Phase II Enzyme Expression in Rat Liver in Response to the Antiestrogen Tamoxifen

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ABSTRACT

The genotoxicity and carcinogenicity of tamoxifen have been attributed to metabolic activation of tamoxifen to an electrophile. Phase II enzymes are known to be involved in the metabolism of the drug and possibly in the formation or elimination of the active metabolite. To determine the effects of tamoxifen on phase II enzyme expression, the drug was administered to F344 rats, and hepatic glutathione S-transferase (GST), UDP-glucuronosyltransferase (UGT), and sulfotransferase (ST) expression was evaluated. Some of the tamoxifen-induced effects, including dramatic suppression of selected GST enzymes and activity, were observed at a dose in rats that is directly equivalent, on a mg/kg b.w. basis, to the doses used for breast cancer treatment. Most of the observed responses are not consistent with the previously described phenobarbital-like properties of tamoxifen and could be the result of the partial agonist activity of tamoxifen at the estrogen receptor. Northern blot analysis was performed with isozyme-specific oligonucleotide probes for rat GST, ST, and UGT. In addition, GST subunit protein levels were assayed by high-performance liquid chromatography. In females, tamoxifen treatment resulted in a 60% suppression of GST Yal mRNA and protein levels and a 40% suppression of GST Ya2 levels. In males, tamoxifen treatment suppressed GST Yal expression approximately 60%, and GST Ya2 expression was suppressed at low doses but induced above control at high doses. Male GST Ycl was induced approximately 80% over control. The expression of all major forms of rat hepatic GST subunit protein, including GST Yb1, Yb2, Yb3, Yp, and Yl, was unaffected by tamoxifen treatment. GST conjugation activity toward 2,3-dihydroxyanthracene-3,17-dione, a GST Yal- and Ya2-specific substrate, was suppressed approximately 40% in both sexes, consistent with our protein and mRNA data. Total GST activity, as measured by the rate of chlorodinitrobenzene conjugation, was not changed. Tamoxifen also produced a dose-dependent increase in UGT2B1 mRNA, a phenobarbital-inducible enzyme; mRNA levels reached 210 and 420% of control in females and males, respectively. In addition, mRNA levels for ST2A2, a female-specific ST gene, were suppressed 50% in females and induced 120% over control in males. mRNA expression for all other forms of rat liver UGT and ST isozymes that were tested was not significantly affected by tamoxifen treatment. Overall, these results demonstrate that tamoxifen has significant effects on hepatic phase II enzyme expression that may have implications for the carcinogenicity and/or therapeutic activity of the drug.

INTRODUCTION

Tamoxifen (TAM) is a nonsteroidal triphenylethylene antiestrogen that has found widespread use in the treatment of all forms of breast cancer (1). The effectiveness of TAM, coupled with a low incidence of acute side effects (2, 3) and clinical data that demonstrate suppression of contralateral disease (4), has led to the initiation of large-scale chemoprevention trials (5, 6). However, the potent carcinogenicity of TAM in rat liver (7–10) and debate concerning the possible carcinogenicity of the drug in human endometrium (11–13), have raised concern over the safety of TAM for prophylaxis in healthy women.

The mechanism by which TAM induces rat liver cancer is unknown. However, it has been suggested that this activity is due to the metabolism of the drug to an activated electrophile (14, 15) that then covalently modifies DNA (16–20). In support of this, TAM can cause hepatic DNA breakage and aneuploidy (21), unscheduled DNA synthesis (17), and micronucleus formation (22). In addition, TAM metabolites can covalently bind to microsomal protein (14, 23) and DNA (16–20).

