mitochondrial subpopulations. This indicates that in starvation, obese rats have a different distribution of UCP between the three mitochondrial subpopulations. The same effect would be seen in controls but is disguised by the diminished levels of UCP in the M15 population, which can be inferred from the total UCP levels in control starved rats.

The pattern of UCP with starvation observed in the pooled BAT of control rats is similar to that described by us previously for individual and pooled BAT depots [5,6,10,14], and by other workers [7,40–42]. The pattern observed is the same as that seen on removal of NA stimulation from mice brown preadipocytes, developed and differentiated in primary cell cultures [14], where the loss of UCP after NA stimulation is observed in the lightest mitochondrial fractions [14]. On the whole, the preceding observations strongly suggest that the specific decrease of UCP in the lightest mitochondria is a response peculiar to BAT, and is independent of the source of the tissue (interscapular, perirenal, periaortic, etc.). Interestingly, BAT from obese rats responds in a different way.

A representative dot–blot analysis of UCP mRNA is shown in Figure 1. The data of UCP mRNA content is presented in Table 4 and is expressed using β-actin mRNA as internal standard, and as total tissue content. Each set of values is shown as a percentage of the value obtained for the control fed rats. In control rats, UCP mRNA levels decreased with fasting, as described previously [7], but this was not the case with obese rats, which was similar to the pattern observed in UCP levels. When the decrease in mRNA is compared with the decrease in total M15 UCP with starvation, the results are similar. This seems to indicate that there is a close relationship between the mRNA levels and UCP content in the M15 subpopulation.

The different timings of UCP appearance in the mitochondrial subtypes with cold acclimatization shown previously, lead us to speculate that the timing of UCP degradation would also be expected to be different when the animals were returned to warm conditions after acute or chronic cold exposure, which could be associated with altered UCP incorporation into the three mitochondrial subtypes [15]. In fact, UCP was first incorporated into the M15 subtype [15], and we have shown in the present study that, with starvation, UCP is lost from this fraction and there is a concomitant reduction in UCP mRNA. It was concluded that newly synthesized UCP is preferentially incorporated into the M15 subpopulation, then a starvation-induced reduction in UCP synthesis results in diminished incorporation into this fraction, which is what happens in control but not in obese rats.

There is, however, the problem of whether the decrease results from specific breakdown of newly synthesized UCP or if it merely represents a lack of influx of UCP into the mitochondrial pool. A marked loss of protein from BAT has been described in fasting mice [8,42,43], which was most likely caused by a fall in protein synthesis and/or from increased intracellular proteolytic activity, since the tissue cellularity (DNA content) remained unchanged during fasting [43,44]. However, very little is known about the biochemical pathways that cause such marked alterations in BAT protein mass and how the proteolytic activity is regulated. Nevertheless, it is clear that BAT contains lysosomal proteases that can rapidly reduce the thermogenic capacity of the tissue, provided that the appropriate signals are received [43]. These do not exclude the contribution of other proteolytic systems in BAT, such as those found in mitochondria [45], which may be involved in some of the more selective changes in the protein composition of the tissue (i.e. loss of uncoupling protein from inner mitochondrial membranes) [8,43]. Yet our present data suggest that proteolytic activity may not be sufficient, with respect to the fall in protein synthesis, to explain the diminished UCP levels that occur with fasting. In fact, it has been shown recently, that UCP content is predominantly regulated pretranslationally [46].

Table 4 Effects of obesity and 24 h fasting on the UCP mRNA content of rat BAT

The data represent the mean $\pm$ S.E.M. (control, control fasted, obese fasted, $n = 6$; obese, $n = 7$) and were analysed by a two- and one-way ANOVA as described in the Materials and methods section. Significant differences ($P < 0.05$): *fed versus fasted; †obese versus control. ANOVA significances ($P < 0.05$): D, effect of diet; S, effect of fasting; D × S, interaction of diet and fasting; NS, not significant.

Parameter	Control fed	Control fasted	Obese fed	Obese fasted	ANOVA
Specific UCP mRNA content (IOD UCP/IOD β-actin) (% of control fed)	100 $\pm$ 25	26.0 $\pm$ 13.0*	128 $\pm$ 26	87.0 $\pm$ 12.1†	S, D, D × S
Total UCP mRNA content (IOD UCP/tissue) (% of control fed)	100 $\pm$ 20	21.8 $\pm$ 7.7*	188 $\pm$ 62	143 $\pm$ 30†	S, D, D × S

Table 5 Effects of obesity and 24 h fasting on the UCP/COX and GDP-binding/UCP ratios in the mitochondrial fractions studied

The data represent the mean ± S.E.M. (control, control fasted, obese fasted, $n = 6$; obese, $n = 7$) and were analysed by a two- and one-way ANOVA as described in the Materials and methods section. Significant differences ($P < 0.05$): *fed versus fasted; †obese versus control; ‡M15 versus M1; §M3 versus M1. ANOVA significances ($P < 0.05$): D, effect of diet; S, effect of fasting; F, effect of mitochondrial fractions; D × S, interaction of diet and fasting; S × F, interaction of fasting and mitochondrial fractions; NS, not significant.

