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Introduction

Combination anti-retroviral therapy (cART) has transformed the prognosis for HIV-infected persons since the late 1990s. However, patients are at risk of mitochondrial toxicity, thought to be mediated very largely through exposure to certain nucleoside analog reverse transcriptase inhibitor (NRTI) anti-retrovirals. NRTIs were the first class of licensed anti-retroviral drug, and several of the older members of this class, zidovudine, stavudine, zalcitabine and didanosine, are known to inhibit the sole mitochondrial DNA (mtDNA) polymerase, pol γ, resulting in chain termination during mtDNA replication. During therapy, the molecular consequence of this inhibition is reduction in cellular mtDNA content (mtDNA depletion). A wealth of previous studies has demonstrated this phenomenon both in vitro, and in a variety of tissues in vivo [1–4]. These older NRTIs are no longer in common usage in industrialized countries owing to concerns over their toxicity profiles, although zidovudine and stavudine have been very extensively used in anti-retroviral therapy ‘roll-out’ programs in developing countries in recent years. Currently used NRTIs, such as tenofovir [a nucleotide RTI] and abacavir, have been shown to be essentially free from pol γ inhibition in vitro and to cause no significant mtDNA depletion in vivo [5,6]. If a patient’s therapy is switched away from a pol γ inhibiting NRTI, the impairment of mtDNA replication is removed and mtDNA content returns to normal [7]. Therefore, although most patients are no longer exposed to pol γ inhibiting NRTIs, a significant cohort of long-term patients will have extensive prior exposure to such drugs. Although such patients do not have persistent mtDNA depletion, it has recently been established that they may have persistent histochemical mitochondrial defects evidenced by an increased proportion of COX (cytochrome c oxidase) deficient skeletal muscle fibers. These COX deficient fibers contain high levels of individual somatic (acquired) mtDNA mutations (princi-
pally large-scale deletion mutations) [8]. The relevance of this persistent cellular and molecular damage on mitochondrial function remains unknown. It is therefore unclear to what extent mitochondria may be functionally impaired in HIV-infected patients treated with contemporary cART.

Phosphorus magnetic resonance spectroscopy ($^{31}$P-MRS) allows the dynamic measurement of in vivo skeletal muscle oxidative function through assessment of ATP (adenosine triphosphate) metabolites as well as acid handling. $^{31}$P-MRS has previously been employed in the longitudinal study of subjects with inherited mitochondrial disorders, both primary mtDNA defects, and secondary mtDNA defects consequent on nuclear gene disorders [9–11]. Limited data also suggests that $^{31}$P-MRS abnormalities in skeletal muscle may be demonstrated in the setting of acute exposure to pol γ inhibiting NRTIs: early in the HIV epidemic, in infected patients exposed to high-dose zidovudine therapy; and in uninfected volunteers treated with stavudine. Such measurements have not been performed in contemporary cART treated patients [12,13].

We have therefore used $^{31}$P-MRS to determine whether patients on contemporary anti-retroviral therapy have abnormal in vivo mitochondrial oxidative function, and whether this correlates with biopsy COX defects.

Methods

Participants

Participants were adult HIV-1 infected patients, receiving ambulatory care at one of four specialist clinics (2 hospital-based, 2 community-based setting). Patients with current active hepatitis B or C co-infection were excluded. Participants were unselected with respect to the presence or absence of complications of HIV or anti-retroviral therapy. Patients with known inherited or non-HIV-associated neuromuscular disease were excluded. Demographic data, surrogate markers (CD4 T lymphocyte count, and HIV-1 RNA plasma viral load) and detailed lifetime anti-retroviral treatment history were obtained by case note review.

HIV-uninfected control subjects for $^{31}$P-MRS studies were age and sex matched to our cases, and we excluded persons with previous muscle injury, or diagnosed neuromuscular disease. Limited data also suggests that $^{31}$P-MRS abnormalities in skeletal muscle may be demonstrated in the setting of acute exposure to pol γ inhibiting NRTIs: early in the HIV epidemic, in infected patients exposed to high-dose zidovudine therapy; and in uninfected volunteers treated with stavudine. Such measurements have not been performed in contemporary cART treated patients [12,13].

We have therefore used $^{31}$P-MRS to determine whether patients on contemporary anti-retroviral therapy have abnormal in vivo mitochondrial oxidative function, and whether this correlates with biopsy COX defects.

