Effect of Stavudine on Mitochondrial Genome and Fatty Acid Oxidation in Lean and Obese Mice

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ABSTRACT

Like other antihuman immunodeficiency virus dideoxynucleosides, stavudine may occasionally induce lactic acidosis and perhaps lipodystrophy in metabolically or genetically susceptible patients. We studied the effects of stavudine on mitochondrial DNA (mtDNA), fatty acid oxidation, and blood metabolites in lean and genetically obese (ob/ob) mice. In lean mice, mtDNA was depleted in liver and skeletal muscle, but not heart and brain, after 6 weeks of stavudine treatment (500 mg/kg/day). With 100 mg/kg/day, mtDNA transiently decreased in liver, but was unchanged at 6 weeks in all organs, including white adipose tissue (WAT). Despite unchanged mtDNA levels, lack of significant oxidative mtDNA lesions (as assessed by long polymerase chain reaction experiments), and normal blood lactate/pyruvate ratios, lean mice treated with stavudine for 6 weeks had increased fasting blood ketone bodies, due to both increased hepatic fatty acid β-oxidation and decreased peripheral ketolysis. In obese mice, basal WAT mtDNA was low and was further decreased by stavudine. In conclusion, stavudine can decrease hepatic and muscle mtDNA in lean mice and can also cause ketoacidosis during fasting without altering mtDNA. Stavudine depletes WAT mtDNA only in obese mice. Fasting and ketoacidosis could trigger decompensation in patients with incipient lactic acidosis, whereas WAT mtDNA depletion could cause lipodystrophy in genetically susceptible patients.

Nucleoside analogs, including zidovudine, stavudine, didanosine, zalcitabine, and lamivudine, are used in combination with human immunodeficiency virus (HIV) protease inhibitors in the treatment of HIV-infected patients (Carpenter et al., 2000). However, the outstanding benefit of these antiretroviral drugs on morbidity and mortality is partly overshadowed by the possible occurrence of serious side effects. Nucleoside analogs can occasionally cause myopathy, cardiomyopathy, pancreatitis, peripheral neuropathy, and microvesicular steatosis of the liver, with lactic acidosis and/or liver failure (Fromenty and Pessayre, 1995; Lewis and Dalakas, 1995; Brinkman et al., 1998). These adverse effects have been mainly ascribed to drug-induced impairment of mitochondrial DNA (mtDNA) replication, causing mtDNA depletion, impaired oxidative phosphorylation, and ATP deficiency (Fromenty and Pessayre, 1995; Brinkman et al., 1998). However, recent data suggest that nucleoside analogs could also impair mitochondrial function and cellular metabolisms independently of any mtDNA depletion (Barile et al., 1997; Garcia de la Asuncion et al., 1998; Szabados et al., 1999; Pan-Zhou et al., 2000).

Another complication seen in treated patients is a lipodystrophy syndrome, with peripheral fat wasting, central adiposity, hyperlipidemia, and insulin resistance (Carr et al., 1999). Although the pathogenesis of this syndrome is unknown, several factors could play a role, including the HIV infection, a putative genetic predisposition, and antiretroviral treatments (Brinkman et al., 1999; Carr et al., 1999). Both HIV protease inhibitors (Carr et al., 1999) and nucleoside analogs (Saint-Marc et al., 1999; Strobel et al., 1999) could modify lipid metabolism in these patients. Although a possible involvement of mitochondrial dysfunction in nucleoside-induced metabolic disorders has been recently suggested (Brinkman et al., 1999), definite data are currently lacking.

Stavudine is currently one of the most widely used nucleoside analogs in numerous countries (Saint-Marc et al., 1999; Carpenter et al., 2000). Like other nucleoside analogs, stavudine has caused neuropathy, pancreatitis, microvesicular steatosis, and lactic acidosis in a few patients (Lenzo et al., 1997; Brinkman et al., 1998; Mokrzycki et al., 2000) and stavudine may also increase the risk of fat wasting and shadowed by the possible occurrence of serious side effects.

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hypertriglyceridemia (Saint-Marc et al., 1999; Strobel et al., 1999). However, to our knowledge, the effects of stavudine on mitochondrial function and lipid metabolism have not been assessed in animal models. We therefore addressed this important issue in lean and obese mice treated for 6 weeks with stavudine.

