Antiviral Therapy 8:323-331

Mitochondrial proliferation, DNA depletion and adipocyte differentiation in subcutaneous adipose tissue of HIV-positive HAART recipients

*Craig S Pace[1], Annalise M Martin[1], Emma L Hammond[1], Cyril D Mamotte[2], David A Nolan[1] and Simon A Mallal[1,2]**

[1]Centre for Clinical Immunology and Biomedical Statistics, Royal Perth Hospital and Murdoch University, Western Australia
[2]Department of Clinical Immunology and Biochemical Genetics, Royal Perth Hospital, Perth, Western Australia

*Corresponding author: Tel: +61 89 244 2899; Fax: +61 89 244 2920; E-mail: S.Mallal@murdoch.edu.au

Objectives: To examine the *in vivo* effects of highly active antiretroviral therapy (HAART) regimens on adipose tissue mitochondrial DNA (mtDNA) depletion, mitochondrial organellar proliferation, and markers of adipocyte differentiation and phenotype.
Design and methods: DNA and mRNA quantification using real-time PCR methods was performed on adipose tissue samples from 31 HIV-infected individuals, of whom 11 were treatment-naive and 20 were receiving HAART. mtDNA depletion was measured as mtDNA copies/cell, and mitochondrial proliferation by quantification of mitochondrial protein mass. Regulation of mitochondrial biogenesis was assessed by NRF-1 and mtTFA mRNA. PPARγ, UCP2 and UCP1 mRNA expression was used to assess adipocyte differentiation and phenotype.
Results: Stavudine-based HAART recipients (*n*=10) displayed significant mtDNA depletion (12.8% of control, *P*<0.001), mildly increased mitochondrial protein mass (2.6-fold of control, *P*=0.032) and decreased expression of PPARγ (53.9% of control, *P*=0.021), UCP2 (62.2% of control, *P*=0.024) and UCP3 (51.8% of control, *P*=0.047) mRNA compared with controls. Zidovudine-based HAART recipients (*n*=7) also displayed significant mtDNA depletion (34.45% of control, *P*=0.031), increased mitochondrial protein mass (5.7-fold of control, *P*=0.009), and markedly increased UCP1 (18-fold of control, *P*=0.009) mRNA. Elevated UCP1 mRNA expression was found to be associated with non-stavudine (zidovudine or abacavir), protease inhibitor (PI)-containing HAART (95-fold of non-stavudine, non-PI-containing HAART, *P*=0.006).
Conclusion: Differential effects of stavudine and zidovudine therapy on mtDNA depletion and expression of adipocyte differentiation markers PPARγ and UCP2 were observed, consistent with increased adipose tissue toxicity associated with stavudine therapy. Increased UCP1 mRNA, a marker of brown adipose tissue phenotype, was associated with non-stavudine, PI-containing HAART, and may represent an adaptive response to the increased fatty acid flux associated with PI therapy, and may contribute to the increased resting energy expenditure reported in such patients.

Introduction

A common adverse effect of antiretroviral therapy, the 'lipodystrophy syndrome' is characterized by peripheral lipoatrophy and/or visceral lipohypertrophy, dyslipidaemia and insulin resistance, and is considered a major determinant of therapy adherence [1,2]. Historically, the emergence of lipodystrophy coincided temporally with the introduction of protease inhibitors (PIs) for the management of HIV infection and led to the conjecture that the PI class of drugs was the causative agent [3]. However, lipoatrophy has also been observed in PI-naive individuals [4,5]. There is now increasing evidence that each of the clinical characteristics of the lipodystrophy syndrome mentioned above arise via distinct aetiopathogenic mechanisms rather than a single cause [3].

Research into the mechanisms of nucleoside reverse transcriptase inhibitor (NRTI) toxicity and lipoatrophy has focused on depletion of mitochondrial DNA (mtDNA). Several *in vivo* studies have shown significant mtDNA depletion in subcutaneous adipose tissue of lipodystrophic NRTI recipients [6–8], strongly suggesting NRTI-mediated mtDNA depletion has a role in the pathogenesis of lipoatrophy [9]. This is supported by clinical studies demonstrating that the choice of NRTI therapy (stavudine versus zidovudine) is the dominant determinant of the severity of lipoatrophy [10,11], and by *in vitro* studies indicating that NRTIs such as stavudine, didanosine and zalcitabine are capable of inducing mtDNA depletion at pharmacological doses [12,13]. While zidovudine has the ability to cause some degree of mtDNA depletion [12],

©2003 International Medical Press 1359-6535/02/$17.00

there is also evidence to suggest that this drug may contribute to mitochondrial dysfunction through alternative mechanisms [14].

The observation that HIV PI therapy contributes to hyperlipidaemia and insulin resistance has prompted numerous *in vitro* studies into the effects of PIs on adipogenesis, the results of which are conflicting. While several studies have demonstrated impaired adipogenesis using suprapharmacological PI concentrations, few studies have demonstrated impaired adipogenesis using pharmacologically relevant PI concentrations [15,16]. In addition, there appear to be distinct differences in the ability of each individual PI to impair adipogenesis, although the clinical relevance of this is not known [17]. Several animal models of lipodystrophy suggest that the insulin resistance and metabolic alterations observed are causally related to the initial loss of subcutaneous adipose tissue [18], although the relevance of these findings to antiretroviral therapy-associated lipodystrophy has not yet been established [19].

In light of this information, we sought to examine the *in vivo* effects of antiretroviral therapy combinations on mtDNA depletion, mitochondrial organellar proliferation and adipocyte differentiation. Previous studies have noted some brown adipocyte-like characteristics in subcutaneous adipose tissue of NRTI-treated individuals [20,21] and NRTI-exposed cell lines [14]. We therefore also examined the influence of the drugs on expression of a brown adipocyte marker (uncoupling protein 1, UCP1). We found that stavudine use was associated with mtDNA depletion, mitochondrial proliferation and decreased expression of peroxisome proliferator activator receptor-γ (PPARγ), UCP2 and UCP3 mRNA, suggestive of impaired adipocyte differentiation. Zidovudine use was also associated with mtDNA depletion and mitochondrial proliferation, though significantly less mtDNA depletion than observed with stavudine. Non-stavudine, PI-containing HAART was associated with markedly increased expression (mRNA) of the brown adipocyte marker, UCP1, that may constitute an adaptive response to the increased fatty acid flux associated with PI therapy and may contribute to the increased resting energy expenditure observed in such patients.