Parameter	Control fed	Control fasted	Obese fed	Obese fasted	ANOVA
UCP/COX ratio (μg UCP/units of COX)					
M1	7.77 ± 2.03	8.25 ± 1.44	6.39 ± 0.47	15.5 ± 2.9*†	D
M3	7.99 ± 2.23	6.23 ± 1.76	11.4 ± 1.9§	10.0 ± 1.6	D × S
M15	8.55 ± 2.10	3.94 ± 1.04	8.21 ± 1.00	8.61 ± 0.63†‡	S × F
GDP/UCP ratio (nmol GDP/μg of UCP)					
M1	2.55 ± 0.55	1.62 ± 0.65	2.51 ± 0.67	1.29 ± 0.58	S
M3	3.11 ± 1.01	0.86 ± 0.38*	1.69 ± 0.52	1.06 ± 0.14	F
M15	1.39 ± 0.24	1.03 ± 0.23	0.96 ± 0.20	0.69 ± 0.10	

Table 5 shows the ratios of UCP/COX and GDP binding/UCP. It is important to point out that the GDP binding/UCP ratio is diminished with starvation in M1 and M3 fractions in control rats but is unaltered in M15 fraction. The same pattern is also present with starvation in obese rats, although in this case it was not significant. It has been shown previously [15] that cold exposure induces the opposite profile, which is in agreement with the present data, because cold exposure induces BAT recruitment while starvation induces atrophy [6,39,40,47]. Based on GDP/UCP ratios, it has been suggested previously that only UCP incorporated into the heavy mitochondrial subtypes is able to unmask GDP-binding sites in response to cold exposure, whereas the increase in GDP binding in the M15 and M3 subpopulations is associated with an increase in UCP levels [15]. In starved rats, we have found that the masking process happens only in the heaviest fractions, and masking does not occur at all in the light fraction. Combination of these results suggests that the size (or maturation) of the mitochondria may be related to the ability to mask or unmask the UCP GDP-binding sites.

In summary, the reductions in UCP mRNA and in UCP levels observed with starvation in control rats are depressed in the obese ones, and would seem to be related mainly to a lack of UCP synthesis. A change in UCP distribution between the mitochondrial subpopulations is produced in response to starvation, with an increase in the heavy, more mature mitochondria (M1 fraction). In conclusion, starvation induces a decrease in mitochondrial turnover in both lean and obese rats. UCP levels are reduced only in the M15 fraction of control rats with a parallel reduction of UCP mRNA, although both reductions appear to be impaired in the dietetic obesity model studied.

This investigation was supported by grants PB 92-0748-C04-00 and PM 94-1178 from DGICYT of the Spanish Government, and also by grant ERBCHRX-CT940490 from the European Commission. J.C.M. and S.Q. were recipients of grants from the Programme FPI (DGICYT) of the Spanish Government.

