Tamoxifen Inhibits Topoisomerases, Depletes Mitochondrial DNA, and Triggers Steatosis in Mouse Liver

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ABSTRACT

Although tamoxifen can trigger steatohepatitis, the mechanism of steatosis is unclear. We hypothesized that this DNA-intercalating, cationic amphiphilic drug could accumulate within mitochondria to impair fatty acid oxidation, respiration, and mitochondrial DNA relaxation and synthesis. We studied the in vitro effects of tamoxifen on topoisomerases and mouse liver mitochondria and its in vivo hepatic effects in mice treated for 1 to 28 days with a daily dose of tamoxifen reproducing the plasma concentrations observed in humans. In vitro, tamoxifen inhibited topoisomerase-mediated plasmid DNA relaxation. It accumulated 40-fold inside mitochondria and inhibited both respiration and fatty acid oxidation. In vivo, a single dose of tamoxifen inhibited palmitic acid oxidation and hepatic lipoprotein secretion. Tamoxifen administration also decreased mitochondrial DNA synthesis and progressively depleted hepatic mitochondrial DNA, down to 40% of control values at 28 days. The decrease in mitochondrial DNA-encoded respiratory complexes sensitized mitochondria to the inhibitory effects of tamoxifen on mitochondrial respiration. Hepatic steatosis was absent at 5 days, mild at 12 days, and moderate at 28 days. The fatty acid synthase protein was normally expressed at 12 days but was decreased by 52% at 28 days. In conclusion, tamoxifen decreases hepatic triglyceride secretion, and it accumulates electrophoretically in mitochondria, where it impairs β-oxidation and respiration. Tamoxifen also inhibits topoisomerases and mitochondrial DNA synthesis and progressively depletes hepatic mitochondrial DNA in vivo. These combined effects could decrease fat removal from the liver, thus causing hepatic steatosis despite a secondary down-regulation of hepatic fatty acid synthase expression.

The nonsteroidal antiestrogen, tamoxifen, is used in women with breast cancer or at risk of developing it (Fisher et al., 2005). Tamoxifen has a 7-day half-life in humans (Lien et al., 1995). Due to faster metabolism, mice have to be treated with 200 mg/kg (0.54 mmol/kg) p.o. daily to reproduce the plasma levels of patients receiving 30 mg of tamoxifen twice daily (Robinson et al., 1991). Plasma tamoxifen levels progressively increase during the first days of tamoxifen administration (0.54 mmol/kg p.o. daily) to reach a plateau after 7 days in mice (Robinson et al., 1991). Tamoxifen is concentrated in tissues, particularly in liver (Robinson et al., 1991). At 7 days in mice, the hepatic tamoxifen content (215 μmol/kg) is 160 times the plasma concentration (1.34 μM) and 8-fold the skeletal muscle content (27 μmol/kg) (Robinson et al., 1991). In treated women, plasma tamoxifen concentrations range from nondetectable to 1.68 μM (Peyrade et al., 1996), and the hepatic concentration was 64 times the plasma concentration in one studied woman (Lien et al., 1991).

Tamoxifen is well tolerated but causes steatosis in 43% of recipients (Nishino et al., 2003). Steatohepatitis can develop, particularly in overweight women (Bruno et al., 2005), and can lead to cirrhosis (Oien et al., 1999). However, the mechanism for tamoxifen-induced steatosis is essentially unknown. Tamoxifen administration decreases fatty acid synthase (FAS) expression in rat liver (Lelliott et al., 2005), and tamoxifen both uncouples and inhibits mitochondrial respiration (Cardoso et al., 2001). However, effects on mitochondrial DNA synthesis and progression of steatosis are unclear. We hypothesized that this DNA-intercalating, cationic amphiphilic drug could accumulate within mitochondria to impair fatty acid oxidation, respiration, and mitochondrial DNA relaxation and synthesis. We studied the in vitro effects of tamoxifen on topoisomerases and mouse liver mitochondria and its in vivo hepatic effects in mice treated for 1 to 28 days with a daily dose of tamoxifen reproducing the plasma concentrations observed in humans. In vitro, tamoxifen inhibited topoisomerase-mediated plasmid DNA relaxation. It accumulated 40-fold inside mitochondria and inhibited both respiration and fatty acid oxidation. In vivo, a single dose of tamoxifen inhibited palmitic acid oxidation and hepatic lipoprotein secretion. Tamoxifen administration also decreased mitochondrial DNA synthesis and progressively depleted hepatic mitochondrial DNA, down to 40% of control values at 28 days. The decrease in mitochondrial DNA-encoded respiratory complexes sensitized mitochondria to the inhibitory effects of tamoxifen on mitochondrial respiration. Hepatic steatosis was absent at 5 days, mild at 12 days, and moderate at 28 days. The fatty acid synthase protein was normally expressed at 12 days but was decreased by 52% at 28 days. In conclusion, tamoxifen decreases hepatic triglyceride secretion, and it accumulates electrophoretically in mitochondria, where it impairs β-oxidation and respiration. Tamoxifen also inhibits topoisomerases and mitochondrial DNA synthesis and progressively depletes hepatic mitochondrial DNA in vivo. These combined effects could decrease fat removal from the liver, thus causing hepatic steatosis despite a secondary down-regulation of hepatic fatty acid synthase expression.
To assess uptake, we energized mitochondria (2 mg of protein/ml) with succinate (10 mM) and incubated them with \(^{3}H\)tamoxifen (25 \(\mu M\); 8.5 \(\mu Ci/ml\) at 30°C for 1 min. An aliquot was taken to measure proteins, and mitochondria were centrifuged for 2 min at 13,000g. Aliquots from the mitochondrial pellet and supernatant were extracted with ethanol and counted for \(^{3}H\) radioactivity. Mitochondrial drug concentrations were estimated assuming a mitochondrial volume of 1 \(\mu l\) per 1 mg of protein (McCormack et al., 1989; Fromenty et al., 1990a).

