Sex Differences in the Activation of Tamoxifen to DNA Binding Species in Rat Liver in Vivo and in Rat Hepatocytes in Vitro: Role of Sulfotransferase Induction

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ABSTRACT

Previous work has indicated that metabolic activation of tamoxifen in rat liver cells involves cytochrome P450-mediated o-hydroxylation, followed by sulfate ester formation, mediated by hydroxysteroid sulfotransferase \( a \) (rHSTa), a member of the SULT2A subfamily, which efficiently metabolizes dehydroepiandrosterone. Because it is known that the expression of rHSTa and other SULT2A forms is substantially higher in female rats than in males, it might be predicted that tamoxifen would be a more potent liver carcinogen in females than in males. Yet tamoxifen has been shown to be equipotent in both sexes. To investigate this paradox, primary cultures of hepatocytes were prepared from Fischer F-344 rats and treated with tamoxifen (10 \( \mu M \)) or o-hydroxytamoxifen (1 \( \mu M \)). Rats were also treated with tamoxifen daily by gavage (0.12 mmol/kg/day) for up to 14 days. DNA was isolated from hepatocytes and liver and analyzed by multiprobe primer PCR technique, it was found that it was primarily rHSTa that was induced. Thus, after prolonged exposure to tamoxifen, DNA adduct formation and rHSTa expression in males are significantly lower than in female cells. Male and female rats showed only very low activity toward ST20, the only known human SULT2A enzyme (also termed hHST2, STa, hydroxysteroid sulfotransferase 40/41, ST-40/41, ST2A2, STI, and rHSST2) is capable of activating o-hydroxytamoxifen (38–40). It is a member of the SULT2A subfamily. Other rat SULT2A enzymes, HST 20 (also termed rHST20, ST-20/21, ST2A1, STHST21) and HST 60 (also termed rHST60, ST-60, ST2A5, or rHSST3) did not activate o-hydroxytamoxifen. Nomenclature for the individual enzyme forms has been proposed but not yet finalized. For this reason, we use the same names in the present paper as we have used in previous publications but add other names at their first appearance. We and others have shown that rat HSTa (also termed rHSTa, STa, hydroxysteroid sulfotransferase 40/41) is the major DHEA sulfotransferase in the liver of adult female rats. It is known, however, that DHEA sulfotransferase activity is not rodent carcinogens will be safer alternatives for women.

INTRODUCTION

Tamoxifen, an antiestrogen widely used in adjuvant therapy for breast cancer (1), has been found to prevent breast cancer in healthy women at high risk of developing the disease (2–4). Concerns over its long-term safety, particularly in chemoprevention strategies, have been raised from the demonstration that it is a potent liver carcinogen in the rat (5–9) and causes an increase in the incidence of endometrial cancer in women (10, 11). To fully assess the long-term safety of the therapeutic and prophylactic use of tamoxifen, it is necessary to elucidate the mechanism(s) of tamoxifen carcinogenesis and whether other antagonists that are not rodent carcinogens will be safer alternatives for women.

Despite giving negative results in standard short-term tests for mutagenicity/carcinogenicity, tamoxifen exhibits genotoxic activity in a number of ways; it forms covalent adducts with DNA in rodent liver (12, 13), it causes gene mutations in vivo in lacI transgenic rats (14), it induces aneuploidy in rat liver (15, 16), and it induces micronuclei in human cells expressing human CYP enzymes (13, 17–20). Moreover, it binds covalently to protein in the presence of human liver microsomal preparations (21, 22).

Tamoxifen is activated to products that bind to DNA in rat liver cells initially by \( o \)-hydroxylation (18, 23–27), followed by sulfate ester formation (28, 29). Some of the DNA adducts formed result from additional \( N \)-demethylation of tamoxifen (30–32).

The xenobiotic-metabolizing sulfotransferases are cytosolic enzymes that are encoded by the SULT superfamily [member(s) of the SULT (cytosolic sulfotransferases) gene/enzyme superfamily; Refs. 33–35]. A classification into subfamilies 1A, 1B, 1C, 1E, 2A, 2B, and 3A according to the degree of the similarities of the deduced amino acid sequences is generally accepted. Nomenclatures for the individual enzyme forms have been proposed but not yet finalized. For this reason, we use the same names in the present paper as we have used in previous publications but add other names at their first appearance. We and others have shown that rat HSTa (also termed rHSTa, STa, hydroxysteroid sulfotransferase 40/41) is the major DHEA sulfotransferase in the liver of adult female rats. It is known, however, that DHEA sulfotransferase activity is not rodent carcinogens will be safer alternatives for women.

The only known human SULT2A enzyme (also termed hHST2, human dehydroepiandrosterone sulfotransferase, or ST2A3) showed only very low activity toward \( o \)-hydroxytamoxifen (38–40). All studies published to date on tamoxifen-DNA adduct formation in rats or rat hepatocytes have been limited to studies of female animals. It is known, however, that DHEA sulfotransferase activity is substantially higher in adult females than in adult males (42, 43). Studies on the protein and enzyme activity level indicate that STI (rHSSTa) is the major DHEA sulfotransferase in the liver of adult female rats. The abbreviations used are: HSTa, hydroxysteroid sulfotransferase \( a \); SMP, senesence marker protein; DHEA, dehydroepiandrosterone; HPLC, high-performance liquid chromatography; ECL, enhanced chemiluminescence; EST, estrogen sulfotransferase; PP, primer pair.

