AZT Treatment Induces Molecular and Ultrastructural Oxidative Damage to Muscle Mitochondria
Prevention by Antioxidant Vitamins

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Abstract
AIDS patients who receive zidovudine (AZT) frequently suffer from myopathy. This has been attributed to mitochondrial (mt) damage, and specifically to the loss of mtDNA. This study examines whether AZT causes oxidative damage to DNA in patients and to skeletal muscle mitochondria in mice, and whether this damage may be prevented by supranutritional doses of antioxidant vitamins. Asymptomatic HIV-infected patients treated with AZT have a higher urinary excretion (355±100 pmol/kg/d) of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) (a marker of oxidative damage to DNA) than untreated controls (asymptomatic HIV-infected patients) (182±29 pmol/kg/d). This was prevented (110±79 pmol/kg/d) by simultaneous oral treatment with AZT plus antioxidant vitamins (C and E).

Introduction
3'-azidothymidine, 3'-azido-2',3'-dideoxymidine (AZT)© is a potent inhibitor of the replication of HIV and one of the drugs of choice for the treatment of AIDS. The incorporation of AZT monophosphate (MP) into viral DNA results in premature termination of DNA synthesis (1). The major limitation in the use of AZT is the occurrence of severe side effects (2). AIDS patients who receive long-term therapy with AZT frequently suffer from toxic mitochondrial (mt) myopathy. Typical features of this myopathy are ragged red fibers and paracrystalline inclusions in mitochondria (3). This has been attributed to damage to mitochondria and to mtDNA depletion (4). mtDNA depletion in muscle is probably due to the AZT-induced inhibition of DNA polymerase gamma, which is responsible for the replication of mtDNA (4). Moreover, decreased mtDNA and mt polypeptide synthesis were found in vitro (5). A single study in 1991 proposed that AZT causes oxidation of deoxyguanosine to yield 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) in DNA from the mitochondria of experimental animals (6). The role of mtDNA in cell physiology and medicine has been emphasized previously (7). Manifestations of mt diseases are thought to be due, at least in part, to increased free-radical formation (7,8). We recently observed that there is a correlation between glutathione oxidation and mtDNA damage and that age-associated mtDNA damage can be prevented by administration of antioxidant vitamins (9).

The aim of this work was to test whether AZT causes oxidative damage to mitochondria and whether this can be prevented by administration of supranutritional doses of antioxidant vitamins.

We have found that AZT causes an increase in the urinary excretion of 8-oxo-dG in humans and mice, and that it also causes an oxidation of mtDNA, mt glutathione, and lipids (measured as malondialdehyde [MDA]). This oxidative damage may be due to an increased peroxide production by muscle mitochondria from AZT-treated animals. Antioxidants revert this oxidative damage and also the ultrastructural changes in mitochondria caused by AZT.

Methods

Chemicals. RNAase (E.C.3.1.27.5) from bovine pancreas was purchased from Boehringer–Mannheim (Mannheim, Germany). Water and methanol (HPLC grade) were from Merck (Darmstadt, Germany). Nuclease P1 (E.C.3.1.30.1) was from P. Citricum. Alkaline phosphatase type III-L (C.E.3.1.30.1) was isolated from Escherichia coli. Glutathione-S-transferase (E.C.2.5.1.18) was isolated from equine liver. GSH, oxidized glutathione (GSSG), 8-oxo-dG, AZT, 3'-azido-3'-deoxymidine) N-ethylmaleimide (NEM), baphenanthrol linedisulfonic acid (BPDS), Mops, BSA, and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Patients. We analyzed urine from 23 30±3-yr-old male adults weighing 50±7 kg. Urine samples were collected from each individual 24 h void and stored at −30°C until analysis. Four groups were studied: (A) healthy controls, (B) HIV-positive patients (who had not received AZT) with 570±261 CD4+/μl, (C) HIV-positive patients treated with AZT (Retrovir®, 250 mg/12 h) over 12–24 mo with 382±141 CD4+/μl, and (D) the same HIV-positive patients who were...
AZT Induces Oxidative Damage to Muscle Mitochondrial DNA

8-oxo-dG was performed using an ESA Coulochem dual coulometric detector (model 5200; ESA, Bedford, MA) equipped with a 5011 analytical cell.

Assays. Standard enzymatic methods were used to measure levels of creatine kinase (CK), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) in mouse serum (20).

Measurement of glutathione. GSH was measured spectrophotometrically using glutathione-S-transferase (21). GSSG was assayed by an HPLC method with detection at 365 nm, which we recently developed to measure GSSG in the presence of a large excess of GSH (22, 23).