Evidence suggests that TAM can induce enzymes responsible for its own activation, or conversely, suppress enzymes responsible for its detoxification. More unscheduled DNA synthesis is produced by TAM in hepatocytes harvested from rats pretreated with the drug than in hepatocytes harvested from untreated rats (17). In addition, other enzyme inducers can potentiate the toxicity of TAM. Rats treated with PB display more TAM-induced 32P-postlabeling adducts (18) and more binding to microsomal protein (14, 24) than rats not pretreated with PB. Similarly, hepatocytes harvested from PB-treated rats are susceptible to TAM-induced superoxide formation, whereas cells harvested from untreated rats are not (25).

The identity of the activated electrophilic metabolite of TAM has not been determined conclusively, but α-hydroxy-TAM, a metabolite found in rat hepatocytes (26) and human liver and serum (15, 27), has been proposed as an intermediate in this activation (15, 28, 29). However, in order for α-hydroxy-TAM to be genotoxic, the α-hydroxy group must first be protonated or conjugated. This would result in a good leaving group, whose loss would generate a highly reactive carbocation (15, 30). This conjugation could be the result of metabolism by phase II enzymes in the liver, such as STs or UGTs.

Phase II enzymes are known to be involved in the metabolism of TAM. Coadministration of TAM and the ST inhibitor pentachlorophenol suppresses the formation of several TAM-DNA adducts (31, 32), suggesting that ST activity is crucial for the formation of these adducts. Paradoxically, pentachlorophenol coadministration enhances the formation of a different subset of DNA adducts (31, 32). Thus, sulfation may be both a detoxication and an intoxication pathway of TAM metabolism in rat liver. In addition to sulfation, glucuronidation is also known to be involved in the metabolism of TAM. McCague et al. (33) have identified 4-hydroxy-TAM β-glucuronide in liver of rats treated with the drug. To date, GST enzymes have not been implicated in the metabolism of TAM. However, the remarkable tolerance of GSTs for a diverse range of substrates (34) suggests that these enzymes may be involved.

Because of the role phase II enzymes may play in the metabolism and genotoxicity of TAM, it is of interest to determine how TAM administration affects the expression of these enzymes. We were interested in ST, UGT, and GST enzymes in particular because members of these families are known to be involved in the maintenance of circulating hormone levels such as thyroid hormone (35–38),
MATeRIALS AND METHODS

Materials. 9[32]P] radionucleotide and Hybond N* nucleic acid transfer membrane were purchased from Amersham (Arlington Heights, IL), Δ3-Androsten-3,17-dione was purchased from Steraloids, Inc. (Wilton, NH). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) and were of molecular biology grade or better.

Animals and Dosing. Six-week-old male and female F344 rats were purchased from Harlan-Sprague-Dawley (Indianapolis, IN) and housed, three animals per cage, on a 12:12 h light-dark cycle. The animals were supplied with water and AIN-76 purified diet (TEKLAD Corp., Madison, WI) ad libitum and were maintained in our facility for 3 weeks prior to the start of the study. TAM citrate was administered at doses of 0.5, 5.0, 20.0, 35.0, or 50.0 mg/kg/day by gavage. Sodium PB and MC were administered at doses of 60 mg/kg/day by gavage or 30 mg/kg/day by i.p. injection, respectively. All drugs were administered in 0.9 to 1.0 nil tricaprylin vehicle daily. The animals were fasted after the last dose until sacrifice 22 h later. Sacrifice was performed by decapitation: the livers were excised quickly, and the remaining liver was frozen in liquid nitrogen in individual Whirl-Pak bags for later RNA preparations.

