TAMOXIFEN INDUCTION OF ARYL SULFOTRANSFERASE AND HYDROXYSTEROID SULFOTRANSFERASE IN MALE AND FEMALE RAT LIVER AND INTESTINE

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(Received October 28, 2002; accepted February 12, 2003)

This article is available online at http://dmd.aspetjournals.org

ABSTRACT:

The antiestrogenic drug tamoxifen (TAM) is widely used in the treatment of breast cancer. Species-specific mutagenic and carcinogenic potencies have been reported and have raised concerns. Sulfotransferases (STs) are important phase II drug-metabolizing enzymes. STs are involved in the sulfation processes of some TAM metabolites (i.e., α-hydroxy tamoxifen and 4-hydroxy tamoxifen). Regulation of drug-metabolizing enzymes is important for the understanding of drug metabolism and detoxification. Studies on ST induction are limited. In the present investigation, protein and mRNA expression of aryl sulfotransferase (AST-IV) and hydroxysteroid sulfotransferase (STα) have been studied in liver and intestine of male and female Sprague-Dawley rats after TAM treatment with either 6.8 or 68 mg/kg/day for 1 or 2 weeks. Enzyme assay and Western blot methods were used for protein level determination; reverse transcription-polymerase chain reaction method was used for mRNA level determination. Here, for the first time, we have demonstrated that AST-IV and STα could be induced in intestine by tamoxifen. Furthermore, intestinal inductions were found to be much greater than the inductions found in the liver, suggesting a distinct potentiality of intestinal cells in TAM metabolism. The impact of induction and regulation of intestinal STs on TAM metabolism with respect to its toxicity has yet to be studied. The role of STs induction and relevant TAM metabolism is discussed in the context of organ- and species-specific variable carcinogenic manifestations.

The antiestrogen tamoxifen (TAM)1 is widely used as an endocrine therapy in the treatment of breast cancer (Jordan 1988). Although it is effective against breast cancer, TAM has been reported to be a possible risk factor for human endometrial cancer (Fisher et al., 1998). TAM-induced hepatogenotoxicity in rats and mice but hepatocarcinogenicity only in rats has been shown (Cartew et al., 1995; Martin et al., 1998; Firozi et al., 2000). Metabolic activation of TAM is believed to be a prerequisite to form an electrophile leading to the formation of DNA adduct, resulting in carcinogenicity (Jarman et al., 1997; Chen et al., 2002). Some reports reveal that, apart from its anticancer and chemoprotective effect (Heel et al., 1978), TAM may be involved in influencing some of the drug-metabolizing enzymes (Hellriegel et al., 1996; Kasahara et al., 2002). In general, the toxicity of tamoxifen is determined by the formation rate of α-hydroxy tamoxifen (α-OH TAM) and 4-hydroxy tamoxifen (4-OH TAM), which is catalyzed by the phase I enzymes cytochrome P450 (Boocock et al., 2000; Kasahara et al., 2002). α-OH TAM formation has been reported to initiate bioactivation (Beland et al., 1999; Boocock et al., 2000), whereas 4-OH TAM formation leads to the detoxification pathway (Crewe et al., 1997; Dehal and Kupfer 1997; Chen et al., 2002). N-Demethylation by CYP3A (Jacolot et al., 1991) and N-oxidation of TAM by flavin monoxygenase (Mani et al., 1993) has also been regarded as a detoxification pathway. In rats, the phase II cytosolic enzyme hydroxysteroid sulfotransferase (STα) is responsible for the O-sulfation of α-OH TAM as shown in vitro and in rat liver (Glatt et al., 1998a). The human hydroxysteroid sulfotransferases showed very low activity toward α-OH-TAM but higher activity toward 4-OH-TAM (Glatt et al., 1998b; Shibutani et al., 1998). On the other hand, lower sulfation ability of rat STs toward 4-OH-TAM (Glatt et al., 1998a; Chen et al., 2002) establishes the carcinogenic potentialities of TAM only in rat liver.