Measurement of MDA. MDA content was determined in skeletal muscle mitochondria using an HPLC method described by Wong et al. (24) that determines MDA formed from lipoperoxides. mt lipoperoxides are hydrolyzed by boiling in diluted phosphoric acid. MDA reacts with thiobarbituric acid to form an adduct, which is detected by HPLC. The calibration curve was assayed using tetramethoxypropane, which undergoes hydrolysis to liberate stoichiometric amounts of MDA (25).

Measurement of peroxide production by mitochondria. After isolation, skeletal muscle mitochondria were suspended in ice-cold respiratory buffer (with 0.3 M sucrose, 1 mM EGTA, 5 mM Mops, 0.1% BSA, and 5 mM KH$_2$PO$_4$ adjusted to pH 7.4 with KOH) and were used to measure the production of peroxides with an Epies Elite II flow cytometer (Coulter Electronics, Hialeah, FL). Dihydrorhodamine 123 was used as fluorochrome on incubation at 37°C for 30 min and was excited with an argon laser tuned at 488 nm. Colorless dihydorhodamine 123 is oxidized outside mitochondria by H$_2$O$_2$-dependent reactions to the fluorescent product rhodamine 123, which stains mitochondria (26, 27). Rhodamine 123 is a cationic fluorescent lipophilic dye with a delocalized positive charge. It is readily taken up by active mitochondria without inducing cytotoxic effects. The mt membrane potential is the driving force for rhodamine 123 uptake by mitochondria (26). This reduced rhodamine has been used to monitor reactive oxygen intermediates in different cellular and subcellular models (27, 28).

The gate was set on logarithm of forward and side scatter to always include mitochondria with the same morphological properties. Thus, changes in fluorescence were independent of the particle size.

Electron microscopy of mouse muscle samples. Skeletal muscle samples (gastrocnemius) were obtained for electron microscopy. Samples were fixed in 0.1 mM phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 2 h, and then postfixed in 0.1 mM phosphate buffer (pH 7.2) containing 2% osmium tetroxide for a further 2 h. After embedding the sample blocks in Epon, ultrathin sections were cut with the ultramicrotome Ultratome III (Ultratome, Uppsala, Sweden) and then contrasted with uranyl acetate and lead citrate for transmission electron microscopy. Electron microphotographs were taken with an electron microscope (JEOL, Tokyo, Japan) JEM 11 B.

Statistics. Results are expressed as the mean ± SD. Statistical analyses were performed by the least significant difference test, which consists of two steps. First an ANOVA was performed. The null hy-

<table>
<thead>
<tr>
<th>8-oxo-dG (pmol/Kg/day)</th>
<th>Healthy</th>
<th>HIV</th>
<th>HIV+AZT</th>
<th>HIV+AZT+antioxid.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(7)</td>
<td>10</td>
<td>60</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>(3)</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>(5)</td>
<td>100</td>
<td>100</td>
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</table>

<table>
<thead>
<tr>
<th>8-oxo-dG (pmol/Kg/day)</th>
<th>Control</th>
<th>AZT</th>
<th>AZT+antioxid.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(6)</td>
<td>6</td>
<td>60</td>
<td>60</td>
</tr>
<tr>
<td>(8)</td>
<td>80</td>
<td>80</td>
<td>80</td>
</tr>
</tbody>
</table>

Figure 1. Levels of 8-oxo-dG in urine from HIV-seropositive patients (A) and from mice (B) treated with AZT and antioxidant vitamins (C and E). Statistical significance is expressed as follows: *P < 0.05 versus the HIV group (untreated patients, A) or versus the control group (B); #P < 0.05 versus the HIV+AZT group (A) or versus the AZT-treated group (B).
pothesis was accepted for all numbers of those set in which F was nonsignificant at the level of $P \leq 0.05$. Then the sets of data in which F was significant were examined by the two-tailed $t$ test using $P \leq 0.05$ as the critical limit.

Results

DNA damage caused by AZT in humans: urinary excretion of 8-oxo-dG in HIV-infected patients treated with AZT. Urinary excretion of 8-oxo-dG is an indicator of oxidative damage to DNA (17, 19). Fig. 1 A shows that asymptomatic HIV patients who receive AZT (250 mg/12 h) have a marked increase of 8-oxo-dG in urine (355±100 pmol/kg/d, $n = 8$) when compared with healthy controls (243±99 pmol/kg/d, $n = 7$, $P < 0.05$). This can be prevented by oral administration of supranutritional doses of antioxidants, which decreases the urinary excretion of 8-oxo-dG to 110±79 pmol/kg/d ($n = 5$), $P < 0.05$ (Fig. 1 A). However, asymptomatic HIV-positive patients who had not received AZT showed 8-oxo-dG values (183±29 pmol/kg/d, $n = 3$) similar to those of the healthy control group.

