

# Cold exposure induces different uncoupling-protein thermogenin masking/unmasking processes in brown adipose tissue depending on mitochondrial subtypes

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The effect of cold exposure on thermogenic parameters such as mitochondrial protein content, GDP-binding and uncoupling protein (UCP) levels in different mitochondrial fractions from rat brown adipose tissue has been investigated. Rats were exposed from 12 h to 5 days at 4 °C, and three mitochondrial fractions were isolated by differential centrifugation: the  $M_1$  fraction (1000 g), the  $M_3$  fraction (3000 g) and the  $M_{15}$  fraction (15000 g). Cytochrome *c* oxidase activity as an index of mitochondrial mass showed an increase during cold exposure. During the first 24 h of cold exposure UCP was incorporated specifically into the  $M_3$  and  $M_{15}$  mitochondrial fractions, and thereafter UCP appeared in the heaviest  $M_1$  fraction. However, specific GDP binding was increased during the first 24 h in the same way in all sub-

populations, and this increase continued up to 72 h of cold exposure. Results suggest that different molecular events are involved during acute and chronic adaptation to cold: during the first 24 h of cold acclimatization, thermogenic activity is increased by an unmasking process of the UCP binding sites in the  $M_1$  mitochondrial fraction as UCP levels were constant and GDP binding increased, but in the  $M_3$  and  $M_{15}$  fraction the increase in thermogenic activity was completely due to an increase in GDP binding induced by a specific incorporation of UCP targeted to these mitochondria. Thus thermogenic parameters change in a different way in the brown-fat mitochondrial subpopulations during cold acclimatization.

## INTRODUCTION

During cold acclimatization, brown adipose tissue (BAT) is recruited by cellular processes such as proliferation and differentiation, leading to the ability for heat production. In this adaptation, the expression of the brown-fat-specific uncoupling protein thermogenin (UCP) and its incorporation into the mitochondria is the rate-limiting factor for thermogenesis [1–4].

UCP activity is usually measured by monitoring GDP binding to isolated BAT mitochondria, as a relationship between these two parameters does exist [5,6]. In some cases differences have been detected in the ability of UCP to bind GDP, which can change rapidly in response to acute cold exposure, noradrenaline and pharmacological agents or dietary manipulations without changing UCP levels [7–11]. The GDP-binding sites can therefore be unmasked and masked, leading to thermogenic or non-thermogenic mitochondria respectively [4]. However, after 24 h cold acclimatization the incorporation of UCP into mitochondria is responsible for the increase in GDP binding [4,11]. A mitochondrial proliferation, evidenced by an increase in cytochrome *c* oxidase (COX) activity, also appears during cold acclimatization [4].

Our previous studies on the topic showed that two different pools of UCP, which have different half-lives, appeared in cultured brown-fat cells after the removal of a noradrenaline stimulus. The newly synthesized UCP pool has a shorter half-life (20 h) than the more stable pool (3 days) [12]. In addition, it has been demonstrated that dietary-induced obesity in rat causes a decrease in the UCP half-life and in the half-life of the other mitochondrial protein [13,14].

All the above results could have an explanation related to

differences within the mitochondrial population. In fact, previous works have reported that the liver mitochondrial population may be fractionated into different subpopulations and that cold exposure influences differentially these mitochondrial fractions, with a more remarkable effect on the lightest fraction [15,16].

To obtain results supporting an explanation of the above-cited hypothesis, we decided to investigate under cold exposure whether the thermogenic parameters of different mitochondrial fractions show differences in UCP incorporation rate and in masking/unmasking mechanism.

## MATERIALS AND METHODS

### Animals

Wistar female rats (3 months old; between 275 and 320 g) were used. They were housed in individual cages in a temperature-controlled room (23 °C), and the light period was 12 h light/12 h dark. Before the experiment four groups of animals were placed in a cold-room at 4 °C, for 12, 24, 72 and 120 h respectively.

Control rats were kept at room temperature (23 °C). The animals were killed by decapitation and the interscapular, cervical, axillar, perirenal and periaortic BAT depots were rapidly removed and carefully dissected free of contaminating muscle, connective tissue and white adipose tissue. All these and subsequent operations were done at ice-melting temperature.