Materials and methods

Patient selection

Thirty-one male Caucasian patients from the Western Australian HIV Cohort [22] who had subcutaneous adipose tissue biopsies were selected and grouped according to their HAART regimen at the time of biopsy. To be included in this study, individuals had to be on the antiretroviral class/agent for which they were grouped for a minimum of 6 months. To assess the influence of PI therapy, individuals were grouped into PI-containing HAART regimens (PI+, *n*=7) and non-PI-containing HAART (non-PI, *n*=13) regimens (Table 1). Similarly, individuals were also grouped into stavudine-based HAART recipients (*n*=10), zidovudine-based HAART recipients (*n*=7) and abacavir-based HAART recipients (*n*=3) to assess the influence of these NRTIs on the aforementioned parameters (Table 2). Informed consent and approval for the use of human genetic material was obtained from all individuals studied and the Royal Perth Hospital ethics committee, respectively. The control group consisted of 11 HIV-seropositive male Caucasians with similar age and BMI who had never received antiretroviral therapy.

Subcutaneous adipose tissue biopsies

Subcutaneous adipose biopsies were obtained from the supra-iliac region following a 5 cm surgical incision down to the adipose layer and direct dissection of adipose tissue; dissected tissues were immediately frozen in liquid nitrogen prior to storage at –70°C.

Quantification of mitochondrial DNA copies/adipocyte

Adipocytes were isolated from adipose tissue by incubation in Hanks balanced salt solution (pH 7.0) containing 3% collagenase solution (Type 1, Sigma C0130) and 1.5% bovine serum albumin at 37°C for 80 min followed by differential centrifugation, as previously described [20]. Total DNA was extracted from isolated adipocytes using QIAamp DNA MIDI Kit (Qiagen, Inc., Chatsworth, Calif., USA) according to the manufacturer's protocol.

Quantification of mitochondrial protein mass

Mitochondrial protein mass was quantified by the Bradford method, as previously described [20].

Quantitation of UCP1, UCP2, PPARγ, mtTFA and NRF-1 mRNA expression by real-time PCR

Total RNA was extracted from adipose tissue using Ultraspec® (Fisher Biotec, Australia) according to the manufacturer's protocol. Total RNA concentration

Table 1. Demographics of the naive, non-PI and PI+ groups

Parameter	Naive	Non-PI	PI+	*P*-value
Number (*n*)	11	13	7	–
Time on regimen*	–	23.5 ±19.5	42.3 ±20.2	0.058
Age (years)	49.0 ±11.0	46.6 ±10.3	45.9 ±8.5	0.780
BMI (kg/m^3)	24.8 ±4.2	23.7 ±3.6	21.0 ±1.8	0.099

All values are expressed as mean ±SD; *months on particular regimen at time of biopsy.

©2003 International Medical Press

Table 2. Demographics of the naive, stavudine, zidovudine and abacavir groups

Parameter	Naive	Stavudine	Zidovudine	Abacavir	*P*-value
Number (*n*)	11	10	7	3	–
Time on regimen*	–	32.2 ±18.9	20.86 ±17.1	44.7 ±34.4	0.058
Age (years)	49.0 ±11.0	48.3 ±9.5	43.9 ±6.3	45.7 ±17.0	0.740
BMI (kg/m^3)	24.8 ±4.2	22.8 ±3.2	24.1 ±2.7	19.3 ±3.2	0.122

All values are expressed as mean ±SD; *months on particular regimen at time of biopsy.

was measured at 260 nm using a Beckman DU 530 Life Science UV/Vis spectrophotometer (USA); 260:280 ratios between 1.7 and 2.0 were considered satisfactorily pure for subsequent reactions. First strand cDNA was synthesized from 1 µg of total RNA using Omniscript (Qiagen, USA) according to the manufacturer's protocol and stored at –20°C.

The sequence of all oligonucleotide primers and fluorogenic probes used in this study are shown in Table 3. Those used for quantification of human UCP1, UCP2, UCP3 and glyceraldehyde-6-phosphate-dehydrogenase (GAPDH) were designed using Primer Express™ software v. 1.5 (Applied Biosystems, Foster City, Calif., USA; ABI), while those used for PPARγ were obtained from Xin *et al.* [23], and those for mitochondrial transcription factor A (mtTFA) and nuclear respiratory factor-1 (NRF-1) were obtained from Miranda *et al.* [24]. The specificity of all oligonucleotides was assessed using BLASTN (http://www.ncbi.nlm.nih.gov/BLAST/) and CLUSTAL W v.1.8 (http://clustalw.genome.ad.jp/). Where possible, primer binding sites were designed to flank intron–exon boundaries; when this was not possible, forward and reverse primer binding sites were designed in separate exons. All probes were labelled at the 5′ end with 6-carboxy-fluorescein (FAM) and at the 3′ end with 6-carboxy-tetramethyl-rhodamine (TAMRA).

Quantitative PCR was carried out in duplicate for each sample on a PE7700 Sequence Detection System (ABI). Each 25 µl reaction contained 50 ng of cDNA, 450 nmol of each forward and reverse primer, 2.5 µl of 10X PCR buffer (Gibco, USA), 2 mM $MgCl_2$, 0.5 µl of 50X ROX reference dye (Invitrogen, USA), 0.5 µl of 40 mM dNTPs, 1U of Platinum® Taq DNA polymerase (Gibco, USA) and sterile, DEPC-treated water pH 8.0. For quantification of UCP1, UCP2, UCP3, PPARγ and GAPDH cDNA, 200 nM of probes was included in the PCR reaction, and for quantification of mtTFA and NRF-1 cDNA, 0.5 µl of 20X SYBR green was included in each reaction. All reactions were carried out using the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Real-time amplifications were analysed using the Sequence Detector software v. 1.5 (ABI) and indirect quantification of the target mRNA was calculated from a standard curve generated by amplification of purified PCR products. The end-point of this assay is the crossing threshold (CT), which represents the number of PCR cycles required to enter the logarithmic amplification phase. As a linear increase in CT thus represents a logarithmic increase in PCR products, all values are expressed and analysed on the logarithmic scale (Figures 1 and 2). For each sample, results were normalized by dividing the amount of target cDNA by the amount of GAPDH. Due to the use of the non-specific dsDNA binding dye, SYBR green, exclusive amplification of mtTFA and NRF-1 was verified by melting curve analysis.

