Biogenesis of thermogenic mitochondria in brown adipose tissue of Djungarian hamsters during cold adaptation

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After cold exposure, cytochrome *c* oxidase (COX) activity increased about 2.5-fold within 2 weeks in the brown adipose tissue (BAT) of Djungarian hamsters. The mRNAs for COX subunits I and III and the 12 S rRNA, encoded on mitochondrial DNA (mtDNA), as well as mRNAs for COX subunits IV, Va and mitochondrial transcription factor A, encoded in the nucleus, were unchanged when expressed per unit of total tissue RNA. However, since total tissue RNA doubled per BAT depot, while total DNA remained unchanged, the actual levels of these transcripts were increased within BAT cells. In contrast, the abundance of mRNA for uncoupling protein was increased 10-fold, indicating specific activation of this gene. In addition, the maximal rate of protein synthesis analysed in a faithful *in organello* system was increased 2.5-fold in mitochondria isolated from BAT after 7 days of cold exposure. We conclude from these data that the biogenesis of thermogenic mitochondria in BAT following cold adaptation is achieved by increasing the overall capacity for synthesis of mitochondrial proteins in both compartments, by increasing their mRNAs as well as the ribosomes needed for their translation. In addition, the translational rate for COX subunits as well as all other proteins encoded on mtDNA is increased. Thus the pool of subunits encoded on mtDNA required for assembly of respiratory chain complexes is provided. By comparison with other models of increased mitochondrial biogenesis, we propose that thyroid hormone (generated within BAT cells by 5′-deiodinase, and induced upon sympathetic stimulation), which is a well known regulator of the biogenesis of mitochondria in many tissues, is also the major effector of these adaptive changes in BAT.

INTRODUCTION

Brown adipose tissue (BAT) is an important site of non-shivering thermogenesis in mammals [1]. In this process, the protonmotive force generated by electron flow via the mitochondrial respiratory chain is dissipated into heat by the presence of a unique polypeptide, the uncoupling protein (UCP), without generation of ATP [2,3]. While BAT is important in all newborn mammals, in many small species the capacity for non-shivering thermogenesis remains functional throughout life. Especially in rodents, seasonal adaptation as well as acute exposure to a cold environment cause an adaptive increase in the capacity for this process in BAT. This is achieved by an increase in mitochondrial mass as well as activation of genes encoding several proteins relevant for thermogenesis, including the UCP [4,5]. Much attention has focused on the latter process, since the UCP is an example of a gene that is expressed in a tightly controlled, cell-specific manner by permissive factors present only in BAT cells [6,7]. At the same time, much progress has been made in our understanding of how the neurohormonal signals acting on the BAT cells during cold adaptation are converted into signals leading to activation of the UCP gene [8].

In contrast, the mechanisms leading to increased biogenesis of thermogenic mitochondria are only poorly understood. This process is complicated by the fact that two genomes, i.e. the nuclear chromosomes and mitochondrial DNA (mtDNA), are involved. mtDNA encodes 13 proteins, which are essential subunits of the large membrane complexes of oxidative phosphorylation, two rRNAs and all the tRNA compounds required for supporting protein synthesis inside the mitochondrial compartment [9]. We have previously studied the expression of nuclear and mitochondrial genes during the synthesis of cytochrome *c* oxidase (COX) [10–12]. This enzyme is composed of three subunits encoded on mtDNA and 10 subunits encoded in the nucleus [13]; it thus represents an excellent model system to study gene regulation and co-ordination between the two genomes during the biogenesis of mitochondria.

In the present study we have investigated the mechanisms leading to increased synthesis of COX in BAT of the Djungarian hamster during cold adaptation. This species is especially suited for this purpose, since it exhibits an extraordinary capacity for cold adaptation due to the low environmental temperatures prevalent in its natural habitat in winter [14]. Animals were cold exposed for up to 4 weeks, and then COX activity, mRNA levels for representative subunits encoded on the two genomes and the translational capacity of isolated mitochondria were analysed in BAT.

MATERIALS AND METHODS

Animals and experimental design

Djungarian hamsters (*Phodopus sungorus*) were bred and raised in the laboratory as described previously [15]. After weaning, hamsters were separated in single cages and kept at 23 °C (thermoneutrality) in a light/dark cycle with 16 h of light per day. They received hamster breeding chow (Hamsterzuchtdiät 7014, Altromin, Germany) and drinking water *ad libitum*. Both male and female hamsters were used for cold acclimation experiments.