STs catalyze the sulfation of different hydroxyl groups. These include endogenous and xenogenic molecules ranging from bioamines, peptides, and hormones to drugs and xenobiotics (Falany and Wilborn, 1994; Matsui and Homma, 1994). In mice, two major ST isoforms catalyze the sulfation of phenols (aryl sulfotransferase, AST-IV) or alcohols (hydroxysteroid sulfotransferase, STα). Hepatic hydroxysteroid and phenol STs show a distinct sex dimorphism in rats (Rajkowski et al., 1997). Higher immunoreactivity of AST-IV and STα proteins has been shown in livers of male and female rats, respectively (Chen et al., 1995; Hellriegel et al., 1996). Most studies of TAM effect on rat STs to date have focused on female STα. Consequently, limited data are available regarding the effect of TAM on STα in male and AST-IV proteins in both male and female rats (Davis et al., 2000). Apart from that, all studies on TAM metabolism...
have been done on either cultured hepatocytes or animal liver tissues, but to the best of our knowledge, no studies have been performed on intestinal cells. Intestinal epithelial cells, the main part of the gastrointestinal tract, represent the first barrier to exogenous compounds of foods or orally administered drugs that are metabolized before occurring in the circulation throughout the whole body. The presence of phenol-catalyzing sulfotransferases (PSTs) in human gastrointestinal tract has been reported (Pacifici et al., 1988; Cappiello et al., 1989; Chen et al., 2003). Polyphenolic flavonoids have been reported to induce phase II enzymes and interact with type II estrogen binding sites in the intestinal cells, thus implying their antineoplastic effects (Gee and Johnson, 2001). Caco-2 cells, a carcinoma cell line and representative of intestinal epithelial cells, have been characterized for phase I (oxidation), phase II (conjugation), and phase III (transmembrane export) activities (Baranczyk-Kuzma et al., 1991; Bjorge et al., 1991; Peters and Roelofs, 1992; Lampen et al., 1998). The patterns seen are similar to those manifested by intestinal epithelial cells.

CYP3A4, a major phase I enzyme highly occurring in human intestine, is also present in Caco-2 cells (Schmiedlin-Ren et al., 1997). Cytochrome P450 families 1 to 4 (CYP1 to CYP4) have been well studied for endogenous hormones, cytokines, and structurally diverse xenobiotic induction (Waxman, 1999). On the other hand, sufficient data are not available describing xenobiotic induction of STs, especially intestinal STs.

In the present investigation, we report the effects of TAM treatment on the expression of AST-IV and STa protein and mRNA in the liver of male and female rats. Here, for the first time, we report the TAM-mediated expression of AST-IV and STa in intestinal epithelial cells of rats of both sexes. This study will be helpful to further assess the extent of dependence of carcinogenic potentialities of TAM-mediated changes in expression of STs and whether tissue-specific differential expression of STs could interfere with these potentialities.

Materials and Methods

Materials. Tamoxifen citrate, β-naphthol, [14C]β-naphthol (4.7 mCi/mmol), p-nitrophenyl sulfate (PNPS), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), and [1,2,6,7-3H]dehyroxyrioproterosterone (60 Ci/mmol) were purchased from Sigma-Aldrich (St. Louis, MO). SDS-polyacrylamide gel electrophoresis reagents were obtained from Bio-Rad (Hercules, CA). Western blot chemiluminescence reagent kits were purchased from Pierce Chemical (Rockford, IL). Nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA) was used in the present investigation.

Animals and Drug Treatment. Male and female Sprague-Dawley rats (Harlan, Indianapolis, IN), 10 to 11 weeks old and 250 to 350 g b.wt., were used for this study. Rats were housed in a temperature- and humidity-controlled room and supplied with rodent chow and water for at least 1 week before use. Two groups of rats having six in each (three males and three females) were taken. One group was assigned for 1-week and other group for 2-week tamoxifen treatment.

