Reverse transcriptase inhibitors alter uncoupling protein-1 and mitochondrial biogenesis in brown adipocytes

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Objective: Human adipose depots contain remnant brown adipocytes interspersed among white adipocytes, and disturbances of brown with respect to white adipocyte biology have been implicated in highly active antiretroviral therapy (HAART)-induced lipomatosis. Brown adipocytes express the uncoupling protein-1 (UCP1) and contain a large number of mitochondria, potential targets of HAART toxicity. The aim of this study was to evaluate the effects of reverse transcriptase inhibitors (RTIs) on primary brown adipocytes differentiated in culture.

Design and methods: We analysed the effects of RTIs, nucleoside analogues (NRTIs: stavudine, zidovudine, didanosine and lamivudine) and non-nucleoside analogues (NNRTIs: nevirapine and efavirenz), on differentiation, mitochondrial biogenesis and gene expression in brown adipocytes.

Results: None of the NRTIs altered brown adipocyte differentiation whereas NNTRIs had differing effects. Efavirenz blocked lipid deposition and expression of adipose marker genes but nevirapine induced lipid accumulation and adipose gene expression, promoted mitochondrial biogenesis and increased UCP1. Stavudine, zidovudine and didanosine reduced mitochondrial DNA (mtDNA) content. However, mitochondrial genome expression was only impaired in didanosine-treated adipocytes. Stavudine, but not zidovudine, induced expression of the mitochondrial transcription factors and this may explain compensatory mechanisms for the depletion of mtDNA by up-regulating mtDNA transcription. Stavudine caused a specific induction of UCP1 gene expression through direct interaction with a retinoic acid-dependent pathway.

Conclusions: Specific disturbances in brown adipocytes in adipose depots may contribute to HAART-induced lipomatosis. Mitochondrial depletion does not appear to be the only mechanism explaining adverse effects in brown adipocytes because there is evidence of compensatory mechanisms that maintain mtDNA expression, and the expression of the UCP1 gene is specifically altered.

Introduction

Highly active antiretroviral therapy (HAART) for HIV-1 usually combines two nucleoside reverse transcriptase inhibitors (NRTIs) with either a non-nucleoside reverse transcriptase inhibitor (NNRTI) or a protease inhibitor (PI). HAART has led to substantial reductions in morbidity and mortality among patients. However, a serious metabolic syndrome has arisen in treated patients, referred to as the HAART-associated lipodystrophy syndrome [1], with a prevalence of about 45% [2]. The main symptoms are body region-specific disturbances of fat distribution: severe loss of peripheral adipose tissue and increased visceral adipose tissue [3]. An estimated 6% of AIDS patients under HAART therapy develop lipomatosis, often characterized by enlarged adipose depots in the dorso-cervical region known as 'buffalo humps' [4]. Other metabolic disturbances include dyslipidaemia, hyperglycaemia and insulin resistance [1,3,5].

Alterations in body fat distribution and lipid metabolism among HIV-infected patients on HAART suggest adipocyte dysfunction. Initial reports implicated PIs in the development of lipodystrophy [5]. Several *in vitro* studies have shown that PIs affect adipocyte differentiation and lipid accumulation in the 3T3 white adipocyte cell lines (see [6] for a review), and alterations in the transcriptional factor SREBP-1c may be responsible for some of these effects [7–9]. It is also widely accepted that RTIs alone can induce the pathogenesis of lipodystrophy [3,10–12]. However, *in vitro* experiments on the effects of RTIs are scarce. NRTIs

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have been reported to interfere to some extent with 3T3 adipocyte differentiation [13,14] and recently, the NNRTI efavirenz (EFV) has been shown to inhibit the SREBP-1c-dependent lipogenic pathway [15]. Nevertheless, NRTIs can induce mitochondrial toxicity via specific inhibition of DNA polymerase-γ [16]. Therefore, it has been suggested that depletion of mitochondrial DNA (mtDNA) content through inhibition of mtDNA synthesis in adipocytes contributes to the development of lipodystrophy [17]. Recently, several studies have shown depletion of mtDNA in subcutaneous fat from NRTI-treated patients with lipodystrophy [18,19]. Furthermore, there is also evidence that NRTIs can affect mitochondrial function through mechanisms not involving inhibition of mtDNA polymerase-γ, such as mtDNA deletions [20] or direct inhibition of mitochondrial respiration [21], probably through the inhibition of ADP/ATP translocase [22].

In mammals, there are two types of adipocyte: 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)] [23]. In humans, brown adipose depots are found in neonates whereas remnant brown adipocytes interspersed among white adipocytes remain in adult adipose depots [24]. The relative amount of brown adipocytes depends on the 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 with respect to white adipose cells [23]. We have recently reported high UCP1 gene expression in lipomas from HAART-treated HIV patients, thus implicating brown adipocytes in the development of HAART-associated lipomatosis [25].

Available data in white adipocytes in culture are of limited value for assessing the disturbances in brown adipocytes potentially implicated in lipomatosis, as brown and white adipocytes have different biological features. Therefore, the aim of this study was to evaluate the effects of RTI drugs used in HAART on primary brown adipocytes differentiated in culture. We determined the effects of RTIs on brown adipocyte differentiation, mitochondrial biogenesis and gene expression.

Materials and methods

Cell culture and treatment

Stavudine (d4T), didanosine (ddI) and EFV were obtained from Bristol-Myers-Squibb (Princeton, NJ, USA), lamivudine (3TC) and zidovudine (AZT) were from GlaxoSmithKline (Greenford, UK), and nevirapine (NVP) was from Boehringer Ingelheim/Roxane (Ridgefield, CT, USA). All NRTIs were dissolved in phosphate buffer saline whereas NNRTIs were dissolved in dimethyl sulphoxide (DMSO). All-*trans* retinoic acid, norepinephrine, ascorbic acid, 3,5,3′-triiodothyronine (T_3), insulin, propanolol and prazosin were purchased from Sigma-Aldrich (St Louis, MO, USA). BRL49653 (rosiglitazone) was kind gift from Dr L Casteilla (Toulouse, France). The specific retinoic acid receptor (RAR) antagonist AGN193109 was kind gift from Dr R Chandraratna (Allergan Pharmaceuticals, Irvine, CA, USA).