Hamsters were exposed to 5 °C for increasing periods of time, ranging from 1 h to 28 days; the light/dark cycle was kept the

Abbreviations used: BAT, brown adipose tissue; COX, cytochrome *c* oxidase; mtDNA, mitochondrial DNA; mtTFA, mitochondrial transcription factor A; T_3, thyroid hormone (3,5,3′-tri-iodothyronine); UCP, uncoupling protein.

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same. In a first experiment, COX activity was measured in individual BAT samples from six hamsters per time point. Next, two separate experiments were performed with three animals per time point. BAT was prepared at each time point and pooled for isolation, quantification and analysis of total protein, RNA and DNA. Day 7 of cold exposure was then chosen for detailed investigation, since the increase in COX activity was found to be linear over time around this time point. Six (control) hamsters were kept at 23 °C, and six hamsters were exposed to 5 °C for 7 days. BAT was prepared from these animals, total RNA was extracted and quantified and expression of COX genes was analysed more precisely by slot blot analysis. Mitochondria were isolated after 1, 3 and 7 days for measurement of their capacity for protein synthesis. Finally, gene expression was analysed by Northern blotting in four animals after 4 h of exposure to 5 °C in order to investigate short-term effects.

Tissue preparation

Distinct depots of BAT were excised from the interscapular and axillary locations, representing approx. 60 % of the total BAT mass of Djungarian hamsters [4]. BAT mass decreases during cold exposure as a result of lipolysis, while the number of cells in individual BAT depots remains unaffected [16]. Therefore all data obtained in the present study were calculated and expressed per total dissected BAT mass. For RNA and DNA isolation and quantification, and for protein determination, BAT was weighed and immediately frozen in liquid nitrogen. Total BAT was powdered in liquid nitrogen for further analysis. For measurement of COX activity and isolation of mitochondria, freshly dissected BAT from individual animals was used.

RNA isolation and blotting

Total RNA was isolated from BAT powder by acidic guanidinium thiocyanate/phenol/chloroform extraction [17]. The final RNA pellet was dissolved in water and the concentration was quantified by measuring its absorbance at 260 nm. Steady-state levels of nuclear and mitochondrial mRNAs and rRNAs were analysed on Northern blots and by slot blotting.

For slot blots, the integrity of the RNA was first checked in 0.8 % agarose gels stained with ethidium bromide. Different amounts of each RNA sample (0.125, 0.25, 0.5 and 1.0 μg) were then dissolved in 100 μl of water, denatured at 70 °C for 15 min with 300 μl of 18 % formaldehyde/10 × SSC, and blotted on to nylon membranes (Hybond N; Amersham Buchler, Braunschweig, Germany) with a slot blot device (Minifold II; Schleicher & Schuell, Dassel, Germany). For Northern blots, RNA was electrophoresed in 1 % agarose/0.66 M formaldehyde gels with 20 mM Mops, 5 mM sodium acetate and 1 mM EDTA (pH 7.0). RNA was blotted to a Hybond N nylon membrane by capillary transfer. Equal loading was confirmed by ethidium bromide fluorescence and final hybridization of blots with a 28 S rRNA probe [10]. RNA was cross-linked on Northern and slot blots with UV light.

DNA isolation and blotting

Genomic DNA was extracted from BAT powder by proteinase K digestion and phenol/chloroform extraction [18]. The DNA yield was quantified by measuring the absorbance of the resulting DNA solution at 260 nm. For quantification of mtDNA, genomic DNA was digested with *Nco*I, which was found to linearize hamster mtDNA, which then appears as a 16 kb band on agarose gels [19]. A 2 μg sample of digested DNA was size fractionated on a 0.4 % agarose gel for 20 h at 4 °C and blotted to nitrocellulose membranes by capillary transfer (Southern blot [20]). Blots were hybridized to a mitochondrial 12 S rRNA cDNA-probe.

Hybridization of DNA and RNA blots

All blots were prehybridized for 2 h and hybridized overnight at 42 °C in formamide-containing buffers; cDNA probes labelled to high specific radioactivities by the random priming method were used [18]. A cDNA for human mitochondrial transcription factor A (mtTFA) was cloned in our laboratory using the published sequence [20], reverse transcription and PCR, and the full length was sequenced. A cDNA for UCP was kindly provided by Dr. Daniel Ricquier. All other probes and hybridization conditions were described in detail previously [10,12].