PNF assay. AST-IV activity from liver cytosols was determined as described previously (Chen et al., 1999, 2000). In brief, sulfation activity was determined in a reaction mixture containing 50 mM Tris buffer, pH 7.2, 5 mM PNPS, 20 μM PAPS, and 0.1 mM β-naphthol. Rat liver cytosol (50 μg) were used as the enzyme source in a total reaction volume of 250 μl of reaction mixture.

Western Blot Analysis. Ten micrograms from liver and 75 μg from intestinal cytosols were used in 10% polyacrylamide gel in an electrophoresis system (Novex, San Diego, CA). After running at 200 V, the protein bands were transferred overnight at 40 V onto a nitrocellulose membrane in an ice-cold water bath. Membranes were blocked for 1 h by 5% nonfat dry milk in phosphate-buffered saline (buffer A). For all assays, 20 μM PAPS was used. Liver (50 μg) or intestine (400 μg) cytosol protein was used as enzyme source in a total volume of 250 μl of reaction mixture. After 30-min incubation at 37°C in a water shaker bath, the reaction was stopped by adding 250 μl of 0.25 M Tris, pH 8.7. The reaction mixtures were read at 401 nm by a spectrophotometer. Specific activity (SA) was expressed as nanomoles per minute per milligram of protein. The data shown in the figures are the average of at least three determinations.

Two different enzyme assay methods have been used in the present investigation. PNPS assay. AST-IV activity from liver cytosols was determined as described previously (Chen et al., 1999, 2002). Other ingredients and reaction conditions were same as the PNPS assay mentioned above. For intestinal AST-IV activity, [14C]β-naphthol (4.7 mCi/mmol; 0.1 mM final concentration) was used as substrate. To determine STa activity in both liver and intestinal cytosols, [1,2,6,7-3H]dehyroxyrioproterosterone (diluted to 0.4 μCi/ml final concentration) was used as substrate. For all assays, 20 μM PAPS was used. Liver (50 μg) or intestine (400 μg) cytosol protein was used as enzyme source in a total volume of 250 μl of reaction mixture. After 30-min incubation at 37°C in a water shaker bath, the reaction was stopped by adding 250 μl of 0.25 M Tris, pH 8.7. Extraction procedure was performed twice by adding 0.5 ml of water-saturated chloroform each time. After final extraction, 50 μl of aqueous phase was used for scintillation counting. The data shown in the figures are the average of at least three determinations.

PAPS was eliminated from the controls of both assay methods. Assays were run in duplicate, and the average of the results used for enzyme activity calculations.

Enzyme Assays. Two different enzyme assay methods have been used in the present investigation. PNPS assay. AST-IV activity from liver cytosols was determined as described previously (Chen et al., 1999, 2000). In brief, sulfation activity was determined in a reaction mixture containing 50 mM Tris buffer, pH 7.2, 5 mM PNPS, 20 μM PAPS, and 0.1 mM β-naphthol. Rat liver cytosol (50 μg) were used as the enzyme source in a total reaction volume of 250 μl of reaction mixture.

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5'-TTCTGGGCTACAGTGAAGGTA-3' (GenBank accession no. X52883), the 299-bp AST-IV cDNA was synthesized (Runge-Morris et al., 1998). The 264-bp STa cDNA was synthesized using the primer pair FP 5'-TCCTCAAAGGATATGTTCCG-3' and RP 5'-CAGTTCCTTCTCCATGAGAT-3' (GenBank accession no. M33329) (Davis et al., 2000). The specificity of all primers was tested using the BLAST of the National Center for Biotechnology Information Open Reading Frame software. cDNA synthesis from total 1 μg of liver RNA was performed in a 50-μl reaction mixture. The concentrations of the different ingredients were used following supplier’s protocol. For control, 500-bp cDNA of rat β-actin was synthesized from the same amount of RNA. The primer pair (FP 5'-GATGTACGTAGCCAATCCA-3' and RP 5'-GTGCAACCAGACAGCA-3') for the synthesis of rat β-actin cDNA was designed in our laboratory using the same software mentioned above.