Primary culture of differentiated murine brown adipocytes was performed as described previously [26]. Precursor cells were grown in 4 ml of Dulbecco's modified Eagle's medium (DMEM)-Ham's F12 medium (1:1, v/v) supplemented with 10% (v/v) fetal calf serum (FCS), 20 nM insulin, 2 nM T_3 and 100 µM ascorbate. At confluence (day 4 of culture), cells were incubated in the absence or presence of each antiretroviral drug until day 8 of culture (long-term treatments). The short-term experiments (24 h or 48 h) with the different RTIs, rosiglitazone at 10 µM, all-*trans* retinoic acid at 1 µM, propanolol plus prazosin at 10 µM and AGN193109 at 1 µM, were performed on day 8 of culture when 80–90% of the cells were considered to be differentiated on the basis of lipid accumulation and acquisition of brown adipocyte morphology. Norepinephrine was added at 0.5 µM for 5 h. Lipid accumulation was quantified by Oil Red-O staining as described elsewhere [27].

Determination of mtDNA abundance

Relative mtDNA abundance was assessed as described elsewhere [28]. Total DNA from preadipocytes or from fully differentiated brown adipocytes in primary culture, treated or not with the various RTIs, was obtained by digesting the cells with proteinase K followed by a phenol/chloroform precipitation. Then 20 µg of total DNA was digested with *Eco*RI endonuclease and analysed by Southern blot. Blots were hybridized with the murine mtDNA fragment encoding 16S rRNA [29], 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 genomic probe [30], which reveals a 4.5 kb band for the corresponding nuclear gene fragment.

RNA isolation and Northern blot analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen Inc, Chatsworth, CA, USA). For Northern blot analysis, 10 µg of total RNA was denatured, electrophoresed on 1.5% formaldehyde-agarose gels, and transferred to positively-charged nylon membranes (N^+; Boehringer Mannheim, Indianapolis, IN, USA).

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Equivalent amounts of ribosomal RNA in the samples were checked by ethidium bromide UV visualization. Prehybridization and hybridization were performed at 55°C in a 0.25M Na_2HPO_4 (pH 7.2), 1 mM EDTA, 20% SDS and 0.5% blocking reagent (Boehringer Mannheim) solution. Blots were hybridized using mtDNA fragments as probes for detection of the mtDNA-encoded cytochrome oxidase subunit II [31] and mitochondrial 16S rRNA [29]. The murine cDNAs for the nuclear-encoded cytochrome oxidase subunit IV (ATCC, Rockville, MD, USA), PGC-1α [32], UCP1 [33], UCP2 [34], adipocyte–fatty acid binding protein aP2/FABP [35], peroxisome proliferator activated receptor γ (PPARγ) [36], CCAAT/enhancer binding protein α (C/EBPα) [37], mitochondrial transcription factor A [38] and the mitochondrial transcription factors B1 and B2 probes [39] were also used as hybridization probes. The DNA probes were labeled using [α-^{32}P]dCTP by the random oligonucleotide-priming method (Amersham Biosciences, Bucks, UK).

Preparation of protein extracts and Western blot analysis

For the protein extracts, fully differentiated brown adipocytes in primary culture treated or not treated with the RTIs were harvested in a buffer containing 100 mM TrisHCl (pH 8), 250 mM NaCl, 1 mM EDTA, 1% IGEPAL, 0.5 mM PMSF, a cocktail of PIs (leupeptin, pepstatin, aprotinin at 1 µg/ml and benzamidin 1 mM) and 0.5 mM DTT. The harvested cells were incubated for 1 h at 4°C and centrifuged at 13 000 rpm for 10 min at 4°C. The final supernatant was the protein homogenate. Protein concentration was measured using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA), using bovine serum albumin as a standard.

For Western blot analysis, samples containing 50 µg of protein extract were mixed with 1/5 volume of a solution containing 50% glycerol, 10% 2β-mercaptoethanol, 0.5% bromophenol blue and 0.5M Tris (pH 6.8), incubated at 90°C for 5 min and electrophoresed on SDS/13% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes (Millipore Corp, Bedford, OH, USA). Blots were probed with a monoclonal antibody for the mtDNA-encoded mouse cytochrome c oxidase subunit I (A-6403; Molecular Probes, Eugene, OR, USA), as well as with the antibodies against the voltage-dependent anion carrier or porin (VDAC) (Calbiochem Anti-Porin 31HL; Calbiochem, Darmstadt, Germany), as a marker of mitochondrial protein loading, and against β-actin (Sigma clone AC-15), as a marker of overall cell protein loading. Immunoreactive material was detected using the enhanced chemiluminiscence method (ECL; Amersham Biosciences).

Transient transfection experiments

The HIB-1B brown adipocyte cell line, kindly provided by Dr B Spiegelman, was cultured in DMEM:F12 (1:1) supplemented with 10% heat-inactivated FCS and 4 mg/l biotin. The transient transfection experiments were performed with HIB-1B preadipocytes at 80% of confluence, using the FuGene6 Transfection Reagent (Roche Molecular Biochemicals, Mannheim, Germany), following the manufacturer's protocol. The plasmid (–4551)UCP1-CAT contains the region –4551 to +110 of the rat UCP1 gene driving the promoterless chloramphenicol acetyltransferase (CAT) gene [26], and the expression vector pRSV-RARα contains the entire open-reading frame of the human RARα protein [40]. Each transfection contained 1 µg of 4.5 kb UCP1-CAT, 0.05 µg of the hRARα expression vector and 0.1 µg of cytomegalovirus β-galactosidase vector, as internal control for variation in transfection efficiency. 24 h after the transfection process, cells were treated or not with d4T or NVP at 20 µM for a further 24 h. CAT and β-galactosidase activities were measured as described elsewhere [41].

Densitometric analysis

Quantification of autoradiographs and ECL signals was performed by densitometric analysis (Phoretics 1D software; Phoretics International Ltd, Newcastle, UK). Only signals in the linear range were analysed.

