ANEXO

VIII. ANEXO

Durante la realización de la presente Tesis Doctoral, la doctoranda ha realizado y colaborado en otros trabajos relacionados con el tema de la Tesis que ha dado lugar a las publicaciones que se presentan a continuación.

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Research Article

Mitochondrial biogenesis in brown adipose tissue is associated with differential expression of transcription regulatory factors

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Abstract. The differentiation of brown adipocytes during late fetal development or in cell culture is associated with enhanced mitochondrial biogenesis and increased gene expression for components of the respiratory chain/oxidative phosphorylation system. We have shown that this is due to a rise in mitochondrial DNA abundance and the corresponding increase in mitochondrial genome transcripts and gene products, as well as to the coordinate induction of nuclear-encoded genes for mitochondrial proteins. We studied how the expression of key components of the transcriptional regulation of mitochondrial biogenesis is regulated during this process. Changes in the expression of nuclear respiratory factor-2/GA-binding protein α and peroxisome proliferator-activated-receptor gamma coactivator-1 (increase) were opposite to those of nuclear respiratory factor-1 and Sp1 (decrease) during the developmental and differentiation-dependent induction of mitochondrial biogenesis in brown fat. These results indicate that the relative roles of transcription factors and coactivators in mediating mitochondrial biogenesis 'in vivo' are highly specific according to the cell type and stimulus that mediate the mitochondriogenic process.

Key words. Brown adipose tissue; mitochondria; nuclear respiratory factor; Sp1; peroxisone proliferator; activated receptor gamma; coactivator-1.

The synthesis of components of the mitochondrial respiratory chain/oxidative phosphorylation (OXPHOS) system in mammalian cells results from the coordinate expression of the mitochondrial genome and nuclear genes for OXPHOS components. Changes in transcriptional rates are considered a major mechanism regulating nuclear gene expression for OXPHOS components [1], although post-transcriptional mechanisms can also be involved [2]. Analysis of the transcriptional regulation of OXPHOS genes in cultured cells led to the identification of transcription factors specifically involved in the control of nuclear genes for mitochondrial proteins which were named nuclear respiratory factors (NRFs). NRF-1 is related to developmental regulatory transcription factors [3]. NRF-2/GABP is a multi-subunit transcription factor that contains the DNA-binding NRF-2/GABPα subunit, a member of the ETS family of transcription factors, and non-DNA-binding subunits, such as NRF-2/GABPβ, which modulate the binding affinity of the overall complex to DNA [4]. NRFs not only control the transcription of multiple genes encoding OXPHOS components, they also regulate gene expression for Tfam, the nuclear-encoded transcription factor determining mitochondrial genome replication and transcription [5]. Many mammalian OXPHOS gene promoters are regulated by

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NRFs, but they usually contain additional regulatory elements that depend on Sp1, a ubiquitous transcription factor [6]. Certain OXPHOS genes do not even contain NRF-1- or NRF-2-dependent regulatory sites and rely on regulation by Sp1. Depending on the OXPHOS target gene and cellular environment, Sp1 acts as an activator or repressor of OXPHOS genes [6, 7]. Moreover, PGC-1, a coactivator capable of interacting with NRF-1 and nuclear hormone receptors, has also been reported to promote mitochondrial biogenesis and OXPHOS gene expression when over-expressed in brown adipocytes or myoblasts [8, 9]. However, the involvement of each of these regulatory factors in the control of mitochondrial biogenesis during developmental processes of tissues and organs is poorly understood.

Brown adipose tissue (BAT) is a mammalian tissue specialized in non-shivering thermogenesis. This function relies on the presence in the brown adipocyte of a large number of mitochondria that are naturally uncoupled owing to the presence of the brown-fat-specific uncoupling protein-1 [10]. When brown adipocytes differentiate, they show the lipid accumulation characteristic of adipose cells and acquire a large content of mitochondria while OXPHOS gene expression is enhanced [11]. This process takes place during late fetal development, in the absence of environmental thermogenic stress, or when precursor cells differentiate into brown adipocytes in cell culture [12, 13]. The study of BAT fetal ontogeny provides an optimal model to identify the molecular mechanisms of mitochondrial biogenesis and OXPHOS gene expression.

In the present study, we determined changes in gene expression of nuclear respiratory factors, Sp1 and the coactivator PGC-1 during BAT ontogeny and brown adipocyte differentiation 'in vitro' and their association with the enhancement of mitochondrial biogenesis during brown fat cell differentiation.

Materials and methods

Swiss female mice were mated with adult males and the day of pregnancy was determined by the presence of vaginal plugs. Fetuses were obtained by Caesarian section of pregnant mice on days 16 and 18 of gestation. Pups remained with their mothers after spontaneous delivery and were killed just after parturition, before they had initiated suckling (birth). Adult mice (2 months old) were also used. Animals were killed by decapitation and interscapular BAT was removed.

Brown adipocytes were differentiated in culture from precursor cells as described elsewhere [14]. Stromal vascular cells were isolated from the interscapular, cervical and axillary depots of BAT of 3-week-old Swiss mice. They were plated (5000 cells/cm^2) and grown in Dulbecco's modified Eagle's medium (Ham's F12 medium (1:1, v/v) supplemented with 10% (v/v) fetal calf serum, 20 nM insulin, 2 nM T3 and 100 µM ascorbate). Cells were harvested after 4, 7 or 10 days of culture.

For transmission electron microscopy analysis (TEM), cultured cells were harvested in phosphate-buffered saline and pelleted by centrifugation at 500 g, for 5 min. Cell pellets and BAT samples were fixed in 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and post-fixed in 1% osmium tetroxide and 0.8% FeCNK in phosphate buffer. After dehydration in a graded acetone series, samples were embedded in Spurr resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi H600AB transmission electron microscopy at 75 kV.

RNA was extracted by a guanidine thiocyanate-based method [15]. For Northern blot analyses, 25 µg of total RNA was denatured, electrophoresed on 1.5% formaldehyde-agarose gels and transferred to nylon membranes (N+; Boehringer Mannheim, Germany). Equivalent amounts of ribosomal RNA in the samples were checked by ethidium bromide UV visualization. Pre-hybridization and hybridization were performed at 55°C in a 0.25 M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 20% SDS, 0.5% blocking reagent (Boehringer Mannheim) solution [16]. Blots were hybridized using mtDNA fragments as probes for detection of the mtDNA-encoded cytochrome oxidase subunit I (COI) and 16S mitochondrial rRNA, as reported elsewhere [17]. The murine cDNAs for the nuclear-encoded COIV (ATCC, Rockville, Md.), NRF-1 [18], NRF-2/GABPα, NRF-2/GABPβ1 [19], PGC-1 [8] and the cDNA for human Sp1 [20] were also used as hybridization probes. The size of the mRNA species detected for every gene was the same as previously reported in murine tissues. Relative mtDNA abundance was assessed as described elsewhere [17]. DNA from interscapular BAT was prepared and digested with *Eco*RI endonuclease. Twenty micrograms of DNA was analyzed by Southern blot and hybridized with the murine mtDNA fragment encoding 16S rRNA, thus leading to a 3.0-kb hybridization signal for mtDNA. As a control for equal loading of nuclear DNA, blots were rehybridized with the murine C/EBPβ probe, which reveals a 4.5-kb band for the corresponding nuclear gene fragment. The DNA probes were labelled using [α^{32} P]dCTP by the random oligonucleotide-priming method. Hybridization signals were quantified using a Molecular Image System GS-525 (BioRad, USA).

Crude protein extracts from isolated mitochondria or nuclei were obtained as reported for developmental studies of brown fat [17, 21]. BAT was homogenized in 10 mM Hepes, pH 7.6, 15 mM KCl, 2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulphonyl fluoride, 2.5 mM benzamidine, 10 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 0.24 M sucrose. After centrifugation at 1500 g for 10 min, pellets were resuspended in the homogenization buffer and centrifuged at 5000 g for 10 min (nuclear protein extract) whereas the supernatants

were centrifuged for 10 min at 8500 g (mitochondrial extracts). Nuclei were lysed and resuspended in the homogenization buffer containing 10% (v/v) glycerol. Protein concentrations were determined by the micromethod of Bio-Rad, using BSA as a standard. Equal amounts of proteins (usually 40 µg) were mixed with 1/5 vol of a solution containing 50% glycerol, 10% SDS, 10% 2-mercaptoethanol, 0.5% bromophenol blue and 0.5 M Tris (pH 6.8), incubated at 90°C for 5 min and electrophoresed on 0.1% SDS/12% (nuclear protein extracts) or 15% (mitochondrial protein extracts) polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, USA). Coomassie Blue staining of gels was performed systematically to check for similar patterns of the major proteins in the various extracts, indicative of a similar overall quality. Blots with mitochondrial protein preparations were probed with monoclonal antibodies for cytochrome oxidase subunits I and IV (A-6403 and A-6409; Molecular Probes). Blots with nuclear protein extracts were probed with rabbit antisera against NRF-2/GABPα, NRF-2/GABPβ (gifts of Dr. S. McKnight) or Sp1 (Santa Cruz, USA). Immunoreactive material was detected by the enhanced chemiluminescence (ECL) detection system (Amersham, UK) and quantified by densitometric analysis (Phoretics; Millipore). The sizes of the proteins detected were estimated using protein molecular-mass standards (Bio-Rad).