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females; this form was not detected and, therefore, is absent or very low in the liver of males (42). Likewise, ST-40/41 (rHSTa) RNA was detected in liver of adult female rats but not in adult male liver (44). Paradoxically, in long-term feeding studies tamoxifen is equipotent as a liver carcinogen in both sexes (6).

Tamoxifen treatment has been shown to alter the levels of expression of a number of phase I and phase II enzymes in rat liver (45–47). In the present study, we have examined the influence of sex on tamoxifen-DNA adducts formation and SULT2A expression in rat liver in vivo and in primary cultures of hepatocytes in vitro. We show that chronic exposure to tamoxifen results in induction of rHSTa in males only, such that DNA adduct levels, initially much lower in males than in females, become similar in both sexes after multiple treatments.

MATERIALS AND METHODS

Isolation and Treatment of Rat Hepatocytes. Primary cultures of hepatocytes were prepared from the livers of untreated male and female Fischer F-344 rats, ages 6–8 weeks, as described previously (24). After 3 h preincubation, the medium was changed (24), and tamoxifen (Sigma Chemical Co., Poole, Dorset, United Kingdom) dissolved in DMSO was added to give a final concentration of 10 μM. The cultures were incubated for 18 h, after which cells were harvested, and DNA was isolated (48). Hepatocytes were also treated with 1 μM α-hydroxysteromifen, synthesized as described previously (49). Experiments were carried out in triplicate on at least two independent hepatocyte preparations in each case.

Treatment of Rats. Male and female Fischer F-344 rats, ages 6–8 weeks, were treated by gavage with 0.12 mmol/kg tamoxifen in triacyclin (1.0 mL/kg; Sigma) daily for 1, 4, 7, or 14 days. Animals were killed (at least three/group) 24 h after their last dose by cervical dislocation, and their livers were removed and stored at −80°C prior to DNA isolation or cytosol preparation. Portions of the thawed livers were homogenized, and DNA was isolated and purified by a phenol/chloroform extraction procedure (48). The liver from each animal was processed and analyzed individually.

32P-Postlabeling Analysis. DNA isolated from rat hepatocytes or liver was subjected to 32P-postlabeling analysis using the nuclease P1 digestion method for sensitivity enhancement and the solvents for TLC on polyethyleneimine-cellulose of the labeled digests essentially as described elsewhere (25). Solvents for chromatography were: D1, 2.3M sodium phosphate, pH 5.8; D2, 2.275 M lithium formate, 5.525 M urea, pH 3.5; D3, 0.52 M LiCl, 0.325 M Tris-HCl, 5.525 M urea, pH 8.0. Chromatography were scanned for radioactivity using an InstantImager (Canberra Packard, Pangbourne, Berks). Relative levels of DNA modification were calculated from the levels of radioactivity in the cDNA using the ECL system, together with Hyperfilm ECL (Amersham). To differentiate between the rat SULT2A forms, specific PPs for each cDNA sequence were selected after a multiple sequence alignment of the cDNA sequences listed in gene databases: PP2 for rHST20/21 (accession no. M31363), 5′-CGTGCCTGGCTTCTCATGCA and 5′-AGTGGCTTCTTTCATGAGGC; PP3 for HSTa (accession no. M33329), 5′-TCTTCAAGAGGATGTTCCG and 5′-CAGTCTCTCTCCATGAGAT; PP4 for rHST60 (accession no. D14989), 5′-TCTTCAAGAGGATGTTCCG and 5′-AAGTGTACTTCCAGTCTT; and PP5 for SULT2A1, 5′-GATCATTAAAGGA-GATCGGTTTAC and 5′-AGAAGCGTTATCCGACATCCGTC. PCR was carried out using 1 unit REDTaq DNA-polymerase (Sigma), 0.4 μM of each primer, and 1 mM deoxynucleotide triphosphates in a final volume of 50 μL. After an initial denaturing step of 5 min at 95°C, 40 cycles (30 cycles in case of β-globin) of denaturing (94°C, 1 min), annealing (varying temperatures, 30 s), and elongation (72°C, 1 min) were performed. For the PCR, the samples were adjusted to amplify equivalent amounts of β-globin cDNA. To monitor if, under the selected conditions for a given PP, exclusive amplification of the regarded cDNA occurred, PCR was carried out in parallel with the cloned cDNAs of each form. Using this control cDNA, the annealing temperature of each PP could be adjusted to amplify only its specific cDNA (PP1 at 59°C, PP2 at 72°C, PP3 at 58°C, and PP4 at 62°C). In the case of SULT2A1, no cloned cDNA was available; thus, various PCRs were carried out with PP5 at different annealing temperatures (50, 55, and 60°C). Subsequently, 10 μl of each PCR reaction were analyzed by 1.5% agarose gel electrophoresis.