Statistical analysis

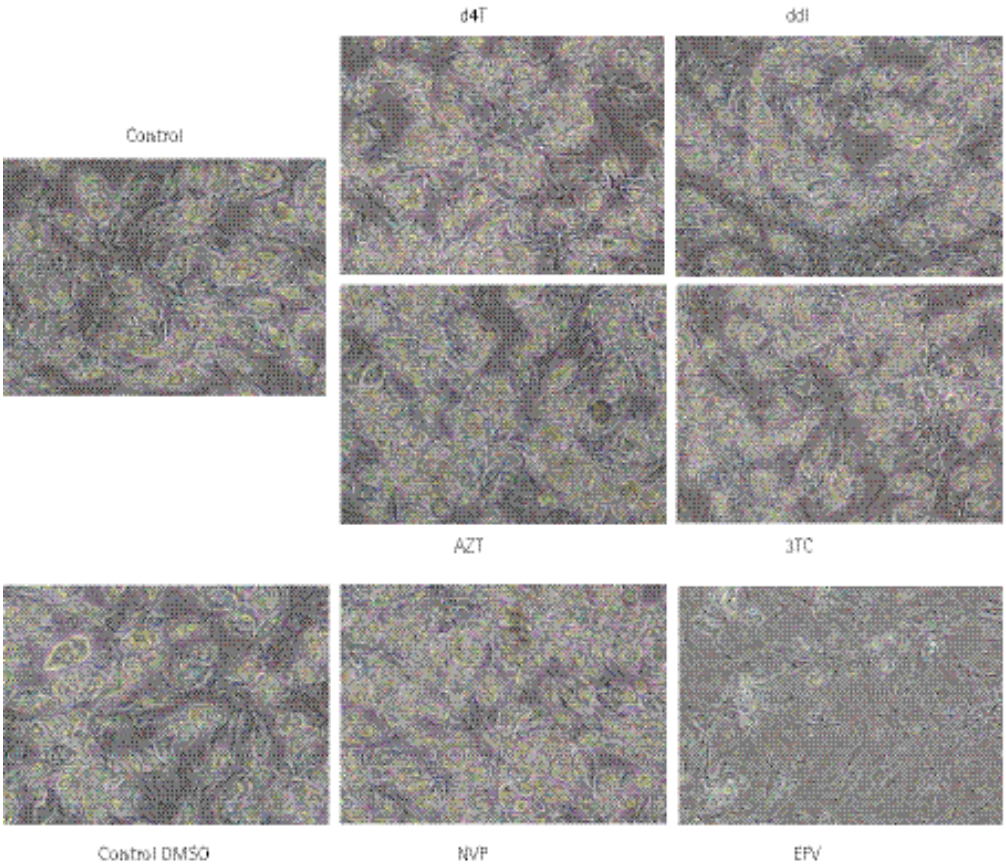
Where appropriate, statistical analysis was performed by Mann–Whitney U test, and significance is indicated on the figure legends.

Results

NVP induced but EFV impaired terminal differentiation of brown adipocytes, whereas NRTIs did not interfere with the process

Brown preadipocytes in primary culture proliferate until day 4, after which they differentiate until day 8, as shown by the acquisition of multilocular lipid accumulation and expression of the UCP1 gene [26]. When d4T, ddI, AZT or 3TC were added to the medium on day 4 of culture, acquisition of differentiated cell morphology at day 8 was unaltered (Figure 1, top). Lipid content quantified by Oil red-O staining was also unaltered (data not shown). The drugs were used at 20 µM, which was in the range of their maximum concentration values and similar to that used in white adipose cell studies [7,13,14,42]. NNRTIs had the opposite effect on brown adipocyte morphological differentiation: NVP slightly increased lipid accumulation whereas cells induced to differentiate in the presence of EFV did not accumulate lipid droplets

Figure 1. Effect of RTIs on morphological differentiation of primary brown adipocyte cultures



Confluent primary brown preadipocytes (day 4) were differentiated in the absence (control and control with solvent DMSO), or presence of various RTIs at a concentration of 20 μM until day 8 of culture. The NRTIs d4T, ddI, AZT and 3TC were dissolved in PBS, whereas the NNRTIs NVP and EFV were dissolved in a final concentration of 0.1% DMSO. Control DMSO cells were differentiated in the presence of 0.1% DMSO. Cells were examined by phase contrast microscopy at x40 magnification on day 8 of culture. The images are representative of at least three independent experiments performed in different primary cultures. 3TC, lamivudine; AZT, zidovudine; d4T, stavudine; ddI, didanosine; EFV, efavirenz; NVP, nevirapine.

(Figure 1, bottom). Lipid content was 144 ±12% in NVP-treated cells and 11 ±1% in EFV-treated cells, respect to solvent-control cells (DMSO).

NRTIs, except 3TC, reduced whereas NVP increased mtDNA content

To analyse whether RTIs affect mitochondrial biogenesis, mtDNA content per cell was assessed by quantifying the relative amount of mtDNA with respect to nuclear DNA. d4T, ddI and AZT reduced mtDNA in brown adipocytes, whereas 3TC and EFV did not (Figure 2A). The relative amount of mtDNA almost doubled from day 4 of culture to day 8, indicating that these NRTIs blocked the differentiation-dependent increase in mtDNA abundance (Figure 2B). In contrast, when brown adipocytes were treated with NVP, mtDNA abundance was double that of solvent-control cells (DMSO).