Statistical Analysis. Student’s t test was performed to calculate the statistical significance with the difference between two means of control and tamoxifen-treated rats.

**Results**

Figure 1 demonstrates that liver AST-IV activity increased in both male and female rats after TAM treatment. The high dose of TAM increased the activity by 43% (p < 0.01) and 79% (p < 0.05) in male liver after 1- or 2-week treatments, respectively. In female rats, AST-IV activity after high dose of treatment increased by 3-fold (p < 0.01) and 4.4-fold (p < 0.001) after 1 and 2 weeks, respectively. The AST-IV protein content was also tested by Western blot and is presented in Fig. 2. After 1-week treatment (A), the protein concentration increased markedly in female as shown by the increased intensity of the protein band. After 2-week drug treatment (B), the increase is more evident from the blot and the corresponding densitometry analysis. The Western blot results agree with activity assays.

STa activity increased in liver of male rats after treatment with both TAM doses (Fig. 3). After 1-week treatment, activity increased in male rat liver by 2.3-fold (p < 0.001) and 3.5-fold (p < 0.001) in either low- or high-dose treatment groups, respectively. TAM treatment for 2 weeks increased the activity by 7-fold (p < 0.001) and 11-fold (p < 0.001) in low- and high-dose groups, respectively. In female rats, STa activity increased by 11 and 42% (p < 0.01) after the treatment of high-dose TAM for either 1 or 2 weeks, respectively. Low-dose TAM treatment also increased the activity by 41% (p < 0.001) in female rat liver in 2-week experiment. In the 1-week experiment, this same low dose did not change the activity. The Western blot analysis of STa protein is presented in Fig. 4. This figure depicts that TAM increased STa protein after 1 week with high-dose and after 2 week with both low- and high-dose treatments. STa protein in liver of female rats increased to some extent, and this has been...
presented by densitometry analysis value. The Western blot analysis is in basic agreement with our enzymatic assay values.

Intestinal AST-IV activity increased both in male and female rats treated with either high or low doses after 1- and 2-week treatment (Fig. 5). In male rats, low-dose TAM treatment increased the activity 3-fold \((p < 0.01)\) and 16-fold \((p < 0.001)\) after 1- and 2-week treatment, respectively. The increase is 2.4-fold \((p < 0.01)\) and 13-fold \((p < 0.001)\) when the drug was administered in its high dose. In female rats, the activity increased by 3.5-fold \((p < 0.001)\) and 20-fold \((p < 0.001)\) after high-dose TAM treatment for 1 and 2 weeks, respectively. The increases after the low-dose treatment were found to be almost 3-fold in the treatment groups of either time period. The induction of enzyme activity after 2-week drug treatment has been demonstrated by increasing protein levels (Fig. 7A). The Western blot and densitometry results indicate a higher increase in male and a moderate increase in female rats.

STa activity increased in intestinal mucosal cells of male rats by 2- and 3.4-fold \((p < 0.01)\) after low-dose TAM treatment for either 1 or 2 weeks, respectively. The increases are 3.4-fold \((p < 0.01)\) and 13-fold \((p < 0.001)\) in high-dose TAM-treated group. In female rats, STa activity increased markedly with the high dosage of TAM after either 1- or 2-week treatment. These increases are 50% \((p < 0.05)\) and 4.9-fold \((p < 0.001)\) (Fig. 6). Figure 7B depicts the Western blot of STa expression in intestinal cytosols after 2-week TAM treatment. Protein expression was found to be higher in male rats as suggested by our enzymatic assay results.

Figure 8 demonstrates the RT-PCR results of AST-IV and STa mRNA in liver after 2-week TAM treatment. AST-IV mRNA expression increased markedly in female liver especially after high-dose TAM treatment. The STa mRNA expression increased both in male and female livers after low- or high-dose TAM treatment. These RT-PCR results agree with the respective protein expressions and enzymatic activities observed in the liver cytosol. The higher induction of STa mRNA in female rats after either TAM doses cannot be correlated with the respective protein expression and enzymatic activities.