Measurement of COX activity

COX (EC 1.9.3.1) activity was assayed polarographically in homogenates of freshly excised BAT samples [21]. Protein concentrations were determined by the method of Bradford [21a] using BSA as a standard.

Isolation of mitochondria

Freshly dissected BAT (approx. 1 g) was minced and homogenized in a 25-fold volume of ice-cold isolation medium containing 250 mM sucrose, 10 mM Tes (pH 7.2), 1 mM EGTA and 5 mg/ml BSA. This was done with four to five strokes in a glass/Teflon Potter–Elvehjem homogenizer. The homogenate was filtered through nylon gauze and mitochondria were isolated in four centrifugation steps beginning with 8500 ***g*** for 10 min at 4 °C. The supernatant was discarded and the walls of the tube were carefully wiped to remove adhering fat. The resuspended pellet was centrifuged at 700 ***g*** to spin down cell debris, nuclei, erythrocytes, etc. The supernatant was decanted into a fresh tube followed by two additional centrifugation steps at 8500 ***g***. After the last centrifugation, mitochondria were resuspended in 75 μl of protein synthesis medium, yielding a protein concentration of approx. 25 mg/ml.

Analysis of mitochondrial protein synthesis

The protein synthesis medium was based on procedures described previously by Desautels and Dulos [22] and McKee et al. [23], and contained 50 mM KCl, 2 mM $MgCl_2$, 1 mM Na_2HPO_4, 10 mM Hepes (pH 7.0), 1 mM EGTA, 10 mM pyruvate, 3 mM malate, 3 mM ATP, 5 μg/ml oligomycin, 0.1 mg/ml cycloheximide, 20 μM methionine, 20 mM glutamate, 0.3 mM of each of the other proteinogenic amino acids, 5 mg/ml BSA and 4 mg/ml mitochondrial protein. Controls contained 0.1 mg/ml chloramphenicol. Incubations were carried out in a 100 μl total volume at 30 °C, and were started by addition of 20 μCi of [^{35}S]methionine. After 0, 20, 40 and 60 min of incubation, 10 μl aliquots were removed from the assays, added to 1 ml of 10 % (w/v) trichloroacetic acid containing unlabelled methionine (5 mM) and incubated for 20 min at 70 °C to hydrolyse labelled aminoacyl-tRNAs. Precipitated proteins were collected by centrifugation at maximal speed in a microcentrifuge for 10 min. Initially, total incorporated radioactivity was measured by liquid scintillation counting. However, in our hands the reproducibility of these measurements was unsatisfactory. Thus proteins were resuspended by sonic disruption (Branson Sonifier B-12) in SDS sample buffer (62.5 mM Tris/HCl, pH 6.8, 8 % SDS, 0.1 M

dithiothreitol, 10% glycerol, 0.1% Bromophenol Blue), dissociated for 1 h at room temperature and separated in an SDS/12.5%-polyacrylamide gel containing 6 M urea. After staining, gels were cut into separate lanes, proteins were extracted with Solvable tissue solubilizer (NEN, Dreieich, Germany) and radioactivity incorporated into mitochondrial proteins was determined by liquid scintillation counting. Radioactivity counted at time zero was subtracted from all values, and incorporation was expressed as pmol of methionine/mg of mitochondrial protein.

Incorporation of [^{35}S]methionine was linear for at least 40 min, and for up to 60 min in most experiments. Rates of protein synthesis were calculated from the value obtained at 40 min, and expressed as pmol of methionine/min per mg of mitochondrial protein.

Fluorographic analysis of labelled proteins

In order to assign labelled proteins to mitochondrial gene products, gels were prepared for fluorography by soaking in EN^{3}HANCE scintillant (NEN). Dried gels were exposed to X-ray film (Kodak Royal X-Omat) at −70 °C for 5–7 days. Translation products detected by fluorography were identified as mitochondrial gene products as judged by their electrophoretic mobility and comparison with similar band patterns obtained from HeLa cells [24].