Mitochondrial genome expression was only impaired in ddI-treated cells

To examine whether changes in mtDNA abundance affect mitochondrial biogenesis, gene expression for the mitochondrial-encoded 16S rRNA and subunits I and II of cytochrome c oxidase (CO), a component of the mitochondrial respiratory system, was assessed (Figure 2C,D). Only ddI treatment reduced 16S rRNA and COII mRNA, thus suggesting a compensatory up-regulation of mtDNA transcription in d4T and AZT-treated cells (Figure 2C). In contrast, increased mtDNA abundance in NVP-treated cells resulted in a significant increase in mitochondrial transcripts 16S rRNA and COII mRNA. When COI protein levels were analysed, a parallel decrease in ddI-treated cells and an increase in NVP-treated cells was observed. These changes were specific for the mitochondrial-genome encoded protein and not related to any general effect upon mitochondrial

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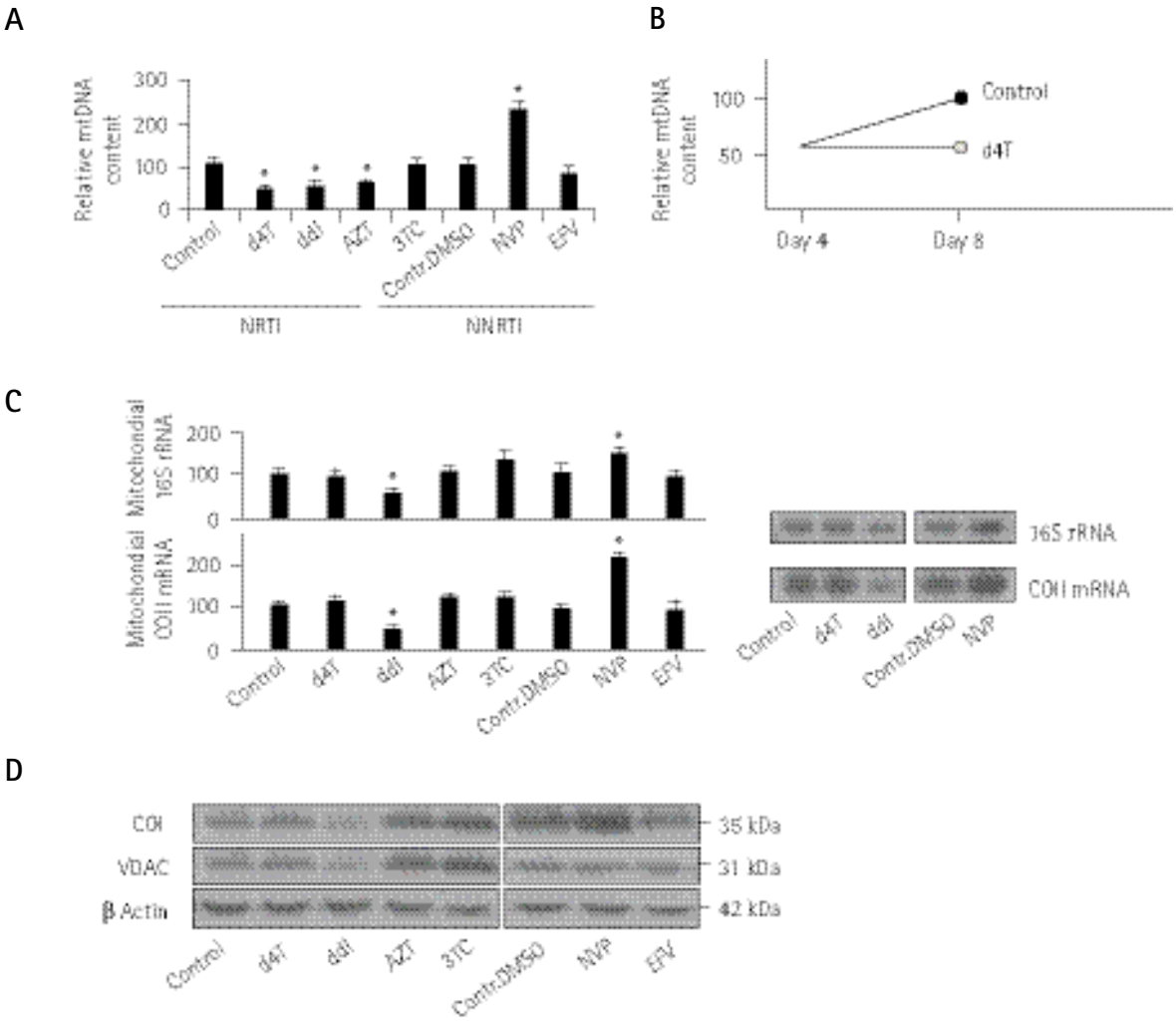
Figure 2. Effect of RTIs on mitochondrial biogenesis in brown adipocytes differentiating in culture



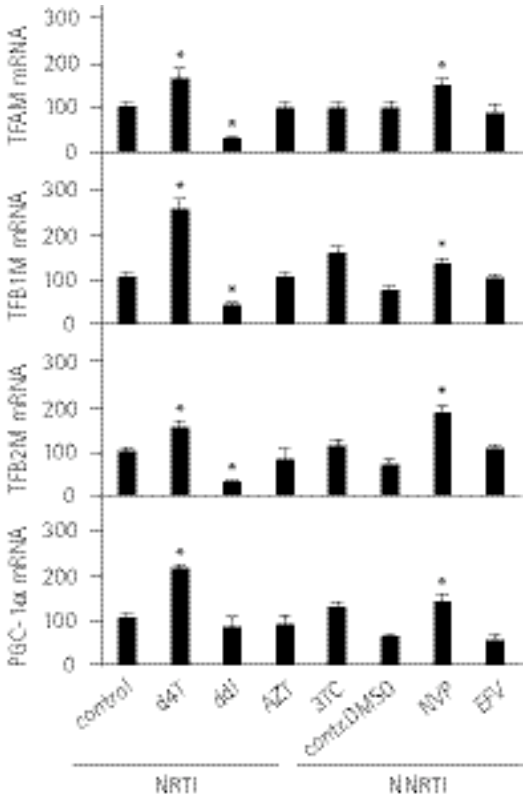
Confluent brown preadipocytes (day 4) were differentiated in the absence (control and control 0.1% DMSO), or presence of the indicated NRTIs (d4T, ddI, AZT and 3TC) or NNRTIs (NVP and EFV) at 20 μM until day 8 of culture. Bars are means ±SEM of two to three independent experiments with duplicate plates and are expressed relative to the untreated control cells, set to 100. Statistical significance of comparisons between treated cells and their respective controls are shown by: *$P \geq 0.05$, **(A)** Relative abundance of mtDNA was assessed by quantifying the relative amount of mtDNA with respect to nuclear DNA by Southern blot analysis. **(B)** Profile of relative mtDNA content during differentiation. Untreated preadipocytes at day 4 of culture were compared with cells at day 8 differentiated from day 4 in the absence (control) or presence of 20 μM d4T. Points are means of two independent experiments and are expressed relative to control cells at day 8, which were set to 100. **(C)** Mitochondrial genome expression as measured by the relative abundance of the mitochondrial transcripts 16S rRNA and cytochrome oxidase subunit II (COII) mRNA as determined by Northern blot analysis, with representative Northern blots shown on the right. **(D)** Relative abundance of cytochrome oxidase subunit I (COI) protein as determined by Western blot analysis of whole-cell lysates, and control of mitochondrial protein (VDAC) and total protein loading (β-actin). A representative immunoblot from three independent experiments is shown. Contr., control; VDAC, voltage-dependent anion carrier porin; AZT, zidovudine; d4T, stavudine; ddI, didanosine; 3TC, lamivudine; EFV,efavirenz; NVP, nevirapine.

