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

SMARAJIT MAITI AND GUANGPING CHEN
Department of Physiological Sciences, College of Veterinary Medicine, Oklahoma State University, Stillwater, Oklahoma

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ABSTRACT:
The antiestrogenic drug tamoxifen (TAM) is widely used in the treatment of breast cancer. Species-specific mutagenic and carcinogenic potentials 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 (STa) 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 STa 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.
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 STα 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 STα 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-nitro-phenyl sulfate (PNPS), 3′-phosphoadenosine-5′-phosphosulfate (PAPS), and [1,2,6,7-3H(naphtol) (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 (Super Signal West Pico Stable Peroxidase, Pierce Chemical, Rockford, IL). Nitrocellulose membrane (Immobilon-P, Millipore Corporation, Bedford, MA) was used during Western blot procedures. Protein assay reagent was purchased from Bio-Rad (Hercules, CA). All other reagents and chemicals were of the highest analytical grade available.

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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.

Tamoxifen citrate suspended in corn oil was administered by gavage at either 68 or 68 mg/kg b.wt./day (Davis et al., 2000) to male and female rats of both groups of either 1- or 2-week treatments. The corresponding control rats received only the vehicle (corn oil). The animals were sacrificed 24 h after the last drug treatment. Their livers were collected; washed with sterile, ice-cold NaCl (0.9%) solution; and kept in dry ice bath. Intestinal lumens were washed carefully with sterile NaCl (0.9%) solution. Luminal cavities were opened and fat particles and small blood vessels were trimmed out. Intestinal mucosal cells were collected by scraping and frozen immediately in dry ice bath. Samples were stored at −80 °C until use.

Cytosolic Sample Preparation. Liver homogenates were prepared with 50 mM Tris buffer containing 0.25 M sucrose, pH 7.5. Intestinal mucosal homogenates were prepared with the same buffer containing 0.01 mg/ml trypsin inhibitor and 10 μg/ml phenylmethylsulfonyl fluoride. All homogenates were spun at 40,000 rpm for 1 h at 4 °C. Cytosol aliquots were collected and preserved at −80 °C for enzymatic assay and Western blot.

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 6.2, 5 mM PNPS, 20 μM PAPS, and 0.1 mM β-naphthol. Rat liver cytosols (50 μg) were used as the enzyme source in a total reaction 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.

Radioactive assay. AST-IV activity in intestinal cytosols and STα activities in both liver and intestinal cytosols were determined by the radioactive assay method described previously (Chen et al., 2002). Other ingredients and reaction conditions were the 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 STα activity in both liver and intestinal cytosols, [1,2,6,7-3H(naphtol) (0.1 M 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 was used for enzyme activity calculations.

Western Blot Analysis. Ten micrograms from liver and 75 μg from intestinal cytosols were used in a 10% polyacrylamide gel in an electrophoresis system (Novex, San Diego, CA). After running at 200 V, the proteins 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 rat liver cytosols, membranes were incubated with either rabbit anti-rat AST-IV or rabbit anti-rat STα (1:5000) in buffer A containing 0.05% Tween 20 (buffer B) for 2 h on a shaker at room temperature. The membranes carrying intestinal samples were incubated in the same antibodies (1:1000) overnight on a shaker at 4 °C. After incubation, all membranes were washed with buffer B for 4 × 15 min and incubated in secondary antibody (horseradish peroxidase-conjugated ImmunoPure goat anti-rabbit IgG; H + L) at 1:5000 dilutions in buffer B for 2 h. The membranes were washed with buffer B for 4 × 15 min and then with phosphate-buffered saline 3 × 5 min. Fluorescent bands were developed with 1 ml of substrate containing same volume of each Super Signal West Pico Luminol Enhancer solution and Super Signal West Pico Stable Peroxidase solution at room temperature for 5 min. The X-ray films were exposed to the membranes and then developed. Films were scanned and the densitometry analysis was performed in an Electronic UV transcibillator from Advanced American Biotechnology and with AAB software (Fullerton, CA).

Extraction of Total RNA and RT-PCR. Total RNA was extracted from liver using RNAeasy mini protection kit from QIAGEN according to the supplier’s guidelines. The concentration and purity of the extracted RNA were checked spectrophotometrically by measuring 260/280 absorption ratios.

The primer pair for AST-IV was designed in our laboratory using the Gene Fisher primer designing and Multialignment software. Using the forward primer (5′-GGTGGCTCATGTGGGTGGTA-3′) and reverse primer (RP)
SA has been expressed as nanomoles per minute per milligram of protein. Liver cytosolic protein (50 μg) was used to determine the enzymatic activity using the PNPS assay method. Tamoxifen citrate was fed as homogenous suspension in corn oil. Only corn oil was administered to corresponding control rats. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

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′-GATGTACGTAGCATTCCA-3′ and RP 5′-GTGCCAACCAGACAGCA-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 < 0.001) 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-
Kasahara et al. (2002) showed that TAM treatment did not alter STα 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 in the liver of male rats after 11-fold TAM treatment with its high dose increased STα mRNA expression in male rat liver (Greaves et al., 1993) might be occurring due to the STα mediated carcinogenic nature (Rutqvist et al., 1995; Hemminki et al., 1996). O-Sulfation of α-OH TAM by rat hydroxysteroid sulfotransferase in liver of TAM-treated rats (Glatt et al., 1998a) has been correlated with the carcinogenic potentialities of TAM. In the present investigation, we report the differential expression of phenol (aryl)-sulfating AST-IV and hydroxysteroid (alcohol)-sulfating STα in liver at protein and mRNA levels. We also, for the first time, report the induction abilities of TAM toward the intestinal mucosal STα. 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 STα levels in female rats and the induction of STα in male rats after tamoxifen treatment suggests that STα 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 STα 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 STα induction and probable bioactivation of TAM metabolic pathway. Contrasting results are available presenting the effects of TAM on STα expression. TAM increased STα 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 STα 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 STα 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 STα 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 STα 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 Chettite, 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 (Aldred et al., 1999), and uterus (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 STs in intestinal mucosal cells. The 10- to 15-fold ($p < 0.001$) increase in AST-IV and ST 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 STs 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 $\beta_1$ and $\beta_2$ 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 STs 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 exhibition 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 are 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.

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