**Topoisomerase-Mediated DNA Relaxation.** We used the pBR322 plasmid to assess topoisomerase-mediated DNA relaxation (Mansouri et al., 2003). The supercoiled plasmid (300 ng) was incubated for 30 min at 30°C with tamoxifen (0–100 \(\mu M\)) and 2.5 units of topoisomerase I or II under conditions specified by the manufacturer.

DNA isoforms were resolved in 0.7% Seakem GTG agarose gels (FMC Bioproducts, Rockland, ME) without ethidium bromide. After staining with ethidium bromide (0.5 \(\mu g/ml\)), DNA bands were visualized by UV light.

**[\(^{3}H\)]Thymidine Incorporation into DNA.** Twenty-four hours after the last dose of tamoxifen, mice received \([\(^{3}H\)]thymidine i.p. (0.03 \(\mu mol/kg\), 0.75 \(\mu Ci/kg\)). Two hours later, the liver was homogenized and centrifuged at 600g for 15 min. We used the nuclear pellet to isolate nuclear DNA (nDNA) and the supernatant to isolate mitochondrial DNA at 7700g (Mansouri et al., 2003). nDNA and mtDNA were recovered with the phenol-chloroform method and counted for \([\(^{3}H\)]thymidine (Mansouri et al., 2003).

**nDNA and mtDNA Levels.** Total hepatic DNA was isolated with QIAGEN Genomic-tip 100 G columns (QIAGEN, Valencia, CA) (Mansouri et al., 1999), and 300 ng was blotted onto Hybond-N\(^+\) nylon membranes (GE Healthcare) and hybridized with a 10.9-kb mtDNA probe (nucleotides 4964–15,896) generated by long polymerase chain reaction and labeled by random priming (Multiprime DNA labelling system; GE Healthcare) (Mansouri et al., 1999). Membranes were stripped and hybridized with a mouse C\(_{\alpha1}\) tet RNA probe (Invitrogen, Cergy Pontoise, France). mtDNA and nDNA were assayed by densitometry analysis of autoradiographs (Mansouri et al., 1999).

**Mitochondrial Complexes.** Hepatic mitochondria were isolated, and submitochondrial particles were prepared by two cycles of freezing/thawing. Complex I was assayed by following the oxidation of NADH in the presence of decylubiquinone and antimycin A (Kwang and Sohal, 2000). Complex II was measured as the phenazine methosulfate-mediated reduction of 2,6-dichlorophenolindophenol in the presence of succinate, rotenone, and antimycin A. Complex IV was assessed by following the reduction of cytochrome c (Kwang and Sohal, 2000).

**Immunoblot Analysis of Selected Respiratory Chain Polypeptides and Lipid Metabolism Enzymes.** Total liver proteins were separated by electrophoresis, blotted, and exposed first to antibodies against COX I, COX 4, FAS, CPT-I, or MCAD, and then a secondary antibody conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Blots were revealed with enhanced chemiluminescence reagents (GE Healthcare), stripped, and exposed to an anti-\(\beta\)-actin antibody.

**In Vivo Formation of \([\(^{14}C\)]\)CO\(_2\) from \([\(^{14}C\)]\)Palmitic Acid.** Mice fasted for 24 h were given a tracer dose of \([\(^{14}C\)]\)palmitic acid (3.7 \(\mu Ci/kg\); 4 nmol/kg) by gastric intubation in 0.2 ml of corn oil and placed in a small plastic cage swept by an air flow of 50 ml/min (Fromenty et al., 1990b). The outflow was bubbled into a 60-ml
mixture of ethanolamine and 2-methoxyethanol (30%/70%, v/v) to trap the exhaled [14C]CO2 (Fromenty et al., 1999).