or cell protein content (assessed by nuclear-encoded voltage-dependent anion carrier (VDAC) or β-actin, respectively) (Figure 2D).

Changes in mitochondrial transcription factors expression as a compensatory mechanism to ensure mtDNA expression

In an attempt to identify how d4T- and AZT-treated cells compensate mtDNA depletion, the expression of the transcription factors that regulate mtDNA transcription was evaluated. As depicted in Figure 3, expression of mitochondrial transcription factor A (TFAM), which is essential for mammalian mtDNA transcription and genome maintenance [43], was decreased in ddI-treated cells, whereas it remained unaltered when cells were exposed to AZT, 3TC or EFV. In contrast, treatment with d4T increased TFAM mRNA expression. A similar profile of expression in

Figure 3. Effect of RTIs on gene expression of mitochondrial transcription factors and co-activator PGC-1α



Messenger RNA expression of mitochondrial transcription factors TFAM, TFB1M and TFB2M and of coactivator PGC-1α were analysed by Northern blot. For details, see the legend to the Figure 2. Contr., control; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, non-nucleoside reverse transcriptase inhibitor.

response to NRTI treatment was found for the recently identified transcription factors involved in the initiation and regulation of mitochondrial transcription, the mitochondrial transcription factor B1 (TFB1M) and B2 (TFB2M) [44]. Thus, a compensatory up-regulation of mitochondrial transcription by increased expression of mitochondrial transcription factors can be proposed in cells treated with d4T whereas it does not explain unaltered levels of mitochondrial transcripts in AZT-treated cells. Next, we analysed the expression of the co-activator PGC-1α which has been demonstrated to induce mitochondrial biogenesis in several tissues including brown fat and, in that tissue, to coordinately activate the thermogenic program including UCP1 gene expression [32,41]. Only d4T induced the expression of PGC-1α mRNA, which was not affected by any other NRTI nor by EFV. In contrast, NVP increased the mRNA expression of TFAM, TFB1M, TFB2M and PGC-1α, in agreement with its global positive effect upon mitochondrial biogenesis. Furthermore, NVP increased the expression of the nuclear-encoded subunit IV of cytochrome c oxidase (COIV) mRNA, while the NRTIs and EFV did not (Figure 4A).

d4T specifically induced UCP-1 gene expression

We next analysed whether the expression of genes that are markers of brown-specific function or adipose differentiation and metabolism was affected by long-term RTI treatment of brown adipocytes. Expression of UCP2 and C/EBPα mRNAs was not affected by any RTI treatment (Figure 4A,B). EFV decreased the expression of the adipogenic marker genes aP2/FABP and PPARγ. In contrast, d4T and NVP induced UCP1 mRNA expression. NVP also induced expression of the adipogenic marker gene aP2/FABP and PPARγ.

The effects of d4T and NVP on differentiated adipocytes on day 8 of culture were examined. NVP had no effect (Figure 5A and data not shown), while d4T caused a twofold induction of UCP1 mRNA expression after 24 h (Figure 5A), which remained after 48 h (data not shown). The effects of d4T on differentiated brown adipocytes were specific for UCP1 since neither COII mRNA (Figure 5A) nor COIV mRNA or aP2/FABP mRNA (not shown) levels were altered. The effects of d4T on UCP1 mRNA expression were dose-dependent, with maximum induction at 20 μM (Figure 5B).

d4T-induced UCP1 gene expression through a retinoic acid-mediated pathway

We next analysed whether the effects of d4T implicate known pathways of regulation of UCP1 gene expression. Noradrenaline directly activates UCP1 gene transcription through a cAMP-response element in the proximal regulatory promoter [45]. Furthermore, noradrenaline highly induces the expression of the co-activator PGC-1α [32], which also contributes to cAMP-mediated up-regulation of UCP1 gene transcription by co-activating nuclear hormone receptors bound to the upstream enhancer of the gene [41]. Among them, all-*trans* retinoic acid receptors (RARs) and 9-*cis*-retinoic acid receptors (RXRs) were responsible for the powerful induction by retinoic acid of UCP1 gene transcription [26,46]. PPARγ and PPARα also activated transcription of the UCP1 gene promoter [41,46]. Addition of adrenergic antagonists (propanolol plus prazosin) did not affect d4T induction of UCP1 gene expression (Figure 6A), although they impaired the activation by noradrenaline (not shown). In contrast, the specific RAR antagonist AGN193109 completely blocked d4T induction of UCP1 gene expression in both the long-term (Figure 6A) and short-term treatment of brown adipocytes (Figure 6B). The inhibitory effects of AGN193109 were specific for retinoic acid induction of UCP1 mRNA expression