Preparation of Liver Cytosolic Fraction. Liver samples (1.5 g) were washed with ice-cold KCl and homogenized by six passages of a Teflon-glass homogenizer in 4 volumes of 0.25 m KPO4 (pH 7.5), 0.15 m KCI, 0.1 mM DTT. Liver cytosolic CDNB conjugation was measured according to previously published procedures (51). The increase in absorbance was monitored at 248 nm. After subtracting nonenzymatic isomerization, activity (in micromoles) of Δ3-androsten-3,17-dione was calculated by the use of A450 = 16,300 m-1 cm-1. Liver cytosolic CDNB conjugation was measured according to previously published procedures (51). Isolation of GSTs. Cytosolic GSTs were isolated by affinity chromatography as described previously (52, 53). Briefly, 500 μg total cytosolic protein were run through a S-hexylglutathione-Sepharose affinity column, and the flow-through fraction was immediately passed through a GSH-Sepharose affinity column. Columns (0.7 X 1.0 cm) were equilibrated with 10 mM Tris/HC1 (pH 7.8) containing 20 mM DTT (buffer A), washed with buffer A containing 0.2 M NaCl (buffer B), and eluted with buffer B containing 5 mM S-hexylglutathione.

Table 1. Isozyme-specific oligonucleotide probes for rat ST mRNA analysis

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<th>Gene</th>
<th>Trivial name</th>
<th>Oligonucleotide probe sequence*</th>
<th>Probe location (bp)</th>
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<th>STIC1</th>
<th>STIE1</th>
<th>STIA2</th>
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* Oligonucleotide probe sequence was selected as indicated in "Materials and Methods." The relative location of the probe sequence, starting from the first bp of the corresponding cDNA, is shown.

Table 2. Isozyme-specific oligonucleotide probes for rat UGT mRNA analysis

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<th>UGT2B2</th>
<th>UGT2B3</th>
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</table>

* Oligonucleotide probe sequence was selected as indicated in "Materials and Methods." The relative location of the probe sequence, starting from the first bp of the corresponding cDNA, is shown.

b, 10 or more mismatches between the probe sequence and the related gene.

and because the expression of ST and GST isozymes is highly sex specific and hormone dependent (39-43).

Recently, it has been shown that TAM is a PB-like inducer of hepatic phase I enzyme expression in the rat (44, 45). The focus of this research was to determine whether the effects of TAM on phase II expression are also PB-like.
Separation, Identification, and Quantitation of GST Subunit Protein by HPLC. HPLC conditions were essentially as described previously (54). Subunits were resolved on a Vydac 30 nm C18 (2.1 mm × 25 cm) reverse-phase column with a linear gradient from 36 to 52% acetonitrile over 70 min, with a 0.25 ml/min flow rate. Solvent A was 0.1% trifluoroacetic acid, and solvent B was 70% acetonitrile and 0.1% trifluoroacetic acid. Absorbance at 214 and 280 nm was monitored. GST subunits were identified by comparison with previously published HPLC profiles (52) and quantitated with the peak area at 214 nm and previously published molar absorption coefficients for the individual subunits (52).

RESULTS

Expression of GST Ya1 Protein and mRNA in Male and Female Rat Liver in Response to TAM Administration. Male and female rats were administered TAM at doses ranging from 0.5 mg/kg to 50 mg/kg, liver cytosol was prepared, and GST subunit protein quantified by HPLC as described in “Materials and Methods.” Fig. 1 depicts a representative HPLC chromatogram of control male rat liver protein. This method of analysis revealed that TAM administration had significant effects on the expression of selected GST protein subunits in rat liver cytosol. GST Ya1 subunit expression (Fig. 2B) was suppressed approximately 50% at all doses in female rats. This suppression was also observed, to a slightly lesser degree, in male rats. Northern blot analysis with an oligonucleotide probe specific for GST Ya1 (49) demonstrated that this suppression was a result of decreased GST Ya1 mRNA levels in both sexes (Fig. 2, A and B). Female and male GST Ya1 mRNAs were maximally suppressed 60 and 70%, respectively. It is of interest to note that suppression of both mRNA and protein was produced at the 0.5 mg/kg dose, a dose directly equivalent, on a mg/kg b.w. basis, to the dose women receive for disease management. As evident in Fig. 2, GST Ya1 is a PB-inducible GST isofrom. Thus, TAM-induced suppression of GST Ya1 cannot be classified as a PB-like effect.