Statistical analysis

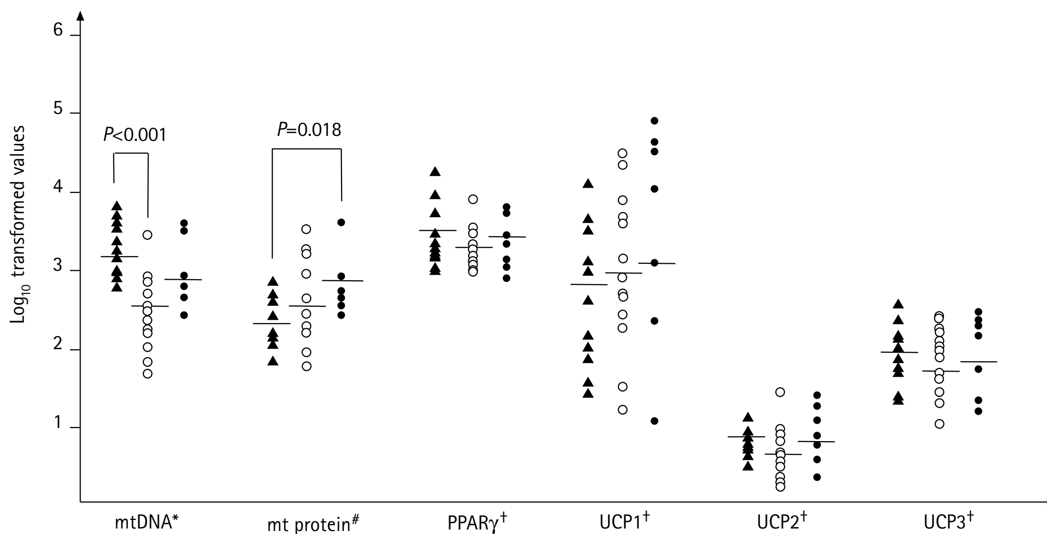
All values are expressed as log transformed data to better approximate normality. For parametric data, means were compared by ANOVA for multiple

Table 3. Oligonucleotide primers and probe sequences used in real-time quantitative PCR

Gene			Amplicon size
UCP1	Forward	5′-CTGCCACTCCTCCAGTCGTT-3′	240 bp
	Reverse	5′-CCGCCTCTCTCAGGATCGGCCT-3′	
	Probe	5′-CCGCCTCTCTCAGGATCGGCCT-3′	
UCP2	Forward	5′-GACCTATGACCTCATCAAGG-3′	288 bp
	Reverse	5′-ATAGGTGACGAACATCACCACG-3′	
	Probe	5′-ACAGATGACCTCCCTTGCCACTTCACT-3′	
UCP3	Forward	5′-ATGGACGCCTACAGAACCAT-3′	311 bp
	Reverse	5′-CTGGGCCACCATCTTTATCA-3′	
	Probe	5′-CCTGTCCAAAGGAACTTTGCCCAACA-3′	
PPARγ	Forward	5′-TTTCACTATGGAGTTCATGCTTGTG-3′	109bp
	Reverse	5′-TTTTTGTGGATCCGACAGTTAAGA-3′	
	Probe	5′-CAAGGGTTTCTTCCGGAGAACAATCAG-3′	
GAPDH	Forward	5′-GAAGGTGAAGGTCGGAGTC-3′	226 bp
	Reverse	5′-GAAGATGGTGATGGGATTTC-3′	
	Probe	5′-CAAGCTTCCCGTTCTCAGCC-3′	
mtTFA	Forward	5′-TATCAAGATGCTTATAGGGC-3′	441 bp
	Reverse	5′-ACTCCTCAGCACCATATTTT-3′	
NRF-1	Forward	5′-GGAGTGATGTCCGCACAGAA-3′	643 bp
	Reverse	5′-CGCTGTTAAGCGCCATAGTG-3′	

Bp, base pairs; note mtTFA and NRF-1 were quantified using the dsDNA binding dye, SYBR green, and as such, did not require fluorogenic probes.

Figure 1. Recipients of non-PI-containing HAART and PI-containing HAART displayed mtDNA depletion and elevated mitochondrial protein, respectively



Horizontal bar indicates mean; ▲, HIV-positive, HAART-naive individuals; 0, non-PI-containing HAART recipients; ●, PI-containing HAART recipients; *mtDNA copies/adipocyte; # mitochondrial protein; † copies/GAPDH copies.

comparisons. For non-parametric data, the Mann-Whitney test was used. The threshold for significance was set at *P*=0.05. Comparisons between groups were performed on standard statistical software (Excel and SPSS), and differences between study groups were expressed as percentage and fold differences using absolute (untransformed) values that were derived from the log transformed output data. These values (that is, $10^{\times}$ where × denotes the log transformed value), therefore, estimate the median values for untransformed data.

Results

The effects of HAART on mitochondrial proliferation, mtDNA depletion, and adipocyte differentiation and phenotype were assessed in subcutaneous adipose tissue biopsies from 31 HIV-seropositive patients on various HAART regimens; gene expression at the mRNA level was compared according to HAART regimen. Parameters that were significantly influenced by NRTIs and PI as a class, or by stavudine, zidovudine or abacavir individually, are displayed in Figures 1 and 2, respectively.

Expression of GAPDH mRNA within subcutaneous adipose tissue was similar amongst non-PI HAART recipients, PI+ HAART recipients and controls (*P*=0.95). In addition, there did not appear to be an affect of stavudine, zidovudine or abacavir on the level of GAPDH mRNA expression (*P*=0.17), suggesting GAPDH is a suitable endogenous control gene within this system, consistent with findings by Gorzelniak and colleagues [25].