Results

Patient Characteristics

23 HIV-infected subjects participated; 78% were male. Mean age was 57.6 years, with age range of 45–74 years. Mean duration of diagnosed HIV infection was 11.8 years. Mean current CD4 T lymphocyte count was 551 cells/μl; mean nadir CD4 count was 183 cells/μl. All subjects were currently receiving cART, of whom 96% had a fully suppressed HIV plasma viral load (<40 HIV-1 RNA copies/ml). In addition to their NRTIs, 70% of treated subjects were receiving a non-nucleoside reverse transcriptase inhibitor (NNRTI) and 33% a protease inhibitor (PI). With respect to the pol γ inhibiting NRTIs, 61% of patients had a prior history of zidovudine exposure, and 48% had didoxynucleoside analog (stavudine, zalcitabine or didanosine) exposure (treatment details of individual patients are shown in Table 1).

Skeletal Muscle Biopsies and Mitochondrial (COX) Histochemistry

Percutaneous lower limb skeletal muscle biopsies were performed under local anesthesia and snap-frozen in the liquid phase of isopentane, cooled in liquid nitrogen within 20 minutes of collection. Sequential COX-SDH (cytochrome c oxidase/succinate dehydrogenase) histochemistry was performed on 20 μm transverse cryo-sections. COX contains respiratory chain subunits encoded by the mitochondrial genome, and fibers stain brown (positive) in the presence of intact respiratory chain activity. SDH contains subunits encoded entirely by the nuclear genome and thus provides an effective counterstain (blue) as activity will be preserved in the presence of a cellular mtDNA defect. Proportional COX defect was determined by counting ≥500 fibers per biopsy.

Statistical Comparisons

Student’s paired t-test was used to compare MRS parameters between cases and controls. Correlation coefficients were calculated between COX and MRS data. All analyses were performed in SPSS 19.

Measures of Muscle ATP and Acid Metabolism by $^{31}$P-MRS

In the resting state, baseline ATP metabolites and pH values were significantly higher in CART-treated HIV-infected subjects compared to age and gender-matched controls (mean ±SD): ADP:ATP ratio, HIV-infected 1.24±0.08×10$^{-5}$, HIV-uninfected 1.16±0.03×10$^{-5}$, p = 0.001; phosphocreatine/ATP (PCr/ATP) ratio, HIV-infected 5.04±1.89, HIV-uninfected 3.75±0.26, p = 0.004; pH, HIV-infected 7.07±0.03, HIV-uninfected 7.04±0.02, p = 0.002. Correspondingly, calculated basal phosphorylation potential was significantly lower in HIV-infected subjects compared with controls: HIV-infected 227±56 mM$^{-1}$, HIV-uninfected 292±53 mM$^{-1}$, p = 0.003 (Figure 1a–c). Further details of calculated $^{31}$P-MRS parameters are shown in the Table S1).

In terms of dynamic oxidative function, mean post-exercise ATP metabolite recovery rates did not differ significantly between HIV-infected subjects and controls. For example, $\tau_1$, ADP: HIV-infected 22.1±9.9 s, HIV-uninfected 18.8±4.4 s, p = 0.09 (Figure 1d). None of the clinical variables (age, duration of diagnosed HIV infection, CD4 T lymphocyte count, or anti-retroviral treatment history) correlated significantly with any of the baseline or post-exercise $^{31}$P-MRS parameters in HIV-infected subjects.
Table 1. Characteristics of HIV-infected subjects.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yrs)</th>
<th>Gender</th>
<th>Duration of diagnosed HIV infection (mo)</th>
<th>Current CD4 count (cells/uL)</th>
<th>Current HIV VL (copies/mL)</th>
<th>Nadir CD4 count (cells/uL)</th>
<th>Duration of ART (mo)</th>
<th>Current HAART</th>
<th>Lifetime HAART</th>
<th>Biopsy COX defect (%)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
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<td>96</td>
<td>177</td>
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<td>&lt;40</td>
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<td>TDF FTC ATV/r</td>
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<td>408</td>
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VL, plasma HIV-1 RNA viral load; (c)ART, (combination) antiretroviral therapy; AZT, zidovudine; d4T, stavudine; ddI, didanosine; ddC, zalcitabine; 3TC, lamivudine; ABC, abacavir; TDF, tenofovir; FTC, emtricitabine; EFV, efavirenz; NVP, nevirapine; ATV, atazanavir; DRV, darunavir; LPV, lopinavir; SQV, saquinavir; NFV, nelfinavir; IDV, indinavir; RTV, ritonavir; ddI, didanosine; ddC, zalcitabine; IDV, indinavir; ddC, didanosine; ddI, didanosine. COX data (but not MRS data) from 5 subjects has been previously described [8].