Hepatic GST Ya2 Expression in TAM-treated Male and Female Rats. Female GST Ya2 subunit expression was suppressed 40% at the 0.5 mg/kg dose but increased steadily from this level to approximately 80% of control over the course of the dose-response curve (Fig. 3B). Male GST Ya2 subunit expression was induced to a maximum of approximately 210% of control at the highest dose tested. Northern blot analysis using a GST Ya2-specific oligonucleotide probe (49) revealed that these changes, in both male and female rats, were the result of similar changes in hepatic GST Ya2 mRNA levels (Fig. 3, A and B). As illustrated in Fig. 3, GST Ya2 is strongly inducible by PB and polycyclic aromatic hydrocarbons. Thus, the suppressive effect of TAM in females is not consistent with the properties of a PB-like inducer.

Hepatic GST Yc1 mRNA and Protein Expression in TAM-treated Rats. Fig. 4 depicts the effects of TAM administration on levels of cytosolic GST Yc1 subunit protein and mRNA levels. GST Yc1 protein was induced in males up to 170% of control at the highest dose tested. Similarly, male GST Yc1 mRNA was induced to approximately 190% of control. Female GST Yc1 protein and mRNA levels were not affected significantly by TAM treatment (data not shown).

Expression of Rat Liver GST Yb1, Yb2, Yb3, Yp, and Yl after TAM Exposure. HPLC analysis revealed that the expression of all the remaining GST subunit isoforms present in rat liver that were detectable with our HPLC technique, including GST Yb1, Yb2, Yb3, Yp, and Yl, were not affected by TAM treatment (data not shown). Similarly, the total expression of all forms combined was unchanged in either sex (data not shown).
TAMOXIFEN AFFECTS PHASE II EXPRESSION

A.

Fig. 3. GST Ya2 mRNA and protein expression in male and female rat liver after TAM administration. Rats were administered TAM, and GST Ya2 mRNA and protein levels were evaluated as described in “Materials and Methods.” A. Northern blot analysis of mRNA, with the dose of TAM administered, in mg/kg b.w., denoted above each lane. Samples from MC- and PB-treated female rats were used as internal standards for interblot comparisons. B. Quantitative changes in mRNA (□) and protein (●). Left Y axis, pmol GST Ya2 subunit protein/mg cytosolic protein; right Y axis, mRNA expression expressed as a percentage of control. The dose of TAM administered, in mg/kg, is indicated on the X axis. Points, the mean of three separate animals; bars, SE (those smaller than the symbol size are not shown). *, significantly different (P < 0.05) from the respective vehicle-treated control group as determined by Dunnett’s t test.

Cytosolic GST Activity toward Δ5-Androstene-3,17-dione and CDNB after TAM Administration. Because TAM treatment resulted in a dramatic suppression of GST Yal protein and mRNA in both sexes, it was of interest to determine whether GST Yal-associated enzyme activity in TAM-treated liver cytosol was also suppressed. Accordingly, GST conjugation activity toward Δ5-androstene-3,17-dione, a GST Yal- and Ya2-specific substrate (55), was assessed. In agreement with the observed suppression of GST Yal and Ya2 protein, the rate of enzymatic conversion of Δ5-androstene-3,17-dione was suppressed 45% in females at 0.5 mg/kg and 30% in males at 20 mg/kg, as shown in Fig. 5. The enzymatic activity data demonstrated a remarkable concordance with the sum of GSTYa1 and Ya2 protein expression data in each sex. The decrease in Δ5-androstene-3,17-dione activity was specific, because total GST activity, as measured by the rate of CDNB conjugation, did not change (Fig. 5). A lack of effect on CDNB conjugation is consistent with the finding that there was no overall change in sum-total cytosolic GST protein in males or females after TAM administration (data not shown).