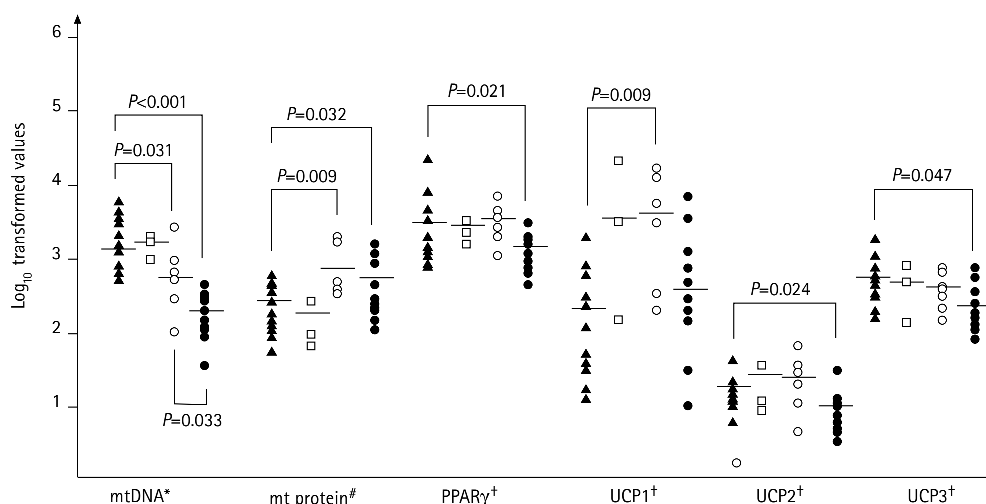
Effect of NRTI therapy versus NRTI and PI combination therapy on mitochondrial proliferation, mtDNA, and adipocyte differentiation and phenotype

As presented in Figure 1, patients on non-PI-containing HAART regimens (non-PI) displayed significantly reduced mtDNA/adipocyte (19.3% of controls, *P*<0.001), suggesting that NRTI therapy alone is sufficient to induce mtDNA depletion. The addition of PIs to NRTI-based HAART regimens (PI+) also tended towards significant mtDNA depletion (43.1% of controls, *P*=0.062). Median mtDNA/adipocyte was not significantly different amongst non-PI and PI regimens (*P*=0.132).

While the PI+ group had significantly increased median mitochondrial protein mass compared to controls (3.3-fold, *P*=0.018), there was no significant difference in the median mitochondrial protein mass of PI+ and non-PI regimens (1.4-fold, *P*=0.315), thus PI therapy did not appear to have an independent significant effect on mitochondrial protein mass. Using linear regression analysis to investigate these associations further, choice of a specific NRTI – zidovudine (*P*=0.010) or stavudine (*P*=0.047), but not abacavir (*P*=0.604) – was found to be the dominant determinant of mitochondrial protein mass. Use of PI therapy did not contribute significantly to the model after adjustment for the effect of these variables (*P*=0.604). In addition, expression of mtTFA and NRF-1 mRNA – key transcription factors involved in mtDNA replication and mitochondrial organellar biogenesis [28,29] – were similar amongst non-PI and PI+ HAART recipients, and

 ©2003 International Medical Press

Figure 2. Stavudine and zidovudine recipients displayed significant mtDNA depletion and elevated mitochondrial protein mass. However, stavudine recipients also displayed reduced expression of PPARγ, UCP2 and UCP3 mRNA, while zidovudine recipients display significantly elevated expression of UCP1 mRNA



Horizontal bar indicates mean; ▲, HIV-positive, HAART-naive individuals; □, abacavir-based HAART recipients; 0, zidovudine-based HAART recipients; ●, stavudine-based HAART recipients; *mtDNA copies/adipocyte; # mitochondrial protein; † copies/GAPDH copies.

in controls (*P*=0.352 and 0.589, respectively). Similarly, there were no significant differences in expression of PPARγ (*P*=0.649), UCP2 (*P*=0.514) or UCP3 (*P*=0.663) mRNA in comparisons of PI+ and non-PI regimens.

Expression of a brown adipocyte-specific marker, UCP1, was only associated with PI therapy once stavudine recipients were excluded from the analysis (Figure 3). Patients receiving either zidovudine or abacavir, and a PI, expressed UCP1 mRNA at levels 96-fold higher than patients receiving either zidovudine or abacavir, and no PI (*P*=0.006), 1500-fold higher than patients receiving stavudine and PI (*P*=0.007), 108-fold higher than patients receiving stavudine no PI (*P*=0.007) and 228-fold higher than naives (*P*<0.001). No significant effect of PIs was observed in stavudine-based HAART recipients (*P*=0.189).

Effects of stavudine, zidovudine and abacavir on mitochondrial proliferation, mtDNA, and adipocyte differentiation and phenotype

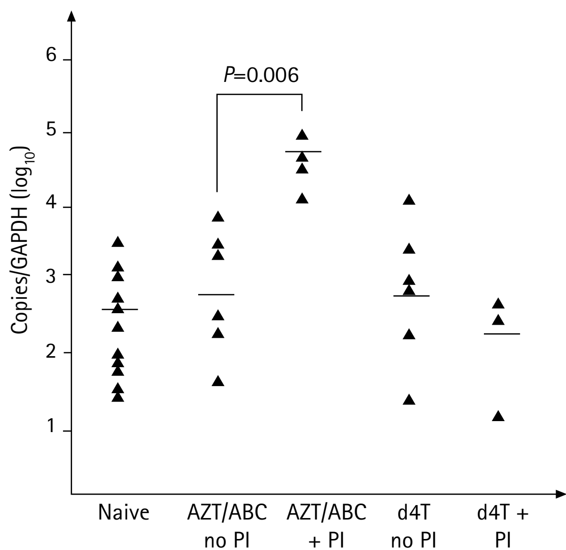
Current use of stavudine or zidovudine therapy was associated with significantly reduced mtDNA/adipocyte (12.8% of controls, *P*<0.001 and 34.4% of controls, *P*=0.031, respectively). However, the mtDNA depletion associated with stavudine therapy was significantly more severe than that associated with zidovudine therapy (37.3% of zidovudine values, *P*=0.033), as presented in Figure 2.