Results

Brown adipocytes differentiate in the late fetal development of mouse. From day 16 of fetal life to birth, they acquire the characteristic morphology of a differentiated brown adipocyte: the appearance of lipid vacuolae and an increase in the amount of mitochondria (fig. 1). En-

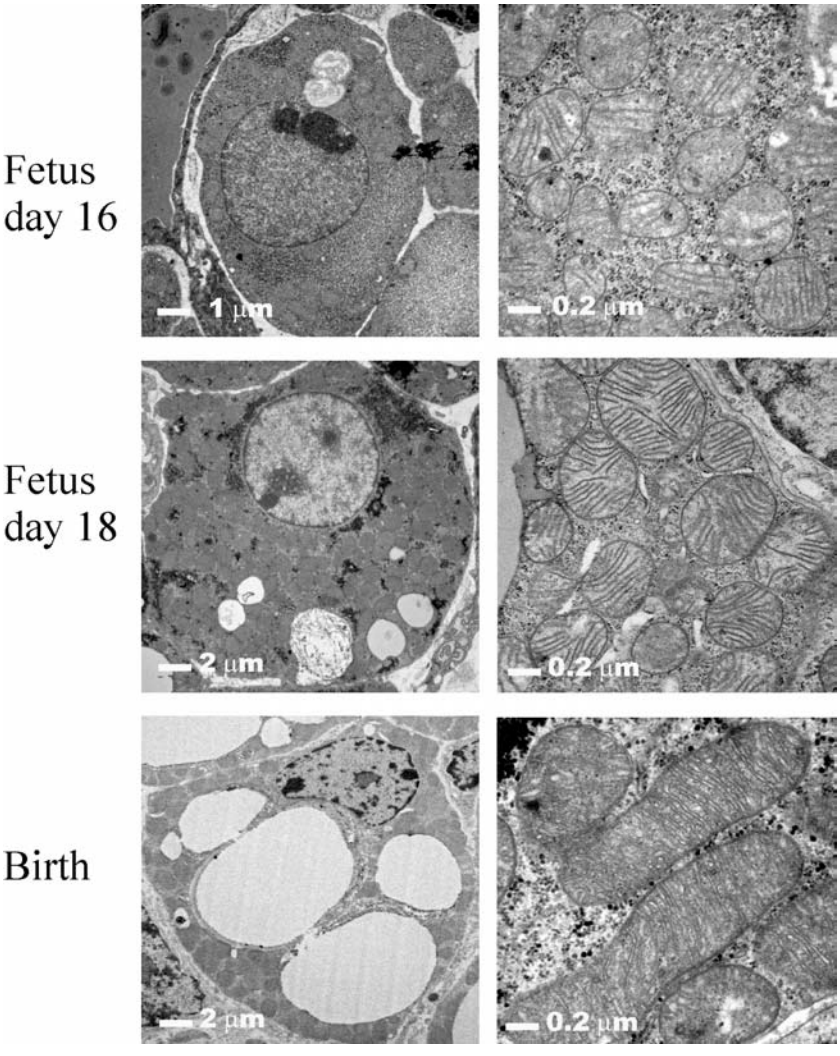

Figure 1. Morphology of brown adipocytes and mitochondria in BAT during fetal development. Transmission electron microscopy analysis of brown adipocytes (left) and mitochondria (right) in BAT of mice at days 16 and 18 of fetal life and at birth. Scale bars show magnification.

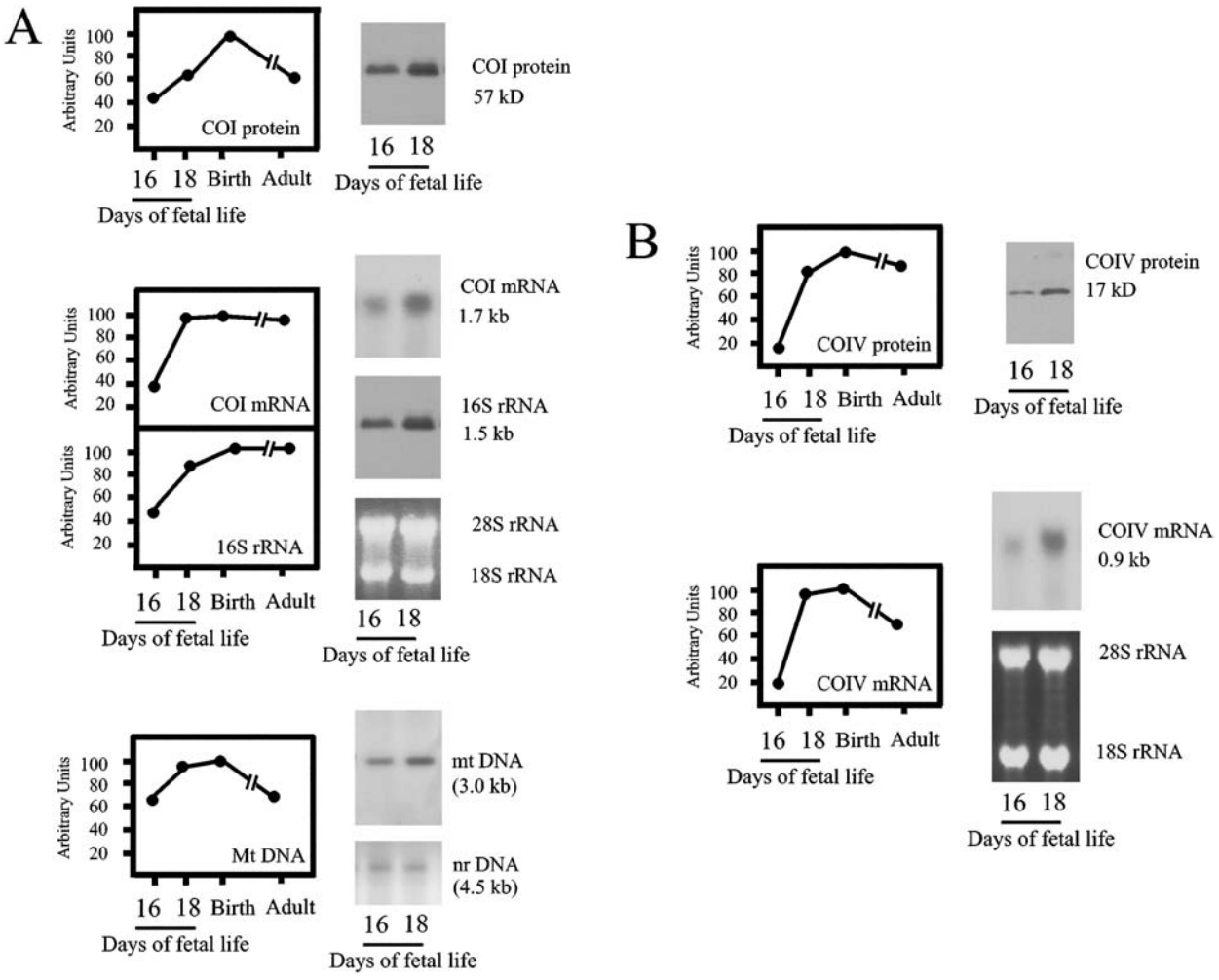

Figure 2. Expression of the mitochondrial genome and nuclear-encoded OXPHOS genes in developing BAT. Points in left panels are means of two to three independent experiments from every time point of development tested and are expressed as percentages of the mean value at the time of maximal expression, which was set to 100 (arbitrary units). (*A*) Mitochondrial genome expression pattern in developing BAT. Top, relative abundance of COI protein as determined by Western blot analysis of 20 µg of mitochondrial protein extracts, with a representative Western blot shown on the right; middle, relative abundance of the mitochondrial transcript COI mRNA and 16S rRNA as determined by Northern blot analysis of 25 µg of RNA, with representative Northern blots and control of RNA loading (nuclear-encoded 28S rRNA and 18S RNA) are shown on the right; bottom, relative abundance of mitochondrial DNA as determined by Southern blot analysis (see Material and methods), with a representative Southern blot for mitochondrial DNA and control of equal loading of nuclear DNA (nr DNA) shown on the right. (*B*) Expression of the nuclear-encoded OXPHOS gene COIV in developing brown fat. Top, relative abundance of COIV protein as determined by Western blot analysis of 20 µg of mitochondrial protein extracts, and a representative Western blot shown on the right; bottom, relative abundance of COIV mRNA, with representative Northern blots and control of RNA loading (nuclear-encoded 28S rRNA and 18S rRNA) shown on the right.

hanced mitochondrial biogenesis is shown both by an increase in the number of mitochondria in the cell and their increase in size, the development of internal membranes (cristae) and the acquisition of a parallel orientation characteristic of mature brown adipocytes (fig. 1, right). The enrichment in mitochondria content in the cells appeared to take place mostly between days 16 and 18, whereas from day 18 to birth, the major changes were the enlargement of mitochondria and the accumulation of lipid droplets in the adipocyte.

Expression of the mitochondrial genome is modified in brown fat during the process of mitochondrial biogenesis (fig. 2 A). The relative amount of mtDNA with respect to nuclear DNA almost doubled from day 16 of fetal life to day 18. Similar changes in the abundance of COI mRNA and 16S rRNA, indicative of messenger and ribosomal transcript products of the mitochondrial genome, were detected: a two- to threefold rise from day 16 to day 18 followed by a further increase until birth for 16S rRNA. This overall induction of mtDNA and mitochondrial transcripts had a direct consequence on the relative amount of mitochondrial genome products, which was also increased when monitored by changes in the mitochondrial COI protein (fig. 2 A, top). The time-course of the increase in

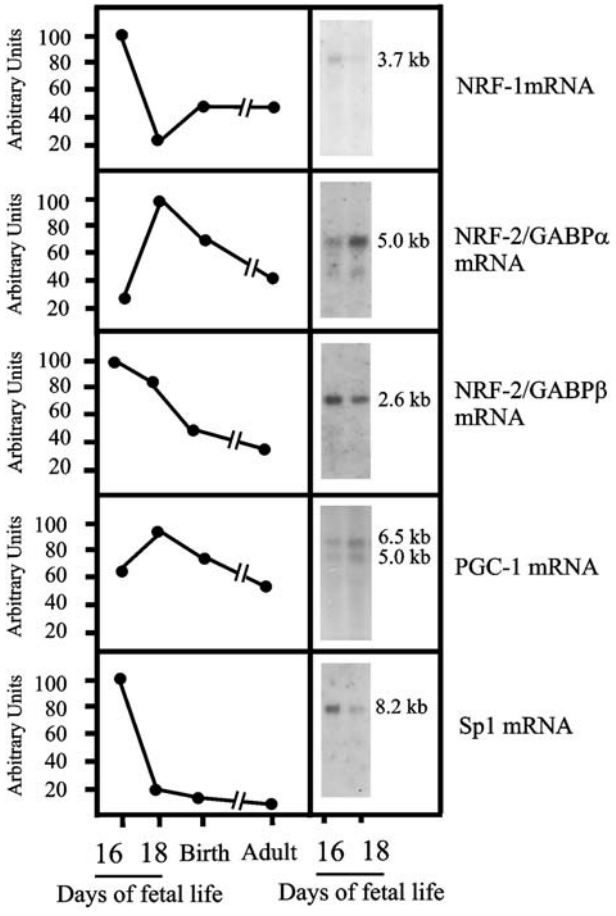

Figure 3. Expression of the mRNAs for transcription factors involved in OXPHOS gene regulation in developing BAT. Points in left panels are means of two to three independent experiments for every time point of development and are expressed as percentages of the mean value at the time of maximal expression, which was set to 100 (arbitrary units). They show the relative abundance of the corresponding mRNAs as assessed by Northern blot analysis of 25 µg of RNA from the interscapular BAT of mice at the indicated stages of development. Representative Northern blots are shown on the right.

the COI protein compared with that of COI mRNA was slightly delayed. The behaviour of COIV protein and COIV mRNA expression, as representative of a nuclear OXPHOS gene, was similar to mitochondrial genome expression but changes were more dramatic: around a five-fold induction was detected between days 16 and 18.