ST mRNA Expression after TAM Administration. To evaluate the effect of TAM on rat liver ST mRNA expression, oligonucleotides specific for the major ST isoforms in rat liver were designed, synthesized, and used for Northern blot analysis as described in “Materials and Methods.” The probes are described in detail in Table 1. Through this method of analysis, it was observed that ST2A2, a female-specific ST, was induced 120% in males and suppressed 50% in females (Fig. 6) after treatment with 50 mg/kg TAM. mRNA expression for ST1A1, ST1C1, and ST1E2, which constitute the remaining major rat liver ST genes, was unaffected by TAM treatment (data not shown).

UGT mRNA Expression after TAM Administration. Similar to our investigation of ST mRNA expression, Northern blot analysis was
TAMOXIFEN AFFECTS PHASE II EXPRESSION

A.

Male

Female

0 0.5 5 20 35 50

0 0.5 5 20 35 50

0 0.5 5 20 35 50

0 0.5 5 20 35 50

0 0.5 5 20 35 50

0 0.5 5 20 35 50

Fig. 6. Effect of TAM administration on ST2A2 mRNA expression in rat liver. TAM was administered, and ST2A2 mRNA levels were assessed by Northern blot analysis, as described in “Materials and Methods.” A, dose of TAM administered, in mg/kg, is indicated above each lane. Samples from MC- and PB-treated female rats were used as internal standards for interblot comparisons. B, quantitative changes in ST2A2 mRNA in males (□) and females (○). The values depicted on the Y axis are the values reported by the PhosphorImager apparatus and represent the average pixel density (× 10^4) of each band. O, misloaded sample (first lane of 20-mg/kg male group), which was not included in the statistical analysis. X axis, dose of TAM administered, in mg/kg. Points, means of three separate animals; bars, SE (those smaller than the symbol size are not shown). *, significantly different (P < 0.05) from the respective vehicle-treated control group as determined by Dunnett’s t-test.

B.

Fig. 7. Increase in UGT2B1 mRNA expression in male and female rats after TAM exposure. Rats were administered TAM, and UGT2B1 mRNA levels were evaluated by Northern blot analysis as described in “Materials and Methods.” A, the dose of TAM administered, in mg/kg, is indicated above each lane. Samples from MC- and PB-treated female rats were used as internal standards for interblot comparisons. B, quantitative changes in UGT2B1 mRNA in males (□) and in females (○). The values depicted on the Y axis are the values reported by the PhosphorImager apparatus and represent the average pixel density (× 10^4) of each band. The X axis is the dose of TAM administered, in mg/kg. Points, means of three separate animals; bars, SE (those smaller than the symbol size are not shown). *, significantly different (P < 0.05) from the respective vehicle-treated control group as determined by Dunnett’s t-test.

performed using oligonucleotides specific for major rat liver UGT isoforms. These oligonucleotides were designed, synthesized, and utilized as described in “Materials and Methods.” Table 2 describes the probes in detail. With this method of analysis, it was determined that TAM administration produced significant effects on UGT2B1 mRNA expression in both sexes. mRNA levels in females and males were maximally induced approximately 100% and 320%, respectively, at the 50 mg/kg dose (Fig. 7). TAM administration was shown to have no effect on UGT106P, UGT2B2, UGT2B3, UGT2B6, or UGT2B12 mRNA (data not shown). Because UGT2B1 is the major PB-inducible UGT isozyme in rat liver (56), these results are consistent with the documented PB-like properties of TAM.

DISCUSSION

Our results show that TAM treatment resulted in significant alterations in rat liver GST expression. Some of these effects, including suppression of GST Ya1, were produced at a dose in rats that is directly equivalent, on a mg/kg b.w. basis, to the doses women receive for disease management. In addition, TAM induced UGT2B1, a PB-inducible enzyme, in both males and females. Further, ST2A2, a female-predominant ST, was induced in males and suppressed in females by TAM.