In addition, stavudine and zidovudine were associated with significantly increased mitochondrial protein mass (2.6-fold, *P*=0.032 and 5.7-fold, *P*=0.009, respectively) compared to controls. However, only stavudine-based HAART recipients had significantly reduced median expression of adipocyte differentiation markers PPARγ (54% of controls, *P*=0.021), UCP2 (62% of controls, *P*=0.024) and of UCP3 (52% of controls, *P*=0.047) mRNA.

As mentioned, the choice of NRTI therapy was the dominant determinant of mitochondrial protein mass. In further regression analysis, the severity of mtDNA depletion was associated with mitochondrial protein levels (*P*=0.04), and after adjusting for this effect, only zidovudine remained independently significant (*P*=0.007). Similar results were obtained in regression analysis of associations with PPARγ expression, in which greater severity of mtDNA depletion was associated with decreased PPARγ (*P*=0.005), while use of zidovudine remained independently associated with higher PPARγ gene expression (*P*=0.049). Thus, while the effects of stavudine on mitochondrial protein mass and PPARγ appear to be exerted through its effects on mtDNA depletion, the effect of zidovudine on these parameters appears at least in part, to be independent of mtDNA depletion.

The only gene whose expression (mRNA) was significantly altered in the zidovudine group was UCP1; 18-fold compared to both controls (*P*=0.009) and stavudine recipients (*P*=0.042). However, as described above, the combination of non-stavudine NRTIs (zidovudine or abacavir) with PIs resulted in the highest and most significant median UCP1 mRNA expression (228-fold higher than controls, *P*<0.001). This was supported by general linear model (GLM)

Figure 3. The addition of PIs to zidovudine- or abacavir-based HAART, but not stavudine-based HAART, is associated with markedly elevated expression of UCP1



Horizontal bar indicates mean; naive, HIV-positive, treatment-naive individuals; AZT/ABC no PI, zidovudine-based, non-PI-containing HAART recipients and abacavir-based, non-PI-containing HAART recipients; AZT/ABC + PI, zidovudine-based, PI-containing HAART recipients and abacavir-based, PI-containing HAART recipients; d4T no PI, stavudine-based, non-PI-containing HAART recipients; d4T + PI, stavudine-based, PI-containing HAART recipients.

analysis, which suggested that in this system, UCP1 mRNA levels are predominantly influenced by an interaction between the use of combined non-stavudine NRTIs and PI therapy (R^2=0.209, P=0.01).

No effect of abacavir on mtDNA (101.1% of controls, P=0.980), mitochondrial protein mass (73% of controls, P=0.541), or expression of PPARγ (100.7% of controls, P=0.987), UCP2 (129% of controls, P=0.268) or UCP3 (89.1% of controls, P=0.812) mRNA was detected, although the statistical power to detect an independent effect of abacavir therapy was limited by the number of samples assessed (n=3). While UCP1 mRNA expression was elevated 10-fold compared to controls, this elevation was statistically non-significant (P=0.174).

Discussion

In this cross-sectional study, stavudine and zidovudine therapy had significant effects on adipose tissue. Use of either stavudine or zidovudine was associated with mtDNA depletion and increased mitochondrial protein mass. However, only stavudine was also associated with reduced expression of PPARγ, UCP2 and UCP3 mRNA; zidovudine did not independently influence expression of any gene examined. PI therapy, when combined with non-stavudine NRTIs (that is, zidovudine or abacavir), was associated with markedly elevated expression (mRNA) of a brown adipocyte marker, UCP1.

The significant mtDNA depletion exhibited by stavudine recipients when compared to both controls (12.8% of controls, P<0.001) and zidovudine recipients (37.2% of zidovudine treated values, P=0.031) is consistent with *in vitro* [26] and *in vivo* studies [27] that associate stavudine with significantly more severe mtDNA depletion than zidovudine.

In addition to severe mtDNA depletion, stavudine recipients also expressed significantly lower levels of PPARγ (53.9% lower, P=0.021), UCP2 (62.2% lower, P=0.024) and UCP3 (51.8% lower, P=0.047) mRNA when compared with controls. PPARγ is a transcription factor expressed predominantly in adipocytes [28], where it is upregulated early in the differentiation process [29,30] and activates transcription of many genes involved in lipid metabolism [31–33]. Similarly, expression of UCP2 mRNA has been shown to increase during differentiation of murine primary cultures and clonal lines [34], and a human brown preadipocyte cell line [30]. UCP2 expression is believed to be augmented by the PPARγ/RXR heterodimer [35] following the identification of two functional peroxisome proliferator response elements within the proximal promoter of the human UCP2 gene [36]. Impaired adipocyte differentiation is consistent with findings from the only other study to date to evaluate adipocyte differentiation in HAART recipients conducted by Bastard and colleagues [37], who reported a 70% reduction in expression of PPARγ protein in lipoatrophic PI-containing HAART recipients when compared to HIV-seronegative controls, as well as comparable reductions in expression of transcription factors C/EBPα, C/EBPβ and SREBP1c, and differentiation markers HSL, GLUT4 and leptin. Interestingly, while they attributed their findings to a specific effect of PI therapy, 21 of the 26 patients in the study were also receiving stavudine treatment.

Stavudine could indirectly contribute to impaired adipocyte differentiation through depletion of mtDNA copy number and subsequent effects on ATP production [38]. In this context, an increase in the cytosolic AMP:ATP ratio promotes the activation of a compensatory response that seeks to restore bioenergetic equilibrium via AMP-activated kinase (AMPK). Artificial activation of AMPK using 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) in differentiating 3T3-L1 adipocytes resulted in inhibited expression of transcription factors C/EBPα and PPARγ, and late markers of differentiation such as fatty acid synthase (FAS) and acetyl-CoA carboxylase [39]. Interestingly, this is believed to be mediated by inhibition of SREBP-1 mRNA expression [40], the proposed mechanism by which PIs impair adipocyte differentiation.