**Materials and Methods**

**Animals and Treatments.** Young (6–10-week-old) male Crl:CD-1(ICR)/BR Swiss mice weighing 28 to 30 g were purchased from Charles River (Saint-Aubin-lès-Elbeuf, France). Young (10-week-old) male obese (ob/ob) mice (C57BL/6-ob) weighing 40 to 44 g were purchased from Janvier (Le-Genest-St-Ise, France). In these ob/ob mice, a nonsense mutation in the leptin gene increases food intake and decreases energy expenditure (Friedman and Halaas, 1998).

Stavudine (Zerit) was kindly provided by Bristol-Myers Squibb (Fontenay sous Bois, France). Unless otherwise indicated, stavudine (100 or 500 mg/kg/day) was given in the drinking water for 6 weeks. The quantity of stavudine (Zerit) added in the drinking water was calculated on the basis of a daily liquid consumption of 5 ml. This daily liquid intake was unaffected by the treatment. Since Zerit also contains sucrose (final concentration in drinking water, 38 mg/ml, with 100 mg/kg/day stavudine), the same amount of sucrose was added to the drinking water of control mice. Mice were either fed ad libitum on a normal diet (A04 biscuits; UAR, Villemoisson-sur-Orge, France) or fasted for the last 24 or 48 h of the stavudine and/or sucrose treatments. In some experiments, mice were treated by zidovudine (100 mg/kg/day in the drinking water) for 2 weeks. All animals received humane care.

**Plasma Stavudine Levels.** Plasma concentrations of stavudine were determined by reversed phase high performance liquid chromatography as previously described (Burger et al., 1992), with minor modifications. Sample preparation was carried out with C18 solid-phase extraction columns (93.5% recovery) and detection was performed by UV absorbance at 254 nm. Between-day and within-day variations of quality control samples of stavudine are lower than 10%. The lower limit of quantification of the assay is 10 ng/ml and linearity is achieved from 10 to 5000 ng/ml.

**Isolation of Total DNA and Slot Blot Hybridization.** Total DNA was isolated from liver, hind limb skeletal muscles, heart, brain, and epididymal white adipose tissue (WAT), using the phenol-chloroform method as previously described (Fromenty et al., 1995). To quantify mtDNA and nuclear DNA (nDNA), slot blot hybridization was performed as previously described (Mansouri et al., 1999). Total DNA (200–400 ng) was blotted onto a Hybond-N+ membrane (Amersham, Les Ulis, France), and hybridized with a 32P-labeled probe generated by long PCR and labeled by random priming (Multiprime DNA labeling system; Amersham). Mitochondria were stripped and hybridized with a mouse C3H-1 nDNA probe (Life Technologies, Cergy Pontoise, France) as previously described (Mansouri et al., 1999). mtDNA and nDNA were assessed by densitometric analysis of autoradiographs (Mansouri et al., 1999).

**Long PCR Experiments.** We used long PCR experiments to look for significant oxidative mtDNA damage, such as strand breaks and apurinic/apyrimidinic (AP) sites, which can hamper the progress of the polymerase (Mansouri et al., 1999; Fromenty et al., 2000). This four-primer, long PCR technique allows the coamplification of a long (8636-bp) and a short (316-bp) mtDNA fragment. PCR reactions were performed with the GeneAmp XL PCR system (PerkinElmer, Cour-taboeuf, France) in a volume of 50 μl, with primers (14–40 pmol), total DNA (50–200 ng), each dNTP (200 μM), magnesium acetate (1.5 mM), and rTth DNA polymerase XL (1.5 units) in MicroAmp reaction tubes (PerkinElmer), using a RoboCycler Gradient 96 PCR apparatus equipped with a heat cover (Stratagene, Montigny-le-Bretonneux, France). The thermocycler profile included initial denaturation at 95°C for 2 min, 26 cycles of 95°C for 45 s, 57°C for 45 s and 68°C for 7.5 min, and final extension at 68°C for 7 min. PCR products (20 μl) were electrophoresed on 1.6% agarose gels (FM BioProducts, Rockland, ME) stained with ethidium bromide. Photographs were taken under UV transillumination, and scanned to determine the respective intensity of the 316- and 8636-bp PCR products.