The total BAT was weighed and homogenized in Hepes/sucrose buffer (250 mM sucrose, 1 mM Hepes, 0.2 mM EDTA; final concn. 15%, w/v) with a Teflon/glass homogenizer (10 strokes).

Different portions of the brown-fat homogenate were used for

determination of COX activity, total protein content and immunoblotting for uncoupling protein thermogenin. The rest of this homogenate was used to isolate the different mitochondrial subpopulations.

### Isolation of BAT mitochondria

The homogenate was filtered through two layers of gauze and centrifuged at 15000 *g* for 15 min at 4 °C. The hard-packed fat layer and supernatant were discarded, and the pellet, consisting of cell debris, nuclei and mitochondria, was resuspended in the original volume in the same medium. 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, each for 10 min, at 1000 *g*, 3000 *g* and 15000 *g* respectively. The pellets (called M<sub>1</sub>, M<sub>3</sub> and M<sub>15</sub> respectively) were washed with the homogenization buffer and resuspended in a minimal volume of the same washing buffer.

Protein concentration of each mitochondrial fraction was measured by the method of Bradford [17], with BSA as a standard. COX activity was measured by a spectrophotometric method [18] to monitor the changes in absorbance during the oxidation of reduced ferrocytochrome. The activity was measured at 37 °C.

### Determination of specific GDP binding to BAT mitochondrial subpopulations

Specific GDP binding to three mitochondrial fractions was measured as described previously [13], with slight modifications. The final mitochondrial pellets were resuspended in the homogenization buffer at a final concentration of 25 mg of protein/ml. Briefly, mitochondrial pellets (2 mg of protein) were incubated at 30 °C in 2 ml of buffer containing 100 mM [U-<sup>14</sup>C]sucrose (6 KBq/ml), 10 μM [8-<sup>3</sup>H]GDP (ammonium salt) (10 kBq/ml), 20 mM Tes, 1 mM EDTA and 5 μM rotenone. Non-specific binding was assessed by addition of unlabelled GDP (25 μl of 10 mM GDP). [U-<sup>14</sup>C]sucrose was used to assay intramitochondrial space. Specific and non-specific GDP binding was performed by filtration through 0.45 μm-pore filters (Sartorius).

### Immunoblotting for UCP thermogenin

Immunoblotting was carried out as previously described [12,13,19]. The source of a rabbit polyclonal antibody against rat UCP made in our laboratory is largely explained in a previous paper [13]. The specificity of the antiserum to the UCP was confirmed by immunoblotting of BAT homogenate proteins, liver homogenate proteins and purified UCP. Samples of BAT homogenates and mitochondrial fractions were denatured by addition of about 10 μl (SDS: protein, 4:1, w/w) of SDS/PAGE sample buffer (containing 62.5 mM Tris base, 4% SDS, 5% 2-mercaptoethanol, 10% glycerol and Bromophenol Blue, pH 6.8). On every gel, a rat liver homogenate (30 μg of protein) prepared in the homogenization buffer was run as a negative control. SDS/PAGE was carried out principally as described by Laemmli [20], with 30 μg for BAT homogenate and 20 μg for mitochondrial fractions per lane. Electrotransfer, blocking and development of the immunoblot were performed as described by Herron et al. [19], except that a Novablott (Pharmacia) was used by the electrotransfer and the final colour development was done with an alkaline phosphatase conjugate substrate kit containing Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl

phosphate (Bio-Rad), according to the manufacturer's instructions.

For quantitative analysis, the bands were scanned with a Bio-Image computing densitometer (Millipore) and the results in each experimental series were expressed as percentages of the level of UCP thermogenin found in the homogenate after 5 days of cold exposure.

### Microcalorimetry in different mitochondrial subtypes

Microcalorimetry measurements were performed in a 2277 Multichannel Thermal Activity Monitor (Thermometric) of the isothermal heat-conduction type working in twin-detector mode. The final mitochondrial pellets from one rat were resuspended in 250 μl of buffer (0.25 M sucrose, 1 mM MgCl<sub>2</sub>, Hepes 1 mM, pH 7.2). Mitochondrial protein (1–2 mg) was suspended in 2.5 ml of the same buffer in sterilized glass ampoules which were sealed at the same time to avoid any difference in seal relaxation, particularly important because the glass ampoules were used for samples producing low μW power outputs. After preparation, the ampoules were pre-heated under dry conditions at 37 ± 1 °C for 15 min by using a constant-temperature block in order to avoid any microcalorimeter thermostated bath disturbance. Samples were loaded into the equilibration position and left in this position for 60 min to equilibrate.