Statistical analysis of data

All values are given as means ± S.E.M. Student's t test was used to evaluate statistical significance, and $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Following acute cold exposure, the total BAT wet mass decreased considerably over time due to lipolysis of the endogenous fat stores (Table 1) [16,25]. Thus we decided to express all parameters per total BAT depots in order to obtain results representing the true changes in single BAT cells. This treatment of data is valid, since the DNA content of the tissue remained stable and therefore no cell proliferation or loss occurred, at least during the first 7 days (Table 1). COX activity and BAT protein both increased linearly during the first 2 weeks of cold exposure (Figure 1; Table 1), with a 50% increase at day 7. Since the specific activity of COX per mg of mitochondrial protein remains unchanged under these conditions ([16]; M. Klingenspor, unpublished work), the same kinetics would also be obtained for total mitochondrial protein. In addition, total tissue RNA accumulated over time (Table 1). It should be noted that very similar values were found in a similar experimental series with six individual determinations per time point, which will be presented elsewhere (M. Klingenspor, unpublished work).

Table 1 Effect of cold exposure on total BAT mass and on DNA, RNA and protein content expressed per total BAT of the animals

Results are means ± range ($n = 2$) of two separate experiments with pooled tissue from three animals per time point. BAT was isolated, weighed and pooled, and DNA, RNA and protein were isolated and quantified as described in the Materials and methods section.

Duration of cold exposure	Total BAT mass (mg)	Total DNA (μg/BAT)	Total RNA (μg/BAT)	Total protein (mg/BAT)
Control	818 ± 6	612 ± 57	141 ± 28	37 ± 2
1 h	670 ± 126	494 ± 66	126 ± 22	36 ± 10
2 h	711 ± 130	487 ± 35	140 ± 1	37 ± 0
4 h	731 ± 191	633 ± 140	168 ± 30	42 ± 4
8 h	634 ± 105	696 ± 52	238 ± 52	38 ± 1
12 h	555 ± 135	491 ± 5	166 ± 10	34 ± 1
24 h	479 ± 73	608 ± 42	209 ± 32	33 ± 4
3 days	411 ± 72	695 ± 47	263 ± 38	41 ± 2
7 days	372 ± 14	672 ± 47	339 ± 27	45 ± 5
14 days	478 ± 112	860 ± 73	407 ± 2	63 ± 3
28 days	483 ± 115	801 ± 255	403 ± 97	64 ± 7

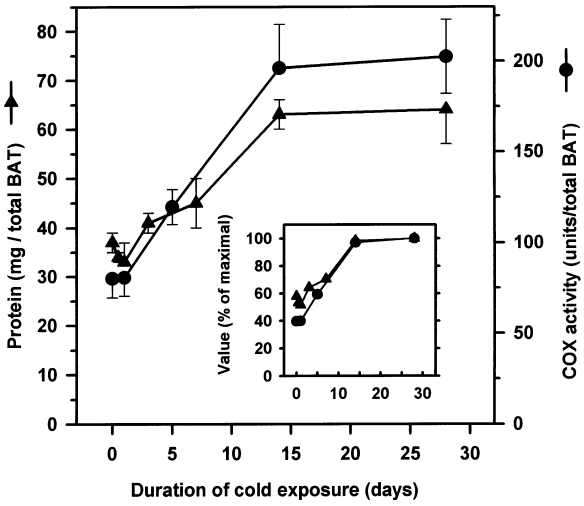

Figure 1 COX activity and total tissue protein in BAT during cold adaptation

Results are means ± S.E.M. for COX activity ($n = 6$) and means ± range of two independent experiments with the pooled BAT of three animals for total protein (see Table 1). Results are expressed per total BAT of the animals in order to correct for the decrease in BAT mass (see Table 1 and text). The inset shows the same values expressed as a percentage of the maximal value measured after 4 weeks.

No changes in the abundance of mtDNA were observed during the first 2 weeks of cold exposure (Figure 2). After 4 weeks, however, some increase in mtDNA was observed. Ethidium bromide staining of the gel showed that equal amounts of DNA had been loaded on to the lanes in Figure 2 (results not shown).

Levels of mRNAs for COX subunit I (mitochondrial), COX III (mitochondrial), COX Va (nuclear), COX IV (nuclear; results not shown) and mtTFA (nuclear), and of mitochondrial 12 S rRNA, were analysed in a first approach by Northern blotting of pooled samples from three animals per time point. No large changes were observed throughout the course of the experiment (Figure 3). Only at 4 weeks were distinct increases in some of these transcripts detected. Hybridization with a probe for mtTFA demonstrated the presence of two transcripts with sizes of about 1.5 kb (major band) and 2.0 kb (minor band), in accordance with sizes reported previously [20]. Hybridization with a 28 S cytosolic rRNA probe showed that the lanes had been loaded with equal amounts of RNA. Similar results were obtained in a second, independent, experimental series (not shown).

