Co-ordinate decrease in the expression of the mitochondrial genome and nuclear genes for mitochondrial proteins in the lactation-induced mitochondrial hypotrophy of rat brown fat

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The relative abundance of the mitochondrial-encoded mRNAs for cytochrome *c* oxidase subunit II and NADH dehydrogenase subunit I was lower in brown adipose tissue (BAT) from lactating rats than in virgin controls. This decrease was in parallel with a significant decrease in mitochondrial 16 S rRNA levels and in the relative content of mitochondrial DNA in the tissue. BAT from lactating rats showed lowered mRNA expression of the nuclear-encoded genes for the mitochondrial uncoupling protein, subunit IV of cytochrome *c* oxidase and the adenine nucleotide translocase isoforms ANT1 and ANT2, whereas mRNA levels for the ATP synthase β -subunit were unchanged. However, the relative

content of this last protein was lower in BAT mitochondria from lactating rats than in virgin controls. It is concluded that lactation-induced mitochondrial hypotrophy in BAT is associated with a co-ordinate decrease in the expression of the mitochondrial genome and nuclear genes for mitochondrial proteins. This decrease is caused by regulatory events acting at different levels, including pre- and post-transcriptional regulation. BAT appears to be a useful model with which to investigate the molecular mechanisms involved in the co-ordination of the expression of the mitochondrial and nuclear genomes during mitochondrial biogenesis.

INTRODUCTION

The biogenesis of the mitochondrial respiratory-chain/oxidative-phosphorylation machinery in mammals depends on two separate genetic systems: the nuclear and the mitochondrial genomes. In the last few years, the whole mitochondrial DNA (mtDNA) sequence of various mammalian species has been described, and the basic mechanisms of replication and transcription are well known. On the other hand, several nuclear genes for proteins of the respiratory-chain/oxidative-phosphorylation system have been cloned and characterized (for reviews see [1,2]). The mitochondrial genome and nuclear genes for mitochondrial proteins must be expressed co-ordinately in order to build the enzymic complexes responsible for ATP production in the mitochondria. However, the molecular mechanisms for the co-ordinate expression of these different genetic systems are poorly known in mammals, especially *in vivo*. This is probably due to the limited number of experimental models in which gene expression leading to mitochondrial biogenesis can be modified in response to physiological or environmental adaptations.

Brown adipose tissue (BAT) is responsible for non-shivering thermogenesis, and it is probably the mammalian tissue in which the mitochondrial content is most modified in response to different environmental and physiological changes. When the thermogenic activity of BAT is increased, as for example in animals exposed to the cold, there is not only an increase in the gene expression of BAT-specific mitochondrial uncoupling protein (UCP), but also a rise in overall mitochondrial biogenesis [3]. However, although the total mitochondrial content of BAT from cold-exposed rats is high, the relative content of mitochondrial

protein with respect to the total protein of the tissue remains unaltered, due to the parallel hyperplasia of the whole tissue [4]. A much more specific effect on the BAT mitochondrial protein content occurs in animals adapting to physiological situations associated with lowered non-shivering thermogenesis. Together with a decrease in UCP gene expression, the relative mitochondrial content of BAT is dramatically lowered when mice or rats are suddenly exposed to thermoneutral temperatures [5], or to acute [6] or chronic underfeeding [7], as well as during late pregnancy and lactation [8,9]. These physiological adaptations constitute potentially useful models in which to study the molecular mechanisms involved in the regulation of mammalian mitochondrial biogenesis *in vivo*. In the present study, the expression of the mitochondrial genome and of nuclear genes for mitochondrial proteins has been studied during lactation-induced mitochondrial hypotrophy of BAT.

MATERIALS AND METHODS

Adult female Wistar rats (*Rattus norvegicus*) corresponding to the B-type population for mitochondrial genome polymorphism [10] were used. They were maintained under standard conditions of illumination (12h light/dark cycle), feeding (A-03 type diet; Panlab, Barcelona, Spain) and environment temperature (21 ± 1 °C). They were mated with adult males, and litter sizes were adjusted at birth to ten pups. Lactating rats were caged singly, whereas virgin controls were caged in pairs. The 15-day-lactating rats and age-matched virgin controls were killed by decapitation, and the interscapular BAT was rapidly dissected.