The major events of coordinate induction of mitochondrial genome and nuclear OXPHOS gene expression in brown fat occurred between days 16 and 18 of fetal development. We next determined how the expression of transcription factors involved in OXPHOS gene regulation was modified during this process (fig. 3). NRF-1 mRNA was down-regulated between days 16 and 18, and remained low at birth and in adults. In contrast, NRF-2/GABPα was dramatically up-regulated from day 16 to day 18 of fetal life, followed by a further reduction. NRF-2/GABPβ mRNA levels were not essentially modified between days 16 and 18 and declined thereafter. The mRNA of the coactivator PGC-1 increased from day 16 to day 18 and also showed a further reduction at birth and in adult mice. Northern blot hybridization with the Sp1 probe yielded a 8.2-kb signal corresponding to Sp1 mRNA in brown fat from fetuses at day 16 of development. This signal was very low in fetuses at day 18 and almost undetectable in adult brown fat.

The changes in NRF-2/GABPα, NRF-2/GABPβ and Sp1 protein abundance in brown fat during the late fetal development of mice were assessed by Western blot analysis (fig. 4). The sizes of the proteins detected using specific antibodies against those transcription factors are in agreement with previous findings in murine tissues [20, 22]. The profiles of changes in the relative abundance of NRF-2/GABPα and NRF-2/GABPβ in nuclear extracts of BAT at the stages of fetal development studied were essentially parallel to those found for the respective mRNAs. Similarly, Sp1 levels were high in fetuses at day 16 and fell dramatically in the last days of fetal life to practically undetectable values in adults. No specific antibodies against NRF-1 are available at present, which precludes the analysis of protein abundance. Western blot analysis of PGC-1 using commercially available antibodies (SC-5816, SC-5815 from Santa Cruz, AB 3242 from Chemicon) did not provide reliable signals for assessment of the relative abundance of this protein in fetal brown fat.

To further establish the extent to which the changes in the expression of transcription factors were associated with the process of mitochondrial biogenesis in the brown adipocyte, an ‘in vitro’ approach based on cell culture was undertaken. Pre-adipocytes from BAT depots of adult mice were obtained and cultured in conditions leading to brown adipocyte differentiation and the evolution of changes in mitochondrial biogenesis and gene expression were monitored during this process. TEM analysis indicated that, on day 4 of culture, cells lacked the morphological signs of the brown adipocyte phenotype: there were no lipid droplets and mitochondria were scarce and poorly developed (fig. 5). After 3 days of culture (day 7), cells acquired the typical brown adipocyte morphology, as shown by the accumulation of lipid droplets and, in particular, by enrichment in the content of mitochondria and their development. Additional culturing for 3 more days (day 10 of culture) merely revealed an increase in the size and amount of lipid droplets but no further modifications in cell morphology or mitochondrial biogenesis (data not shown). Parameters related to OXPHOS gene expression were coordinately induced during this period: relative mtDNA abundance almost doubled between days 4 and 7, similar inductions were observed for the abundance of the mitochondrial transcripts COI mRNA and 16S rRNA and a similar rise took place for the nuclear-

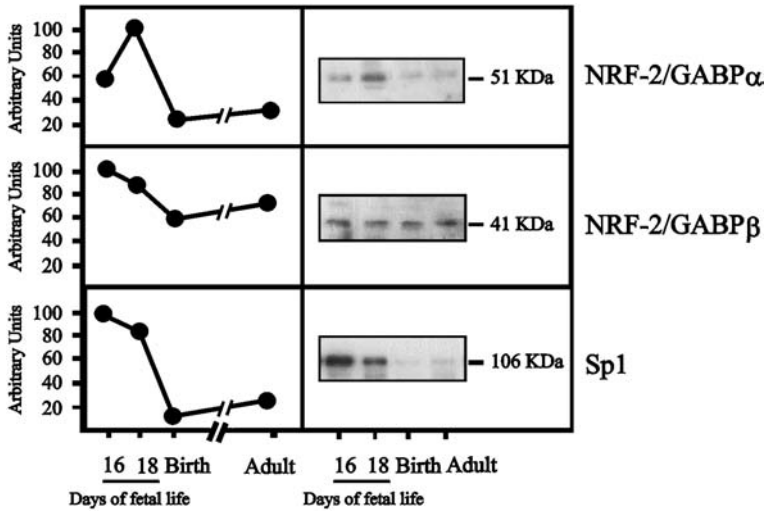

Figure 4. Content of NRF-2/GABPα, NRF-2/GABPβ and Sp1 in developing BAT. Forty micrograms of nuclear protein extracts from interscapular BAT of mice at the indicated stages of development analysed by Western blot using specific antibodies for the indicated proteins. Results (left) are means of two to three independent analyses from different samples and are expressed as percentages of the mean value at the time of maximal expression which was set to 100. Representative Western blot analyses showing the sizes of the proteins detected are depicted on the right.

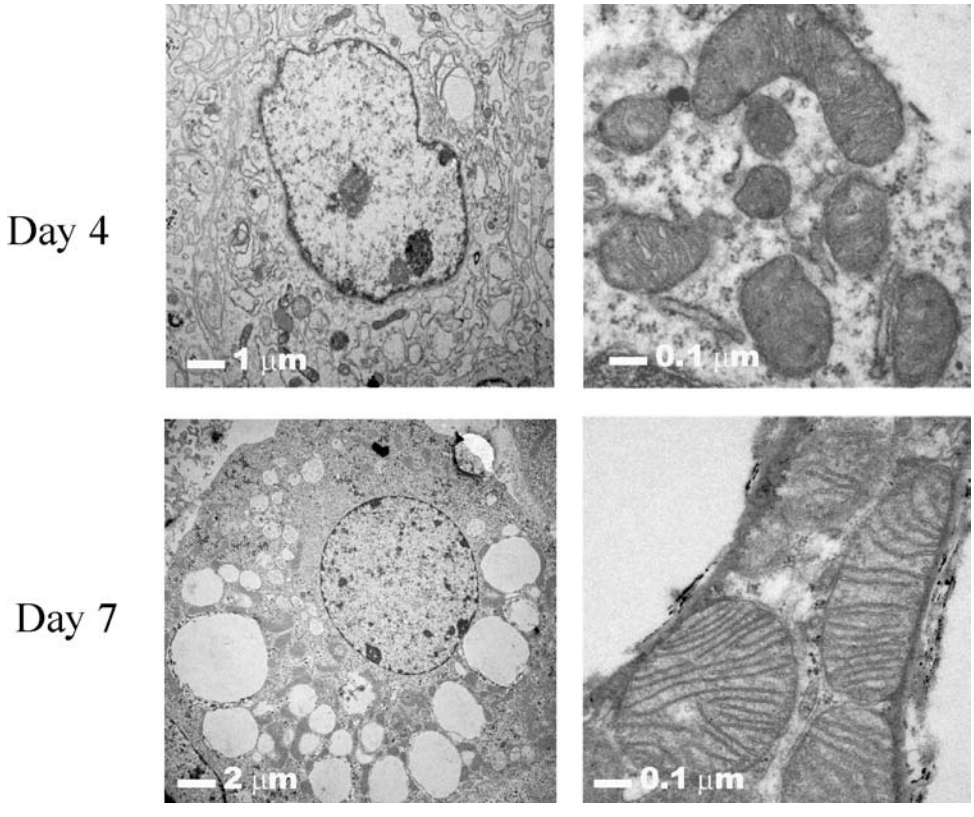

Figure 5. Morphology of cells and mitochondria in brown adipocytes differentiating in culture. Transmission electron microscopy analysis of murine brown adipocyte precursor cells (left) and mitochondria (right) after 4 or 7 days of culture. Scale bars show magnification.

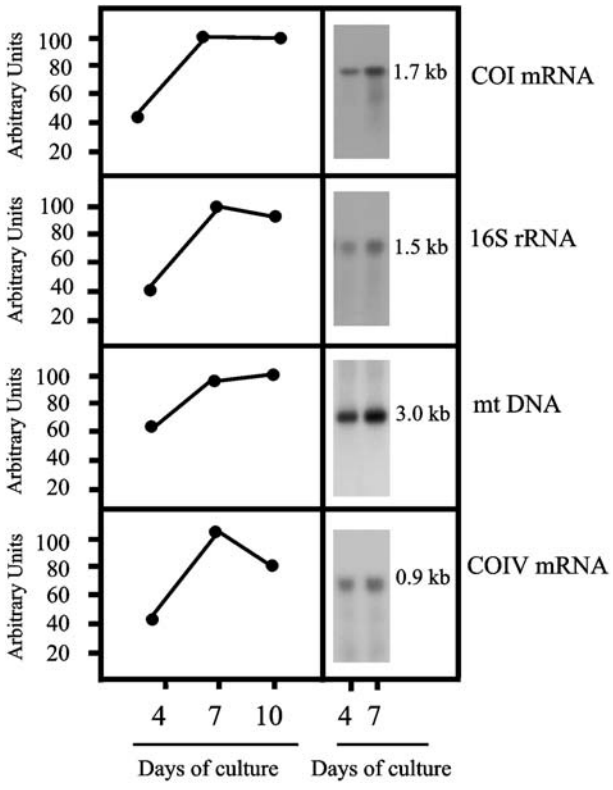

Figure 6. Expression of the mitochondrial genome and nuclear-encoded OXPHOS genes in brown adipocytes differentiating in culture. Points in left panels are means of two to three independent experiments from every time point of culture and are expressed as percentages of the mean value at the time of maximal expression, which was set to 100 (arbitrary units). The relative abundance of the mitochondrial transcripts COI mRNA and 16S rRNA and the nuclear-encoded transcript COIV mRNA was determined by Northern blot analysis of 25 µg RNA; representative Northern blots are shown on the right. Controls of equal loading of RNA by ribosomal RNA staining were established as in figure 2. The relative abundance of mtDNA was determined by Southern blot analysis (see Material and methods); a representative Southern blot for mtDNA is shown on the right. Controls of equal loading of nuclear DNA were established as in figure 2.