 ©2003 International Medical Press

Current use of zidovudine was associated with moderate mtDNA depletion (34% of control, *P*=0.031 and 37% of stavudine, *P*=0.033) and elevated mitochondrial protein mass. Using linear regression analysis, the elevated mitochondrial protein mass in zidovudine recipients was found to be attributed, in part, to an independent effect of zidovudine, rather than through its effects on mtDNA depletion. This may be due to the immediate effects zidovudine exerts on both mitochondrial function and proliferation, in the absence of mtDNA depletion [14,41].

Initial analysis revealed zidovudine recipients expressed (mRNA) a brown adipocyte-specific marker, UCP1, at levels 18-fold higher than controls (*P*=0.009) or stavudine recipients (*P*=0.042). That zidovudine induces uncoupling of oxidative phosphorylation is contrary to indirect evidence [42,43]. A similar pattern was observed in the few abacavir recipients in our study (10-fold of controls, *P*=0.174) and on subsequent analysis we found that zidovudine- or abacavir-based, PI-containing HAART recipients expressed UCP1 mRNA 96-fold that expressed by zidovudine- or abacavir-based, non-PI containing HAART recipients (*P*=0.003). Elevated UCP1 mRNA expression in recipients of relatively non-toxic (compared to stavudine-based regimens), PI-containing HAART regimens may represent an adaptive response to the increased free fatty acid flux associated with PI therapy. As proposed previously by our group [44], by increasing UCP1 during times of increased fatty acid availability, the generation of reactive oxygen species is minimized, mitochondrial metabolic processes that may otherwise become inhibited by excessive substrate availability and/or ATP production are maintained, and fatty acids are removed from the mitochondrial matrix where they may be deleterious. The origin of the increased UCP1 expression is unclear; it may result from a proliferation and differentiation of brown preadipocytes known to exist in traditional white adipose tissue depots [45] or from a phenotypic conversion of pre-existing white adipocytes [34,46].

While measurements of mtDNA and mitochondrial protein were performed on isolated adipocytes, gene expression was assessed at the adipose tissue level and thus reflect mRNA expression of a heterogenous cell population that includes vascular cells, fibroblasts and adipocytes [47]. This limits interpretation of the gene expression data for a number of reasons. First, the use of GAPDH (a broadly expressed cellular marker) as an endogenous control allows for the possibility that reduced expression of adipocyte-specific markers such as PPARγ may reflect either reduced adipocyte PPARγ expression (suggestive of impaired adipocyte differentiation) or reduced adipocyte number relative to the overall cell population. In the latter scenario reduced PPARγ expression associated with stavudine therapy could result from selective adipocyte cell death within fat tissue, a possibility supported by studies investigating apoptosis markers in fat samples from lipoatrophic patients [48,49]. Secondly, whilst these preliminary data indicate that antiretroviral therapy influences the expression of other non-adipocyte-specific markers such as UCP2, it is uncertain which cell populations within adipose tissue are involved in these effects. Whilst these limitations could be overcome through the use of collagenase digestion, we have not been able to obtain a collagenase protocol that maintained the integrity of the 18S and 28S RNA. We therefore propose to focus on protein expression in collagenase-digested samples as a more appropriate end-point in future longitudinal studies.

In conclusion, stavudine use was associated with significant mtDNA depletion, increased mitochondrial mass and reduced expression of PPARγ, UCP2 and UCP3 mRNA. Among these gene expression markers, PPARγ is adipocyte-specific so that decreased levels may signify either reduced adipocyte mass per adipose tissue mass or reduced adipocyte differentiation. The combination of decreased PPARγ and UCP2 is suggestive of impaired adipocyte differentiation, with evidence that decreased gene expression is specifically associated with the severity of mtDNA depletion. Zidovudine therapy was associated with less severe mtDNA depletion and similar mitochondrial protein mass as stavudine therapy. However, the absence of a similar reduction in gene expression suggests that the less severe mtDNA depletion observed in zidovudine recipients may be below a threshold required for toxicity. Therapies that are not associated with severe mtDNA depletion, and coupled with PI therapy, are associated with statistically and biologically significant elevations in expression (mRNA) of a brown adipocyte marker, UCP1, which may represent an adaptive response to increased fatty acid flux.

Acknowledgements

The authors gratefully acknowledge: The Department of Haematology, Royal Perth Hospital, for use of the PE7700; Anothai Pocathikorn for technical advice; Prof Ian James, Dr Elizabeth McKinnon and Susan Herrmann (RN) for statistical expertise.

References

1. Duran S, Saves M, Spire B, Cailleton V, Sobel A, Carrieri P, Salmon D, Moatti JP & Leport C. Failure to maintain long-term adherence to highly active antiretroviral therapy: the role of lipodystrophy. *AIDS* 2001; **15**:2441–2444.
2. Bogner JR, Vielhauer V, Beckmann RA, Michl G, Wille L, Salzberger B & Goebel FD. Stavudine versus zidovudine and the development of lipodystrophy. *Journal of Acquired Immune Deficiency Syndrome* 2001; **27**:237–244.

3. John M, Nolan D & Mallal S. Antiretroviral therapy and the lipodystrophy syndrome. *Antiviral Therapy* 2001; **6:**9–20.

4. Tsekes G, Chrysos G, Douskas G, Paraskeva D, Mangafas N, Giannakopoulos D, Papanikolaou M, Georgiou E & Lazanas MC. Body composition changes in protease inhibitor-naive HIV-infected patients treated with two nucleoside reverse transcriptase inhibitors. *HIV Medicine* 2002; **3:**85–90.

5. Boufassa F, Dulioust A, Lascaux AS, Meyer L, Boue F, Delfraissy JF, Sobel A & Goujard C. Lipodystrophy in 685 HIV-1-treated patients: influence of antiretroviral treatment and immunovirological response. *HIV Clinical Trials* 2001; **2:**339–345.

6. Shikuma CM, Hu N, Milne C, Yost F, Waslien C, Shimizu S & Shiramizu B. Mitochondrial DNA decrease in subcutaneous adipose tissue of HIV-infected individuals with peripheral lipoatrophy. *AIDS* 2001; **15:**1801–1809.

7. Mallal SA, John M, Moore CB, James IR, & McKinnon EJ. Contribution of nucleoside analogue reverse transcriptase inhibitors to subcutaneous fat wasting in patients with HIV infection. *AIDS* 2000; **14:**1309–1316.