Abbreviations used: COII, cytochrome *c* oxidase subunit II; NDI, NADH dehydrogenase subunit I; BAT, brown adipose tissue; mtDNA, mitochondrial DNA; UCP, uncoupling protein; COIV, cytochrome *c* oxidase subunit IV; ANT, adenine nucleotide translocase; ANT1 and ANT2, ANT isoforms 1 and 2; ATPsyn β , ATP synthase β -subunit; LPL, lipoprotein lipase.

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Half the tissue was frozen in liquid nitrogen for further preparation of RNA and DNA, and the other half was used to isolate mitochondria.

Total RNA was prepared by a modified phenol/chloroform extraction procedure [11], and the quality of RNA preparations was always checked in ethidium bromide-stained agarose gels and by the A_{260}/A_{280} ratio method. Northern-blot analysis of RNA was performed with 10 μ g of RNA, as described elsewhere [12]. Probes to assess the levels of the mitochondrial-genome-encoded mRNAs for cytochrome *c* oxidase subunit II (COII) and NADH dehydrogenase subunit I (NDI) and the 16 S rRNA have been described previously [13,14]. Probes for nuclear-encoded mitochondrial proteins were: the rat cDNA for UCP [15], a genomic DNA fragment corresponding to 288 bp of the first exon of human ATP synthase β -subunit (ATPsyn β) gene [16], the cDNA of human cytochrome *c* oxidase subunit IV (COIV) (A.T.C.C., Rockville, MD, U.S.A.), and the cDNA for the bovine ANT1 [17] and the cDNA for the human ANT2 [18] isoforms of adenine nucleotide translocase (ANT). The mRNA levels for the non-mitochondrial protein lipoprotein lipase (LPL) were determined as a control, since they are unchanged in BAT during lactation [19]. A guinea-pig cDNA probe for LPL was used [20]. Probes were labelled by the random oligo-priming method using [α - 32 P]dCTP (Amersham). Prehybridization, hybridization and washing conditions were as reported previously [12]. The sizes of the mRNAs detected were determined approximately by reference to rRNA bands and to the positions of the previously characterized hybridization signals for UCP mRNA, COII mRNA and NDI mRNA [12,14]. Determination of mtDNA abundance was performed essentially as previously reported [14]. Briefly, total DNA from interscapular BAT was prepared [21] and, after digestion with *Bam*HI endonuclease, 20 μ g of DNA was subjected to Southern-blot analysis, using the COII cDNA as labelled probe [14].

Mitochondria were isolated from interscapular BAT as previously described [22], and the protein content of mitochondria preparations was determined [23]. For Western-blot analysis, samples containing equal amounts of mitochondrial protein were mixed with equal volumes of 2 $\times$ SDS loading buffer, incubated at 90 °C for 5 min and electrophoresed on SDS/12%-polyacrylamide gels [24]. Proteins were transferred to polyvinylidene difluoride membranes (Millipore) and probed with rabbit antisera raised against either bovine heart ATPsyn β or bovine heart ANT, kindly provided by Dr. J. Kopecky and Dr. G. Brandolin respectively. Immunoreactive material was detected by the enhanced chemiluminescence (ECL) detection system (Amersham) according to the manufacturer's instructions. The sizes of the proteins detected were estimated by using protein molecular-mass standards (Bio-Rad). Quantification of autoradiographs and ECL signals was performed by scanning densitometry (LKB). Means $\pm$ S.E.M. were calculated from the densitometry arbitrary units; values from controls were set at 100%, and values from lactating rats were transformed to percentages of the virgin control values.

Statistical analysis was performed by Student's *t* test, and significance is indicated in the Tables.

RESULTS AND DISCUSSION

The expression of the mRNAs for two mitochondrial-genome-encoded proteins, COII and NDI, was decreased in BAT from lactating rats (see Table 1 and Figure 1a). A decrease in COII mRNA has been previously reported in this situation [12]. The assessment of mitochondrial rRNA expression, as shown by 16 S rRNA levels (see Table 1), indicated a parallel decrease in BAT

Table 1 Changes in the mitochondrial genome expression and relative content of mtDNA in BAT from lactating rats

Northern-blot analyses of 10 μ g of RNA (COII mRNA, NDI mRNA and 16 S rRNA) or Southern-blot analyses of 20 μ g of DNA (mtDNA) obtained from the interscapular BAT of virgin or 15-day-lactating rats were used for quantification. Results are expressed as percentages of virgin control values and are means $\pm$ S.E.M. for four rats: * difference statistically significant ($P < 0.05$) compared with virgin controls.