In order to exclude the possibility that small but significant increases in transcript amounts had escaped our notice, BAT from animals exposed to cold stress for 7 days was analysed in more detail. We chose this time point because the linear increase

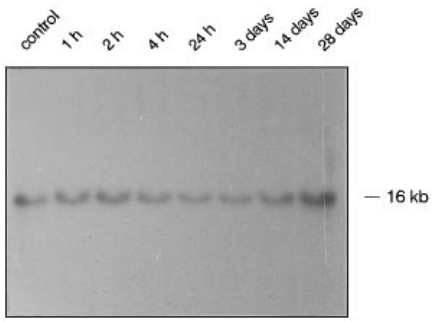

Figure 2 mtDNA levels in BAT during cold adaptation

Animals were cold-adapted for the time periods shown. Total tissue DNA (2 μg) was digested with *Nco*I, size fractionated by agarose gel electrophoresis, transferred to a nitrocellulose membrane and probed with a cDNA for mitochondrial 12 S rRNA.

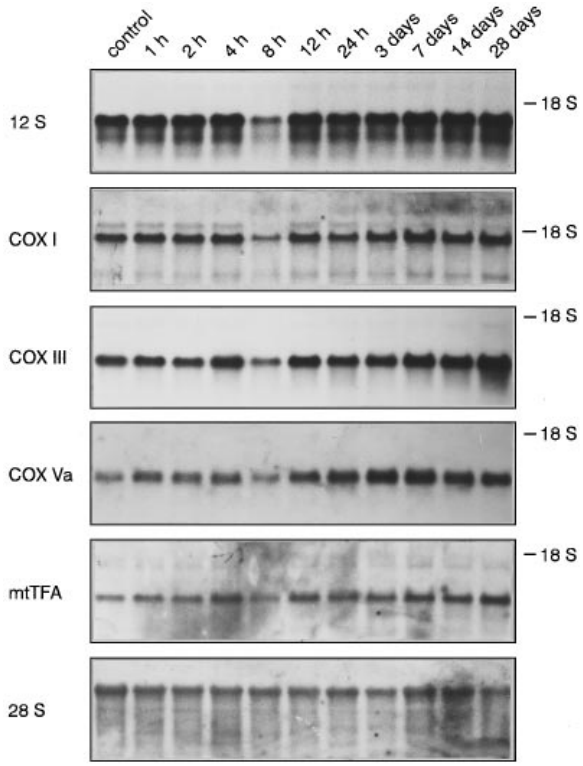

Figure 3 Northern blot analysis of mRNAs for COX subunits I, III and Va, mtTFA mRNA and 12 S rRNA in BAT during cold adaptation

Animals were cold-adapted for the time periods shown. RNA (10 μg) from pooled BAT of three animals per time point was size fractionated by agarose gel electrophoresis, transferred to a nylon membrane and probed consecutively with radioactively labelled cDNAs. The position of 18 S rRNA relative to specific bands is indicated. The blot was also probed with a cDNA for 28 S rRNA in order to confirm that equal amounts of total RNA had been blotted. The results are representative of two independent experiments.

in COX activity indicated maximal synthesis rates (Figure 1). Slot blot analysis confirmed the results presented in Figure 3, i.e. no significant increase in the transcripts relative to total RNA had occurred after 7 days in cold-adapted BAT. However, total RNA had increased considerably in this tissue, from 326 ± 31 μg/BAT to 583 ± 43 μg/BAT ($P < 0.001$; $n = 6$). Thus the densitometric values derived from slot blots were multiplied by the value of total RNA per BAT in order to obtain values representing the actual levels of the transcripts in the BAT cells (Table 2). The mRNA for UCP had increased approx. 10-fold within the same time period, indicating that this gene was specifically activated, as expected [3].

Since a specific increase in mitochondrial mRNAs has been reported within the first 1 h of cold exposure in BAT of rats [26], RNA from four animals exposed to 4 °C for 4 h was analysed by Northern blotting. No significant changes in the abundance of COX III mRNA or 12 S rRNA were found (results not shown).

The increase in COX activity (Figure 1) seemed to be caused simply by an unspecific increase of mitochondrial and nuclear transcripts encoded by the representative genes chosen by us within the background of an overall increased cellular RNA pool. We next asked whether any post-transcriptional mechanisms are involved in the increased synthesis of thermogenic mitochondria during cold acclimation. Mitochondria were therefore isolated from BAT and their protein synthetic capacity was quantified by measuring the amount of labelled protein generated *in organello*.