**Oxygen Consumption and β-oxidation in Isolated Hepatic Mitochondria.** Liver mitochondria were prepared from control or treated mice, and polarographic measurements of mitochondrial respiration were performed at 30°C, as previously described (Fromenty et al., 1990a; Mansouri et al., 1999). Mitochondria were energized with either glutamate and malate (5 mM each), providing electrons to complex I of the respiratory chain, or with succinate (10 mM), feeding electrons into complex II. Respiration was measured after addition of 0.2 mM ADP (state 3 respiration) and after total consumption of ADP (state 4 respiration). The respiratory control ratio was calculated as the rate of oxygen consumption in state 3 to that in state 4. The ADP/O ratio was calculated as the ADP consumed per oxygen atom consumed during the whole state 3 period.

[U-14C]Palmitic acid β-oxidation was assessed as previously described (Fromenty et al., 1989, 1990b). Mitochondria (2 mg of protein) were preincubated at 30°C with 0.2 mM ATP, 50 μM l-carnitine, and 15 μM coenzyme A, with or without 2 mM KCN (which blocks mitochondrial β-oxidation). After 5 min, [U-14C]palmitic acid (final concentration, 40 μM; 0.05 μCi/2 ml) was added with albumin, and the reaction was carried out for 10 min at 30°C. After addition of 5% perchloric acid and centrifugation at 4000g for 10 min, 14C-acid-soluble β-oxidation products were counted in the supernatant. These products mainly represent ketone bodies and, to a small extent, citric acid cycle intermediates (Fromenty et al., 1989, 1990b).

**In Vivo Formation of [14C]CO2 from [1-14C]Fatty Acids or [1-14C]Acetate.** The generation of [14C]CO2 from [1-14C]palmitate, [1-14C]palmitate, or [1-14C]acetate was assessed in mice as previously described (Fromenty et al., 1989, 1990b). A tracer dose of [1-14C]palmitate (3.7 μCi/kg; 4 nmol/kg), [1-14C]palmitate (15 μCi/kg; 274 nmol/kg), or [1-14C]acetate (4 μCi/kg; 69 nmol/kg) was given by gastric intubation in 0.2 ml of corn oil. Mice were placed in a small plastic cage swept by an airflow of 50 ml/min. The outflow was bubbled into 60 ml of an ethanolamine-2-methoxyethanol mixture (30/70%, v/v), and an aliquot (3 ml) was removed at different times and counted for [14C]CO2 activity. The in vivo formation of [14C]CO2 from [1-14C]acetate was similarly assessed, after intraperitoneal administration of [1-14C]acetate (90 μCi/kg; 2 nmol/kg) (Favarger and Favarger, 1975).

**Plasma Ketone Bodies, Lipids, Glucose, Lactate, and Pyruvate.** Plasma β-hydroxybutyrate and acetocetate concentrations were measured as previously described (Fromenty et al., 1990b). Plasma triglycerides, phospholipids, and total cholesterol were measured with an automated analyzer (Hitachi 717). Plasma free fatty acids were determined with a commercial kit (Nefa C Wako kit 46551). Concentrations of blood glucose, lactate, and pyruvate were assessed with commercial kits (Sigma diagnostics kits 510-DA, 826, and 726, respectively).

**Hepatic Lipids and Liver Histology.** Liver total lipids and triglycerides were measured as previously described (Fromenty et al., 1990b). Liver fragments were given to the pathologist for Oil red O staining of hepatic fat.

**Assay of Tricarboxylic Acid Cycle Enzymes in Liver and Skeletal Muscles.** Liver or muscle fragments were homogenized at 4°C in a Tris-HCl (10 mM) buffer, pH 7.4, containing EDTA (2 mM) and succrose (250 mM). After centrifugation at 750g for 20 min, pellets were discarded and Triton X-100 (0.1%) was added to the supernatants. Activities of several enzymes of the tricarboxylic acid cycle, namely, citrate synthase, aconitate, isocitrate dehydrogenase, fumarase, and malate dehydrogenase were subsequently assayed as previously described (Robinson et al., 1987).