**Hepatic Secretion of TG and Apo B.** Plasma TG and Apo B were determined with commercial kits (Biotrol A 01549, Chennervières-les-Louvois, France and Sigma kit no. 357, respectively) 4 h after the administration of Triton WR-1339, which inhibits lipoprotein lipase activity and blocks the peripheral removal of TG (Lettéron et al., 2003). In 24-h-fasted animals (with no intestinal fat absorption), the increase in TG mostly reflects hepatic TG secretion (Lettéron et al., 2003).

**Plasma Chemistry, Cytokines, Alanine Aminotransferase Activity, Caspase Activities, and Assessment of nDNA Fragmentation.** Plasma β-hydroxybutyrate, glucose, TG, cholesterol, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-β, and alanine aminotransferase (ALT) activity were measured with commercial kits.

Livers were minced and homogenized in 1 mMEDTA, 50 mM HEPES, 0.1% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 5 mM dithiothreitol, 4 mg/ml leupeptin, and 4 mg/ml pepstatin, pH 7.4. After centrifugation at 14,000g, caspase-3 activity was measured in the supernatant with a fluorescent assay kit (Biomol, Plymouth Meeting, PA). Caspase-9 activity was measured with 25 μM Ac-LEHD-AFC (Calbiochem, Darmstadt, Germany).

Hepatic DNA (5 μg) was loaded on 2% agarose gels (Mansouri et al., 1999). After electrophoresis for 4 h at 50 V, DNA was transferred to a nylon membrane (Hybond-N+, Amersham, Arlington Heights, IL) and used with the Tag peroxidase in situ apoptosis detection kit (Chemicon International, Temecula, CA) following the manufacturer’s recommendations.

A liver fragment for light microscopy was fixed with 10% formalin in phosphate-buffered saline, dehydrated in an alcoholic series, placed in toluene baths, and embedded in paraffin. Three-micrometer-thick sections were prepared for hematoxylin-eosin staining. In situ detection of apoptosis was performed with the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay (Biovision, Mountain View, CA) following the manufacturer’s recommendations.

A liver fragment was cut into 1-mm3 blocks and fixed in phosphate-buffered saline with 2.5% glutaraldehyde. After dehydration in an alcoholic series, blocks were placed in propylene oxide solutions and embedded in epoxy resin. Semithin sections were stained with toluidine blue and examined on light microscopy for orientation. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 electron microscope (Tokyo, Japan).

**Statistical Analysis.** Data were analyzed with one-way analysis of variance and Fischer’s tests. The significance level was set at P < 0.05. Results in mice treated with saline for 5, 12, or 28 days did not differ from each other. For clarity, they were pooled into a single control group in the table and figures showing results at different treatment times.

**Results**

**Tamoxifen Is Electrophoretically Taken up by Mitochondria.** We isolated mitochondria, energized them with succinate, and added [3H]tamoxifen (25 μM) (Table 1). Tamoxifen was concentrated 40-fold in mitochondria, reaching a concentration of 640 μM compared with a residual concentration of 16 μM in the medium. The electrophoretic uptake of tamoxifen was decreased by valinomycin (70 nM), a potas-

<table>
<thead>
<tr>
<th>Final Tamoxifen Concentration</th>
<th>Mitochondria/Medium Concentration Ratio</th>
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<tr>
<td>Medium</td>
<td>Mitochondria</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>Tamoxifen and valinomycin</td>
<td>21 ± 4</td>
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<sup>*</sup> Different from the incubation without valinomycin, P < 0.05.

**Table 1** Mitochondrial uptake of tamoxifen

Mouse liver mitochondria (2 mg proteins/ml) were incubated at 30°C for 1 min with succinate (10 mM) and [3H]tamoxifen (25 μM; 8.5 μCi/ml), with or without valinomycin (70 nM). Mitochondria were centrifuged for 2 min, and the concentrations of tamoxifen in the mitochondrial pellet and the supernatant (medium) were determined. Results are means ± S.E.M. for five to eight determinations.

**Tamoxifen Impairs Mitochondrial Respiration and Fatty Acid β-Oxidation.** At 50 μM, tamoxifen increased basal (state 4) respiration (Supplemental Table S1), indicating an uncoupling effect. At 150 μM, tamoxifen no longer stimulated basal respiration but inhibited the high respiratory rate triggered by the uncoupler, 2,4-dinitrophenol (Supplemental Table S1), suggesting that tamoxifen impairs electron flow in the respiratory chain. At an intermediate concentration (100 μM), tamoxifen inhibited ADP-stimulated respiration but did not significantly inhibit 2,4-dinitrophenol-stimulated respiration. The more severe impairment of ADP-stimulated respiration may suggest additional inhibitory effect(s) on the adenine nucleotide translocator and/or ATP synthase. Both are involved in ADP-stimulated respiration but not in 2,4-dinitrophenol-uncoupled respiration.