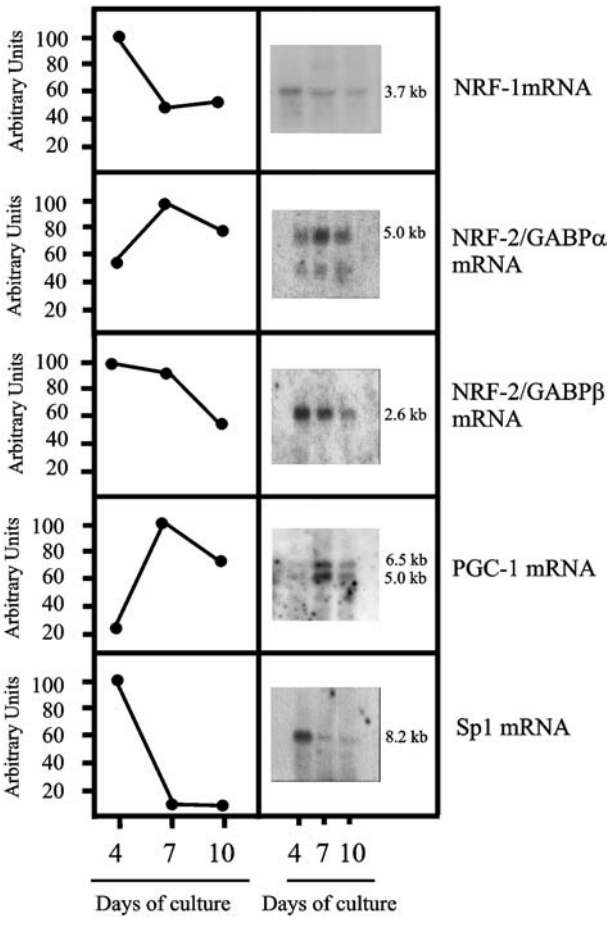

Figure 7. Expression of the mRNAs for transcription factors involved in OXPHOS gene regulation in differentiating brown adipocytes. Points in left panels are means of two to three independent experiments for every time point of culture and are expressed as percentages of the mean value at the time of maximal expression, which was set to 100 (arbitrary units). They show the relative abundance of the corresponding mRNAs as assessed by Northern blot analysis of 25 µg of RNA. Representative Northern blots are shown on the right. Controls of equal loading of RNA by ribosomal RNA staining were established as in figure 2.

encoded OXPHOS transcript (fig. 6). OXPHOS gene expression was not further enhanced from day 7 to day 10. When gene expression for transcription factors was determined between days 4 and 7 of cell culture, the profile of reciprocal changes observed was similar to that found in BAT differentiating in vivo during fetal life: NRF-1 mRNA and Sp1 mRNA abundance was reduced from day 4 to day 7, whereas NRF-2/GABPα and PGC-1 mRNAs were up-regulated and NRF-2/GABPβ mRNA was essentially not modified (fig. 7).

Discussion

The present TEM results show that mitochondrial biogenesis is mostly induced in BAT in a precise period of mouse development, between days 16 and 18 of fetal life. This is associated with a rise in the relative abundance of mtDNA and in mitochondrial-genome-encoded transcripts. The increase in transcript abundance was slighlty higher than in mtDNA abundance, thus suggesting that enhanced mitochondrial DNA replication accounts for most of the rise in the expression of mitochondrial genome products. Several physiological and experimental models of enhanced mitochondrial genome expression in mammals have shown that multiple mechanisms can be used to induce regulatory changes in mtDNA expression. For example, when mtDNA expression in the liver is triggered by the stress of birth [23] or after a cold-environment exposure [24], enhanced mtDNA transcription increases mtDNA gene product synthesis without changes in mtDNA replication. In contrast, electric stim-

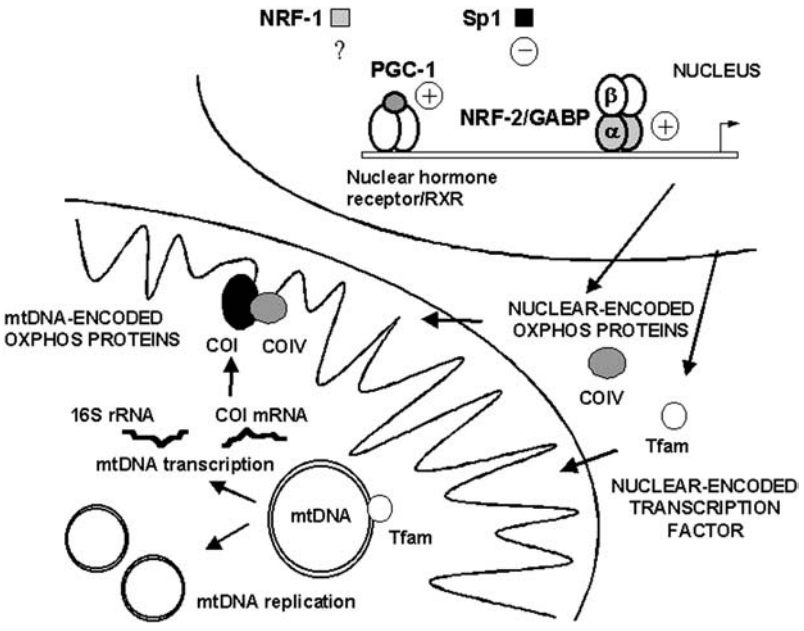

Figure 8. Model of the transcriptional regulation of mitochondrial biogenesis in differentiating brown adipocytes. NRF-2/GABP, as a transcription factor, and PGC-1 as a co-activator of nuclear hormone receptors, induce the transcription of genes for OXPHOS proteins, such as COIV, and for Tfam. Tfam induces mtDNA gene replication and transcription thus leading to enhanced synthesis of mtDNA-encoded proteins, such as COI. In this situation, Sp1 and NRF-1 levels would be low and do not interact with OXPHOS or Tfam gene promoters. The putative repressor role of Sp1 on these promoters would be relieved.

ulation of skeletal muscle raises the relative mtDNA content, which, at a similar rate of transcription, accounts for an overall increase in mitochondrially encoded protein synthesis [25]. Regulation of mitochondrial genome expression in differentiating brown adipocytes appears to be closer to this last mechanism. In this regard, lactation-induced mitochondrial hypotrophy in BAT and PGC-1-induced mitochondrial biogenesis in brown adipocytes are associated with a reduction and an increase, respectively, in the relative amount of mtDNA [8, 26]. The molecular mechanisms determining whether mtDNA replication or transcription is regulated to meet the need of higher mtDNA expression in a given physiological situation or cell type are not known, as the major transcription factor involved, the nuclear-encoded Tfam protein, participates in the control of both processes [27]. In any case, this process parallels the enhancement of nuclear-encoded OXPHOS gene expression, which suggests the involvement of NRFs or other transcriptional regulators in the control of OXPHOS nuclear genes and, through Tfam, of mitochondrial DNA replication.

The transcriptional regulators whose expression was up-regulated in association with enhanced OXPHOS gene expression in differentiating brown adipose cells were NRF-2/GABPα and the co-activator PGC-1. NRF-2/GABPβ, the accessory subunit of the NRF-2/GABP complex, was not modified in the key periods of enhanced OXPHOS and NRF-2/GABPα gene expression. In fact, the subunits of the NRF-2/GABP complex that limit building of the functionally active NRF-2/GABP transcription factor have not been identified in any cell type, but the present findings suggest that regulation of the α subunit is mostly associated with differentiation, at least in the brown adipocyte.

NRF-2/GABP regulates multiple nuclear OXPHOS genes [28], e. g. expression of the ATP synthase β-subunit gene during brown adipocyte differentiation depends on NRF-2/GABP interaction with the gene promoter [12]. Thus, our present results strongly suggest that a rise in NRF-2/GABP leads to enhanced OXPHOS gene expression and mitochondriogenesis during BAT differentiation. This regulatory role of NRF-2/GABP may be specific for BAT, at least when considering the other mammalian cell type in which differentiation is mostly associated with enhanced mitochondrial biogenesis. i. e. muscle cells. In these cells, there is no evidence for active NRF-2/GABP cis-acting elements in OXPHOS promoters [29] and no changes in NRF-2/GABP subunit expression have been detected in the transition from myoblasts to myotubes [30].

PGC-1 up-regulation, in association with mitochondria enrichment in brown adipocytes, is consistent with the known effects of PGC-1 over-expression in these cells or in myoblasts in culture promoting mitochondrial biogenesis [8, 9]. As a co-activator, PGC-1 can trigger the high expression of OXPHOS genes by interacting with NRF-1 [9], by inducing NRF-2 [9] or by interacting with thyroid hormone receptors [31], which can mediate the mito-

chondriogenic effect of thyroid hormones. Although none of these three mechanisms can be ruled out, opposite regulation of PGC-1 and NRF-1 mRNA expression when mitochondriogenesis is enhanced during BAT fetal development or in cell culture suggests a major relevance of the last two mechanisms. Thyroid hormone receptors and thyroid hormone accumulate in brown fat between days 16 and 18 of mouse fetal development [17] or in equivalent periods of rat fetal development [32], which indicates that thyroid-mediated mechanisms for mitochondrial biogenesis may be highly relevant in this period and that PGC-1 induction may contribute to this effect. On the other hand, thyroid hormones induce NRF2/GABPα gene transcription [33] pointing to cross-talk between hormones and transcription factors in the control of brown-fat mitochondrial biogenesis during late fetal life. Moreover, PGC-1 stimulates gene transcription of the brown-fat-specific protein UCP-1 [8] and the rise in PGC-1 mRNA expression parallels the initiation of UCP1 gene transcription during BAT ontogeny [17].

In addition to the up-regulation of NRF-2/GABPα and PGC-1, we observed that NRF-1 and Sp1 gene expression were down-regulated. The parallel reduction in NRF-1 mRNA expression during BAT differentiation in the fetal period and in cell culture was an unexpected finding, because it suggests that NRF-1 expression is not only unrelated but even opposite to mitochondrial biogenesis in the brown adipocyte. Multiple OXPHOS gene promoters are regulated by NRF-1, whose expression is transiently induced in skeletal muscle [34] and cardiac myocytes in culture [35] when exposed to stimuli leading to mitochondrial biogenesis. Either the specific program of mitochondrial biogenesis in the brown fat cell does not require high levels of NRF-1 or the levels of NRF-1 gene expression at day 16 of fetal life or day 4 of cell culture are high enough to trigger the mitochondriogenic process, although they are immediately down-regulated upon mitochondrial biogenesis. In fact, the targeted disruption of NRF-1 is lethal in the very early process of mouse embryogenesis and a more general role in embryo development and cell growth has been proposed for NRF-1 in addition to its function as regulator of mitochondrial biogenesis [36].

Although Sp1 is considered a highly ubiquitous, poorly regulated, transcription factor, the data from the two models of brown adipocyte differentiation studied indicate a strong regulation. Sp1-binding sites are regarded as major regulatory elements when OXPHOS gene promoters are analysed in the context of transformed cells [6, 7] and, for certain OXPHOS genes like cytochrome c, lacking NRF-dependent regulation, it is the only transcriptional regulator identified so far [6]. Moreover, cell transformation enhances Sp1 expression [37]. This would be consistent with a major role for Sp1 in determining the high expression of OXPHOS gene mRNAs characteristic of transformed cells [38] but not when the expression of OXPHOS genes is enhanced owing to the differentiation to a specialized cell type such as the brown adipocyte. In this regard, the levels of Sp1 are high in cells differentiating to brown adipocytes only when they proliferate and initiation of differentiation is associated with Sp1 down-regulation. Moreover, Sp1 has also been shown to inhibit the expression of some OXPHOS gene promoters [6], consistent with the down-regulation of Sp1 and enhanced mitochondrial biogenesis. On the other hand, the role of Sp1 in gene regulation is more widespread than the control of OXPHOS genes. For example, Sp1 has been reported to repress the adipogenic program of differentiation in white adipocytes in cell culture [39] and present findings of Sp1 down-regulation agree with a parallel role during brown adipocyte differentiation. The adipogenic and mitochondriogenic programs of differentiation in the brown adipocyte are closely related. For example, fetal BAT mitochondrial biogenesis is impaired in mice lacking C/EBPα, an essential adipogenic transcription factor which does not trans-activate OXPHOS genes, and this is associated with reduced expression of NRF-2 and PGC-1 [17].