8. Walker UA, Bickel M, Lutke Volksbeck SI, Schofer H, Setzer B, Rickerts V & Staszewski S. Decrease in mitochondrial DNA content in adipose tissue of HIV-1-infected patients treated with NRTIs. *2nd International Workshop on Adverse Drug Reactions & Lipodystrophy in HIV*, Toronto, Canada, 13–15 September 2000; Abstract O6.

9. Walker UA & Brinkman K. An argument for mitochondrial toxicity in highly active antiretroviral therapy-induced lipoatrophy. *AIDS* 2001; **15:**1450–1452.

10. Chene G, Angelini E, Cotte L, Lang JM, Morlat P, Rancinan C, May T, Journot V, Raffi F, Jarrousse B, Grappin M, Lepeu G & Molina JM. Role of long-term nucleoside-analogue therapy in lipodystrophy and metabolic disorders in human immunodeficiency virus-infected patients. *Clinical Infectious Diseases* 2002; **34:**649–657.

11. Saves M, Raffi F, Capeau J, Rozenbaum W, Ragnaud JM, Perronne C, Basdevant A, Leport C & Chene G. Factors related to lipodystrophy and metabolic alterations in patients with human immunodeficiency virus infection receiving highly active antiretroviral therapy. *Clinical Infectious Diseases* 2002; **34:**1396–1405.

12. Walker UA, Setzer B & Venhoff N. Increased long-term mitochondrial toxicity in combinations of nucleoside analogue reverse-transcriptase inhibitors. *AIDS* 2002; **16:**2165–2173.

13. Arpadi SM, Cuff PA, Horlick M, Wang J & Kotler DP. Lipodystrophy in HIV-infected children is associated with high viral load and low CD4+ lymphocyte count and CD4+ lymphocyte percentage at baseline and use of protease inhibitors and stavudine. *Journal of Acquired Immune Deficiency Syndrome* 2001; **27:**30–34.

14. Cazzalini O, Lazze MC, Iamele L, Stivala LA, Bianchi L, Vaghi P, Cornaglia A, Calligaro A, Curti D, Alessandrini A, Prosperi E & Vannini V. Early effects of AZT on mitochondrial functions in the absence of mitochondrial DNA depletion in rat myotubes. *Biochemical Pharmacology* 2001; **62:**893–902.

15. Zhang B, MacNaul K, Szalkowski D, Li Z, Berger J & Moller DE. Inhibition of adipocyte differentiation by HIV protease inhibitors. *Journal of Clinical Endocrinology & Metabolism* 1999; **84:**4274–4277.

16. Caron M, Auclair M, Vigouroux C, Glorian M, Forest C & Capeau J. The HIV protease inhibitor indinavir impairs sterol regulatory element-binding protein-1 intranuclear localization, inhibits preadipocyte differentiation, and induces insulin resistance. *Diabetes* 2001; **50:**1378–1388.

17. Carr A, Samaras K, Thorisdottir A, Kaufmann GR, Chisholm DJ & Cooper DA. Diagnosis, prediction, and natural course of HIV-1 protease-inhibitor-associated lipodystrophy, hyperlipidaemia, and diabetes mellitus: a cohort study. *Lancet* 1999; **353:**2093–2099.

18. Reitman ML, Mason MM, Moitra J, Gavrilova O, Marcus-Samuels B, Eckhaus M & Vinson C. Transgenic mice lacking white fat: models for understanding human lipoatrophic diabetes. *Annals of The New York Academy of Sciences* 1999; **892:**289–296.

19. Shimomura I, Hammer RE, Richardson JA, Ikemoto S, Bashmakov Y, Goldstein JL & Brown MS. Insulin resistance and diabetes mellitus in transgenic mice expressing nuclear SREBP-1c in adipose tissue: model for congenital generalized lipodystrophy. *Genes & Development* 1998; **12:**3182–3194.

20. Nolan D, Hammond E, Martin A, Taylor L, Herrmann S, McKinnon E, Metcalf C, Latham B, & Mallal S. Mitochondrial DNA depletion and morphologic changes in adipocytes associated with nucleoside reverse transcriptase inhibitor therapy. *AIDS* 2003; **17:**1329–1338.

21. Fessel WJ, Follansbee SB & Barker B. Ultrastructural findings consistent with brown adipocytes in buffalo humps of HIV-positive patients with fat redistribution syndrome. *2nd International Workshop on Adverse Drug Reactions & Lipodystrophy in HIV*, Toronto, Canada 13–15 September 2000; Abstract P1.

22. Mallal SA. The Western Australian HIV Cohort Study, Perth, Australia. *Journal of Acquired Immune Deficiency Syndrome & Human Retrovirology* 1998; **17(Suppl. 1):**S23–S27.

23. Xin X, Yang S, Kowalski J & Gerritsen ME. Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis *in vitro* and *in vivo*. *Journal of Biological Chemistry* 1999; **274:**9116–9121.

24. Miranda S, Foncea R, Guerrero J & Leighton F. Oxidative stress and upregulation of mitochondrial biogenesis genes in mitochondrial DNA-depleted HeLa cells. *Biochemical & Biophysical Research Communications* 1999; **258:**44–49.

25. Gorzelniak K, Janke J, Engeli S & Sharma AM. Validation of endogenous controls for gene expression studies in human adipocytes and preadipocytes. *Hormone & Metabolic Research* 2001; **33:**625–627.

26. Birkus G, Hitchcock MJ & Cihlar T. Assessment of mitochondrial toxicity in human cells treated with tenofovir: comparison with other nucleoside reverse transcriptase inhibitors. *Antimicrobial Agents & Chemotherapy* 2002; **46:**716–723.

27. Cherry CL, Gahan ME, McArthur JC, Lewin SR, Hoy JF & Wesselingh SL. Exposure to dideoxynucleosides is reflected in lowered mitochondrial DNA in subcutaneous fat. *Journal of Acquired Immune Deficiency Syndrome* 2002; **30:**271–277.

28. Tai TA, Jennermann C, Brown KK, Oliver BB, MacGinnitie MA, Wilkison WO, Brown HR, Lehmann JM, Kliewer SA, Morris DC & Graves RA. Activation of the nuclear receptor peroxisome proliferator-activated receptor gamma promotes brown adipocyte differentiation. *Journal of Biological Chemistry* 1996; **271:**29909–29914.