We next assessed the β-oxidation of [U-14C]palmitic acid by isolated mitochondria (Fig. 1A). β-Oxidation was decreased by 44, 70, 92, and 97% by 100, 150, 200, and 250 μM tamoxifen, respectively. A severe impairment of mitochondrial respiration can decrease the reoxidation of FADH2 and NADH into FAD and NAD+ (Table 2), thus inhibiting fatty acid β-oxidation, which requires these oxidized cofactors (Fromenty and Pessayre, 1995). To assess whether tamoxifen inhibits β-oxidation directly or only as a consequence of the inhibition of respiration, we added ferriencium hexafluorophosphate and acetoacetate to the β-oxidation medium (Fig. 1B). Ferriencium accepts the electrons coming from the enzyme-bound FADH2 in acyl-CoA dehydrogenases (Lettéron et al., 1997), whereas acetoacetate reoxidizes NADH into NAD+, which is required by 3-hydroxy-acyl-CoA dehydrogenases (Fromenty and Pessayre, 1995). Although acetoacetate and ferriencium in combination partly relieved the inhibition of mitochondrial β-oxidation that was caused by rotenone or antimycin A, they did not relieve the inhibition caused by tamoxifen (250 μM) (Fig. 1B), suggesting that tamoxifen directly inhibits the β-oxidation process.

CPT-I partly controls the mitochondrial β-oxidation rate of long-chain fatty acids by modulating their entry into mitochon-
Tamoxifen Also Inhibits Hepatic TG Secretion In Vivo. To assess hepatic TG secretion, we measured the increase in plasma TG 4 h after administration of Triton WR-1339, which blocks peripheral TG utilization (Lettéron et al., 2003). Tamoxifen (1 mmol/kg) inhibited the increase in TG by 68% but did not significantly blunt the increase in Apo B (Fig. 2), suggesting that tamoxifen impairs Apo B lipida tion.

Tamoxifen Inhibits Topoisomerases, Impairs mtDNA Synthesis, and Depletes mtDNA. We used the supercoiled pBR322 plasmid to assess the effects of tamoxifen on topoisomerase activities (Fig. 3). Tamoxifen (20–100 μM) inhibited DNA relaxation mediated by topoisomerases I and II.

Intercalcating topoisomerase inhibitors can inhibit DNA replication (Li and Liu, 2001; Wang, 2002; Mansouri et al., 2003). To assess DNA synthesis, we treated mice with tamoxifen for 12 or 28 days, injected [3H]thymidine 24 h after the last dose, and killed the animals 2 h later (Fig. 4A). [3H]Thymidine incorporation into mtDNA was decreased by 32% at 12 days and by 62% at 28 days. In contrast, [3H]thymidine incorporation into nDNA was unchanged at 12 days and was decreased by only 25% at 28 days.

Tamoxifen administration progressively decreased hepatic mtDNA level without affecting nDNA content (Fig. 4B). We used the mtDNA/nDNA hybridization ratio to assess mtDNA changes (Fig. 4C). This ratio was unchanged after 5 days of treatment but was decreased by 31% at 12 days and by 60% at 28 days (Fig. 4C).

Tamoxifen Administration Decreases mtDNA-Encoded Respiratory Complexes and Sensitizes Mitochondria to the Inhibitory Effects of Tamoxifen. Although the mtDNA-encoded COX 1 polypeptide was decreased by 66% in mice treated with tamoxifen for 28 days, the nDNA-encoded COX 4 was unchanged (Fig. 5). To assess the activities of mitochondrial complexes, we isolated mouse liver mitochondria and prepared submitochondrial particles (Table 2). The washings and dilutions involved in the isolation of mitochondria can remove drugs present in the liver of treated animals at the time of sacrifice, thus suppressing their in vivo inhibitory effects on mitochondrial respiration and/or β-oxidation (Fromenty and Pessayre, 1995). Indeed, the activities of mitochondrial complexes were unchanged in the submitochondrial particles isolated from mice killed 24 h after the last dose of tamoxifen, 0.5 mmol/kg daily for 7 days (Table 2). In contrast, the activities of complexes I and IV were decreased by 32 and 20%, respectively, in mice treated for 28 days (Table 2). The deterioration occurring between days 7 and 28 is unlikely to be due to tamoxifen accumulation because plasma concentrations already reach a plateau at day 7 (Robinson et al., 1991). Instead, the impairment of complexes I and IV after 28 days, without significant impairment of complex I, may be due to progressive mtDNA depletion. Although complex II is encoded by nDNA, some of the polypeptides of complexes I and IV are mtDNA-encoded (Fromenty and Pessayre, 1995).