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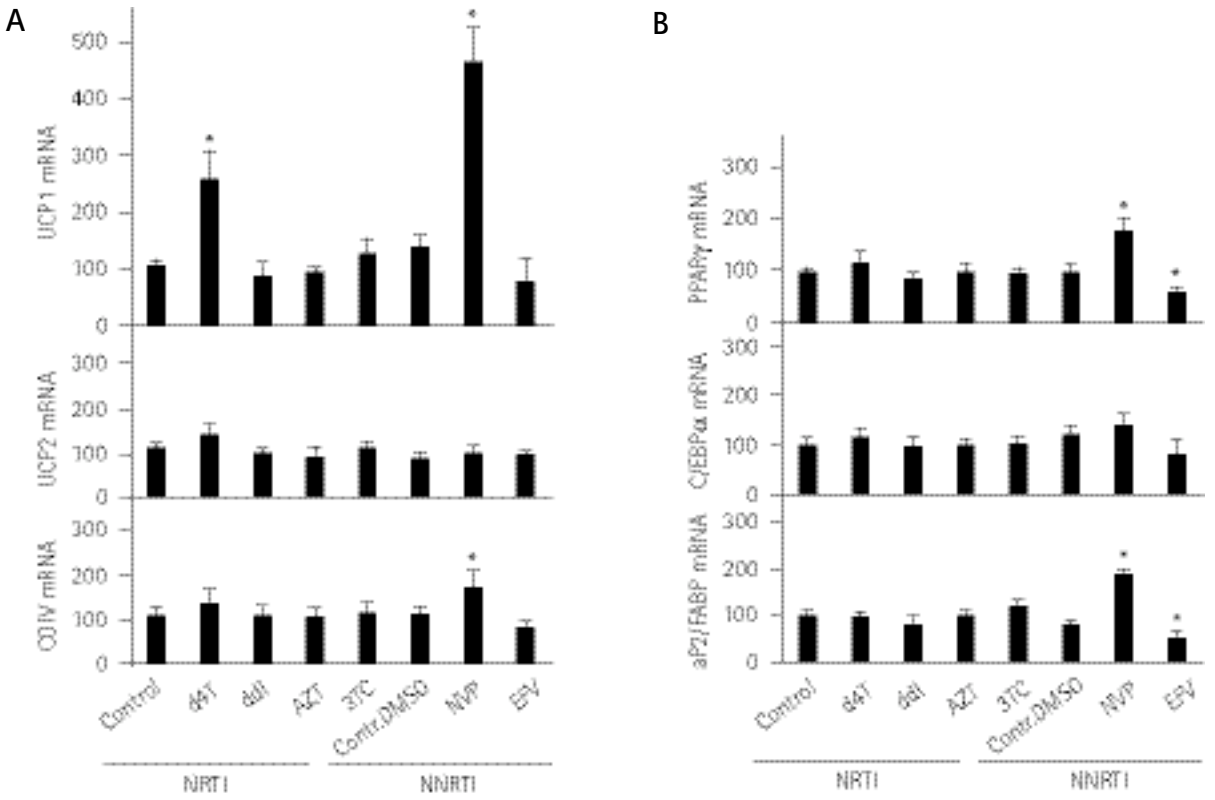
Figure 4. Effect of RTIs on the expression of uncoupling proteins, mitochondrial and adipose gene markers



(A) mRNA expression of uncoupling proteins UCP1 and UCP2, and of nuclear-encoded mitochondrial COIV. **(B)** mRNA expression of adipose marker adipocyte-fatty acid–binding protein (aP2/FABP) and adipogenic transcription factors PPARγ and C/EBPα was analysed by Northern blot. For details, see the legend to the Figure 2. COIV, cytochrome oxidase subunit IV; Contr., control; RTI, reverse transcriptase inhibitor; UCP1/2, uncoupling protein-1/2; AZT, zidovudine; d4T, stavudine; ddI, didanosine; 3TC, lamivudine; EFV,efavirenz; NVP, nevirapine.

without interfering with its induction by noradrenaline or the PPARγ-agonist rosiglitazone (Figure 6C). Thus, induction of UCP1 gene expression by d4T involves a retinoic acid-mediated pathway.

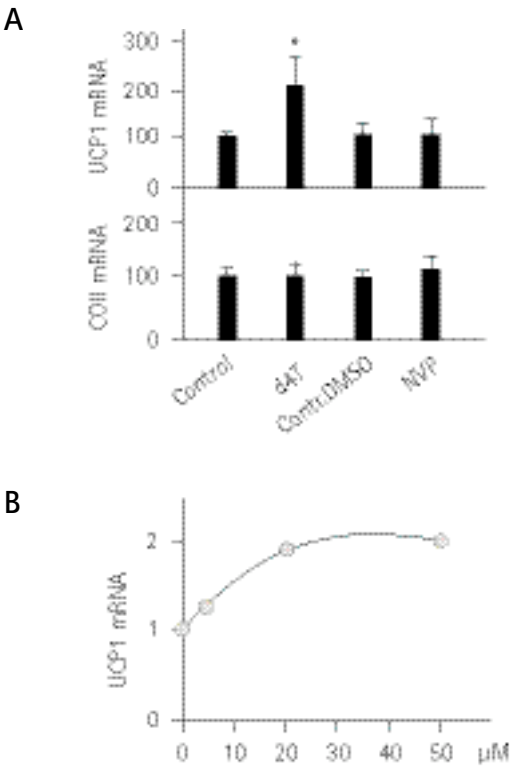
To further investigate d4T regulation of UCP1 gene expression, brown adipocyte derived HIB-1B cells were transiently transfected with a plasmid containing the upstream 4.5 kb of the UCP1 gene fused to a CAT reporter gene. d4T, but not NVP, significantly increased 4,5UCP1-CAT activity only when the expression vector for RAR was transfected (Figure 7), further indicating an involvement of an RAR-mediated signalling pathway in UCP1 gene induction by d4T.

Discussion

In humans, remnant brown adipocytes are interspersed among white adipocytes in adult adipose depots and it has been recently reported that brown adipocytes are involved in the development of HAART-induced lipomatosis [25]. Here we report that RTIs have direct effects on brown adipocytes by interfering with mitochondrial biogenesis, differentiation and specific expression of the brown adipocyte gene marker UCP1.