**Reactive Oxygen Species and Thiobarbituric Acid Reactants.** Reactive oxygen species were assessed in liver homogenates.
with the fluorescent probe, dichlorofluorescein diacetate, which oxidizes into a fluorescent product in the presence of \( \text{H}_2\text{O}_2 \) or other peroxides (Szabados et al., 1999). Liver fragments (50 mg) were homogenized at 4°C in 3 ml of Tris-HCl (20 mM) buffer, pH 7.4, containing KCl (150 mM), EDTA (0.5 mM), MgCl\(_2\) (1 mM), glucose (5 mM), and octanoic acid (0.5 mM). Dichlorofluorescein diacetate (5 \( \mu \text{M} \)) was added, and the homogenate was incubated at 37°C for 30 min. The reaction was stopped with 0.1 mM HCl in cold 70% ethanol. After centrifugation at 3000 \( \times g \) for 15 min, the supernatant was neutralized with NaHCO\(_3\) and centrifuged at 6000 \( \times g \) for 15 min. Fluorescence of the supernatant was measured with excitation at 502 nm and emission at 523 nm (Szabados et al., 1999). Thiobarbituric acid-reactants (TBARs) were assayed to evaluate the extent of hepatic lipid peroxidation as previously described (Letéron et al., 1996).

**Statistical Analysis.** The Student's *t* test for independent data or the Mann-Whitney test was used to assess the significance of differences between means, as appropriate.

### Results

**Stavudine Concentrations.** Plasma concentrations were assessed in lean mice treated for 6 weeks with stavudine (100 mg/kg/day). Because mice mostly drink during the night, blood was collected between 9:00 and 10:00 AM on the last day of stavudine administration. Plasma concentrations of stavudine (mean \( \pm \) S.E.M. for 15 mice) were 323 \( \pm \) 149 ng/ml.

mtDNA. For these investigations, lean mice were treated with either 100 or 500 mg/kg/day stavudine for 1 to 6 weeks; mtDNA and nDNA levels were determined by slot blot hybridization in different tissues, and the mtDNA/nDNA hybridization ratio was used to assess mtDNA changes (Mansouri et al., 1999). Figure 1 shows the time course of mtDNA/nDNA ratio in both liver and skeletal muscles in mice treated with stavudine. In mice treated with 500 mg/kg/day stavudine, mtDNA levels were significantly decreased as soon as the first and second week of treatment, in liver and skeletal muscles, respectively (Fig. 1), whereas mtDNA levels were unchanged in brain or heart throughout the treatment (data not shown). In mice treated with 100 mg/kg/day stavudine, the hepatic mtDNA/nDNA ratio was significantly decreased by 32% after 1 week, and by 25% after 2 weeks, but had returned to normal after 6 weeks of stavudine administration; muscle and WAT mtDNA levels remained unchanged, whatever the duration of treatment (Fig. 1; data not shown).

We also looked for oxidative mtDNA lesions hampering the progress of the polymerase in long PCR experiments (Mansouri et al., 1999; Fromenty et al., 2000). We used a four-primer technique that coamplifies a long (ca. 8.6-kb) and a short (ca. 0.3-kb) PCR fragment. Since random DNA lesions are more likely to hamper the progression of the polymerase and decrease PCR amplification for long DNA fragments than short DNA fragments, a reduction in the relative yield of the long PCR product reflects the presence of blocking DNA lesions, such as strand breaks and AP sites (Mansouri et al., 1999; Fromenty et al., 2000). Hepatic DNA was isolated after 2 and 6 weeks of stavudine (100 mg/kg/day) treatment, and skeletal muscle DNA after 6 weeks. Whatever the tissue or the duration of treatment, the yield of the long PCR product in six to nine treated mice was not significantly different from control mice (data not shown), indicating the absence of severe oxidative lesions able to hamper the progress of the polymerase.

Because neither mtDNA depletion nor severe oxidative mtDNA damage was detected after 6 weeks of treatment with 100 mg/kg/day stavudine, this treatment was selected for further investigations aimed at looking for metabolic effects unrelated to mtDNA alterations.

**Respiration of Hepatic Mitochondria.** Mitochondrial respiration was assessed ex vivo on hepatic mitochondria isolated from mice treated for 6 weeks with stavudine. State 3 mitochondrial respiration and the ADP/O were not modified in stavudine-treated mice (Table 1). State 4 mitochondrial respiration was slightly increased, albeit not significantly (Table 1). The respiratory control ratio (i.e., state 3/state 4 respiratory ratio) was significantly decreased (Table 1). Taken together, these data suggest that the stavudine treatment slightly uncouples oxidative phosphorylation in mouse liver mitochondria.

