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Underlying mitochondrial dysfunction triggers flutamide-induced oxidative liver injury in a mouse model of idiosyncratic drug toxicity

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ABSTRACT

Flutamide, a widely used nonsteroidal anti-androgen, but not its bioisostere bicalutamide, has been associated with idiosyncratic drug-induced liver injury. Although the susceptibility factors are unknown, mitochondrial injury has emerged as a putative hazard of flutamide. To explore the role of mitochondrial sensitization in flutamide hepatotoxicity, we determined the effects of superimposed drug stress in a murine model of underlying mitochondrial abnormalities. Male wild-type or heterozygous *Sod2*$^{+/-}$ mice were injected intraperitoneously with flutamide (0, 30 or 100 mg/kg/day) for 28 days. A kinetic pilot study revealed that flutamide (100 mg/kg/day) caused approximately 10-fold greater exposure than the reported therapeutic mean plasma levels. Mutant (5/10), but not wild-type, mice in the high-dose group exhibited small foci of hepatocellular necrosis and an increased number of apoptotic hepatocytes. Hepatic GSSG/GSH, protein carbonyl levels, and serum lactate levels were significantly increased, suggesting oxidant stress and mitochondrial dysfunction. Measurement of mitochondrial superoxide in cultured hepatocytes demonstrated that mitochondria were a significant source of flutamide-enhanced oxidant stress. Indeed, mitochondria isolated from flutamide-treated *Sod2*$^{+/-}$ mice exhibited decreased aconitase activity as compared to vehicle controls. A transcriptomics analysis using MitoChips revealed that flutamide-treated *Sod2*$^{+/-}$ mice exhibited a selective decrease in the expression of all complexes I and III subunits encoded by mitochondrial DNA. In contrast, *Sod2*$^{+/-}$ mice receiving bicalutamide (50 mg/kg/day) did not reveal any hepatic changes. These results are compatible with our concept that flutamide targets hepatic mitochondria and exerts oxidant stress that can lead to overt hepatic injury in the presence of an underlying mitochondrial abnormality.

Introduction

Flutamide is a widely used nonsteroidal anti-androgen for the treatment of prostate cancer. Although generally considered safe, flutamide therapy has been associated with rare cases of idiosyncratic liver injury and therefore received a black box warning label (Wysowski and Fourcroy, 1996; Aizawa et al., 2003; Thole et al., 2004; Osculati and Castiglioni, 2006). The hepatic effects can either present as cholestasis or overt hepatocellular injury, sometimes developing to fulminant hepatitis (Hart and Stricker, 1989; Moller et al., 1990; Corkery et al., 1991; Dankoff, 1992; Wysowski et al., 1993). In contrast, bicalutamide, a bioisosteric analog of flutamide, has not been associated with idiosyncratic hepatotoxicity (Thole et al., 2004). The mechanisms of how flutamide can damage liver cells in susceptible patients are poorly understood. From experimental models there is evidence that reactive intermediates generated by CYP3A4, CYP1A2, and CYP2C19 might be involved, based on findings that flutamide underwent conjugation with glutathione (GSH) (Kang et al., 2007; Kostrubsky et al., 2007; Kang et al., 2008) or produced protein adducts from *N*-hydroxylation of a flutamide metabolite (Ohbuchi et al., 2008). Indeed, high concentrations (≥1 mM) of flutamide decreased cellular GSH concentrations in rat hepatocytes and were cytotoxic (Fau et al., 1994). However, the exact mechanisms by which these flutamide metabolites might injure hepatocytes have not been elucidated. One possibility is redox cycling of the intermediates formed from reduction of the nitroaromatic group (Coe et al., 2007; Wen et al., 2008). In addition, flutamide has been shown to inhibit the bile salt export pump (ABCB11) in rats and to inhibit the transport of taurocholate in isolated membrane vesicles (Iwanaga et al., 2007).

Abbreviations: ALT, alanine aminotransferase; BIC, bicalutamide; DILI, drug-induced liver injury; FLU, flutamide; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; SOD2, superoxide dismutase-2 (Mn-SOD); TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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Finally, flutamide has been found to act as a non-classical activator of the aryl hydrocarbon receptor (AhR) in rat liver (Coe et al., 2006; Hu et al., 2007), but the significance of this pathway is not clear. Thus, despite these multiple effects associated with flutamide in various models, a causal link to drug-induced liver injury (DILI) in patients has not been convincingly demonstrated.

Another putative mode of action by which flutamide can injure hepatocytes is mitochondrial liability. In fact, there is substantial experimental evidence that flutamide can disrupt mitochondrial function in vitro. For example, in isolated rat liver mitochondria, flutamide inhibited respiration primarily with glutamate/malate as substrate (pointing to inhibition of complex I) (Fau et al., 1994). This has been further confirmed by direct demonstration of complex I inhibition (and uncoupling effects at low concentrations) (Hynes et al., 2006) and by morphological changes in mitochondria in a liver cell line, as well as ATP depletion following exposure to flutamide (Coe et al., 2007). Taken together, these findings clearly identified a mitochondrial hazard, based on the concept that flutamide increased oxidant stress in mitochondria.