Heat-production values were calculated by using the difference between the value in μW after 30 min from the introduction of the ampoules and the baseline value during the equilibration.

## RESULTS

### Effect of cold exposure on BAT weight and protein content in different mitochondrial subtypes

As shown in Table 1, total BAT wet weight from different depots increased after 72 h and 120 h of cold exposure by approx. 0.5 g compared with the control (not cold-exposed) situation. Acute or short-term (12 and 24 h) cold exposure did not affect significantly the BAT weight. Total homogenate protein content (Table 1) increased by 20 mg after 24 h cold exposure, and after 120 h the value was twice that of control rats. Total mitochondrial protein recovered was significantly increased after 12 h cold exposure in the three mitochondrial fractions investigated.

### Effect of cold exposure on COX activity in different mitochondrial fractions

As shown in Table 2, specific COX activity was constantly increased 3-fold from 0 h to 120 h cold exposure. However, the changes in specific COX activity differed between mitochondrial fractions (Table 2). In the M<sub>1</sub> fraction the increase began at 12 h, and at 120 h was double the control values. Specific COX activity of M<sub>3</sub> and M<sub>15</sub> fractions were maximal at 72 h and 120 h respectively.

Total COX activity (Table 2) was also increased 5-fold from 0 h to 120 h of cold exposure. Total COX activity was higher in the M<sub>1</sub> fraction than in M<sub>3</sub> and M<sub>15</sub>, but the amount in all three fractions increased during cold exposure (Table 2).

### Effect of cold exposure on GDP binding and UCP levels in different mitochondrial fractions

Specific GDP binding (Table 3) was increased during cold acclimatization up to 4- or 5-fold at day 3, and showed some decrease at day 5 compared with day 3. Except for the M<sub>15</sub>

**Table 1 Effect of cold acclimatization on BAT wet weight, total and mitochondrial protein from different mitochondrial fractions**

Rats which had been exposed to cold for different periods of time were killed at the indicated time, and the wet weight and protein content of brown fat (BAT) were determined. Values represent means  $\pm$  S.E.M. of five rats per group. Mitochondrial fractions  $M_1$ , 1000 g;  $M_3$ , 3000 g;  $M_{15}$ , 15000 g. Significant differences: \* $P < 0.05$ , \*\* $P < 0.01$  for time of cold acclimatization versus control. Individual differences from mean values were assessed by Student's  $t$  test.

	Time of cold acclimatization (h)				
	0	12	24	72	120
BAT wet weight (g)	1.02 $\pm$ 0.09	0.911 $\pm$ 0.10	1.017 $\pm$ 0.02	1.564 $\pm$ 0.18*	1.406 $\pm$ 0.12*
Total protein (mg)	75.4 $\pm$ 3.77	78.5 $\pm$ 2.56	95.7 $\pm$ 2.66**	119.0 $\pm$ 6.20**	143.0 $\pm$ 4.50**
Total protein recovered (mg)					
$M_1$	10.68 $\pm$ 0.21	11.82 $\pm$ 0.35*	12.16 $\pm$ 0.16**	13.20 $\pm$ 0.82**	16.16 $\pm$ 0.17**
$M_3$	7.55 $\pm$ 0.15	8.16 $\pm$ 0.10*	8.55 $\pm$ 0.4*	9.79 $\pm$ 0.25**	11.22 $\pm$ 0.13**
$M_{15}$	3.91 $\pm$ 0.04	3.44 $\pm$ 0.15*	3.12 $\pm$ 0.03*	5.51 $\pm$ 0.09**	6.06 $\pm$ 0.12**

**Table 2 Effect of cold acclimatization on COX activity from different mitochondrial fractions**

Specific homogenate and mitochondrial and total homogenate and mitochondrial COX activities were measured in the brown fat obtained from the rats described in Table 1. Abbreviation: H, homogenate. Values represent means  $\pm$  S.E.M. from five animals per group. Units are given as  $\mu$ mol of ferrocytochrome oxidized/min. Significant differences: \* $P < 0.05$ , \*\* $P < 0.01$  for time of cold acclimatization versus control. Individual differences from mean values were assessed by Student's  $t$  test.