The activity of mitochondrial complexes has to be severely inhibited (beyond a 70–80% threshold) for mitochondrial respiration to decrease (Rossignol et al., 1999). In keeping with the moderately impaired activities of complexes I and IV, respiration was unchanged in the mitochondria isolated from mice treated with tamoxifen for 28 days (Fig. 6). As already mentioned, the isolation of mitochondria can remove drugs present in the liver at the time of sacrifice so that their in vivo inhibitory effects on mitochondrial function are no longer observed ex vivo (Fromenty and Pessayre, 1995). To
reproduce the in vivo situation, we added tamoxifen to some incubation flasks (Fig. 6). Combined with the depletion of mtDNA-encoded respiratory polypeptides, the acute inhibitory effects of tamoxifen may then hamper enough the flow of electrons within the respiratory chain to significantly decrease mitochondrial respiration. Indeed, adding a small concentration of tamoxifen (15 μM) to the mitochondria of mice treated with tamoxifen for 28 days markedly inhibited the respiration that was supported by malate and glutamate, which donate electrons to the partially mtDNA-encoded complex I (Fig. 6). In contrast, in mice treated for only 7 days, tamoxifen (15 μM) failed to inhibit respiration (Fig. 6), probably due to the lack of significant mtDNA depletion at that time. Collectively, these data indicate that the depletion of mtDNA-encoded polypeptides after prolonged tamoxifen administration sensitizes mitochondria to the acute inhibitory effects of a small concentration of tamoxifen on mitochondrial respiration.

Western Blot Analysis of Selected Proteins Involved in Fatty Acid Metabolism and Effects on ex Vivo MCAD Activity. The expression of FAS and CPT-I were similar in control mice and mice treated with tamoxifen for 5 or 12 days (Fig. 7). However, at 28 days, FAS protein expression decreased by 52% in treated mice, and CPT-I expression increased by 22%. MCAD protein expression did not change, whatever the duration of treatment (Fig. 7). We also determined the effect of tamoxifen administration on ex vivo MCAD activity. MCAD activity (mean ± S.E.M. for five mice) was not significantly decreased in mice treated with tamoxifen (0.5 mmol/kg daily) for 28 days (18 ± 4 nmol/min/mg protein in tamoxifen-treated mice versus 26 ± 2 in control mice).

Effects of Tamoxifen on Plasma β-Hydroxybutyrate, Glucose, Cholesterol, TG, TNF-α, TGF-β, and ALT Activity. The β-oxidation of fatty acids generates ketone bodies, the most abundant of which is β-hydroxybutyrate (Fromenty and Pessayre, 1995). In fed mice treated with tamoxifen for 28 days, plasma β-hydroxybutyrate was decreased by 64% (Table 3), thus confirming the decreased β-oxidation. Plasma glucose was unchanged, whereas plasma cholesterol was decreased by 27% (Table 3).

We also measured plasma β-hydroxybutyrate, TG, TNF-α, and TGF-β levels and ALT activity in mice treated for 28 days and fasted for the last 24 h (Table 3). Plasma β-hydroxybutyrate was decreased by 25% and TG by 55% in tamoxifen-treated mice (Table 3). Plasma TNF-α was decreased by 43%, whereas TGF-β was unchanged. Plasma ALT activity was increased by 87% in treated mice (Table 3).

Tamoxifen Triggers Hepatic Steatosis and Ultrastructural Mitochondrial Abnormalities but No Apoptosis. Liver histology was evaluated in control mice (n = 11) and in mice treated with tamoxifen for 5 (n = 4), 12 (n =
tamoxifen inhibits mtDNA synthesis. We treated mice for 12 or 28 days with saline or TAM (0.5 mmol/kg daily) and administered [3H]thymidine 24 h after the last dose. Two hours after [3H]thymidine administration, we isolated hepatic mtDNA and nDNA from mitochondria and nuclei, respectively, and determined the amount of incorporated [3H]thymidine (means ± S.E.M. for 6 to 14 mice). *, different from control mice, P < 0.05; ***, P < 0.001. B and C, tamoxifen depletes mtDNA. We treated mice with saline or tamoxifen (0.5 mmol/kg) for 5, 12, or 28 days and killed the animals 24 h after the last dose. Total hepatic DNA (300 ng) was blotted onto a nylon membrane, hybridized with a 10.9-kb mtDNA probe, stripped, and hybridized with a mouse Cot-1 nDNA probe. B, representative slot blots in two mice for each treatment time. Although nDNA does not change, mtDNA decreases progressively. C, mtDNA/nDNA hybridization ratio. For each mouse, blot intensities were determined by densitometry analysis, and the mtDNA/nDNA hybridization ratio was calculated. In each individual experiment, the mtDNA/nDNA ratio in treated mice was expressed as the percentage of the mean control value. Results are means ± S.E.M. for 9 to 22 treated mice per group and 28 control mice. ***, different from concomitantly studied control mice, P < 0.01; **, P < 0.001.