Proteins labelled *in organello* by [^{35}S]methionine were separated by SDS/PAGE and their identity with the 13 products encoded by mtDNA was confirmed by estimating their molecular masses as well as by comparison of the band pattern with previously published gels from HeLa cells (Figure 4) [24]. Protein synthesis rates were calculated from the radioactivity incorporated into these proteins; they were linear in our assay system up to 60 min and could be completely blocked by chloramphenicol (Figures 4 and 5). Protein synthesis rates calculated from values obtained by counting radioactivity in total precipitable material were approx. 10-fold higher, and similar to values obtained by this method reported by Desautels and Dulos [22] for mouse BAT mitochondria (results not shown).

The incorporation rate of [^{35}S]methionine was markedly higher in mitochondria isolated from BAT after 7 days of cold acclimation of the animals. Protein synthesis rates were 0.64 ± 0.09 pmol of methionine/h per mg of protein in warm-adapted animals and 1.63 ± 0.33 pmol of methionine/h per mg of protein in cold-adapted animals, i.e. the translational capacity was increased by a factor of 2.5 ($P < 0.05$). In contrast, no differences were found between mitochondria isolated from warm- and cold-acclimated BAT after 1 or 3 days of cold exposure (results not shown).

DISCUSSION

Our results indicate that at least two mechanisms are responsible for the increase in COX activity, which is representative of total mitochondrial protein, during cold adaptation in BAT. First, there is an increase in total RNA per BAT depot, but the amounts of mRNAs for COX subunits encoded on nuclear and mtDNA, as well as of mitochondrial 12 S rRNA, are increased in constant proportion to the rise in total RNA. Thus the overall capacity for synthesis of COX subunits is increased in both the cytosolic and the mitochondrial compartments. Secondly, the activity of the mitochondrial translation machinery is specifically stimulated. At the end of these adaptive processes, which last about 2 weeks, COX activity has increased by a factor of 2.5 (Figure 1). This increased respiratory capacity, accompanied by a pronounced stimulation of UCP gene expression (Table 2), elevates the capacity of BAT for non-shivering thermogenesis.

In order to study the two mechanisms of COX synthesis in more detail, day 7 of cold exposure was chosen for more extensive analysis, when the total RNA content and thus also all

Table 2 Levels of COX subunit mRNAs, mitochondrial 12 S rRNA and UCP mRNA in BAT after 7 days of cold adaptation

Results are means ± S.E.M. ($n = 6$) of individual densitometric values obtained from autoradiograms of slot blots, corrected using the 28 S signal of the same blot and multiplied by the total RNA content of the individual samples. After this treatment, derived values for warm-adapted animals were set to 100 densitometric units. RNA was blotted in four dilutions (two-fold steps) and care was taken that the signal was in the linear range of intensity over blotted RNA. Significant differences from warm-adapted animals: $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.0001$.

	RNA level (units)					
	COX I	COX III	12 S rRNA	COX IV	COX Va	UCP
Warm adapted (23 °C)	100 ± 14	100 ± 10	100 ± 14	100 ± 16	100 ± 18	100 ± 19
Cold adapted (5 °C)	195 ± 23**	164 ± 24*	182 ± 18**	261 ± 35**	198 ± 19**	938 ± 81***

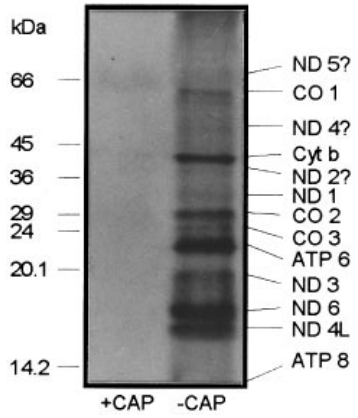

Figure 4 Fluorography of proteins labelled *in organello* by mitochondria isolated from BAT

Mitochondria were incubated for 60 min in the presence of [^{35}S]methionine, and labelled proteins were precipitated, size fractionated by SDS/PAGE, treated for fluorography and exposed to X-ray film. Bands were assigned names according to the known molecular masses of proteins encoded by human mtDNA as well as published band patterns. Mitochondria treated with chloramphenicol (+CAP) were used as a control. Abbreviations of mitochondrial proteins: ND 1–6: subunits 1–6 of NADH dehydrogenase; CO 1–3, subunits of COX; Cyt b, cytochrome *b*; ATP 6/8, subunits 6 and 8 of ATP synthase. Some bands, which were only weakly labelled (consistent with previous work [24]), are marked in the Figure with a question mark.