In summary, we have established that enhanced mitochondrial biogenesis in BAT is related to an increase in mtDNA content and the subsequent rise in the expression of mtDNA-encoded gene products, as well as to the coordinate induction of nuclear-encoded OXPHOS gene expression. This process is associated with reciprocal changes in the expression of NRF-2 and PGC-1 (increase) compared with NRF-1 and Sp1 (decrease) (see fig. 8). This pattern of behaviour is a common feature of brown adipose cells upon differentiation during fetal development or in cell culture. These results suggest that the relative roles of transcription factors and co-activators in mediating mitochondrial biogenesis 'in vivo' are highly specific for the cell type and stimuli that mediate the mitochondriogenic process.

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Uncoupling Protein and Brown Adipocyte Mitochondria as Potential Targets of Reverse Transcriptase Inhibitor-induced Lipodystrophy

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Summary

Human adipose depots contain remnant brown adipocytes widespread among white adipocytes. The aim of this study was to evaluate the effects of reverse transcriptase inhibitors in a cell model of brown adipocytes. Stavudine, zidovudine and didanosine, but not lamivudine, caused a reduction in mtDNA content. In contrast, mtDNA-encoded transcripts (16S rRNA, COII mRNA) were not essentially reduced, thus suggesting compensatory up-regulation of mtDNA transcription. Nuclear-encoded COIV mRNA was unaltered. Nevirapine caused an overall increase in mtDNA content and expression. Stavudine, lamivudine and nevirapine induced the expression of the specific brown adipocyte-gene marker UCP1, indicating that disturbances in brown adipocytes may contribute to HAART-induced lipodystrophy.

Introduction

Highly active antiretroviral therapy (HAART) for human immunodeficiency virus type-1 combine protease inhibitors with nucleoside reverse transcriptase inhibitors (NRTIs), or NRTIs with non-nucleoside reverse transcriptase inhibitors (NNRTIs). HAART has led to substantial reductions in morbidity and mortality among patients. However, a serious metabolic syndrome has arisen in treated patients, referred as the HAART-associated lipodystrophy syndrome (1). The main clinical signs of this syndrome are

region-specific disturbances of fat distribution: severe loss of peripheral adipose tissue and central adiposity (abdomen, cervical fat-pads, and breast hypertrophy) (2). Other metabolic disturbances include dyslipideamia, hyperglycemia, and insulin resistance (1, 2).

Alterations in body fat distribution and lipid metabolism among HIV-infected patients on HAART suggest adipocyte dysfunction. There are two types of adipocytes: white adipocytes (which store metabolic energy as fat) and brown adipocytes (which dissipate metabolic energy as heat due to the presence of the uncoupling protein-1, UCP1) (3). In humans, brown adipose depots are found in newborns whereas remnant brown adipocytes widespread among white adipocytes remain in adult adipose depots. The relative amount of brown adipocytes is different depending on the adipose anatomical site (peripheral *vs* visceral fat depots). Brown adipocytes have a high metabolic rate and contain a large number of mitochondria, potential targets of HAART toxicity. The abundance and activity of brown adipocytes can be monitored by the expression of the UCP1 gene, a qualitative marker of brown respect to white adipose cell (3). Here we determined the specific effects of RTI drugs used in HAART on brown adipocyte mitochondrial biogenesis and UCP1 gene expression.

Materials and Methods

NRTIs stavudine and didanosine (Bristol-Myers-Squibb), lamivudine and zidovudine (GlaxoWellcome), and NNRTI nevirapine (Boehringer Ingelheim/Roxane) were used. All NRTIs were dissolved in PBS whereas nevirapine was dissolved in dimethyl sulfoxide (DMSO). Brown adipocyte precursor cells obtained from brown fat from three-week-old Swiss mice were plated (7500 cells/cm^2) and grown in Dulbeccos's modified Eagle's medium-Ham's F12 medium (1:1) supplemented with 10% fetal calf serum, 20 nM insulin, 2 nM T_3 and 100 μM ascorbate (4). At confluence (day 4 of culture), cells were incubated in the absence or presence of drugs at a concentration of 20 μM. Cells were harvested on day 9 of culture. Total RNA was extracted using the Rneasy Mini Kit (Quiagen). For Northern blot analysis, 10 μg of total RNA was electrophoresed and transferred to nylon$^+$ membranes. Blots were hybridized with probes for the mitochondrial-genome encoded 16S rRNA and cytochrome oxidase subunit II (COII), the nuclear-encoded cytochrome oxidase subunit IV (COIV) and uncoupling protein-1 (UCP1). Mitochondrial DNA abundance was determined by Southern blot analysis of 20 μg of total DNA after digestion with *EcoR*I endonuclease and using the 16S rRNA probe. Autoradiographs were quantified by densitometry.

Results

Brown preadipocytes in primary culture proliferate until day 4 and

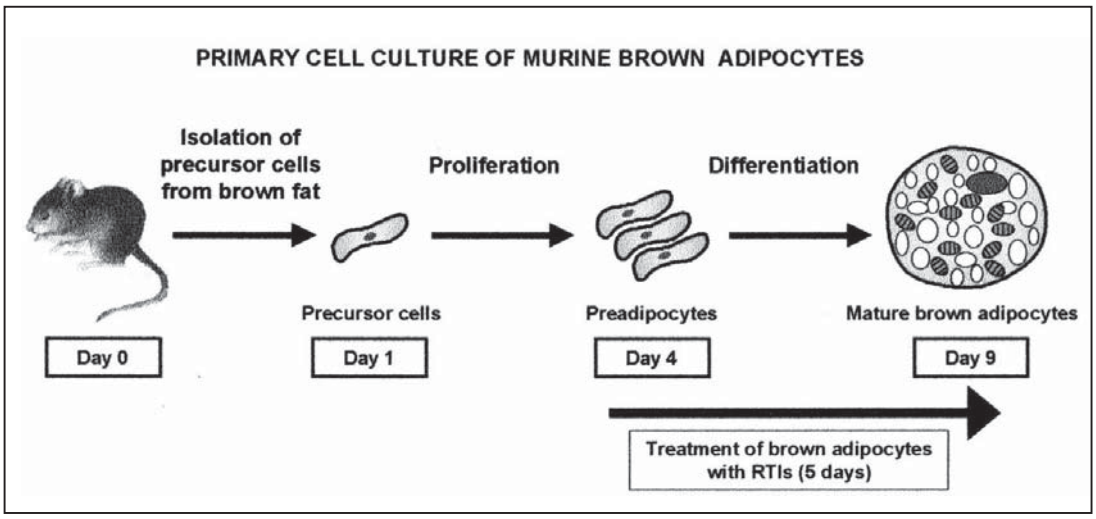

Figure 1. Schematic model of the primary culture of brown adipocytes

thereafter they differentiate until day 9 (Fig. 1), as shown by the acquisition of multilocular lipid accumulation and expression of the UCP1 gene (4). When NRTIs were added to the medium at day 4 of culture, cell morphology was unaltered whereas a minor positive action on morphological differ-

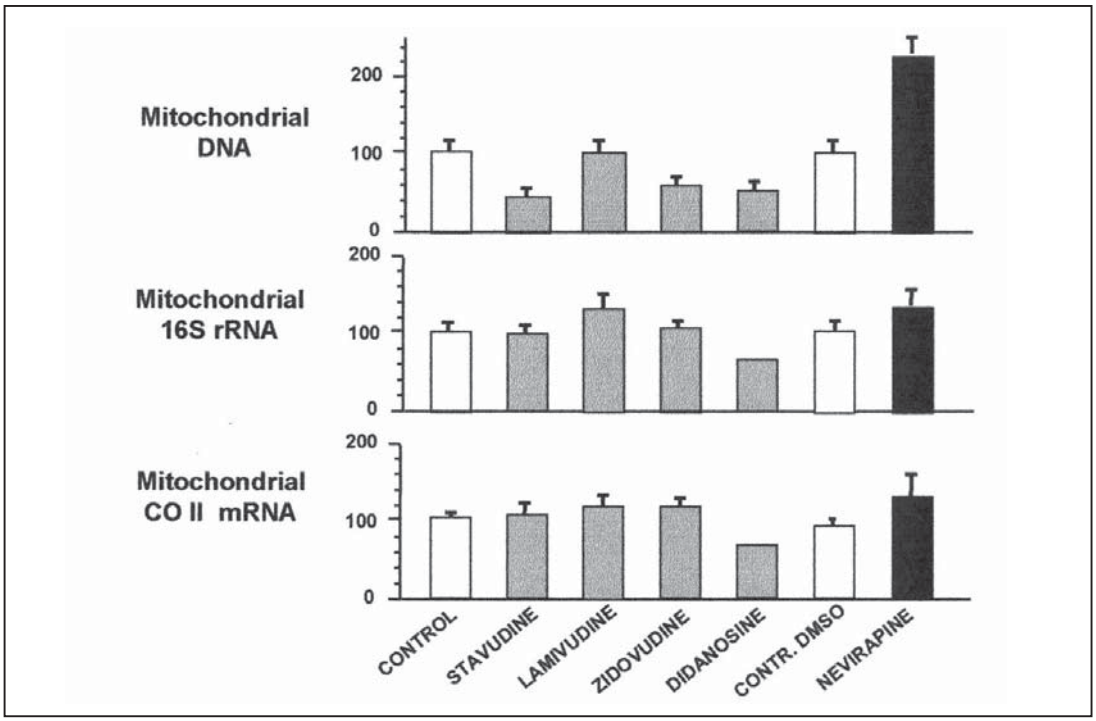

Figure 2. Effect of RTIs on mtDNA content, and 16S rRNA and COII mRNA levels. Data are expressed as means ± SEM of at least two independent experiments, and represented relative to the control value (which is set to 1).

entiation was observed for the NNRTI nevirapine (data not shown). To analyze whether RTIs affect mitochondrial biogenesis, mtDNA content and gene expression for mitochondrial-encoded 16S rRNA, and mitochondrial (COII mRNA) and nuclear-encoded (COIV mRNA) components of the mitochondrial respiratory system were assessed. Stavudine, zidovudine and didanosine caused a reduction in the amount of mtDNA in brown adipocytes, whereas lamivudine did not (Fig. 2). However, only didanosine treatment caused a minor decrease in the levels of 16S rRNA and COII mRNA, thus suggesting a compensatory up-regulation of mtDNA transcription in stavudine and zidovudine-treated cells. In contrast, when brown adipocytes were treated with the NNRTI nevirapine, mtDNA abundance was found to be 2-fold higher respect to solvent-control cells (DMSO). Furthermore, nevirapine-treated cells also showed an increase in mitochondrial transcripts 16S rRNA and COII mRNA. Expression of nuclear-encoded COIV mRNA was also increased by nevirapine whereas it was unaltered in NRTI-treated cells (Fig. 3). Expression of UCP1 mRNA was increased in nevirapine-treated brown adipocytes. Furthermore, a specific induction of UCP1 mRNA expression due to stavudine and to a lesser extent to lamivudine was also observed.