**\( \beta \)-Oxidation of [U-\( ^{14} \text{C} \)Palmitic Acid by Liver Mitochondria.** Uncoupling of oxidative phosphorylation can increase mitochondrial fatty acid oxidation (Fromenty et al., 1993; Argyropoulos et al., 1998). We therefore assessed [U-\( ^{14} \text{C} \)Palmitic acid \( \beta \)-oxidation in liver mitochondria isolated from stavudine-treated, lean mice. Because mitochondrial preparations contain peroxisomes, fatty acid \( \beta \)-oxidation was measured with or without KCN, and mitochondrial \( \beta \)-oxidation was assessed as the KCN-inhibitable activity (Fromenty et al., 1989).

In a first series of experiments, investigations were performed in mice that were fed until sacrifice. In stavudine-treated animals, total, mitochondrial, and peroxisomal \( \beta \)-oxidation were increased by 25, 28, and 21%, respectively,
**TABLE 1**

Ex vivo effects of stavudine on hepatic mitochondrial respiration

Lean mice were treated with stavudine (100 mg/kg/day) for 6 weeks. Liver mitochondria were isolated and oxygen consumption was measured by polarography. After addition of malate and glutamate (5 mM each) or succinate (10 mM), oxygen consumption was measured in state 3 (after addition of 0.2 mM ADP) and then state 4 (after consumption of ADP). The respiratory control ratio (RCR) is the ratio of state 3 respiration over state 4 respiration. ADP/O is the ratio of ADP consumed to oxygen consumed during the whole state 3 period. Results are means ± S.E.M. for eight control mice and seven mice treated with stavudine.

<table>
<thead>
<tr>
<th></th>
<th>Oxygen Consumption</th>
<th>RCR</th>
<th>ADP/O</th>
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<tbody>
<tr>
<td></td>
<td>State 3</td>
<td>State 4</td>
<td></td>
</tr>
<tr>
<td><strong>Glutamate and Malate</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Controls (n = 8)</td>
<td>154 ± 25</td>
<td>40 ± 4</td>
<td>3.8 ± 0.4</td>
</tr>
<tr>
<td>Stavudine (n = 7)</td>
<td>150 ± 27</td>
<td>48 ± 5</td>
<td>3.0 ± 0.3*</td>
</tr>
<tr>
<td><strong>Succinate</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 8)</td>
<td>201 ± 28</td>
<td>63 ± 5</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>Stavudine (n = 7)</td>
<td>202 ± 40</td>
<td>79 ± 10</td>
<td>2.4 ± 0.2*</td>
</tr>
</tbody>
</table>

Data are nmol O_{2} · min⁻¹ · mg protein⁻¹ 

O. atom of oxygen.

* Significant difference between control and treated mice (P < 0.05).

Compared with control mice, but these differences were not statistically significant (data not shown). In a second series of experiments, investigations were performed on hepatic mitochondria isolated from mice fasted for the last 48 h of the stavudine treatment. In these fasted mice, total β-oxidation activity (assayed in the absence of KCN) was increased by 43% in stavudine-treated mice compared with control mice (Table 2). This higher total activity was mainly due to a 47% increase in mitochondrial β-oxidation (KCN-inhibitable), whereas peroxisomal β-oxidation (KCN-insensitive) exhibited a nonsignificant 29% increase (Table 2). These data suggest that treatment with stavudine enhances hepatic mitochondrial β-oxidation, especially after starvation.

**Hepatic Triglycerides and Liver Histology.** Increased fatty acid oxidation could change the hepatic amount of lipids and triglycerides. Therefore, we measured total lipids and triglycerides in the liver of mice treated with stavudine for 6 weeks and fasted for the last 48 h. Total lipids and triglycerides were decreased by 4 and 12%, respectively, in mice treated with stavudine compared with controls, but these mild differences were not statistically significant. Liver histology showed no difference in the amount of microvesicular fat between mice treated with stavudine and control mice (data not shown).

**Plasma Ketone Bodies.** In the fasted state, hepatic fatty acid β-oxidation mainly generates ketone bodies. We measured plasma ketone bodies in lean mice treated with stavudine for 6 weeks and fasted for the last 48 h. In the stavudine-treated mice, β-hydroxybutyrate and acetoacetate were increased by 107 and 73%, respectively, compared with control mice, with a mild (19%) increase in the β-hydroxybutyrate/acetoacetate ratio (Table 3).