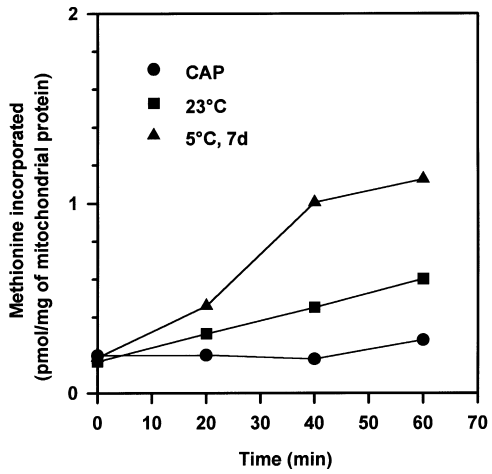

Figure 5 Incorporation of [^{35}S]methionine into protein by mitochondria isolated from BAT after 7 days of cold adaptation

Mitochondria were incubated for the indicated times. Labelled protein was precipitated, size fractionated by SDS/PAGE and dissolved, and incorporation of radioactivity was determined by liquid scintillaton counting. ●, Mitochondria treated with chloramphenicol (CAP) as a control; ■, mitochondria isolated from warm-adapted animals; ▲, mitochondria isolated from cold-adapted animals.

COX transcripts under investigation have approximately doubled per BAT cell (Tables 1 and 2). Such an increase in total RNA without specific changes in transcripts, derived from two genomes, is far from being trivial. It necessitates a co-ordinated increase in the activities of nuclear RNA polymerases I and II, together with all of the basic transcription factors necessary for transcription of genes encoding proteins targeted to mitochondria, and the activity of mitochondrial polymerase. Two putative nuclear transcription factors (NRF-1/NRF-2), which regulate transcription of nuclear genes encoding mitochondrial proteins by binding to highly conserved promoter sequences, have been described [27,28]. Mammalian mitochondrial polymerase, on the other hand, needs only one additional protein for efficient and correct initiation of transcription *in vitro*, i.e. mtTFA, which is probably also responsible for regulation of mitochondrial transcription *in vivo* [29,30]. However, the mtTFA gene was obviously activated in concert with all the other genes being investigated here (Figure 3), since we found no specific changes in mtTFA transcript levels. Interestingly, the mtTFA gene is tightly controlled by NRF-1, both in transfected cells and in *in vitro* transcription assays [31]. Thus the biogenesis of thermogenic mitochondria in BAT may turn out to be a good model system to test which nuclear factors play a physiological role in the concerted activation of nuclear genes encoding mitochondrial proteins as well as in the co-ordination between the two genomes.

Adding even more complexity, both classes of transcripts, i.e mitochondrial mRNAs and a mitochondrial rRNA, which are all encoded on the heavy strand of mtDNA downstream of the heavy strand promoter, were up-regulated to the same extent in the mitochondrial compartment. In contrast, specific increases in mitochondrial mRNA encoding COX II and in 16 S rRNA were reported in rat liver after cold exposure [26]. However, in the same study no long-term changes in these transcript levels relative to tissue RNA were found in BAT, supporting our findings.

In accordance with results from many other systems (e.g. [11,12,26]), no changes in the copy number of mtDNA were observed in our model (Figure 2), at least not during the period of maximal COX synthesis (the first 2 weeks). This indicates that up-regulation of mtDNA levels does not play a major role in increasing the amounts of mitochondrial transcripts, and increases in mtDNA and mitochondrial RNAs observed at late

stages are coincidental rather than indicative of a causal relationship ([32], but see also [33]).