REFERENCES

1 Shrago, E. and Strieleman, P. J. (1987) World Rev. Nutr. Diet. **53**, 171–217
2 Himms-Hagen, J. (1989) Prog. Lipid Res. **28**, 67–115
3 Lean, M. E. J. and James, W. P. T. (1986) in Brown Adipose Tissue (Trayhurn, P. and Nicholls, D. J., eds.), pp. 339–365, Edward Arnold, London
4 Rothwell, N. and Stock, M. J. (1979) Nature (London) **281**, 31–35
5 Puigserver, P., Lladó, I., Palou, A. and Gianotti, M. (1991) Biochem. J. **279**, 575–579
6 Puigserver, P., Gianotti, M. and Palou, A. (1992) Int. J. Obes. **16**, 255–261
7 Champigny, O. and Ricquier, D. (1990) J. Nutr. **120**, 1730–1736
8 Trayhurn, P. and Jennings, G. (1988) Am. J. Physiol. **254**, R11–R16
9 Picó, C., Herron, D., Palou, A., Jacobson, A., Cannon, B. and Nedergard, J. (1994) Biochem. J. **302**, 81–86
10 Puigserver, P., Herron, D., Gianotti, M., Palou, A., Cannon, B. and Nedergaard, J. (1992) Biochem. J. **284**, 393–398
11 Goglia, F., Liverini, G., Lanni, A., Iossa, S. and Barletta, A. (1988) Biochem. Biophys. Res. Commun. **151**, 1241–1249
12 Goglia, F., Liverini, G., Lanni, A., Iossa, S. and Barletta, A. (1986) Comp. Biochem. Physiol. **85B**, 869–873
13 Lanni, A., Moreno, M., Lombardi, A. and Goglia, F. (1996) Int. J. Biochem. Cell Biol. **3**, 337–343
14 Bonet, M. L., Serra, F., Matamala, J. C., García-Palmer, F. J. and Palou, A. (1995) Biochem. J. **311**, 327–331
15 Moreno, M., Puigserver, P., Llull, J., Gianotti, M., Lanni, A., Goglia, F. and Palou, A. (1994) Biochem. J. **300**, 463–468
16 Lladó, I., Proenza, A. M., Serra, F., Palou, A. and Pons, A. (1991) Int. J. Obes. **15**, 415–419
17 Gianotti, M., Roca, P. and Palou, A. (1988) Horm. Metab. Res. **20**, 208–212
18 Picó, C., Pons, A., Gianotti, M. and Palou, A. (1991) Arch. Int. Physiol. Biochim. Biophys. **99**, 345–348
19 Lladó, I., Picó, C., Palou, A. and Pons, A. (1995) Physiol. Behav. **58**, 513–519
20 Serra, F., Bonet, L. and Palou, A. (1987) Arch. Int. Physiol. Biochim. Biophys. **95**, 263–268
21 Bradford, M. M. (1976) Anal. Biochem. **72**, 248–254
22 Wharton, D. C. and Tzagoloff, A. (1967) in Oxidation and Phosphorylation (Estabrook, R. W. and Pullman, M. E., eds.), pp. 245–250, Academic Press, Orlando
23 Gribskov, C. L., Henningfield, M. F., Swick, A. G. and Swick, R. W. (1986) Biochem. J. **233**, 743–747
24 Jacobsson, A., Stadler, U., Glotzer, M. A. and Kozak, L. P. (1985) J. Biol. Chem. **260**, 16250–16254
25 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
26 Engler-Blum, G., Meier, M., Frank, J. and Müller, G. A. (1993) Anal. Biochem. **210**, 235–244
27 Rohr, H. P. and Riede, U. N. (1973) Curr. Top. Pathol. **58**, 1–48
28 Aw, T. Y. and Jones, D. P. (1989) Annu. Rev. Nutr. **9**, 229–251
29 Heaton, G. M., Wagenvoord, R. J., Kemp, A. and Nicholl, A. (1978) Eur. J. Biochem. **82**, 515–521
30 Swick, A. G., Kemnitz, J. M., Houser, W. D. and Swick, R. W. (1986) Int. J. Obes. **10**, 241–244
31 Swick, A. G. and Swick, R. W. (1986) Am. J. Physiol. **251**, E192–E195
32 Milner, R. E., Wilson, S., Arch, J. R. S. and Trayhurn, P. (1988) Biochem. J. **249**, 759–763
33 Peachey, T., French, R. R. and York, D. A. (1988) Biochem. J. **249**, 451–457
34 Nedergaard, J., Rassmaja, A. and Cannon, B. (1984) Biochem. Biophys. Res. Commun. **122**, 1328–1336
35 Nedergaard, J. and Cannon, B. (1985) Am. J. Physiol. **248**, C365–C371
36 Nedergaard, J. and Cannon, B. (1987) Eur. J. Biochem. **164**, 681–686
37 Nedergaard, J., Jacobsson, A. and Cannon, B. (1989) in Hormones, Thermogenesis and Obesity (Lardy, H. and Stratman, F., eds.), pp. 105–116, Elsevier, New York
38 Ricquier, D., Mory, G., Bouillaud, F., Thibault, J. and Weissenbach, J. (1984) FEBS Lett. **178**, 240–244
39 Rothwell, N. J., Saville, M. E. and Stock, M. J. (1984) Biosci. Rep. **4**, 351–357
40 Trayhurn, P. and Jennings, G. (1986) Biosci. Rep. **6**, 805–810
41 Levin, I. and Trayhurn, P. (1987) Am. J. Physiol. **252**, R987–R993
42 Desautels, M. (1985) Am. J. Physiol. **249**, 99–106
43 Desautels, M., Michalska, E. and Mozaffari, B. (1990) Biochem. Cell Biol. **68**, 441–447
44 Ohno, T., Ohinata, H., Ogawa, K. and Kuroshima, A. (1994) J. Therm. Biol. **19**, 135–140
45 Wonko, N. and Desautels, M. (1988) FASEB J. **2**, A1425
46 Jacobsson, A., Mülhleisen, M., Cannon, B. and Nedergaard, J. (1994) Am. J. Physiol. **267**, R999–R1007
47 Hayashi, M. and Nagasaka, T. (1983) Am. J. Physiol. **245**, E582–E586

Received 7 May 1996; accepted 15 May 1996