In another series of experiments, we also assessed plasma ketone bodies in treated mice that were fasted for only 24 h at the end of the treatment. In this metabolic situation, plasma acetoacetate and β-hydroxybutyrate were slightly but not significantly increased in mice treated with stavudine compared with control mice (data not shown). Altogether, these results suggest that treatment with stavudine significantly increases plasma ketone bodies only after a prolonged fast.

**Blood or Plasma Levels of Other Metabolites.** Blood or plasma levels of various compounds were measured in lean mice treated with stavudine for 6 weeks. In a first set of investigations, mice were fasted for the last 48 h. Blood levels of glucose, lactate, or pyruvate and plasma concentrations of free fatty acids, triglycerides, total cholesterol, and phospholipids were not significantly modified in treated mice compared with controls (Table 3; data not shown). In a second series of investigations, some biochemical parameters were measured in fed mice. We found no difference between treated mice and controls for blood glucose, lactate, and pyruvate and for plasma triglycerides (data not shown).

**In Vivo Formation of [14C]CO_{2} from 14C-Fatty Acids.** Since mitochondrial β-oxidation was increased in the liver of fasted lean mice treated with stavudine, we asked whether this effect was accompanied by change in the in vivo oxidation of different 14C-labeled fatty acids. In a first series of investigations, measurements were performed in mice fasted for 48 h. In this metabolic condition, we found that exhalation of [14C]CO_{2} from [U-14C]palmitate, [1-14C]palmitate, and [1-14C]octanoate was decreased by 30, 28, and 23%, respectively, in mice treated with stavudine compared with control mice (Fig. 2).

In another series of experiments, in vivo investigations were carried out in mice treated with stavudine but fed until the end of the treatment. In this metabolic situation, oxidation of [U-14C]palmitic acid was not impaired in treated mice (data not shown). Altogether, these results suggest that impairment of fatty acid oxidation in mice treated with stavudine arises only in the fasting state when an increased load of fatty acids is delivered to the liver and extensively transformed into ketone bodies.

**In Vivo Formation of [14C]CO_{2} from [1-14C]Acetic Acid and Enzyme Activities of the Tricarboxylic Acid Cycle.** Since the in vivo determination of the oxidation of
14C-labeled fatty acids evaluates both the β-oxidation process (which transforms 14C-fatty acids into [14C]acetyl-CoA moieties) and the tricarboxylic acid cycle (which oxidized [14C]acetyl-CoA into [14C]CO2), we also assessed the in vivo oxidation of [1-14C]acetic acid by the tricarboxylic acid cycle in mice fasted for 48 h at the end of the treatment. No difference was found in the exhalation of [14C]CO2 from [1-14C]acetic acid between treated and control mice fasted for 48 h (Fig. 2), suggesting that inhibition of the tricarboxylic acid cycle was not involved in the impairment of the in vivo oxidation of 14C-labeled fatty acids observed in mice treated with stavudine. Indeed, none of the enzyme activities of the tricarboxylic acid cycle that were measured in liver and skeletal muscles (namely, citrate synthase, aconitase, isocitrate dehydrogenase, fumarase, and malate dehydrogenase) were impaired in stavudine-treated mice (data not shown).

Reactive Oxygen Species and Hepatic TBARs. It has been recently suggested that nucleoside analogs may induce an oxidative stress in different tissues (Sabados et al., 1999; Skuta et al., 1999). Thus, we asked whether treatment with stavudine was able to increase reactive oxygen species generation and/or lipid peroxidation in mouse liver. Hepatic levels of peroxides (H₂O₂ and other peroxides) were assessed with the fluorescent probe dichlorofluorescein diacetate in mice treated with stavudine for 6 weeks and in control mice. Peroxide-mediated fluorescence was slightly but significantly increased by 32% in treated mice compared with control mice.