	Time of cold acclimatization (h)				
	0	12	24	72	120
Specific COX activity (units/mg of protein)					
H	0.47 $\pm$ 0.01	0.71 $\pm$ 0.01**	0.76 $\pm$ 0.01**	0.90 $\pm$ 0.01**	1.32 $\pm$ 0.03**
$M_1$	1.02 $\pm$ 0.021	1.12 $\pm$ 0.035**	1.12 $\pm$ 0.016**	1.85 $\pm$ 0.082**	2.29 $\pm$ 0.017**
$M_3$	0.56 $\pm$ 0.01	0.61 $\pm$ 0.01**	0.74 $\pm$ 0.01**	1.04 $\pm$ 0.03**	0.64 $\pm$ 0.01**
$M_{15}$	0.38 $\pm$ 0.01	0.83 $\pm$ 0.02**	0.74 $\pm$ 0.01**	0.54 $\pm$ 0.01**	0.54 $\pm$ 0.02**
Total COX activity (units/tissue)					
H	35.5 $\pm$ 1.2	55.9 $\pm$ 1.5**	72.60 $\pm$ 1.4**	107.5 $\pm$ 5.7**	188.6 $\pm$ 3.9**
$M_1$	11.0 $\pm$ 0.31	13.1 $\pm$ 0.47**	13.6 $\pm$ 0.17**	24.4 $\pm$ 0.77**	37.0 $\pm$ 0.45**
$M_3$	4.24 $\pm$ 0.08	5.03 $\pm$ 0.10**	6.53 $\pm$ 0.22**	10.1 $\pm$ 0.19**	7.18 $\pm$ 0.22**
$M_{15}$	1.49 $\pm$ 0.03	2.83 $\pm$ 0.07**	2.52 $\pm$ 0.05**	2.95 $\pm$ 0.07**	3.25 $\pm$ 0.06**

**Table 3 Effect of cold acclimatization on GDP binding from different mitochondrial fractions**

Specific and total GDP binding were measured in the mitochondrial brown-fat fractions obtained from the rats described in Table 1. Values represent means  $\pm$  S.E.M. from five animals per group. Significant differences: \* $P < 0.05$ , \*\* $P < 0.01$  for time of cold acclimatization versus control. Individual differences from mean values were assessed by Student's  $t$  test.

	Time of cold acclimatization (h)				
	0	12	24	72	120
Specific GDP binding (nmol of GDP/mg mitochondrial protein)					
$M_1$	0.057 $\pm$ 0.02	0.114 $\pm$ 0.02	0.171 $\pm$ 0.02**	0.214 $\pm$ 0.03**	0.189 $\pm$ 0.02**
$M_3$	0.092 $\pm$ 0.02	0.118 $\pm$ 0.01*	0.196 $\pm$ 0.03**	0.277 $\pm$ 0.04**	0.192 $\pm$ 0.02**
$M_{15}$	0.066 $\pm$ 0.01	0.133 $\pm$ 0.02*	0.161 $\pm$ 0.01**	0.376 $\pm$ 0.05**	0.214 $\pm$ 0.03**
Total GDP binding (nmol of GDP/total tissue)					
$M_1$	0.613 $\pm$ 0.212	1.35 $\pm$ 0.19*	2.08 $\pm$ 0.23**	2.85 $\pm$ 0.50**	2.63 $\pm$ 0.33**
$M_3$	0.694 $\pm$ 0.160	0.794 $\pm$ 0.130*	1.59 $\pm$ 0.33**	2.74 $\pm$ 0.37**	1.94 $\pm$ 0.15**
$M_{15}$	0.256 $\pm$ 0.050	0.462 $\pm$ 0.051*	0.443 $\pm$ 0.14**	2.08 $\pm$ 0.35**	1.27 $\pm$ 0.17**

fraction at 72 h, the increase was similar in the three fractions analysed, and no differences were found between mitochondrial subpopulations (Table 3). After cold exposure, total GDP

binding (Table 3) was different for different mitochondrial fractions, being higher in the  $M_1$  fraction than in the lightest fractions.