4), or 28 (n = 9) days. No steatosis, or minimal steatosis, was observed in control mice (Fig. 8A) or mice treated for 5 days (Fig. 8B). Three of the four mice treated for 12 days had mild periportal steatosis (Fig. 8C). Eight of the nine mice treated for 28 days exhibited moderate, periportal, macrovacuolar, and microvesicular steatosis, as well as ballooning of periportal hepatocytes, without apoptosis. One of these animals also exhibited a few necrotic hepatocytes, without inflammation and without apoptosis (Fig. 8D). Although moderate steatosis was observed in most mice treated with tamoxifen for 28 days (Fig. 8D), TG and cholesterol contents measured in total liver extracts were not different in six control mice and five mice treated with tamoxifen for 28 days (12 ± 1 mg of TG/g liver in control mice versus 13 ± 3 in tamoxifen-treated mice; 2.7 ± 0.7 μmol of cholesterol/g liver in control mice versus 2.2 ± 0.2 μmol in tamoxifen-treated mice). This discrepancy is thought to be due to the fact that treated mice only exhibited moderate steatosis, which was selectively localized to the periportal region of the liver (Fig. 8D), whereas TG and cholesterol were determined in whole-liver extracts. Electron microscopy at 28 days showed small lipid droplets (Fig. 9, B and C) and elongated, hypertrophied, or swollen mitochondria with dilatation of cristae (Fig. 9, E and F).

Fig. 5. Tamoxifen increases mtDNA-encoded COX 1 polypeptide but not the nDNA-encoded COX 4 polypeptide. Mice were treated with saline or TAM (0.5 mmol/kg daily) for 28 days. A hepatic homogenate was prepared, and proteins (100 μg) were separated by SDS-polyacrylamide gel electrophoresis, blotted, probed with an anti-COX 1 antibody, stripped, probed with an anti-COX 4 antibody, stripped again, and probed with an anti-β-actin antibody. A, representative Western blot of COX 1, COX 4, and β-actin. B, densitometric analysis of COX 1 and COX 4 (means ± S.E.M. for six mice). ***, different from concomitantly studied control mice, P < 0.01.

TABLE 2
Ex vivo effects of tamoxifen on the activity of respiratory complexes I, II, and IV
We treated mice with tamoxifen (0.5 mmol/kg daily) or saline for 7 (n = 4) or 28 (n = 12) days and killed the animals 24 h after dosing. We isolated hepatic mitochondria, prepared submitochondrial particles, and measured the activities of respiratory complexes I, II, and IV. Results (means ± S.E.M.) are expressed as the percentage of the mean value in control mice.

<table>
<thead>
<tr>
<th>Activity</th>
<th>Complex I</th>
<th>Complex II</th>
<th>Complex IV</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 7</td>
<td>100 ± 9</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>Tamoxifen, 7 days</td>
<td>96 ± 9</td>
<td>104 ± 22</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>Tamoxifen, 28 days</td>
<td>68 ± 6*</td>
<td>84 ± 9</td>
<td>80 ± 6*</td>
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* Different from control, P < 0.05.

Discussion
Although tamoxifen administration causes hepatic steatosis in 43% of recipients (Nishino et al., 2003), the mecha-
In the acidic intermembrane space of mitochondria, the weak base, tamoxifen (pKₐ, 8.5), is protonated. Like other cationic amphiphilic drugs (Fromenty et al., 1990a, b; Berson et al., 1998), tamoxifen is electrophoretically transported into the mitochondrial matrix, thus achieving high intramitochondrial concentrations (Table 1). In the alkaline matrix, protonated tamoxifen may then partly dissociate into a proton and uncharged tamoxifen. This protonphoric effect may decrease the mitochondrial membrane potential (Cardoso et al., 2001), thus unleashing the flow of electrons in the respiratory chain and increasing basal mitochondrial respiration (Supplemental Table S1). Although tamoxifen increases basal respiration, it decreases the high respiratory rates triggered by ADP or 2,4-dinitrophenol (Supplemental Table S1). Tamoxifen partitions into biomembranes and decreases membrane fluidity (Custódio et al., 1993). It could thus impair the interactions between respiratory chain components and the diffusional mobility of membrane proteins (Custódio et al., 1993), thus forbidding high rates of electron flow and oxidative phosphorylation.

The present study shows for the first time that tamoxifen directly inhibits the mitochondrial β-oxidation of fatty acids, in part by inhibiting CPT-I (Fig. 1). CPT-I allows the entry of long-chain fatty acids into mitochondria and thus controls the mitochondrial β-oxidation rate (Froment and Pesseyre, 1995). CPT-1 activity was significantly inhibited by tamoxifen in a concentration-dependent manner (Fig. 1C). Yet, the level of CPT-1 protein was slightly but significantly increased at 28 days, although not at 5 or 12 days (Fig. 7). The secondary up-regulation of CPT-1 may mitigate the inhibitory effect of tamoxifen on CPT-1. Like the secondary down-regulation of hepatic FAS expression (discussed further on), the secondary up-regulation of CPT-1 may be an adaptive response tending to prevent further fat accretion. The present work also identifies for the first time mtDNA as an important target in tamoxifen-induced mitochondrial toxicity (Fig. 4). Tamoxifen intercalates between DNA bases (Snyder and Brown, 2002), and several DNA-intercalating drugs inhibit topoisomerases (Lin and Castora, 1991; Mansouri et al., 2003). At concentrations (20–100 μM) in the range of those present in the liver of treated mice and some treated women (Lien et al., 1991; Robinson et al., 1991; Peyrade et al., 1996), tamoxifen inhibited the relaxation of a supercoiled plasmid by topoisomerases I or II (Fig. 3). Like this plasmid, mtDNA is a supercoiled molecule (Mansouri et al., 1999), and mitochondria contain both topoisomerase I and a bacterial-like topoisomerase II activity (Lin and Castora, 1991; Zhang et al., 2001).