The potential role that these specific TAM-induced changes in phase II enzyme expression may play in the carcinogenicity or therapeutic activity of the drug is not known. The difficulty in assessing this question lies with the fact that the exact isoenzymes responsible for the metabolic activation and excretion of TAM have not been identified. However, it is clear that TAM and PB can induce enzymes responsible for at least some of the metabolic activation of TAM, or conversely, suppress enzymes responsible for some of its detoxication (14, 17, 18, 24, 25). The results from this study, in combination with work on phase I expression (44, 45), provide a comprehensive picture of TAM-induced changes in liver enzyme expression. Taken in sum, these data allow us to suggest several possible isoenzymes that might be involved in the induction or suppression pathways.

TAM is an effective inducer of CYP1I1, CYP1I2, CYP1I1A (44, 45), and UGT2B1. Thus, based on the extent of current data, these isoenzymes are the most likely candidates involved in a putative induction pathway. CYP1I1A is known to be involved in the metabolism of TAM (24, 57, 58) and may be responsible for the metabolic activation of the drug to a reactive electrophile (24). Of further interest is that this subfamily is inducible in humans (59). The CYP1I1 subfamily may also play a role in the metabolism of TAM (58), and CYP1I1C6 is PB inducible (60), but it is not known whether it is also inducible by TAM. In the case of UGT2B1, this isozyme could be involved in the TAM-induced genotoxicity pathway via glucuronidation of α-hydroxy-TAM, resulting in formation of the reactive carbocation.
versely, induction of UGT2B1 could result in increased clearance of the drug via conjugation of 4-hydroxy-TAM (33).

Because GST Yal is the only gene that is suppressed in both sexes, this isozyme is the most likely candidate for the "supression of detoxication" hypothesis. Other possibilities include ST2A2 and GST Ya2, although these enzymes are less likely candidates because the suppression was sex specific, whereas the carcinogenicity of TAM is not (7). TAM treatment results in decreased cellular glutathione levels and increased peroxidation potential of microsomal preparations (61), possibly as a result of superoxide anion formation (25). It is known that GSTs can provide protection against reactive oxygen species and the breakdown products of oxidized DNA and lipids (62, 63). Thus, the increased susceptibility to oxidative stress in the liver after TAM treatment (25, 61), coupled with or as a result of significant suppression of specific GST isoforms, could be involved in the carcinogenicity of TAM.

Data from the present investigation, combined with previously published findings (44, 45), suggest that the effects of TAM on enzyme expression can be grouped into two general categories: PB-like and non-PB-like. PB-like properties of TAM include induction of GST Ya2 and Yc1 in males and CYPIIB1, CYPIIB2, CYPIIA1, epoxide hydrolase, aldehyde dehydrogenase (44), and UGT2B1 induction in both sexes. Properties of TAM that are not PB-like include GST Ya1 suppression (both sexes) and GST Ya2 suppression (females). In addition, mRNA levels for CYPIIC7* and ST2A2 were suppressed in females and induced slightly in males after TAM treatment, but are not affected by PB administration. In the case of CYPIIC7 and ST2A2, changes in mRNA expression could be due to the partial agonist activity of TAM at the estrogen receptor; mRNA levels in females decreased, whereas levels in males were increased. This supposition is supported by the fact that both genes are female predominant, and the responses were sexually dimorphic. The mechanism by which TAM affects GST expression is unknown. However, treatment with the anticancer drug cisplatin results in a very similar pattern of GST expression as the pattern produced by TAM administration. Both drugs cause a dramatic suppression of GST Ya1, induction of GST Yc1, and no change in GST Yb1 or Yb2 (49). In addition, both cisplatin (42) and TAM cause suppression of CYPIIC7 in females and induction in males. Cisplatin treatment results in decreased serum testosterone (42, 64), which in turn can cause a suppression of circulating growth hormone (65), a known regulator of GST Yal, the profile in male and female rats (67), suggesting that this hormone is relevant to dosing regimens used in breast cancer patients.

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4 Unpublished observations.

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