**Table 4 Effect of cold acclimatization on UCP content, GDP/UCP and UCP/COX ratios from different mitochondrial fractions**

Specific homogenate and mitochondrial and total homogenate and mitochondrial UCP content were measured as described in the Materials and methods section in the brown fat obtained from the rats described in Table 1. Abbreviation: H, homogenate. Values represent means  $\pm$  S.E.M. from five animals per group. The values were calculated relative to the value of brown-fat homogenate after 120 h in the cold. Significant differences: \* $P < 0.05$ , \*\* $P < 0.01$  hours of cold acclimatization versus control. Individual differences from mean values were assessed by Student's  $t$  test.

	Time of cold acclimatization (h)				
	0	12	24	72	120
Specific UCP content (% of homogenate at 120 h)					
H	14.5 $\pm$ 2.14	37.6 $\pm$ 7.70*	43.4 $\pm$ 7.06*	91.7 $\pm$ 19.0*	100
M <sub>1</sub>	77.2 $\pm$ 15.0	79.6 $\pm$ 20.6	69.7 $\pm$ 14.7	164 $\pm$ 30.0*	160 $\pm$ 28.0*
M <sub>3</sub>	45.7 $\pm$ 9.60	89.6 $\pm$ 11.4*	79.0 $\pm$ 19.0*	101 $\pm$ 21.4*	118 $\pm$ 17.5**
M <sub>15</sub>	34.4 $\pm$ 6.67	52.2 $\pm$ 5.90*	114 $\pm$ 22.3**	61.4 $\pm$ 8.40*	70.3 $\pm$ 16.2*
Total UCP content (% of homogenate at 120 h)					
H	14.1 $\pm$ 2.93	20.6 $\pm$ 4.17	29.2 $\pm$ 5.76*	79.4 $\pm$ 18*	100
M <sub>1</sub>	9.62 $\pm$ 0.79	6.76 $\pm$ 1.12	8.97 $\pm$ 1.77	21.5 $\pm$ 5.21*	30.9 $\pm$ 4.47**
M <sub>3</sub>	3.67 $\pm$ 0.74	7.65 $\pm$ 1.28*	6.53 $\pm$ 1.25*	10.2 $\pm$ 1.27**	13.5 $\pm$ 1.97**
M <sub>15</sub>	1.39 $\pm$ 0.03	1.89 $\pm$ 0.26	3.66 $\pm$ 0.93*	3.55 $\pm$ 0.74*	4.41 $\pm$ 0.92**
GDP/UCP ratio (arbitrary units)					
M <sub>1</sub>	67 $\pm$ 15	205 $\pm$ 33**	315 $\pm$ 72**	160 $\pm$ 30*	130 $\pm$ 17*
M <sub>3</sub>	250 $\pm$ 33	212 $\pm$ 58	291 $\pm$ 58	275 $\pm$ 57	200 $\pm$ 24
M <sub>15</sub>	380 $\pm$ 74	337 $\pm$ 60	208 $\pm$ 41	293 $\pm$ 26	236 $\pm$ 29
UCP/COX ratio (arbitrary units)					
M <sub>1</sub>	1.00 $\pm$ 0.25	0.95 $\pm$ 0.25	0.89 $\pm$ 0.22	0.85 $\pm$ 0.20	1.09 $\pm$ 0.20
M <sub>3</sub>	0.77 $\pm$ 0.24	1.41 $\pm$ 0.25*	1.25 $\pm$ 0.14*	1.23 $\pm$ 0.15*	1.62 $\pm$ 0.14*
M <sub>15</sub>	0.81 $\pm$ 0.20	0.76 $\pm$ 0.17	1.58 $\pm$ 0.25*	1.21 $\pm$ 0.20	1.48 $\pm$ 0.25*