DNA damage caused by AZT in mice: urinary excretion of 8-oxo-dG in mice treated with AZT. We also tested the effect of AZT (at doses of 10 mg/kg body wt/d, similar to those taken by patients) on the excretion of 8-oxo-dG in the urine of mice. Controls excreted 18.0±9.2 pmol/g body wt/d ($n = 6$) whereas AZT-treated mice excreted 72.8±59.2 pmol/g body wt/d ($n = 10$, $P < 0.05$). This was prevented by antioxidants: mice treated with AZT and vitamins C and E excreted 15.3±4.1 ($n = 8$, $P < 0.05$) (Fig. 1 B).

Table I. Effects of AZT and Antioxidant Vitamins (C and E) on GSH and GSSG from Total Homogenate of Mouse Skeletal Muscle (Quadriceps)

<table>
<thead>
<tr>
<th></th>
<th>GSH</th>
<th>GSSG</th>
<th>(GSSG/GSH) x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol/g</td>
<td>nmol/g</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>948±109 (9)</td>
<td>18.6±8.8 (7)</td>
<td>1.89±0.85 (7)</td>
</tr>
<tr>
<td>AZT</td>
<td>902±110 (8)</td>
<td>59.8±26.1 (8)*</td>
<td>6.59±2.75 (8)*</td>
</tr>
<tr>
<td>AZT and antioxidant vitamins</td>
<td>992±98 (8)</td>
<td>26.0±9.4 (8)†</td>
<td>2.53±0.97 (8)†</td>
</tr>
</tbody>
</table>

Data are means±SD, with the number of observations in parentheses. Statistical significance is expressed as follows: *$P < 0.05$ versus the control group; †$P < 0.05$ versus the AZT-treated group.

Serum activity of muscle damage marker enzymes in AZT-treated mice: effect of antioxidant vitamins (C and E). Classic marker enzymes of skeletal muscle damage were measured in serum from three experimental groups. CK activity was increased from 1,645±966 ($n = 8$) to 2,407±558 U/liter ($n = 12$) ($P < 0.05$). AST activity was also statistically increased from 93±34 ($n = 8$) to 135±43 U/liter ($n = 12$) ($P < 0.05$). Finally, LDH activity was also statistically increased from 389±187 ($n = 7$) to 707±243 U/liter ($n = 13$) ($P < 0.05$). Vitamins C and E prevented the increase in enzyme activities caused by AZT (Fig. 2).

Antioxidant vitamins (C and E) decrease AZT-induced glutathione oxidation in homogenate from mouse skeletal muscle. Treatment with AZT did not cause a significant decrease in GSH in total muscle homogenate. However, GSSG concentration increased significantly in the AZT-treated group when compared with controls. These results show that AZT induces an oxidation of glutathione in mouse skeletal muscle. Treatment with antioxidant vitamins prevented the AZT-induced glutathione oxidation (Table I). The effect of AZT and antioxidant vitamins (C and E) on the skeletal muscle GSSG/GSH ratio (values for the ratio show GSSG as a percentage of GSH) followed a similar pattern. Values for the AZT-treated group (6.59±2.75, $n = 8$) show an increase when compared with the control group (1.89±0.89, $n = 7$, $P < 0.05$). Treatment with antioxidant vitamins prevented the AZT-induced increase in GSSG in muscle homogenate (Table I).

AZT causes an increase in peroxide production by skeletal muscle mitochondria. To investigate the cause of oxidative damage that we observed in skeletal muscle mitochondria, we measured the effect of AZT on the rate of peroxide production in mitochondria from skeletal muscle. AZT causes a marked increase in peroxide production in mitochondria from skeletal muscle (137±26 fluorescence units, $n = 3$) when compared with the control group (12.4±1.8 fluorescence units, $n = 3$, $P < 0.001$). This effect was completely abolished (22.1±14.8 fluorescence units, $n = 4$, $P < 0.001$) when mice were fed a diet supplemented with vitamins C and E (Fig. 3).

AZT induces an oxidation of mt glutathione in mouse skeletal muscle. Table II shows that mitochondria from mouse skeletal muscle treated with AZT have an increased level of GSSG; oral administration of antioxidant vitamins (C and E) prevents this change. Changes in the GSSG/GSH ratio followed a similar pattern, showing that AZT induces a prooxidative status in mitochondria. Values of the AZT-treated group (4.24±0.45, $n = 3$) are higher than those of controls (2.19±
AZT causes an increase in mt MDA levels from mouse skeletal muscle. AZT causes an increase in lipoperoxide production in mitochondria that is detected as an increase in MDA levels in skeletal muscle mitochondria. This effect was abolished when mice were fed a diet supplemented with vitamins C and E (Table III).