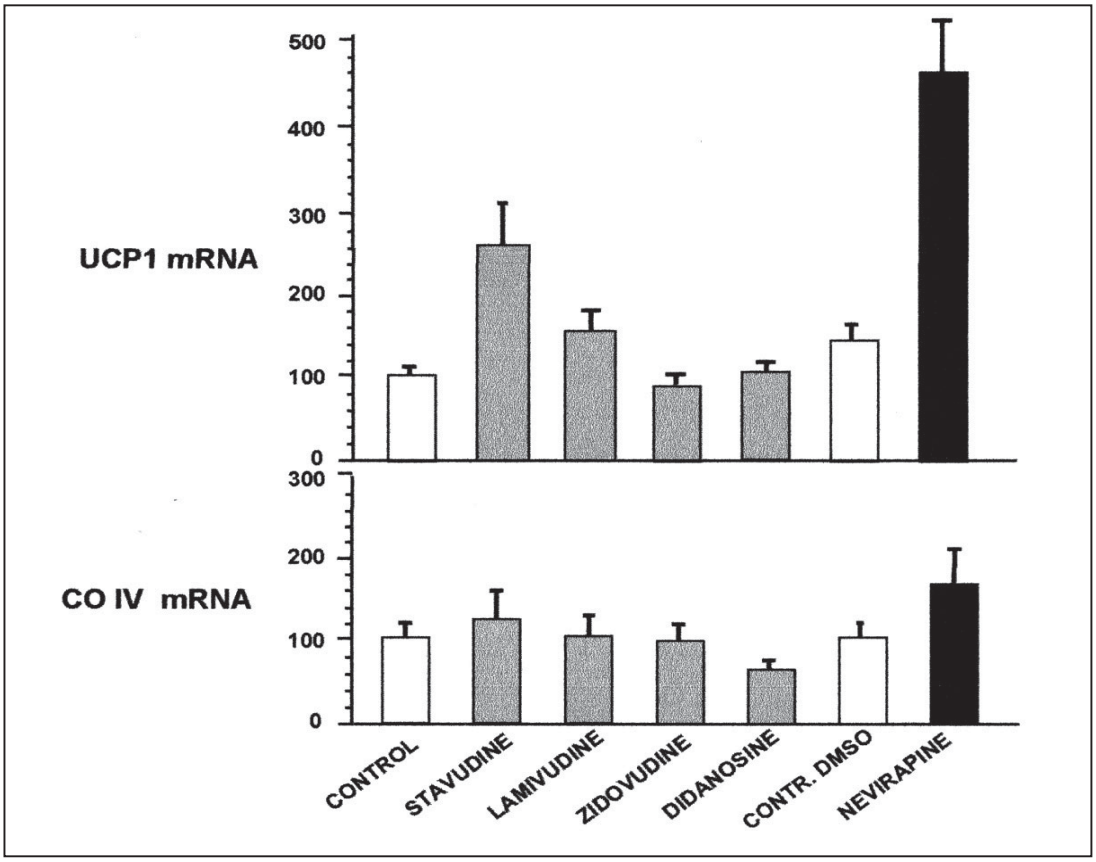

Figure 3. Effect of RTIs on UCP1 and COIV mRNA levels. As in the Fig 2 legend.

Conclusions

Despite a reduction in mtDNA content caused by some NRTIs, gene expression for the mitochondrial oxidative machinery is not essentially impaired, and it is even increased by NNRTI nevirapine.

Several RTIs cause an abnormal induction of UCP1 gene expression, pointing to brown adipocytes as specific targets of RTI drugs used in HAART. A specific disturbance of cells from the brown adipocyte lineage respect to the white one in the adipose depots appears as a future research direction in the assessment of the basis of HAART-induced lipodystrophy.

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Upregulatory Mechanisms Compensate for Mitochondrial DNA Depletion in Asymptomatic Individuals Receiving Stavudine Plus Didanosine

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Summary: Nucleoside analogue use is often related to mitochondrial DNA (mtDNA) depletion, but mitochondrial function is preserved in most asymptomatic patients. We determined whether homeostatic mechanisms are able to compensate for this mtDNA depletion in patients receiving stavudine plus didanosine (d4T + ddI), an antiretroviral combination with great in vitro and in vivo capacity to decrease mtDNA. We included 28 asymptomatic HIV-infected individuals: 17 subjects (cases) on a first-line antiretroviral regimen consisting of d4T + ddI as the nucleoside backbone plus nevirapine or nelfinavir for at least 6 months (mean: 16 ± 8 months) and 11 naive subjects (controls). We assessed the following in peripheral blood mononuclear cells: mitochondrial mass by citrate synthase activity, mtDNA content by real-time polymerase chain reaction, cytochrome c oxidase subunit II (COX-II) expression by Western blot analysis, and COX activity by spectrophotometry. The mitochondrial mass and mtDNA content of cases decreased when compared with controls, whether normalized per cell or per mitochondrion. Conversely, COX-II expression and COX activity were similar in cases and controls. COX-II expression was constant and independent of the mtDNA content, whereas it was closely related to COX activity. We concluded that treatment with dd4T + ddI is associated with decreased mitochondrial mass and mtDNA content but that COX-II expression and COX activity remain unaltered. These data suggest that upregulatory transcriptional or posttranscriptional mechanisms compensate for mtDNA depletion caused by d4T + ddI before profound mtDNA depletion occurs.

Key Words: peripheral blood mononuclear cells, mitochondrial DNA, cytochrome c oxidase subunit II, nucleoside reverse transcriptase inhibitors, stavudine, didanosine, DNA γ-polymerase

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Mitochondrial DNA (mtDNA) depletion is a secondary effect inherent to most of the nucleoside reverse transcriptase inhibitors (NRTIs). The main reason for this fact is that NRTI triphosphates are also able to inhibit DNA γ-polymerase, the only enzyme that replicates mtDNA.[1] Some reports have attributed a pathogenic role to mtDNA depletion in the long-term adverse effects associated with the use of NRTIs, especially hyperlactatemia[2,3] and lipodystrophy.[4–8] However, the cumulated lifetime doses of NRTIs at which these adverse effects develop have not been established. In fact, the reasons why some people do not develop these adverse effects, despite large doses of NRTIs, remain unknown, and few studies have evaluated the functional relevance of mtDNA depletion at the level of the mitochondrial respiratory chain (MRC). Thus, many questions arise as to the real role of mtDNA depletion in these adverse effects.[9–11]

One factor that may contribute to the inconsistent presentation of adverse effects, despite the constant presence of mtDNA depletion, may be the development of homeostatic mechanisms to compensate for this depletion. At least before profound mtDNA depletion occurs, these mechanisms may act at transcriptional or posttranscriptional levels to compensate for the mild to moderate decrease of the mtDNA content. The final objective of these hypothetic changes would be to maintain the MRC capacity unaltered, because all the mtDNA-encoded genes are devoted to the synthesis of proteins of MRC complexes. To extend this mechanism, we assessed the effects of the antiretroviral therapy beyond mtDNA content by determination of the expression of the human cytochrome c oxidase subunit II (COX-II, 1 of the 3 subunits of COX encoded by mtDNA) and COX activity. We chose HIV-infected individuals undergoing treatment with stavudine and didanosine

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(d4T + ddI) as the nucleoside backbone of a highly active antiretroviral therapy (HAART) regimen because of the great in vitro[12] and in vivo[13,14] capacity of this antiretroviral combination to decrease the mtDNA content.

METHODS

We designed a cross-sectional and observational study including HIV-infected patients on a first-line HAART regimen consisting of d4T + ddI as the nucleoside backbone (plus nelfinavir or nevirapine) for at least 6 months. A control group consisting of antiretroviral-naive HIV-infected patients was also studied. All patients were symptom-free regarding HIV disease and/or antiretroviral therapy at the time of inclusion in the study. None of the individuals receiving treatment had clinical data of lipodystrophy. Clinical and demographic data for each patient, including age, gender, $CD4^+$ T-cell count, HIV-1 RNA copies, and duration of HAART, were recorded at the time of inclusion. Patients with a personal or familial history suggestive of mitochondrial disease or neuromuscular disorder were excluded. As reference values for all the mitochondrial experiments, we used data from 20 healthy individuals matched by age and gender who were not infected with HIV and had been previously assayed in our laboratory. The protocol was approved by the institutional ethics committee of each hospital, and all the patients provided written informed consent.

Obtaining Samples

A total of 20 mL of venous blood was extracted from each patient, and peripheral blood mononuclear cells (PBMCs) were immediately isolated by Ficoll density gradient centrifugation (Histopaque-1077; Sigma Diagnostics, St. Louis, MO). The platelet count after the PBMC extraction procedure was confirmed to be less than 200 per PBMC in each case. Total DNA was obtained by a standard phenol-chloroform extraction procedure from an aliquot of PBMCs and was used for genetic studies. The remaining PBMCs were resuspended in 100 to 150 μL of phosphate-buffered saline (PBS) and used for Western blot studies and enzyme assays. Protein content was measured according to the protein-dye binding principle of Bradford.[15]

Mitochondrial Mass

The quantity of mitochondria was estimated by means of spectrophotometric measurement at 412 nm (UVIKON 922; Kontron, Basel, Switzerland) of the citrate synthase (CS) activity (Enzyme Code (EC) 4.1.3.7), a mitochondrial matrix enzyme of the Krebs cycle, which remains highly constant in mitochondria and is considered to be a reliable marker of mitochondrial content.[16–18] CS activity was expressed as nanomoles of reduced substrate per minute and per milligram of cell protein. The complete methodology has been described elsewhere.[19]

Mitochondrial DNA Quantification

For each DNA extract, the housekeeping r18S nuclear gene and the highly conserved mitochondrial ND2 gene were quantified separately by quantitative real-time polymerase chain reaction (PCR; LightCycler FastStart DNA Master SYBR Green I; Roche Molecular Biochemicals, Mannheim, Germany). Detailed conditions of the experiments have been reported extensively elsewhere.[19] The results were expressed as the ratio of the mean mtDNA value of duplicate measurements to the mean nuclear DNA value (nDNA) of duplicate measurements (mtDNA/nDNA).[20,21] The results of mtDNA content using the methodology described are related to cells. To normalize these results by the cellular mitochondrial content, we also calculated mtDNA content per mitochondria by dividing the mtDNA/nDNA ratio by CS activity.