Topoisomerase inhibition by tamoxifen should keep mtDNA in its supercoiled form, which, together with intercalation, may hamper the progression of DNA polymerase γ during mtDNA replication (Nelson et al., 1984; Mansouri et al., 2003). Indeed, tamoxifen decreased [3H]thymidine incorporation into mtDNA, with limited effects on nDNA (Fig. 4A), probably because mtDNA is exposed to higher tamoxifen concentrations than nDNA. Liver mitochondria may be particularly targeted because of the higher tamoxifen concentrations in the liver than other organs (Robinson et al., 1991).

Even in tissues whose cells do not divide or rarely divide, such as the liver, there is a basal turnover of mitochondria. Therefore, drugs that decrease mtDNA synthesis can progressively deplete mtDNA (Rowe et al., 2001; Mansouri et al., 2003). Indeed, tamoxifen administration progressively de-
A decrease in respiratory chain function can decrease the reoxidation of NADH into NAD + (15), which is required to sustain β-oxidation (Fromenty and Pessayre, 1995). This added effect could further explain why steatosis was absent at 5 days, mild at 12 days, and more prominent at 28 days (Fig. 8).

**TABLE 3**
Effects of tamoxifen on plasma β-hydroxybutyrate, TG, cholesterol, glucose, TNF-α, TGF-β, and ALT activity

<table>
<thead>
<tr>
<th></th>
<th>Fed/Fasted for the Last 24 h</th>
<th>Control Mice</th>
<th>Tamoxifen-Treated Mice</th>
</tr>
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<tbody>
<tr>
<td>β-Hydroxybutyrate (mM)</td>
<td>Fed</td>
<td>0.22 ± 0.05 (8)</td>
<td>0.08 ± 0.01* (8)</td>
</tr>
<tr>
<td>Glucose (mM)</td>
<td>Fed</td>
<td>8.8 ± 0.5 (5)</td>
<td>8.6 ± 0.4 (8)</td>
</tr>
<tr>
<td>Cholesterol (μM)</td>
<td>Fed</td>
<td>3.0 ± 0.4 (5)</td>
<td>2.2 ± 0.2* (8)</td>
</tr>
<tr>
<td>β-Hydroxybutyrate (mM)</td>
<td>Fasted</td>
<td>1.18 ± 0.09 (12)</td>
<td>0.88 ± 0.04* (12)</td>
</tr>
<tr>
<td>TG (mM)</td>
<td>Fasted</td>
<td>1.95 ± 0.20 (12)</td>
<td>0.87 ± 0.08* (12)</td>
</tr>
<tr>
<td>TNF-α (pg/ml)</td>
<td>Fasted</td>
<td>23 ± 2 (8)</td>
<td>13 ± 2* (8)</td>
</tr>
<tr>
<td>TGF-β (ng/ml)</td>
<td>Fasted</td>
<td>66 ± 10 (8)</td>
<td>74 ± 7 (8)</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>Fasted</td>
<td>31 ± 4 (12)</td>
<td>58 ± 7* (12)</td>
</tr>
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* Different from control mice, P < 0.05.

Tamoxifen also decreased hepatic TG secretion (Fig. 2). Although the mechanism of this effect remains to be ascertained, we previously reported that several statogenic drugs inhibited not only fatty acid oxidation but also microsomal triglyceride transfer protein activity and very low-density lipoprotein secretion, thus concomitantly decreasing the two pathways removing fat from the liver (Lettéron et al., 2003).