AZT causes oxidative damage to mtDNA from mouse skeletal muscle. The fact that urinary excretion of 8-oxo-dG (Fig. 1) is increased in patients and mice who receive AZT prompted us to study the possible effect of this drug on oxidative damage to mtDNA from skeletal muscle in mice. Table III shows that AZT causes an increase of >100% in the levels of 8-oxo-dG when compared with controls (controls: 0.795±0.383 pmol/μg mtDNA, n = 4; AZT-treated: 1.748±0.568 pmol/μg mtDNA, n = 5, P < 0.05). This change is completely prevented by the oral administration of antioxidant vitamins (0.396±0.156 pmol/μg mtDNA, n = 4, P < 0.01).

Morphological analysis of mouse skeletal muscle by electron microscopy: antioxidants prevent ultrastructural damage to mitochondria caused by AZT. Fig. 4 shows that treatment with AZT causes considerable damage to muscle mitochondria, which includes swelling and severe disorganization of the internal mt architecture with cristae disruption. Treatment with antioxidant vitamins prevented this damage. These morphological observations are in keeping with the protective effect of antioxidants on AZT-induced oxidative damage to mtDNA and other biochemical parameters we have observed.

Table II. Effects of AZT and Antioxidant Vitamins (C and E) on GSH and GSSG of Isolated Mitochondria from Mouse Skeletal Muscle

<table>
<thead>
<tr>
<th></th>
<th>GSH (pmol/mg protein)</th>
<th>GSSG (pmol/mg protein)</th>
<th>(GSSG/GSH) × 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>603±406 (3)</td>
<td>8.1±3.0 (3)</td>
<td>2.19±0.28 (3)</td>
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<tr>
<td>AZT</td>
<td>320±177 (3)</td>
<td>13.0±2.0 (4)*</td>
<td>4.24±0.45 (3)*</td>
</tr>
<tr>
<td>AZT and antioxidant vitamins</td>
<td>414±49 (3)</td>
<td>7.6±1.5 (3)</td>
<td>1.85±0.33 (3)</td>
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</tbody>
</table>

Data are means±SD, with the number of observations in parentheses. Statistical significance is expressed as follows: *P < 0.05 versus the control group; †P < 0.05 versus the AZT-treated group.

Table III. Effects of AZT and Antioxidant Vitamins (C and E) on mt MDA and on Levels of 8-oxo-dG in mtDNA from Mouse Skeletal Muscle

<table>
<thead>
<tr>
<th></th>
<th>MDA (pmol/mg protein)</th>
<th>8-oxo-dG (μmol/μg mtDNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>28.6±11.7 (3)</td>
<td>0.78±0.38 (4)</td>
</tr>
<tr>
<td>AZT</td>
<td>52.1±4.1 (4)*</td>
<td>1.75±0.57 (5)*</td>
</tr>
<tr>
<td>AZT and antioxidant vitamins</td>
<td>41.5±2.0 (3)†</td>
<td>0.40±0.16 (4)†</td>
</tr>
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</table>

Data are means±SD, with the number of observations in parentheses. Statistical significance is expressed as follows: *P < 0.05 versus the control group; and †P < 0.05, or ‡P < 0.01 versus the AZT-treated group.
mtDNA, mtRNA, and mt polypeptide synthesis (5). However, the mechanism of such myopathy was previously unknown. Here we show that AZT causes an oxidation of mtDNA, as evidenced by increased levels of 8-oxo-dG in mtDNA isolated from mouse skeletal muscle (Table III), and that this correlates with changes in the urinary excretion of 8-oxo-dG. Other signs of oxidative stress, such as MDA formation (32–35), or glutathione oxidation (9), also occur in mitochondria isolated from the skeletal muscle of AZT-treated animals (Table II) (see Fig. 3B). As a consequence, AZT may affect whole muscle cells as indicated by an increase in plasma activity of cytoplasmic enzymes such as CK or LDH (see Fig. 2).

These effects are probably due to an increased peroxide formation by mitochondria from the muscle of AZT-treated animals. Indeed, the increase in peroxide production (see Fig. 3) may explain the signs of oxidative damage to mt macromolecules such as DNA, mt lipids (MDA), and the oxidation of glutathione.

Several studies have documented that AZT induces inhibition of DNA polymerase gamma, which is responsible for mtDNA replication (36). A possible link between this decrease in DNA replication and the increase in free-radical production that we find is that as a result of lowered mtDNA replication, mt turnover is decreased. Thus, mitochondria will not be properly renewed in the cell, and old mitochondria will remain in the cell. We have found that mitochondria from old animals produce more peroxides than those from young ones and suffer from oxidative stress (28). In any case, oxidative stress directly or indirectly contributes to mtDNA damage caused by AZT, and treatment with antioxidant vitamins prevents such mt damage.

In summary, treatment of animals with vitamins C and E protects against signs of ultrastructural mt damage (see Fig. 4) and prevents the oxidation of muscle intra-mt components induced by AZT. These protective effects provide biochemical bases for preventing AZT-induced mt myopathy using oral supplementation with antioxidant vitamins such as C and E.

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References