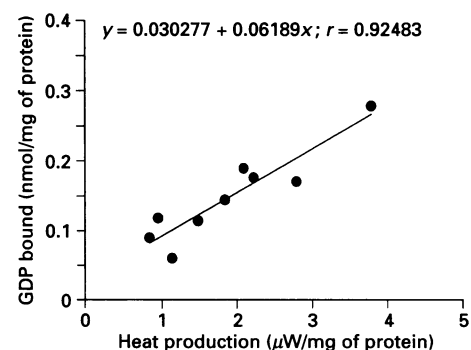
Specific UCP levels (Table 4) increased in the homogenate and in the three fractions, but this increase differed with respect to the time of cold exposure. In the M<sub>3</sub> and M<sub>15</sub> fractions UCP levels increased in the first 24 h, but it was observed that in the heaviest fraction an increase in UCP levels did not appear until 72 h of cold exposure. The UCP distribution between the mitochondrial fractions gave a similar picture in control, 72-h and 5-days-cold-acclimatized rats, with the heavy fraction having more UCP than the other fractions. However, in the transition period of 12 and 24 h of cold exposure, the picture was different, with practically the same values in all fractions. Total UCP levels (Table 4) gave practically the same pattern as did specific values.

From GDP binding and UCP levels the ratio between both parameters has been calculated (Table 4), in order to evaluate how GDP-binding sites in different mitochondrial subpopulations can be masked and unmasked during cold exposure. GDP/UCP ratio in a control situation was up to 6 times higher in the light compared with the heavy mitochondrial fraction. However, acute or short-term cold exposure for 12 and 24 h induced an increase of practically 6-fold only in the M<sub>1</sub> fraction, which was decreased to half at 72 h and 5 days of cold acclimatization.

From UCP levels and COX activity values the ratio between both parameters has been calculated (Table 4) to see whether these are specific increases in the thermogenic parameter compared with general mitochondrial parameters. The changes observed in the UCP/COX ratio are practically opposite to those on GDP/UCP; no changes were observed in the M<sub>1</sub> fraction, but significant increases were found in M<sub>3</sub> and M<sub>15</sub> fractions during cold acclimatization.

### Correlation between GDP binding and heat production in different mitochondrial fractions

In Figure 1 direct evidence is provided for a correlation between GDP binding and heat production in the three different mitochondrial fractions from rat brown adipose tissue. In Figure 1, although there is a significant correlation between GDP binding and heat production, some individual variations appear that could suggest that for future studies we need to determine other factors that could influence heat production.



**Figure 1. Correlation between GDP binding and heat production in different mitochondrial fractions from rat BAT**

GDP-binding was measured in the mitochondrial brown-fat fractions obtained from the rats described in Table 1, and from identical rats in the same conditions heat production was also measured in the different mitochondrial fractions. GDP binding and heat production were measured as described in the Materials and methods section. Each point represents an individual measurement from different mitochondrial fractions from one rat.

## DISCUSSION

The increase in BAT size and protein content in response to cold exposure agrees with previous studies [4,21]. COX activity, an index of mitochondrial mass and thus of oxidative capacity, increased during the first 24 h of cold exposure as previously described [4,22], indicating that mitochondrial proliferation starts as soon as the animals are put in the cold.