None of the NRTIs tested impaired brown adipocyte differentiation. This is similar to data from the few studies in the murine white adipose cell lines 3T3-L1 [7,42] and 3T3-F442A [13,14], although d4T and AZT have very recently been reported to alter terminal lipid deposition in 3T3-F442A cells [14]. The NNRTIs studied have the opposite effect on the differentiation of primary brown adipocytes: whereas EFV blocked lipid accumulation, NVP had a global positive effect. The negative effect of EFV on brown adipocyte differentiation was similar to that previously reported in white adipose cell lines and primary cultures of human preadipocytes [15]. In contrast, NVP enhanced the differentiation of primary brown adipocytes: it induced lipid deposition and expression of adipose markers, promoted mitochondrial biogenesis and increased the expression of the UCP1 gene. These effects depend on long-term treatment of cells during differentiation and,

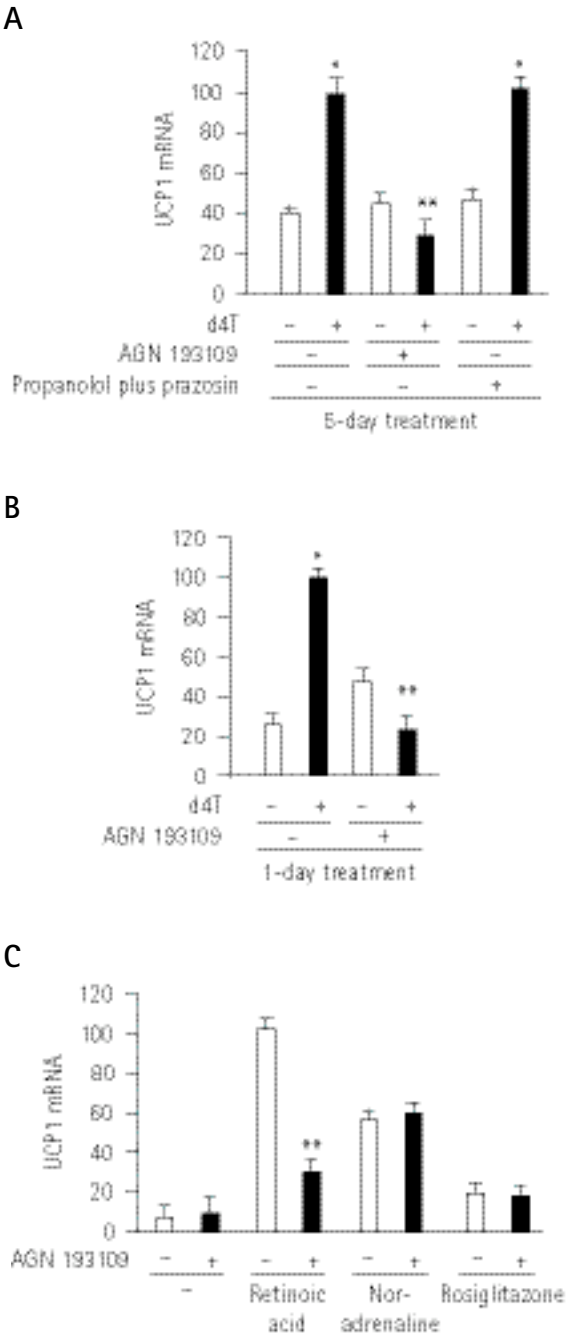
Figure 5. Acute induction of UCP1 gene expression by stavudine



(A) Differentiated brown adipocytes at day 8 of culture were maintained for 24 h in the absence (control and control 0.1% DMSO), or presence of either 20 μM stavudine or 20 μM nevirapine in 0.1% DMSO. Bars are means ±SEM of two to three independent experiments with duplicate plates and are expressed as relative to the untreated control cells which was set to 100. Statistical significance of comparisons between treated cells and their respective controls are shown by: * $P \leq 0.05$, **(B)** Dose–response curve for the effects of the NRTI d4T on UCP1 gene expression. Differentiated brown adipocytes (day 8) were exposed to the indicated concentrations of d4T for 24 h. Points are means from two to three independent experiments with duplicate plates, in which the variation within the experimental groups is less than 15%. COII, cytochrome oxidase subunit II; Contr., control; UCP1, uncoupling protein-1; d4T, stavudine; NVP, nevirapine.

in contrast to the effects of d4T (see below), they did not occur in acute treatment, thus further supporting that the effects of NVP occur through a positive global effect upon brown adipocyte differentiation. Mangiacasale *et al.* [47] have recently reported that NVP inhibits proliferation and promotes differentiation in several normal and transformed cell types by inhibiting the endogenous non-telomeric reverse transcriptase. Further studies are needed to determine whether this or other molecular mechanisms contribute to NVP induction of brown adipocyte differentiation, but whatever the mechanism involved, present data should be taken in consideration regarding the potential action of NVP in the induction of HAART-associated lipomatosis.

Figure 6. Effect of the RAR antagonist AGN193109 on the induction of UCP1 gene expression by stavudine



(A) Confluent brown preadipocytes (day 4) were differentiated in the absence or presence of 20 μM stavudine and/or adrenergic antagonists propanolol (10 μM) plus prazosin (10 μM), or 1 μM AGN193109 (a specific RAR-antagonist) until day 8 of culture. **(B)** Differentiated brown adipocytes (day 8) were exposed to 20 μM d4T and/or 1 μM AGN193109 for 24 h, or **(C)** to the activators of UCP1 gene transcription all-*trans*-retinoic acid (1 μM), noradrenaline (0.5 μM) or rosiglitazone (10 μM), in the presence or not of 1 μM AGN193109 for 24 h. UCP1 mRNA expression was analysed by Northern blot. Bars are means ±SEM of two to three independent experiments with duplicate plates and are expressed relative to the point of maximal expression, which was set to 100. Statistical significance of comparisons is shown by: *$P \leq 0.05$, for the effect of d4T and **$P \leq 0.05$, for the effect of AGN193109. RAR, retinoic acid receptor; UCP1, uncoupling protein-1; d4T, stavudine.