**Discussion**

The chemotherapeutic antiestrogenic drug TAM has received attention in recent years because of its potential to cure breast cancer yet also induce endometrial cancer (Fisher et al., 1998). Mutagenicity in mice and hamsters and carcinogenicity in rats (Carthew et al., 1995; Martin et al., 1998) suggest the differential extents of toxicity of this drug. Presumably, these differences are due to organ- and species-specific metabolic patterns producing different redox active metabo-
treatment with no signs of liver carcinogenesis, but suppressed the activity in the liver of female rats after 1-day, 2-week, or 12-week.

Kasahara et al. (2002) showed that TAM treatment did not alter STa expression in females (Nuwaysir et al., 1996). TAM increased STa mRNA expression in male rat liver (Greaves et al., 1993) might be occurring due to the STa protein and mRNA levels. We also, for the first time, report the induction abilities of TAM toward the intestinal mucosal STs. We anticipate our investigation will be helpful in evaluating the metabolic nature of exogenously administered drugs or xenobiotics in intestinal mucosal cells.

Our finding of high STa levels in female rats and the induction of STa in male rats after tamoxifen treatment suggests that STa-mediated α-OH TAM sulfation could be a major route for TAM-DNA adduct production and carcinogenesis as reported by Davis et al. (2000). In this study, 2-week TAM treatment with its high dose increased STa activity 11-fold (p < 0.001) and 42% (p < 0.01) in the liver of male and female rats, respectively. This increase has been confirmed at protein and mRNA expression level. TAM-induced carcinogenesis in male rat liver (Greaves et al., 1993) might be occurring due to the STa induction and probable bioactivation of TAM metabolic pathway. Contrasting results are available presenting the effects of TAM on STa expression. TAM increased STa mRNA expression in male rat liver but decreased expression in females (Nuwaysir et al., 1996). Kasahara et al. (2002) showed that TAM treatment did not alter STa activity in the liver of female rats after 1-day, 2-week, or 12-week treatment with no signs of liver carcinogenesis, but suppressed the activity significantly after 52-week treatment with signs of hyperplastic nodules occurring in the liver. The relationship between TAM-induced expression of STa leading to bioactivation of TAM and carcinogenesis has been shown by several investigators. Here, we have to consider that the rate of both detoxification and bioactivation processes impact xenobiotic disposition and ultimate clinical outcome. Our previous work (Chen et al., 2002) suggested that the 4-OH TAM sulfation-mediated detoxification pathway is highly active in human liver and intestinal tissues but almost inactive in rat tissues. This metabolic disparity in 4-OH TAM sulfation explains at least partially the resistant nature of human liver against TAM-mediated carcinogenesis. Like STa induction in male rats as observed by Davis et al. (2000) and confirmed in the present investigation, estrogen sulfotransferase (EST) was induced in male rats after TAM treatment (Hellriegel et al., 1996). Unlike STa induction in rats, TAM-mediated EST (the expression of this gene is normally regulated by estrogen) induction exerts its beneficial effects against estrogen-dependent carcinogenesis in humans. This mechanism is unrelated to TAM’s ability to antagonize cell surface estrogen receptor. Hellriegel et al. (1996) also showed that female rats neither have the basal level of EST protein nor do they express it after TAM treatment. 4-OH TAM also exerts a protective effect by inhibiting sulfatase (Pasqualini and Chetrite, 1999), which catalyzes the hydrolysis of estrone sulfate. The plasma level of estrone sulfate is balanced by both sulfatase and EST, which catalyzes the sulfation of estrogen to make it biologically inactive. Here, we extended our study to investigate the effect of TAM treatment on AST-IV protein in liver and intestinal mucosal cells. TAM treatment (high dose) in both the 1- and 2-week group increased
AST-IV activity in liver of female rats by 3.0-fold ($p < 0.01$) and 4.4-fold ($p < 0.001$), respectively, but in male liver the increase was 40 to 70%. In humans, the role of phenol-catalyzing sulfotransferase (SULT1A1), the counterpart to that of AST-IV in rat, was shown to have 4-OH TAM sulfation ability, which was absent in the liver of male and female rats (Chen et al., 2002). Nishiyama et al. (2002) showed that mainly SULT1A1 and to some extent EST (SULT1E1) are responsible for TAM metabolism in hepatic cytosolic fractions from cancer patients. Seth et al. (2000) demonstrated that 4-OH TAM could induce phenol sulfotransferase (SULT1A) in ZR75–1, a breast cancer cell line. They also hypothesized that inherited variability in SULT1A activity due to the polymorphic nature in sulfotransferase genes might influence the risk of breast cancer. Higher variability in the sulfotransferase activities in human intestinal cytosols has been demonstrated by Chen et al. (2003). We found innately high levels of AST-IV in male rat livers and an inducible level of the same enzyme in female rat livers treated with TAM. Davis et al. (2000) also treated rats with TAM and found it to be hepatocarcinogenic. Given these findings, AST-IV is likely unable to counteract the negative effects of TAM. The precise role of AST-IV and other isoforms toward TAM or its metabolites needs to be further investigated.