### UCP synthesis and targeting into mitochondria

UCP is synthesized in the cytoplasm and, in contrast with other mitochondrial proteins, has no leader sequence to be incorporated into the mitochondria [23,24]. Herron et al. [19] showed in brown-fat-cell cultures that after 24 h noradrenaline treatment UCP was completely incorporated into mitochondria. Our results suggest that, as soon as UCP is synthesized in the cytoplasm, it appears in the lightest mitochondrial subpopulations and the increase that appears in the total homogenate could be accounted for practically by the increase in these mitochondrial fractions. It seems that newly synthesized UCP is rapidly incorporated into the mitochondria and, at least during the first 24 h of cold acclimatization, it appears exclusively in the  $M_3$  and  $M_{15}$  fractions. Thereafter UCP appears mainly in the heaviest fraction, and small increases are found in the other subtypes of mitochondria. These molecular events can be explained in two non-exclusive ways. Firstly, possibly only  $M_3$  and  $M_{15}$  fractions were able to incorporate newly synthesized UCP, and the increase found in the heaviest fraction could be explained, as previously hypothesized [15,16], by a mitochondrial differentiation from precursors of  $M_{15}$  and  $M_3$  to heavy mitochondria, which would be accelerated after at least 24 h of cold acclimatization. Secondly, it would seem that the ability to incorporate newly synthesized UCP by the heaviest mitochondrial subtypes would appear after a delay period of cold acclimatization. Our previous studies [12] in brown-fat-cell cultures stimulated acutely or chronically with noradrenaline, which closely resembles cold acclimatization [25], showed two different pools of UCP in the brown-fat cells which have different half-lives when noradrenaline is removed. Then, the newly synthesized UCP pool has a shorter half-life (20 h) compared with the more stable pool (3 days). However, whether or not these two pools are associated with different mitochondrial subtypes was not determined. Similarly, two pools of UCP can also be identified in cold-acclimated mice [12]. Our present results showed different timing of appearance of the UCP in the different mitochondrial subtypes. Thus, different timing of UCP degradation could take place when the animals are returned to a warm temperature after acute or chronic cold exposure that could be associated with this different UCP incorporation into the different mitochondrial subtypes. Then, it could be speculated that when UCP appears in the heavy mitochondrial fraction it is more stable and is not degraded as quickly as when it appears in the lightest mitochondrial fractions. However, nothing can be currently concluded as to why UCP appears firstly in the lightest mitochondrial subpopulations, or which molecular mechanisms are involved in the incorporation of UCP into these possible precursor mitochondria, or even whether or not they are related to the different degradative pools described [12].

### Masking/unmasking processes in different mitochondrial subpopulations

GDP binding has been widely used as a method of assessing the thermogenic state of BAT mitochondria *in vitro*, and in this

study a direct correlation exists between GDP binding and heat production in different mitochondrial subpopulations. However, whether binding studies are a measure of the concentration of UCP has been extensively discussed [26–28]. We have previously shown that rats with diet-induced obesity can change GDP binding in response to fasting without any change in UCP levels, indicating a masking of the GDP-binding sites, whereas in control rats the decrease in GDP binding parallels the UCP decrease [14]. It has been previously indicated that the ability of UCP to bind GDP can rapidly change in response to cold exposure [9,11,26], and that the binding sites can therefore be unmasked. Our results provide a new insight, showing that in response to cold exposure only the UCP incorporated into the heavy mitochondria subtypes is able to unmask their GDP-binding sites, whereas the increase in GDP binding in  $M_{15}$  and  $M_3$  mitochondria is associated with an increase in UCP levels. Then, perhaps the size or maturation of the mitochondria could be related to the ability of masking or unmasking the GDP-binding sites of UCP. In the present study, on the basis of the sucrose-inaccessible space (d.p.m. of [ $^{14}$ C]sucrose per mg of protein; the means are 3652 for  $M_1$ , 5542 for  $M_3$  and 15820 for  $M_{15}$ ), there is a different volume in the three mitochondrial fractions,  $M_1 > M_3 > M_{15}$ . Other authors have suggested [15,16] that unmasking of GDP-binding sites could result from mitochondrial swelling, as indicated by changes in the volume of the mitochondrial matrix. However, Swick and Swick [9] were unable to demonstrate any changes in GDP binding associated with mitochondrial swelling. Thus it would seem that it is not the size of the heavy mitochondria which is involved in masking/unmasking processes, and it would be some specific characteristic of the subtype of mitochondria which confers the ability to mask or unmask UCP, leading to non-thermogenic or thermogenic mitochondria.

Our present results in BAT indicate that the lightest mitochondria are more unmasked (see their higher GDP/UCP ratios) than heavy mitochondria. When UCP was related to COX activity (i.e. UCP levels per mitochondrial mass), cold induced in  $M_3$  and  $M_{15}$  fractions an increase in UCP/COX ratio, suggesting that the capacity to uncouple the proton gradient is higher in lighter mitochondrial subtypes compared with heavy mitochondria. This is in good agreement with previous results showing that in the liver the lightest mitochondrial fraction is less coupled than the heaviest one [15]. However, the heaviest mitochondria have a higher total thermogenic activity (GDP binding) and capacity (UCP levels) compared with the other mitochondria, which contribute a smaller percentage to heat production in BAT during cold acclimatization.

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