Measurement of the Cytochrome C Oxidase Subunit II of Cytochrome C Oxidase

Crude protein extracts containing 20 μg of protein were mixed with a 1:5 volume of a solution containing 50% glycerol, 10% sodium dodecyl sulfate (SDS), 10% 2-mercaptoethanol, 0.5% bromophenol blue, and 0.5 M of Tris (pH 6.8), incubated at 90°C for 5 minutes and electrophoresed on 0.1% SDS/13% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were probed with a monoclonal antibody for the mtDNA-encoded human COX-II (A-6404; Molecular Probes, Eugene, OR) as well as with antibodies against voltage-dependent anion carrier (VDAC) or porin (Calbiochem Anti-Porin 31HL; Darmstadt, Germany) as a marker of mitochondrial protein loading and against β-actin (Sigma clone AC-15; St. Louis, MO) as a marker of overall cell protein loading. Immunoreactive material was detected by the enhanced chemiluminescence detection system and resulted in a 25-kd band for COX-II, a 31-kd band for VDAC, and a 47-kd band for β-actin as expected (Fig. 1). The intensity of signals was quantified by densitometric analysis (Phoretics 1D Software; Phoretics International LTD, Newcastle, England). The content of the COX-II subunit was normalized by the content of β-actin signal to establish the relative COX-II abundance per overall cell protein and by the nuclear-encoded mitochondrial

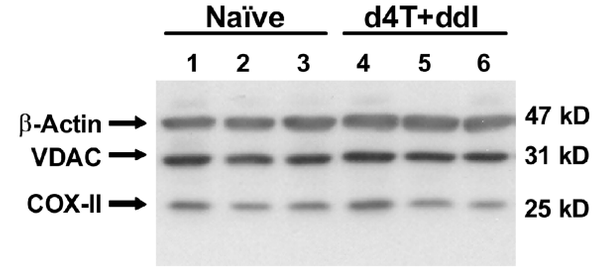

FIGURE 1. Example of immunoblot analysis of cytochrome c oxidase subunit II (COX-II) protein abundance in protein extracts from peripheral blood mononuclear cells of naive (1–3) and stavudine plus didanosine (d4T + ddI)–treated (4–6) patients. Each lane corresponds to 20 μg of protein, and arrows indicate the specific immunoreactive signals for COX-II, voltage-dependent anion channel (VDAC), and β-actin proteins. The COX-II/β-actin and COX-II/VDAC ratios were calculated using densitometric analysis of the chemoluminescence specific signal obtained in Western blots probed with the specific antibodies (see Methods section).

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protein VDAC to establish the relative COX-II abundance compared with overall mitochondria.

Cytochrome C Oxidase (Enzyme Code (EC) 1.9.3.1) Activity

The measurement of the specific enzyme activity was performed spectrophotometrically (UVIKON 922) at 37°C according to Rustin et al[22] and was slightly modified for minute amounts of biologic samples.[18,23] COX activity was expressed as nanomoles of oxidated substrate per minute and per milligram of cell protein. It was also calculated per mitochondrion by dividing absolute COX activity by CS activity.

Statistical Analysis

Qualitative data were expressed as percentages and quantitative data as mean ± SD. Comparisons were carried out by using the χ^2 test and unpaired Student t test for qualitative and quantitative variables, respectively. In the Student t test, normality of the distribution was ascertained using the Kolmogorov-Smirnov test before applying parametric tests. Linear regression analysis was performed to evaluate the relation between quantitative variables. In all cases, P values less than 0.05 were considered statistically significant.

RESULTS

We included 11 consecutive HIV-infected treatment-naive individuals (controls) and 17 consecutive HIV-infected individuals (cases) receiving a first-line HAART regimen containing d4T + ddI as the nucleoside backbone (9 of them receiving nevirapine and 8 of them taking nelfinavir as the third drug of the antiretroviral combination). The clinical characteristics are shown in Table 1. The 2 groups only differed in viral load, which was greater in untreated individuals.

CS activity was 126 ± 19 nmol/min/mg of protein for naive individuals and 92 ± 31 nmol/min/mg of protein for individuals receiving treatment (73% of control activity; $P < 0.001$), indicating decreased mitochondrial mass in patients on d4T + ddI. Individuals receiving d4T + ddI also showed a significant decrease in mtDNA content, which was expressed per cell (60% of control content; $P < 0.01$) or per mitochondrion (72% of control content; $P < 0.05$). Conversely, the expression of the COX-II subunit of COX (encoded by mtDNA) was similar in the 2 groups, regardless of expression

TABLE 1. Clinical Characteristics of Individuals Included in the Study

	Naive (n = 11)	d4T + ddI (n = 17)	*P*
Age (y), ±SD	39 ± 10	42 ± 12	NS
Male gender (%)	91	77	NS
$CD4^+$ lymphocyte count ($cells/mm^3$), ±SD	304 ± 196	446 ± 206	NS
Log_{10} viral load (copies/mL)†, ±SD	5.2 ± 0.5	2.0 ± 0.6	<0.001
Months on treatment, ±SD	—	16 ± 8	—

*$P < 0.05$ was considered to be statistically significant with respect to the controls.
†Viral load <50 copies/mL was considered to be 49 copies/mL.
NS indicates not significant.

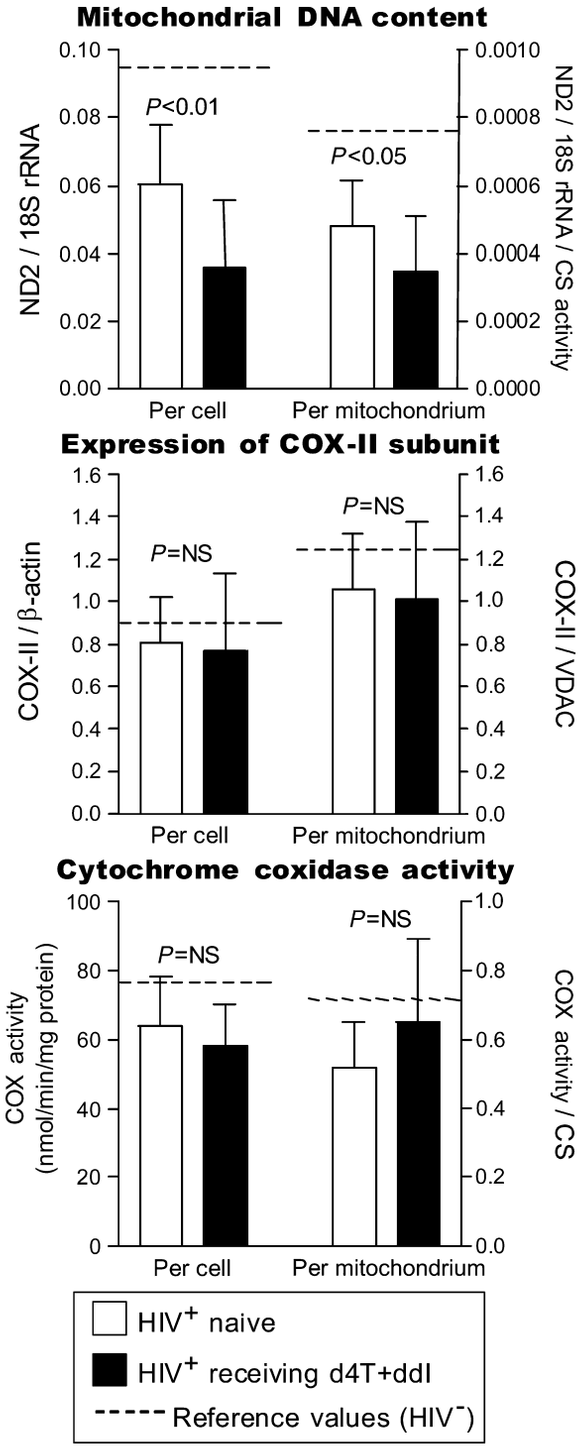

FIGURE 2. Mitochondrial DNA content and expression of cytochrome c oxidase subunit II (COX-II) and COX activity assessed in peripheral blood mononuclear cells of naive and stavudine plus didanosine (d4T + ddI)–treated patients. CS indicates citrate synthase; VDAC, voltage-dependent anion channel.

per cell or per mitochondrion. Similarly, patients receiving d4T + ddI did not show a decrease in COX activity expressed per cell or per mitochondrion (Fig. 2). When we analyzed the cases according to the treatment that they were receiving, nevirapine or nelfinavir in combination with d4T + ddI, we did not find differences between the 2 subgroups for any of the studied mitochondrial parameters (data not shown). It is remarkable that although mtDNA content decreased in HIV-positive treatment-naive patients in comparison to uninfected individuals, the expression of COX-II and COX activity did not differ between the 2 groups.

Expression of the COX-II subunit was independent of the amount of mtDNA for the 2 groups of individuals, and this absence of a relation was confirmed per cell and per mitochondrion. In contrast, expression of the COX-II subunit and COX activity showed a close relation, being stronger in treatment-naive individuals than in subjects undergoing treatment with d4T + ddI (Fig. 3).

The time on antiretroviral treatment was associated with a significant decrease in mtDNA content, whereas the expression of COX-II was mild and not statistically significantly decreased and the activity of COX remained unaltered over time (Fig. 4). Interestingly, when we assessed the effects of HIV infection itself (by means of viral load) and immunologic status (by means of $CD4^+$ T-cell count) on COX-II expression in the absence of antiretrovirals, we found a significantly greater decrease in the expression of COX-II in patients with greater viremia and poorer immunologic status (Fig. 5).