Rats	COII mRNA	NDI mRNA	16 S rRNA	mtDNA
Virgin	100 $\pm$ 13	100 $\pm$ 12	100 $\pm$ 15	100 $\pm$ 18
Lactating	46 $\pm$ 10*	54 $\pm$ 8*	62 $\pm$ 4*	55 $\pm$ 8*

from lactating rats compared with virgin controls. A concurrent change in mitochondrial mRNA and rRNA, like that observed here in BAT from lactating rats, has previously been reported in the liver of rats following long-term cold exposure [14] or in immortalized and foetal-calf-serum-treated cultured cells [13,25]. Moreover, parallel decreases in mitochondrial mRNAs and rRNAs occur in denervated rat muscle [26] or in brain from tetrodotoxin-treated monkeys [27]. The mitochondrial genome is expressed through polycistronic RNAs, including the mitochondrial rRNAs, with or without the mitochondrial mRNAs, which results in the different individual RNA species after post-transcriptional processing [1]. Thus, parallel changes in mitochondrial mRNAs and rRNAs such as those observed here are usually attributed to changes in the overall transcription rate of mtDNA. However, other possibilities should be considered. The molecular mechanisms eliciting parallel changes in mitochondrial mRNAs and rRNAs *in vivo* may be due not only to a lower transcription of the mtDNA, but also to a change in the relative mitochondrial gene abundance, which has been reported to constitute the main adaptive mechanism for long-term modifications in mitochondrial genome expression in chronically stimulated muscle [21]. There is a decrease in the relative content of mtDNA with respect to total DNA in BAT of lactating rats, as shown in Table 1 and Figure 1(b). This indicates that the mitochondrial hypotrophy of BAT during lactation is associated with a decrease in the mitochondrial gene dosage of the tissue. Therefore, the decreased expression of the mitochondrial genome in BAT from lactating rats could be viewed as a consequence of lower amounts of mtDNA templates that elicit decreased mitochondrial mRNA and rRNA levels without the necessity of lower transcription rates. However, the extent to which modifications in mtDNA abundance may account for changes in gene expression for mitochondrial proteins has been questioned [28,29], and further studies will be needed to ascertain the precise molecular mechanisms of the changes in the mitochondrial genome expression reported here.

Changes in the mRNAs for nuclear-encoded mitochondrial proteins are shown in Table 2 and Figure 1(c). UCP mRNA is dramatically decreased in BAT from mid-lactating rats, in agreement with previous reports [12], and concomitant with the lower thermogenic activity and UCP content of BAT mitochondria in mid-lactation [8,9,30]. The levels of mRNAs for non-tissue-specific mitochondrial proteins are also shown in Table 2: the mRNA levels for COIV, ANT1 and ANT2 were significantly decreased in lactating rats, whereas ATPsyn β mRNA levels were not modified. The mRNA levels for the non-mitochondrial protein LPL were unchanged in BAT from lactating rats with respect to virgin controls, in agreement with previous reports [19]. The decrease in the mRNA for COIV, taken together with

Figure 1 Representative Northern-blot analyses of the expression of the mitochondrial genome and nuclear genes for mitochondrial proteins, and Southern-blot analysis of the relative mtDNA content, in BAT from virgin and lactating rats

Northern-blot analyses of (a) mitochondrial-genome-encoded mRNAs (COII and NDI) and rRNA (16 S rRNA) and (c) nuclear-genome encoded mRNAs for mitochondrial proteins (UCP, COIV, ATPsyn β , ANT-1 and ANT-2) were performed with 10 μ g of RNA from the interscapular BAT. (b) Southern-blot analysis of 20 μ g of *Bam*HI-digested DNA from interscapular BAT, probed with the COII cDNA probe. V, virgin rats; L, 15-day-lactating rats.

Table 2 Changes in the expression of the mRNAs for nuclear-encoded mitochondrial proteins in BAT from lactating rats

Northern-blot analyses of 10 μ g of RNA obtained from the interscapular BAT of virgin or 15-day-lactating rats were used for quantification. Northern blots were probed for the mRNA expression of the mitochondrial proteins UCP, COIV, ATPsyn β , ANT-1 and ANT-2, and the non-mitochondrial control LPL. Results are expressed as percentages of virgin control values and are means $\pm$ S.E.M. for four rats: *difference statistically significant ($P < 0.05$) compared with virgin controls.