In Vivo Mitochondrial Function in HIV-Infected Subjects.
Mitochondrial (COX) Histochemistry and Correlation with ATP Metabolism

A wide range of COX defects were observed across the subject group (0 to >10% of muscle fibers affected per biopsy). Interestingly, we observed significant COX defects both in patients with prior exposure to pol γ inhibiting NRTIs, and in some patients without such exposure (COX defects for individual patients are shown in Table 1). Resting state ADP/ATP ratio showed a moderate correlation with biopsy proportional COX defect (Kendall’s $\tau = 0.34$, $p = 0.034$) (Figure 2a). There was no correlation between dynamic ATP metabolism, for example as estimated by $\tau_{t}$, ADP, and biopsy COX defect (Figure 2b).

Discussion

In our study, most anti-retroviral treated HIV-infected subjects demonstrated dynamic in vivo tissue mitochondrial function comparable with uninfected control subjects, whereas it is generally impaired in inherited mitochondrial disorders. Our subject group included patients with very long durations of HIV infection and extensive anti-retroviral drug treatment histories, including past exposure to pol γ inhibiting NRTIs. Interestingly, in the present study we observed COX defects both in subjects with prior exposure to pol γ inhibiting NRTIs, and in some subjects without such exposure. This observation contrasts with our previous work in younger HIV-infected patients (all aged ≤50 years), where COX defects appeared to be attributable almost entirely to exposure to pol γ inhibiting NRTIs [8]. In the present study, the heterogeneous COX defects are most likely to reflect the significantly older subject age range (45–74 years). In this age group it is expected to see some COX defects due to normal aging [17], although it is also possible that there are other unmeasured HIV or treatment-associated factors driving COX defects in some of these patients. As with NRTI-associated COX defects, these COX deficient fibers also contain high levels of individual somatic mtDNA mutations [18]. What is therefore the likely explanation of

Figure 1. Phosphorus magnetic resonance spectroscopy. Resting state metabolic parameters differed significantly between HIV-infected subjects (HIV+) and HIV-uninfected controls (HIV−): ADP/ATP (adenosine diphosphate/ATP) ratio (a), phosphorylation potential (b), and pH (c) ($n = 23$ each; ADP/ATP, $p = 0.001$; phosphorylation potential, $p = 0.003$; pH, $p = 0.002$). In contrast, the rate of ATP re-synthesis (estimated as $\tau_{t}$, ADP) following exertion was not significantly impaired in HIV-infected subjects compared with controls ($p = 0.09$) (d).

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The decreased basal phosphorylation potential, as we have observed in our patients, implies an increased rate of ATP synthesis at rest [19]. Cytosolic ATP concentration is tightly buffered, and results from the balance of the ATP hydrolysis required for the maintenance of cellular integrity and the synthesis of ATP from oxidative phosphorylation [16]. The rate of ATP synthesis is strongly dependent on the phosphorylation potential in resting muscle, with a decreased phosphorylation potential, as we have observed in our patients, implying an increased rate of ATP synthesis [19]. This notion suggests that there is a requirement for an increased basal rate of intracellular ATP hydrolysis in HIV-infected subjects, and an increased basal rate of ATP synthesis is therefore required to maintain ATP homeostasis. Although this might imply increased basal energy expenditure in these patients compared with healthy subjects, given that dynamic in vivo mitochondrial function is unimpaired, the physiological significance of this observation remains uncertain. Further work should therefore examine correlates of this finding, such as fatigue [20].

In conclusion, in a cohort of predominantly older HIV-infected patients with longstanding cART, we observed frequent histochemical COX defects both in patients with and without prior exposure to pol γ inhibiting NRTIs. It is, however, broadly reassuring that in vivo whole tissue mitochondrial function in most contemporary anti-retroviral treated patients appears to be largely maintained, despite the presence of this frequent mitochondrial damage within individual cells.

Supporting Information

Table S1  Phosphorus magnetic resonance data. Calculated 31P-MRS parameters, in resting state and during recovery from sub-maximal exercise.

(LOCX)

Author Contributions

Conceived and designed the experiments: BAIP KGH DAP MT PFC. Performed the experiments: BAIP KGH. Analyzed the data: BAIP KGH. Contributed reagents/materials/analysis tools: JB EW VL DAP. Wrote the paper: BAIP PFC. Revised the manuscript: KGH JB EW VL DAP MT.

References