Table 3

<table>
<thead>
<tr>
<th></th>
<th>Glucose</th>
<th>Lactate</th>
<th>β-OH-Butyrate</th>
<th>Acetoacetate</th>
<th>β-OH-Butyrate/Acetoacetate</th>
<th>FFA</th>
<th>TG</th>
<th>TC</th>
<th>PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>0.68 ± 0.04 (10)</td>
<td>4.45 ± 0.49 (9)</td>
<td>3.30 ± 0.10 (11)</td>
<td>0.41 ± 0.04 (12)</td>
<td>3.49 ± 0.19 (12)</td>
<td>4.37 ± 0.30 (11)</td>
<td>2.35 ± 0.15 (10)</td>
<td>1.20 ± 0.05 (32)</td>
<td>6.00 ± 0.16 (13)</td>
</tr>
<tr>
<td>Stavudine</td>
<td>0.50 ± 0.04 (10)</td>
<td>3.60 ± 0.25 (9)</td>
<td>3.20 ± 0.10 (11)</td>
<td>0.50 ± 0.04 (12)</td>
<td>3.20 ± 0.19 (12)</td>
<td>4.07 ± 0.30 (11)</td>
<td>1.70 ± 0.15 (10)</td>
<td>0.71 ± 0.05 (32)</td>
<td>4.20 ± 0.16 (13)</td>
</tr>
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</table>

Asterisks indicate significant differences between control and treated mice (*P < 0.05; **P < 0.01).
(data not shown). In contrast, hepatic TBARs were not increased in treated mice, suggesting the absence of significant lipid peroxidation.

**Effect of Stavudine in Obese (ob/ob) Mice.** Since the effect of stavudine seems to depend on the metabolic status of the animals, we next used genetically obese (ob/ob) mice that present many disturbances of fatty acid, lipid, and carbohydrate metabolism (Friedman and Halaas, 1998), as well as mitochondrial dysfunction (Chavin et al., 1999).

In a first series of investigations, levels of mtDNA were assessed in liver, skeletal muscles, and WAT of obese mice. Unlike its lack of effect in lean mice, stavudine significantly decreased WAT mtDNA levels by 45% in obese mice (Fig. 3). In contrast, mtDNA levels were increased, albeit not significantly, by 61 and 44%, respectively, in liver and skeletal muscles (Fig. 3). Importantly, we noted that ob/ob control mice had low basal levels of mtDNA in WAT compared either with liver and skeletal muscles (Fig. 3), or with WAT from lean control mice (data not shown).

We next assessed the in vivo oxidation of [U-14C]palmitic acid in treated or control obese mice fasted for the last 48 h. We found that the in vivo exhalation of [14C]CO2 from [U-14C]palmitic acid was not significantly different between control and treated ob/ob mice (data not shown). Finally, we also measured several blood metabolites in obese mice treated with stavudine and fasted for the last 48 h. We found that plasma β-hydroxybutyrate, acetoacetate, and β-hydroxybutyrate/acetocetate ratio were significantly increased by 77, 23, and 47%, respectively, in obese mice treated with stavudine in comparison with the controls (Table 4). Finally, we found that plasma triglycerides, total cholesterol, and phospholipids were not significantly different between treated and control ob/ob mice (Table 4). However, plasma levels of free fatty acids were significantly increased by 19% in obese mice treated with stavudine compared with the controls (Table 4).

**Effect of Zidovudine on mtDNA Levels and Plasma Ketone Bodies.** In a last series of experiments, we assessed the effect of zidovudine (100 mg/kg/day) for 2 weeks on hepatic and muscle mtDNA levels and plasma ketone bodies.

Zidovudine significantly decreased hepatic mtDNA levels by 50% \( (P < 0.01) \) but did not change skeletal muscle mtDNA in 10 treated mice compared with 20 controls (data not shown). In seven mice treated with zidovudine and fasted for the last 48 h, total plasma ketone bodies were significantly increased by 47% \( (P < 0.05) \) compared with control mice. Acetoacetate was significantly increased by 55%, whereas plasma β-hydroxybutyrate was also increased by 44%, albeit not significantly.

**Discussion**

We studied the effects of stavudine on mtDNA and lipid metabolism in lean and obese mice. Stavudine's effects on mtDNA depended on the dose, the duration of treatment, the tissue, and the genetic background (Figs. 1 and 3). Whereas hepatic mtDNA was stably decreased in lean mice treated with 500 mg/kg/day stavudine, a secondary improvement occurred in mice treated with 100 mg/kg/day (Fig. 1). Adaptive mechanisms can restore mtDNA levels in response to different insults (Mansouri et al., 1999; Tang et al., 2000), and mitochondria harbor a β-like DNA polymerase that is less sensitive to dideoxynucleotide triphosphate than polymerase γ (Nielsen-Preiss and Low, 2000). Conceivably, this resistant polymerase could progressively substitute for the inhibited DNA polymerase γ, which could restore mtDNA levels when polymerase γ is only partially inhibited, as may occur with the 100-mg daily dose. mtDNA levels were also unchanged in HepG2 cells incubated for 14 days with 10 or 50 μM stavudine, whereas zalcitabine caused marked mtDNA depletion (Pan-Zhou et al., 2000).