The available data pointing to potential mitochondrial toxicity induced by flutamide, however, cannot easily explain idiosyncratic DILI. In the vast majority of patients, flutamide does not produce hepatic injury at therapeutic dosage. Commensurately, flutamide even at a high dose is not hepatotoxic in normal healthy laboratory rodents (Friry-Santini et al., 2007). We have previously hypothesized that those patients who develop DILI from mitochondria-targeting drugs may exhibit some underlying mitochondrial abnormalities that would greatly sensitize them to the hepatotoxic effects of these drugs (Boelsterli and Lim, 2007). Such a correlation with genetic or acquired abnormalities in patient liver mitochondria has, however, not yet been established. On the search for an animal model that combines underlying genetic abnormalities (leading to oxidant stress) in mitochondria and superimposed drug stress, we recently adapted and further developed a mouse model that features a mild underlying oxidative stress in mitochondria due to a heterozygous deficiency of mitochondrial superoxide dismutase-2 (*Sod2*) (Ong et al., 2006; Ong et al., 2007; Boelsterli and Hsiao, 2008; Boelsterli and Lee, 2008; Lee et al., 2008a). The aim of this study was to use the *Sod2*$^{+/-}$ mouse model to study the mechanisms of flutamide-induced oxidative injury that leads to mitochondrial toxicity and hepatic toxicity. The systems toxicological approach involving pathobiological, biochemical, and toxicogenomics endpoints revealed that, in the sensitized mouse model of clinically silent oxidant stress, cumulative mitochondrial dysfunction led to focal hepatocellular demise, while normal-wild-type mice were resistant to flutamide's potential hepatic adverse effects.

Methods

Chemicals and reagents

All chemicals and reagents used in this study were of the highest analytical grade available and purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. Bicalutamide was obtained from Pfizer, Inc., San Diego, CA.

Sod2 knockout mice

All protocols involving animals were in compliance with the Institutional Animal Care and Use Committee and in accordance with the guidelines of the National Advisory Committee for Laboratory Animal Care and Research. Heterozygous *Sod2*^{tm1Leb}/*J* mice (breeding pairs), congenic in the C57BL/6 background, were obtained from the Jackson Laboratory (Bar Harbor, ME). A breeding colony was established by crossing male *Sod2*$^{+/-}$ with female *Sod*$^{+/+}$ mice. The F_1 littermates were genotyped before weaning as described (Ong et al., 2007) and subsequently used for the experiments. The mice were kept under controlled environmental conditions (22 ± 2 °C, 75 ± 5% relative humidity, 12/12 h dark–light cycle) and had free access to Laboratory Rodent Diet No. 5001 (PMI Feeds, Inc., St. Louis, MO) and water. All animals were 8–16 weeks old at the start of drug treatment.

Drug administration and experimental design

Flutamide was dissolved in Solutol HS-15 (9% in phosphate-buffered saline) and filtered through 0.22 μm syringe filters. Solutol HS-15 (BASF, Ludwigshafen, Germany) is a nontoxic solvent used for parenteral administration of water-insoluble compounds and is composed of polyglycol mono- and di-esters of 12-hydroxystearic acid and 30% free polyethylene glycol. Flutamide (30 or 100 mg/kg body weight) or vehicle (10 μL/g body weight) was injected intraperitoneally daily at 9am for 28 days. The high flutamide dose used here in mice is similar to the commonly used therapeutic human dose (750 mg/day) if corrected for interspecies differences with the dose scaling factor of 12 (Ong et al., 2007). The intraperitoneal route was selected in an attempt to target the drug to the liver and to guarantee maximal absorption. Bicalutamide was suspended in methyl cellulose (0.5%) and administered by oral gavage (0 or 50 mg/kg/day) daily for 28 days. The oral route was selected due to the poor solubility of bicalutamide. A pilot study had confirmed that bicalutamide was well absorbed in mice after peroral administration (data not shown). The dose was calculated from the human dose and exposure data (Cockshott, 2004).

Heterozygous *Sod2*$^{+/-}$ mice or wild-type (*Sod2*$^{+/+}$) mice were randomly assigned to three groups consisting of vehicle control, low-dose (30 mg/kg/day), and high-dose (100 mg/kg/day) flutamide. The study was performed in three parts; the main study (A) was used to analyze the histopathology and serum chemistry, while the repeat study (B), involving fewer animals per dose group, was used to analyze the mitochondrial transcriptomics and to assess mitochondrial oxidative stress. A separate study (C) was conducted to isolate hepatic mitochondria ex vivo. The overnight food-deprived mice were anesthetized with CO_2. Blood was collected by cardiac puncture, and serum was prepared. The liver was quickly excised; one portion was used for histopathology, and pieces of the remaining tissue were snap-frozen in liquid nitrogen for biochemical and transcriptomics analysis. Protein content was determined with the Bradford reaction using albumin as reference protein.

LC-MS analysis of flutamide plasma levels

Wild-type mice were injected a single dose of flutamide (100 mg/kg, i.p.). Blood samples were obtained by retroorbital puncture at 1, 2, 4, and 8 h post dose. The mice were euthanized at 24 h post-dose and blood was obtained by cardiac puncture. Flutamide mouse plasma levels were quantified utilizing an LC/MS/MS method. Briefly, plasma (25 μL) was incubated with acetonitrile/methanol (75/25, v/v) to precipitate proteins. A standard curve for flutamide was prepared in blank mouse plasma. Samples and standards were diluted with water and then analyzed by an LC/MS/MS system consisting of a CTC-HTS-PAL autosampler, a binary Shimadzu LC-10AD HPLC and a Sciex API4000 mass spectrometer. The mobile phase consisted of solvent A (0.1% formic acid in water) and solvent B (0.1% formic in acetonitrile). The gradient was as follows: solvent B was held for 0.8 min at 5%, linearly ramped from 5 to 90% in 1.0 min, held at 90% for 0.5 min and immediately returned to 5% at 2.4 min for column re-equilibration. Total run time was 3.4 min with a flow rate of 0.600 mL/min. Flutamide retention time was 1.79 min. Peak areas were determined using Analyst® software and individual sample concentrations were determined using a LIMS software system (Watson, version 7.2). Quantitation was performed by linear regression with a $1/x^2$ weighting.