Rats	UCP mRNA	COIV mRNA	ATPsyn β mRNA	ANT-1 mRNA	ANT-2 mRNA	LPL mRNA
Virgin	100 $\pm$ 18	100 $\pm$ 16	100 $\pm$ 16	100 $\pm$ 9	100 $\pm$ 8	100 $\pm$ 15
Lactating	16 $\pm$ 3*	41 $\pm$ 8*	123 $\pm$ 18	52 $\pm$ 10*	48 $\pm$ 4*	95 $\pm$ 11

Table 3 Changes in the content of ATPsyn β and ANT in BAT mitochondria from lactating rats

Western-blot analyses of 25 μ g of pooled (2–3 different animals/sample) mitochondrial protein from virgin or 15-day-lactating rats were used for quantification. Results are expressed as percentages of virgin control values and are means $\pm$ S.E.M. for four samples: *difference statistically significant ($P < 0.05$) compared with virgin controls.

Rats	ATPsyn β	ANT
Virgin	100 $\pm$ 5	100 $\pm$ 12
Lactating	61 $\pm$ 11*	33 $\pm$ 12*

the lowered COII mRNA levels described above, is consistent with the overall decrease in cytochrome *c* oxidase activity in BAT from lactating rats [8,31]. The lack of change in ATPsyn β

Figure 2 Representative Western-blot analyses of the ATPsyn β and ANT content in BAT mitochondria from virgin and lactating rats

Western-blot analyses were performed with 25 μ g of mitochondrial protein from interscapular BAT. V, virgin rats; L, 15-day-lactating rats.

mRNA abundance was unexpected, but changes in gene expression for this protein could be achieved by regulatory events after the establishment of a steady-state level of its mRNA. Indeed, translational regulation of the ATPsyn β gene expression has been reported [32], and it is known to be especially relevant in BAT [33]. To test this possibility, BAT mitochondria were prepared from virgin and lactating rats and assayed for their content of ATPsyn β . The study was extended to the determination of the relative abundance of ANT.

As shown in Table 3 and Figure 2, Western-blot analysis showed a significant decrease in the content of ATPsyn β and ANT in BAT mitochondria from lactating rats. These findings indicate that the expression of ATPsyn β is lower in BAT from lactating rats, due to mechanisms that operate after the establishment of mRNA levels. Moreover, the present results indicate that the mitochondrial hypotrophy of BAT during lactation involves not only a decrease in the overall mitochondrial

mass and a specific decrease in its thermogenic-related components (UCP and components of the respiratory chain), but also a decrease in components of the mitochondrial respiratory-chain/oxidative-phosphorylation apparatus which are unrelated to thermogenesis, such as ATPsyn β or ANT.

Several regulatory factors might be responsible for the decrease in gene expression for mitochondrial protein in BAT from lactating rats. The decreased sympathetic activity on BAT during lactation [34,35] is likely to be responsible for the low expression of the UCP gene, which is mainly regulated by cyclic-AMP-mediated noradrenergic signals [36]. Sympathetic activity might also be involved in the adaptive decrease in gene expression for non-BAT-specific mitochondrial proteins in lactating rats, due to its effects on the thyroid status of BAT [37]. Thyroid hormones are the biological signals that have the most powerful mitochondriogenic effects in mammals [38]. The content of thyroid hormones is dramatically lowered in BAT from lactating rats as a consequence of a decrease in the iodothyronine 5'-deiodinase activity in the tissue [39,40]. Thyroid hormones are known to increase mitochondrial genome expression [41,42], as well as the expression of the ATPsyn β [32] and ANT2 mRNA [43]. Therefore, the tissue hypothyroidism occurring in BAT during lactation may constitute a major biological signal for the adaptive mitochondrial hypotrophy in this physiological situation.

In summary, the mitochondrial hypotrophy elicited by lactation in BAT is associated with a decrease in the expression of both the mitochondrial genome and nuclear genes for mitochondrial proteins. The mitochondrial genome expression may be lowered as a consequence of the decrease in mtDNA abundance, although changes at the transcription and/or post-transcription levels cannot be ruled out. Nuclear gene expression for mitochondrial proteins is lowered via different mechanisms, depending on each particular gene. Such mechanisms may involve either a decrease at the mRNA level or other regulatory mechanisms, probably translational, after the establishment of steady-state mRNA levels. Recent research on the mechanisms that co-ordinate gene expression for mitochondrial biogenesis has led to the identification of several transcription factors specifically involved in this function [16,44,45]. The present findings, together with previous reports [32,33], indicate that future research will also be needed to identify the molecular mechanisms not only for the co-ordinate transcription but also for post-transcriptional events involved in the co-ordinate gene expression leading to mitochondrial biogenesis.

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