Since mtDNA levels were unchanged in liver, muscles, and WAT of lean mice treated for 6 weeks with 100 mg/kg/day stavudine, and since long PCR experiments did not detect oxidative lesions hampering the progress of the polymerase (such as strand breaks and AP sites), this treatment offered an opportunity to look for effects of stavudine on fatty acid metabolism that would be independent of mtDNA alterations. Fasting conditions are often used to disclose moderate alterations in lipid metabolism, because the oxidation of stored lipids then becomes the main source of energy (Kersten et al., 1999). During food deprivation, WAT triglycerides are hydrolyzed into free fatty acids that are directly oxidized in extrahepatic tissues or are first transformed by the liver into ketone bodies that are then taken up by peripheral tissues and further oxidized by the tricarboxylic acid cycle. Plasma ketone bodies concentrations therefore depend on both their hepatic generation rate (ketogenesis) and their extrahepatic oxidation rate (ketolysis).

Both the hepatic mitochondrial β-oxidation of fatty acids and plasma ketone bodies were increased in stavudine-treated lean mice fasted for the last 48 h compared with untreated fasted mice, suggesting enhanced in vivo hepatic fatty acid β-oxidation and increased ketogenesis. The increased hepatic β-oxidation could be due, at least in part, to the uncoupled state of hepatic mitochondrial respiration (Table 1), since uncoupling enhances fatty acid β-oxidation (Fromenty et al., 1993; Argyropoulos et al., 1998). In addition to increased ketogenesis, decreased peripheral ketolysis may contribute to the increased ketonemia of stavudine-treated mice. Indeed, contrasting with a 40% increase in hepatic β-oxidation, there was a disproportionally higher increase...
creased in vivo generation of CO2 from fatty acids may be facilitated by only 25%. Thus, the murine model used in this study could prove useful in the future to compare the in vivo effects of stavudine and mitochondrial dysfunction, incipient lactic acidosis causes vomiting, diarrhea, nausea, anorexia, decreased food intake, and weight loss, i.e., a situation alike to fasting (Chariot et al., 1999; Brivet et al., 2000). In this study, stavudine decreased the peripheral oxidation of fatty acids and increased ketonemia during fasting conditions. This may aggravate the energy deficit caused by mtDNA depletion and enhance metabolic acidosis in these patients. The stavudine-mediated decrease in WAT mtDNA in obese mice, but not lean mice, is also intriguing (Fig. 3). Lipotoxicity often occurs on a background of central adiposity, and recent clinical data suggest that mtDNA depletion could be the basis for peripheral lipotoxicity in patients receiving diverse nucleoside reverse transcriptase inhibitors (Walker et al., 2000).

### References


**TABLE 4**

Effect of stavudine on plasma metabolic substrates and lipids in genetically obese mice fasted for 48 h

<table>
<thead>
<tr>
<th></th>
<th>mmol/l</th>
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<th>mmol/l</th>
<th>mmol/l</th>
<th>mmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (n)</td>
<td>Stavudine (n)</td>
<td>mmol/l</td>
<td>mmol/l</td>
<td>mmol/l</td>
<td>mmol/l</td>
<td>mmol/l</td>
<td>mmol/l</td>
</tr>
<tr>
<td>[U-14C]palmitic acid</td>
<td>3.74 ± 0.47 (7)</td>
<td>6.61 ± 0.38** (7)</td>
<td>0.80 ± 0.08 (7)</td>
<td>0.98 ± 0.02** (7)</td>
<td>4.63 ± 0.39 (7)</td>
<td>6.80 ± 0.41** (7)</td>
<td>1.13 ± 0.03 (7)</td>
<td>1.34 ± 0.10* (6)</td>
</tr>
</tbody>
</table>

Asterisks indicate significant differences between control and treated mice (*P < 0.05; **P < 0.01).
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