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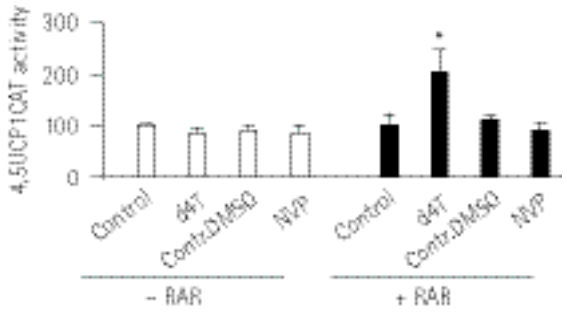
Figure 7. Effects of d4T on the transcriptional activity of the UCP1 gene promoter



Transient transfection experiments of HIB-1B brown preadipocytes were performed with 1 µg plasmid containing the upstream 4.5 kb of the UCP1 gene promoter fused to a CAT reporter gene (4,5UCP1CAT). When indicated, 0.05 µg of the expression vector for the RARα (pRSV-RARα) was co-transfected. Cells were exposed to 20 µM d4T or NVP for 24 h or not (control or 0.1% DMSO solvent control for NVP). Results are expressed as CAT activity relative to control, which is set to 100, and are means ±SEM of two independent experiments, each performed in duplicate. CAT, cloramphenicol acetyltransferase; Contr., control; RAR, retinoic acid receptor; UCP1, uncoupling protein-1; d4T, stavudine; NVP, nevirapine.

Present results indicate that NRTIs, except 3TC, reduced the content of mtDNA in long-term treated brown adipocytes. However, only ddI decreased the levels of mitochondrially encoded transcripts, thus indicating that transcriptional mechanisms compensate for the depletion of mtDNA in d4T- and AZT-treated cells. NRTI effects are specific to mitochondrial genome expression since expression of the nuclear-encoded genes for mitochondrial proteins such as COIV and UCP2 were unaltered in NRTI-treated cells. Present data constitute one of the first reports on the NRTI effects on mitochondrial biogenesis and gene expression in an *in vitro* adipose cell model. A previous report indicates that large doses of d4T and AZT also reduce mtDNA concentration in 3T3-L1 adipocytes [48], and a recent report indicates that d4T and AZT, in contrast to ddI, increase mitochondrial mass but lower the mitochondrial membrane potential in differentiating 3T3-F442A adipocytes [14]. On the other hand, other mitochondrial targets not yet studied in adipocytes (that is, direct inhibition of mitochondrial respiration [21], inhibition of ADP/ATP translocase [22]) can also be affected. In this context, mitochondrial disturbances may be particularly relevant to HAART-induced lipomatosis, because multiple lipomatosis is also a distinctive symptom of mitochondrial dysfunction due to some mtDNA mutations [49].

In ddI-treated cells, the expression of mitochondrial transcription factors TFAM, TFB1M and TFB2M was decreased in agreement with lack of compensation of mitochondrial genome expression. In contrast, d4T, but not AZT, induced expression of mitochondrial transcription factors, thus normalizing mtDNA-encoded transcripts and proteins. Compensatory mechanisms for mtDNA depletion have been adduced to explain the lack of correlation between time on NRTI treatment and the probability of developing adverse effects. Similarly to what happens in patients with mtDNA defects of genetic origin, mitochondrial dysfunction in HAART-treated patients may become evident only after a profound reduction in mtDNA content. In peripheral blood cells from asymptomatic NRTI-treated patients, high levels of mtDNA depletion have been reported to be compatible with the maintenance of mtDNA expression by homeostatic compensatory mechanisms [50]. The intensity of such compensatory mechanisms can differ between individuals and/or between treatments, and identification of the molecular mechanisms involved would be of interest in order to minimize mitochondrial dysfunction. Further studies are required to establish whether mitochondrial transcription factors explain compensation, and if so, to identify potential strategies to promote their expression to compensate mtDNA depletion through up-regulation of mtDNA expression.

The brown adipocyte cell linage has been implicated in HAART-induced lipomatosis due to the expression of the UCP1 gene in the lipomas [25]. Here we report that d4T, but not other NRTIs, induces UCP1 gene expression in brown adipocyte cultures. The effect correlates with increased expression of PGC-1α, which is a potent transcriptional coactivator of UCP1 gene transcription, besides its role in regulating mitochondrial biogenesis. In contrast, the effects of d4T on white adipocytes have been linked to lipoatrophy both *in vivo* [51,52] and *in vitro* [14]. Furthermore, d4T did not significantly alter UCP1 gene expression in subcutaneous fat biopsies of lipodystrophic patients [25,53].

Among the known transcriptional regulators of the UCP1 gene, we demonstrate that d4T specifically mimics the action of the retinoic acid pathway. The hypothesis that drugs used in HAART may affect vitamin A metabolism and/or retinoid-mediated signalling pathways has been proposed, but more related to HIV PIs. PIs were believed to interfere with retinoid metabolism [54] and indinavir with the retinoic acid pathway [55]. More recently, it has been described that some patients under HAART treatment have alterations in the plasma retinol levels, which has been related to changes in retinoic acid synthesis [56]. In fact, white and brown adipose tissues, together with the liver, constitute the main active depots of vitamin A in the body [57]. Whether d4T interferes with vitamin A metabolism and/or retinoic acid signaling pathway specifically in brown adipocytes or this is a more wide effect remains to be determined.

In conclusion, brown adipose mitochondria are direct targets of the NRTIs d4T, AZT and ddI in an *in vitro* brown adipose model. However, transcriptional mechanisms compensate for the loss of mtDNA content, which results in unaltered levels of mitochondrial expression in d4T- and AZT-treated cells. At least for d4T, the increased expression of the mitochondrial transcription factors TFAM, TFB1M and TFB2M may be involved in such compensatory mechanisms. Identification of UCP1 as a specific target of d4T through retinoic-mediated pathways, together with the overall action of NVP in promoting brown adipocyte differentiation, may contribute to the appearance of brown adipocyte-like features in HAART-associated lipomas. In a previous study, in which UCP-1 gene expression was found to be high in a limited number of lipomas, treatments always included d4T [25]. A population-based study on the appearance of dorsocervical fat pads indicated that NRTIs but not PIs were always present in the treatment regime of affected patients [4]. However, a systematic study relating the appearance of lipomatosis to individual components of the treatment, for instance d4T, is not available. On the other hand, further studies are needed to assess whether fat accumulation in the intra-abdominal region implicates brown adipocytes, and therefore, present findings may also be relevant to this feature of HAART-associated lipodystrophy.

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