Being the first target to counteract with different kinds of orally fed...
exogenous substances, intestinal mucosal cells may play an important role in drug metabolism and xenobiotic detoxification. The occurrence of extrahepatic STs has been reported in platelets (Harris et al., 2000), brain (Alred et al., 1999), and uteri (Parker et al., 1999). There have also been reports on intestinal STs (Sundaram et al., 1989; Harris et al., 2000; Chen et al., 2003). To our best knowledge, the induction of intestinal STs is basically unknown. In the present study, for the first time, we report that TAM could induce AST-IV and STa in intestinal mucosal cells. The 10- to 15-fold (p < 0.001) increase in AST-IV and STa activity might suggest that these proteins are much more inducible in intestinal mucosal cells than in liver after TAM treatment. Although the impact of induction of these two enzymes has not been tested in the present investigation, our data suggest that phase II drug-metabolizing enzyme STs could be induced in intestinal mucosal cells, perhaps to a much greater extent than in liver.

Further studies are necessary to investigate the impact of TAM-mediated induction of AST-IV and STa in intestinal mucosal cells, whether this induction is related to the metabolism of TAM, or whether the induction could lead to carcinogenesis by DNA adduct formation. Some recent reports reveal that sex-specific expression of estrogen receptors β1 and β2 in intestinal mucosal cells, and differential estrogen signaling might have a role in colon carcinogenesis in male and female (Campbell-Thompson et al., 2001; Witte et al., 2001). Because TAM is antiestrogenic and can induce STs in intestinal cells even to a greater extent than in liver, sulfation-mediated TAM metabolism is possibly occurring in intestinal epithelial cells. We found STa mRNA expression to be very high in female liver after tamoxifen treatment, which does not directly correlate with the corresponding enzyme activity and protein expression. We have to take into account that the rate of transcription and translational processes are not the only determining factors for the extent of expression of a protein activity. Posttranscriptional, posttranslational modifications, and even the metabolic demands of the intracellular milieu, can also interfere with the activity of a protein. It will be relevant to investigate the changes in the activity of each enzyme isoform with regard to tamoxifen metabolism. The activity and protein expression of hepatic drug-metabolizing enzymes is known to sometimes be independent of the level of corresponding mRNA.

More studies are necessary to elucidate the role of intestinal mucosal cells in the metabolism of different drugs and xenobiotics, including their detoxification and/or bioactivation abilities compared with that of liver. The impact of STs induction in intestinal mucosal cells after TAM treatment should be investigated further.

Acknowledgments

We appreciate the generous gift of AST-IV and STa antibody from Dr. Michael W. Duffel. We thank Sharon M. Baker for proofreading the manuscript.

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