DISCUSSION

The present study demonstrates that although mtDNA depletion is clearly present in asymptomatic HIV-infected individuals treated with antiviral regimens containing d4T + ddI, this depletion is not associated with a decrease in expression of the COX-II subunit (encoded by mtDNA) or with a decrease in COX activity (complex IV of the MRC). Identical conclusions are achieved if the results are normalized per cell or per mitochondrion, which reasonably excludes any masking effect caused by changes in the whole mitochondrial content of the cell as a result of antiretroviral drugs. Thus,

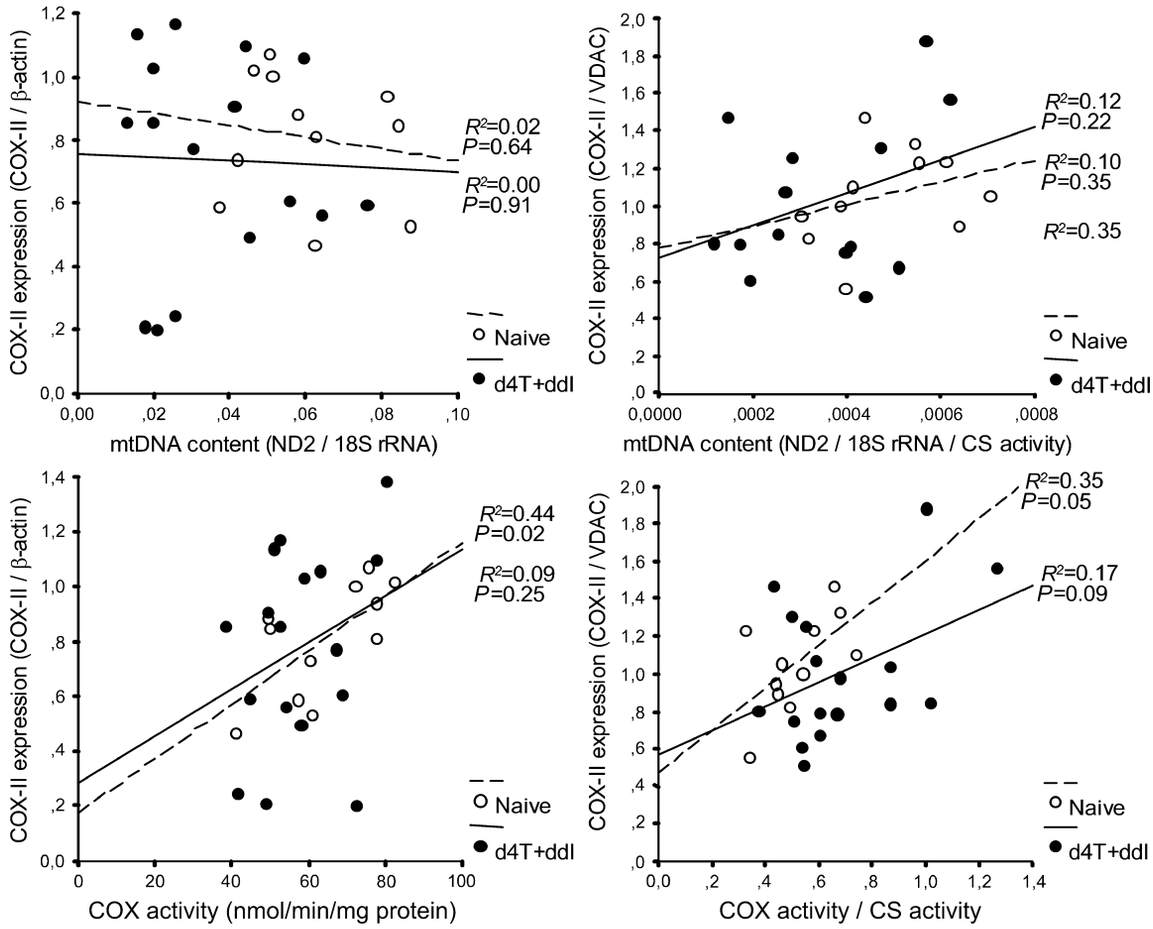

FIGURE 3. Relation between the expression of cytochrome c oxidase subunit II (COX-II) and mitochondrial DNA (mtDNA) content (upper) and COX activity (lower) in peripheral blood mononuclear cells of naive and stavudine plus didanosine (d4T + ddI)–treated patients. In all cases, these relations are assessed per cell (left) and per mitochondrion (right). CS indicates citrate synthase; VDAC, voltage-dependent anion channel.

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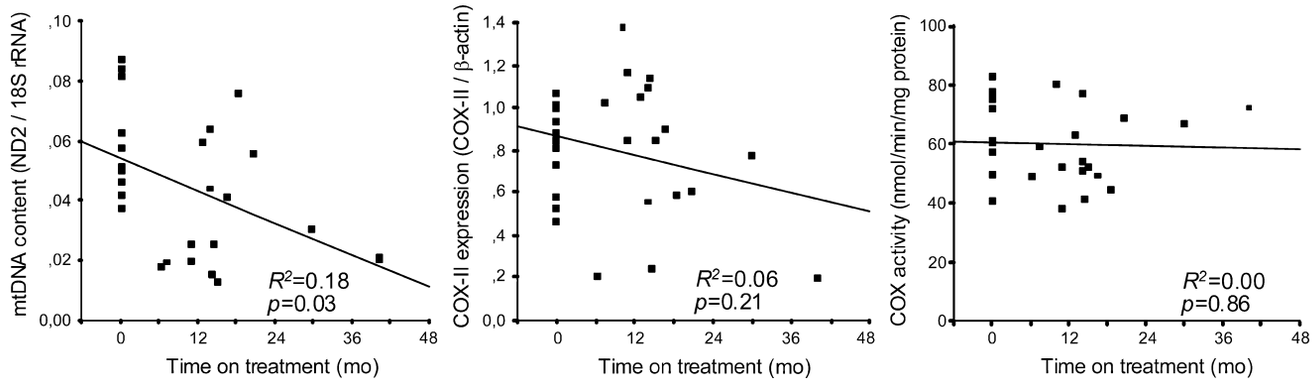

FIGURE 4. Relation between time receiving antiretroviral therapy, including stavudine plus didanosine (d4T + ddI), and mitochondrial DNA (mtDNA) content (left), expression of cytochrome c oxidase subunit II (COX-II; center), and COX activity (right).

these data suggest that transcriptional (increased transcription of mRNA from mtDNA) or posttranscriptional (increased synthesis of protein from mRNA) mechanisms act to compensate for the loss in the number of mtDNA copies. Figure 2 is highly illustrative of the supposition that the amount of COX-II is maintained irrespective of the mtDNA content.

Our results are exclusively related to the conditions of the study (ie, to PBMCs of individuals receiving d4T + ddI for a mean of 16 months as a first-line therapy with no clinical evidence of drug-related long-term secondary effects). Therefore, our data do not mean that mtDNA depletion does not play a role in the adverse effects that may develop during long-term d4T + ddI treatment. PBMCs are not the target of such adverse effects; accordingly, they only represent a conservative estimate of what really occurs at target tissues. In addition, in patients receiving antiretrovirals for longer periods or those who have developed lipodystrophy or hyperlactatemia, the expression of mitochondrially encoded proteins and/or the activity of such proteins may be dramatically impaired. In fact, we found a tendency to a decline in COX-II expression in PBMCs in relation to the length of time on antiretroviral drugs. The results of the present study agree with the finding that in patients suffering from zidovudine myopathy, mtDNA depletion at the skeletal muscle level is accompanied by a clear decrease in COX-II expression in the sarcoplasm.[24] In vitro studies have also shown a time- and dose-dependent mtDNA depletion caused by d4T and ddI on human hepatocellular carcinoma cell line (HepG), which preceded or coincided with a decline in COX-II expression.[25] In any case, we believe that the absence of downstream effects caused by mtDNA depletion emphasizes the efficiency of mitochondria in compensating for antiretroviral toxicities, at least when mtDNA depletion is mild to moderate. This is in accordance with the hypothesis that only mtDNA defects involving more than 80% of the genetic material are able to induce MRC dysfunction.[26]

Compensatory mechanisms for mtDNA depletion have been proposed as an explanation for the lack of a close correlation between time on treatment and the probability of developing adverse effects. The intensity of such compensatory effects may vary from patient to patient according to risk

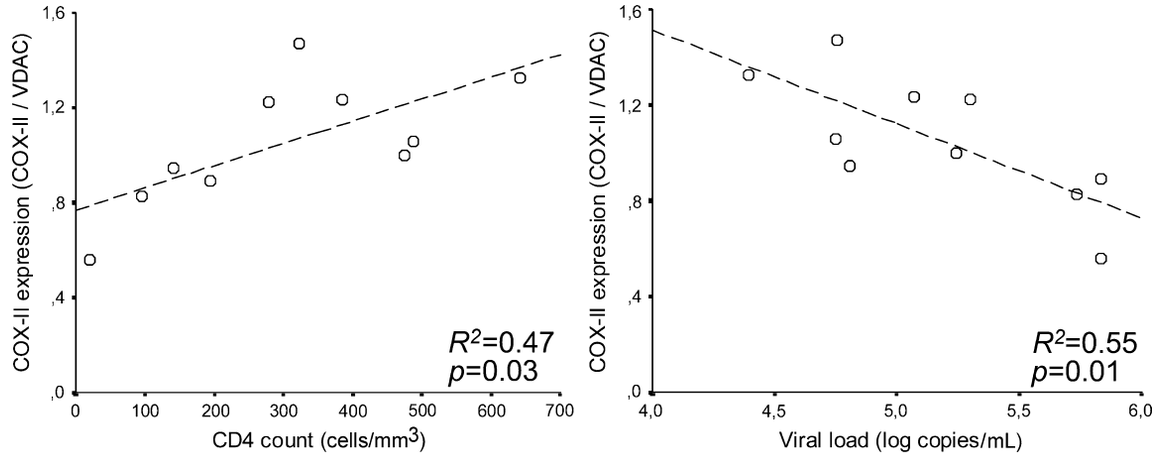

FIGURE 5. Relation between the expression of cytochrome c oxidase subunit II (COX-II) and $CD4^+$ T-cell count (left) and viral load (right) in the absence of antiretroviral therapy (naive patients). VDAC indicates voltage-dependent anion channel.

factors such as the time on antiretroviral therapy, immunologic status, and/or degree of viremia. Interestingly, we found that although the expression of COX-II only showed a weak correlation with the first factor (time on antiretroviral therapy), the latter 2 factors (immunologic status and degree of viremia) were significantly associated with this expression. These findings suggest that toxic mitochondrial effects are not only limited to the inhibition of DNA γ-polymerase caused by antiretrovirals but to the combined effects of being immune compromised and having HIV infection and that receiving antiretrovirals could have a cumulative effect. Nowadays, it has been widely reported that HIV itself is also increasingly implicated in diverse and extensive mitochondrial disturbances,[3,14,27–32] most of which are triggered by mitochondrially dependent apoptotic mechanisms. Consistent with those reports, the present study also postulates that HIV-infected patients naive to antiretrovirals have a decreased amount of mtDNA compared with uninfected people. Conversely, the expression of COX-II and COX activity seems to be less influenced than mtDNA content by the effects of HIV infection itself. Accordingly, adaptive mechanisms may be effective in withholding the decline in MRC function caused by mtDNA depletion as a result of administration of d4T + ddI in certain circumstances; however, in other cases, the collateral actions of antiretrovirals and/or HIV itself against mitochondria may be the final determinants leading to mitochondrial failure. In any case, the demonstration of up-regulatory mechanisms compensating for mtDNA depletion is an additional argument for the need to be cautious when using mtDNA quantification as the only tool to monitor the clinical relevance of the mitochondrial toxicity of antiretroviral drugs.

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