29. Hamm JK, Park BH & Farmer SR. A role for C/EBPbeta in regulating peroxisome proliferator-activated receptor gamma activity during adipogenesis in 3T3-L1 preadipocytes. *Journal of Biological Chemistry* 2001; **276:**18464–18471.

30. Strobel A, Siquier K, Zilberfarb V, Strosberg AD & Issad T. Effect of thiazolidinediones on expression of UCP2 and adipocyte markers in human PAZ6 adipocytes. *Diabetologia* 1999; **42:**527–533.

31. Rosen ED, Walkey CJ, Puigserver P & Spiegelman BM. Transcriptional regulation of adipogenesis. *Genes & Development* 2000; **14:**1293–1307.

32. Adams M, Montague CT, Prins JB, Holder JC, Smith SA, Sanders L, Digby JE, Sewter CP, Lazar MA, Chatterjee VK & O'Rahilly S. Activators of peroxisome proliferator-activated receptor gamma have depot-specific effects on human preadipocyte differentiation. *Journal of Clinical Investigation* 1997; **100:**3149–3153.

33. Tontonoz P, Hu E & Spiegelman BM. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* 1994; **79:**1147–1156.

 ©2003 International Medical Press

34. Aubert J, Champigny O, Saint-Marc P, Negrel R, Collins S, Ricquier D & Ailhaud G. Up-regulation of UCP-2 gene expression by PPAR agonists in preadipose and adipose cells. *Biochemical & Biophysical Research Communications* 1997; **238:**606–611.

35. Kelly LJ, Vicario PP, Thompson GM, Candelore MR, Doebber TW, Ventre J, Wu MS, Meurer R, Forrest MJ, Conner MW, Cascieri MA & Moller DE. Peroxisome proliferator-activated receptors gamma and alpha mediate *in vivo* regulation of uncoupling protein (UCP-1, UCP-2, UCP-3) gene expression. *Endocrinology* 1998; **139:**4920–4927.

36. Tu N, Chen H, Winnikes U, Reinert I, Marmann G, Pirke KM & Lentes KU. Molecular cloning and functional characterization of the promoter region of the human uncoupling protein-2 gene. *Biochemical & Biophysical Research Communications* 1999; **265:**326–334.

37. Bastard JP, Caron M, Vidal H, Jan V, Auclair M, Vigouroux C, Luboinski J, Laville M, Maachi M, Girard PM, Rozenbaum W, Levan P & Capeau J. Association between altered expression of adipogenic factor SREBP1 in lipoatrophic adipose tissue from HIV-1-infected patients and abnormal adipocyte differentiation and insulin resistance. *Lancet* 2002; **359:**1026–1031.

38. Brinkman K, Smeitink JA, Romijn JA & Reiss P. Mitochondrial toxicity induced by nucleoside-analogue reverse-transcriptase inhibitors is a key factor in the pathogenesis of antiretroviral-therapy-related lipodystrophy. *Lancet* 1999; **354:**1112–1115.

39. Habinowski SA & Witters LA. The effects of AICAR on adipocyte differentiation of 3T3-L1 cells. *Biochemical & Biophysical Research Communications* 2001; **286:**852–856.

40. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, Wu M, Ventre J, Doebber T, Fujii N, Musi N, Hirshman MF, Goodyear LJ & Moller DE. Role of AMP-activated protein kinase in mechanism of metformin action. *Journal of Clinical Investigation* 2001; **108:**1167–1174.

41. Benbrik E, Chariot P, Bonavaud S, Ammi-Said M, Frisdal E, Rey C, Gherardi R & Barlovatz-Meimon G. Cellular and mitochondrial toxicity of zidovudine (AZT), didanosine (ddI) and zalcitabine (ddC) on cultured human muscle cells. *Journal of the Neurological Sciences* 1997; **149:**19–25.

42. Hobbs GA, Keilbaugh SA, Rief PM & Simpson MV. Cellular targets of 3′-azido-3′-deoxythymidine: an early (non-delayed) effect on oxidative phosphorylation. *Biochemical Pharmacology* 1995; **50:**381–390.

43. Keilbaugh SA, Hobbs GA & Simpson MV. Effect of 2′,3′-dideoxycytidine on oxidative phosphorylation in the PC12 cell, a neuronal model. *Biochemical Pharmacology* 1997; **53:**1485–1492.

44. Nolan D & Mallal S. Getting to the HAART of insulin resistance. *AIDS* 2001; **15:**2037–2041.

45. Cousin B, Cinti S, Morroni M, Raimbault S, Ricquier D, Penicaud L & Casteilla L. Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *Journal of Cell Science* 1992; **103(Pt 4):**931–942.

46. Tsukiyama-Kohara K, Poulin F, Kohara M, DeMaria CT, Cheng A, Wu Z, Gingras AC, Katsume A, Elchebly M, Spiegelman BM, Harper ME, Tremblay ML & Sonenberg N. Adipose tissue reduction in mice lacking the translational inhibitor 4E-BP1. *Nature Medicine* 2001; **7:**1128–1132.

47. Ntambi JM & Kim YC. Symposium: adipocyte function, differentiation and metabolism. *Journal of Nutrition* 2000; **(Suppl.):**3123S–3126S.

48. Domingo P, Matias-Guiu X, Pujol RM, Francia E, Lagarda E, Sambeat MA & Vazquez G. Subcutaneous adipocyte apoptosis in HIV-1 protease inhibitor-associated lipodystrophy. *AIDS* 1999; **13:**2261–2267.

49. Thompson K, McComsey G, Paulsen D, Cherry C, Lonergan T, Hessenthaler S, Williams V, Fisher R, Wesselingh S, Hernandez J & Ross L. Improvements in body fat and mitochondrial DNA levels are accompanied by decreased adipose tissue cell apoptosis after replacement of stavudine therapy with either abacavir or zidovudine. *10th Conference on Retroviruses & Opprotunistic Infections*, Boston, Mass., USA, 10–14 February 2003; Poster 728.

Received 28 February 2003; accepted 22 April 2003