Finally, as previously reported (Lelliott et al., 2005), tamoxifen administration decreased hepatic FAS expression (Fig. 7). This effect is not due to the antiestrogenic effect of tamoxifen, which should have the opposite effect (D’Eon et al., 2005). Although an agonistic effect is possible, the late down-regulation of FAS (at 28 but not at 12 days) rather suggests an adaptive response to tamoxifen-induced steatosis. Indeed, in a previous study, a high-fat diet increased hepatic TG 2.5-fold and decreased hepatic FAS mRNA 2.5-fold in mice (Kim et al., 2004). In another study, an Apo B antisense oligonucleotide administered twice weekly for 6 weeks doubled the mean hepatic TG content (albeit not significantly) and decreased hepatic FAS protein expression (Crooke et al., 2005). Finally, a diet deficient in methionine and choline triggered hepatic steatosis and severely down-regulated hepatic FAS mRNA expression (Tomita et al., 2006). It is difficult to predict what may be the consequences of this secondary down-regulation of FAS on the outcome of tamoxifen-mediated steatosis. On the one hand, the secondary down-regulation of FAS may increase hepatic malonyl-CoA and inhibit CPT-I activity (Lelliott et al., 2005), which could further decrease hepatic fat oxidation. On the other hand, by decreasing hepatic fatty acid synthesis, FAS down-regulation may prevent further fat accretion. A new steady state may thus be achieved, whereby the decreased hepatic fatty acid synthesis may now equilibrate the decreased hepatic fatty acid oxidation and decreased TG secretion, resulting in stable and moderate periportal steatosis. In a previous study, the predominance of steatosis in periportal hepatocytes after valproate administration has been ascribed to a lower rate of oleate oxidation into ketone bodies in periportal than in pericentral hepatocytes and also to a relatively more severe inhibition of ketogenesis by valproate in periportal than in central hepatocytes (Olson et al., 1986).

Although TGF-β was unchanged, plasma TNF-α was decreased by 43% in tamoxifen-treated mice (Table 3). A possible mechanism could be that tamoxifen is not a pure antiestrogenic compound but also displays some estrogenic activity. Estrogens have been shown to decrease TNF-α gene expression by blocking JNK activity and the expression of c-Jun and JunD (Srivastava et al., 1999).
Effects of tamoxifen on hepatic caspase-9 and -3 activities

Mice were treated with tamoxifen (0.5 mmol/kg daily) or saline for 28 days and killed 24 h after the last dose. We centrifuged a liver homogenate at 14,000g and measured caspase activities in the supernatant. Results are means ± S.E.M. for 8 (caspase-9) or 23 (caspase-3) mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Caspase-9 Activity</th>
<th>Caspase-3 Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline, 28 days</td>
<td>100 ± 17</td>
<td>100 ± 9</td>
</tr>
<tr>
<td>Tamoxifen, 28 days</td>
<td>111 ± 10</td>
<td>177 ± 37*</td>
</tr>
</tbody>
</table>

* Different from control mice, P < 0.05.

Although hepatocyte ballooning was observed in tamoxifen-treated mice, inflammation, apoptosis, or necrosis were absent, with the exception of a single mouse, which had mild liver cell necrosis. Likewise, most patients treated with tamoxifen may only have steatosis (Nishino et al., 2003). Tamoxifen-associated steatohepatitis seems to occur mostly in obese persons (Bruno et al., 2005). Although tamoxifen decreases the removal of fat from the liver, obesity leads to insulin resistance in muscle, thus increasing plasma glucose and/or insulin, which induce hepatic FAS expression and fatty acid synthesis (Pessayre and Fromenty, 2005). Due to different targets, tamoxifen and obesity in combination could cause more steatosis than tamoxifen alone or obesity alone.

Furthermore, the hepatic steatosis associated with obesity can lead to mitochondrial lesions, mtDNA depletion, and decreased activities of respiratory chain complexes (Pessayre and Fromenty, 2005). Combined with the effects of tamoxifen, the mitochondrial effects of obesity could severely hamper mitochondrial respiration, possibly leading to reactive oxygen species formation, cytokine induction, and steatohepatitis lesions (Pessayre and Fromenty, 2005).

The present study and a few others have identified mtDNA as a new target for drug-induced hepatotoxicity (Rowe et al., 2001). Rapid mtDNA depletion occurs after high doses of alcohol or paracetamol, which trigger mtDNA degradation (Mansouri et al., 1999; Cover et al., 2005), whereas progressive mtDNA depletion occurs during prolonged treatments with tacrine or nucleoside reverse transcriptase inhibitors, which impair mtDNA replication (Gaou et al., 2001; Mansouri et al., 2003).

In conclusion, tamoxifen inhibits hepatic TG secretion and concentrates within mitochondria, where it inhibits β-oxidation and respiration. Tamoxifen also inhibits topoisomerases and impairs mtDNA synthesis. After 4 weeks of treatment with a dose reproducing human plasma concentrations, tamoxifen depletes mtDNA in mice. These combined effects may decrease fat removal from the liver and cause steatosis, despite a secondary down-regulation of hepatic FAS expression.

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References


Fig. 9. Electron microscopy. Liver fragments were processed for electron microscopy in control mice (A and D) and mice treated with tamoxifen (0.5 mmol/kg daily) for 28 days (B, C, E, and F). Steatosis (S) droplets and elongated or swollen mitochondria (arrows) with dilation of cristae were observed in treated mice. N, nucleus; m, mitochondria. Original magnification, A to C, ×5000; D to F, ×30,000.
Tamoxifen and Mitochondria


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