In addition to the unspecific increase in the protein biosynthetic capacity in both compartments, the translational activity of mitochondria was specifically stimulated 2.5-fold on cold adaptation (Figure 5). This cannot be explained simply by higher amounts of RNA/mg of protein of isolated mitochondria used in the assay. After 7 days COX activity per BAT, and thus also mitochondrial protein/BAT, had increased by 50 % (Figure 1). At the same time, total RNA/BAT had increased by 100 %, including mitochondrial mRNAs and mitochondrial 12 S rRNA (Table 2). Thus mitochondria isolated from cold-adapted animals contained up to 50 % more RNA/protein compared with mitochondria from control hamsters. Protein synthesis rates, however, were increased to 250 % of controls. Leung and McKee [34] have shown a similar stimulation of mitochondrial translational activity in rat heart following thyroid hormone (T_3) injection (see below), and Desautels and Dulos [22] demonstrated decreased mitochondrial translation rates in BAT of mice following fasting. We propose that, once again, cold adaptation in BAT will be a useful model to study the regulation of this process *in vivo*, with special regard to putative key factors involved in initiation and elongation of mitochondrial translation [35,36].

We decided to study specifically the process of mitochondrial, and not cytosolic, translation because there is evidence that the synthesis of proteins encoded on mtDNA may not only be permissive, but limiting for the assembly of the multisubunit complexes of the respiratory chain. Nelson and co-workers [37] have shown that T_3 application to hypothyroid rats increased the abundance of all proteins encoded on mtDNA, but accumulation of only a few nuclear-encoded gene products was found, among these COX subunits IV, Va-c and VIa-c [11]. In the work of Leung and McKee [34], cytoplasmic translation rates for mitochondrial proteins were increased marginally, whereas the mitochondrial translation rate was increased rapidly and dramatically in rat hearts following T_3 injection. Thus stimulation of mitochondrial protein synthesis seems to be necessary to provide the pool of subunits encoded on mtDNA which is required for the assembly of functioning respiratory chain complexes.

Finally, the intracellular cascade between well known changes in physiological signals following cold adaptation and expression of mitochondrial genes remains to be elucidated. However, an increase in sympathetic tone seems to be the primary stimulus for initiation of all adaptive events in BAT cells [38]. Unilateral denervation of BAT almost completely inhibits the increase in COX activity following cold exposure [39]. At the same time, denervation blunts the cold-induced activation of BAT 5′-deiodinase that is responsible for the intracellular conversion of circulating thyroxine into the active form, T_3 [40,41]. This hormone acts mostly through stimulation of transcription and is one of the most potent stimulators of mitochondrial biogenesis [11,42]. Thus one may speculate that T_3 generated within BAT cells following sympathetic stimulation is the key effector stimulating expression of mitochondrial genes in concert with other genes relevant for thermogenesis [43]. Moreover, the same T_3 may be liberated from stimulated BAT cells [44] and may increase the expression of mitochondrial genes in other tissues, e.g. in liver [26]; however, this does not seem to be a major site of thermoregulatory heat production.

This speculation is supported by many remarkable similarities between cold adaptation and hyperthyroidism, not only on the whole animal level with regard to metabolic rate but also when looking at mitochondrial biogenesis on the molecular level. T_3 increases COX synthesis, and does so specifically in liver and muscle [11], whereas in the heart the specific activity of COX remains unchanged, but within considerably enlarged myocytes [12]. On the other hand, cold adaptation causes increased accumulation of COX in BAT ([16]; the present study), but also in liver [45] and muscle [46]. In the study of Bourhim et al. [45], even hypertrophy of the heart was reported after cold exposure, emphasizing again how increased circulating T_3 may act physiologically on other organs. Such an enlargement of the heart may help to increase cardiac output during blood flow redistribution in non-shivering thermogenesis [47].

Thus BAT and the heart seem to use similar mechanisms in both situations, namely an increase in total RNA without changes in relative amounts of transcripts coding for COX subunits (the present study; [12]) as well as a specific stimulation of mitochondrial translational activity (the present study; [34]). In liver and muscle, on the other hand, specific increases in COX mRNAs were found after T_3 injection [11], comparable with the specific increase in the amounts of mitochondrial transcripts in the liver following cold adaptation [26].

In conclusion, T_3 generated locally may be the major effector of stimulation of mitochondrial biogenesis in a specialized tissue such as BAT following sympathetic stimulation. At the same time, increased circulating hormone levels may stimulate this process in other tissues, such as muscle, liver and heart. Depending on the cell type, however, mitochondrial biogenesis seems to be achieved by two possible mechanisms: either by specifically increasing the amounts of transcripts encoding mitochondrial proteins, as in liver and muscle, or by increasing all mRNAs together with ribosomes in both compartments, but at the same time also increasing the translational capacity inside mitochondria, This latter mechanism, which appears to occur in BAT and the heart, will provide the pool of subunits required for the assembly of respiratory chain complexes.

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