Statistical Analysis. Differences between results with males and females were tested for statistical significance by the Mann-Whitney U test (two-tailed). A paired t test was used to compare relative amounts of different tamoxifen-derived adducts. Changes in sulfotransferase activity in males and females were tested for by one-way ANOVA.

RESULTS

Tamoxifen-DNA Adduct Formation in Rat Hepatocytes. Treatment of female rat hepatocytes with 10 μM tamoxifen for 18 h resulted in a 32P-postlabeling pattern of DNA adducts on TLC (not shown) similar to that reported previously (24, 25). The pattern obtained with male hepatocytes was qualitatively similar, but the level of adducts formed was 11-fold lower (Fig. 1). Similarly, treatment with 1 μM α-hydroxysteromifen resulted in a 14-fold lower level of DNA adducts in male hepatocytes compared with female hepatocytes (Fig. 1); the pattern of adducts observed (not shown) was the same as seen with tamoxifen and as reported previously (24, 25).

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Tamoxifen-DNA Adduct Formation in Rat Liver. Twenty-four h after administration of a single oral dose of tamoxifen to rats, the level of liver DNA adducts detected in males was less than one-sixth the level formed in females (Fig. 2). There was an increase in adduct levels in both sexes after four daily doses, but the males still had a 5-fold lower level than the females. After 7 days of daily dosing, still higher levels were obtained, with the males having one-third of the adduct level of the females. At each of these time points, the difference between males and females is statistically significant (P < 0.01). After 14 days of multiple treatment, adduct levels in males were 87% of those in females, a
fig. 1. DNA adduct formation in male and female rat hepatocytes treated with tamoxifen (0.12 mmol/kg by gavage daily). Columns, results from at least three cultures; bars, SD.

Fig. 2. DNA adduct formation in liver of male and female Fischer F-344 rats treated with tamoxifen (0.12 mmol/kg by gavage daily). Columns, results from three animals; bars, SD.

difference that was not statistically significant ($P > 0.1$). Thus, in males the adduct levels were 60-fold higher after 14 days than they were after 1 day, whereas in females, the increase was only 11-fold.

**HPLC Analysis of Tamoxifen-DNA Adducts.** HPLC analysis of $[^3]P$-labeled digests of DNA from liver cells that have been exposed to tamoxifen in vivo or in vitro reveals two major peaks and a number of minor ones (31). Of the two major peaks, the earlier eluting one (peak 2) derives from the binding of the $\alpha$-position of $N$-desmethyltamoxifen, probably to the $N^2$-position of guanine moieties in DNA, whereas the later eluting peak (peak 3) derives from the binding of the $\alpha$-position of tamoxifen itself (31).

After a single treatment with tamoxifen, 2.3 times more adducts in female rat liver are derived from tamoxifen than from $N$-desmethyltamoxifen ($\pm 30\%$; $n = 3$; $P = 0.04$, paired $t$ test, two-tailed), but after 14 days the latter accounts for 1.4 times as much adduct as the former ($\pm 5\%$; $n = 3$; $P = 0.0019$). After 14 days of treatment, the ratio of these adducts in male liver is similar to that in females. At earlier time points, the ratios were more variable, possibly because of the fact that significantly lower levels of adducts were formed, making the determination of the relative peak sizes less precise.

**DHEA Sulfotransferase Activity in Rat Liver.** After a single treatment with tamoxifen, DHEA sulfotransferase activity in male rat liver was less than one-seventh of that in female liver (Fig. 3), a difference that was statistically significant ($P = 0.027$). With subsequent treatment, activity rose in males, and after 14 days of treatment, it had increased 5-fold (highly significant, $P < 0.001$). In females, there was a slight increase up to 7 days, but at 14 days, the increase over 1 day was $< 30\%$, which was not statistically significant ($P = 0.09$). Furthermore, after 14 days, the mean level of sulfotransferase activity in males had risen to 62% of that in females, from being only 14% after 1 day. Thus, tamoxifen induces DHEA sulfotransferase activity in liver of male rats but not in females.

**Levels of SULT2A Proteins in Rat Liver.** Tamoxifen treatment of male rats caused a time-dependent increase in SULT2A protein levels (Fig. 4A, lower band). In contrast, SULT2A expression in female rat liver (Fig. 4B) was high at the beginning of the treatment and only marginally increased by tamoxifen treatment. Expression of members of the SULT1 family, for example rat ST1C1 (Fig. 4A, upper band), or rat EST (Fig. 4C) was not affected in males. These enzymes are expressed very weakly in female rats and could not be detected with AB1 or AB2 in females at any time point. Thus, the induction of SULT2A by tamoxifen in male rats is highly specific. The different SULT2A proteins show very similar electrophoretic mobilities and immunoreactivities. Therefore, the induced SULT2A form(s) could not be identified conclusively by immunoblotting.

**Identification of Individual SULT2A Forms at the RNA Level.** For the identification of the induced SULT2A form(s), RT-PCR with specific primers for each form was carried out with the male rat samples of day 1 and day 7. The analysis showed a fragment of rHSTa cDNA in the liver of those males that were treated for 7 days with tamoxifen but not in animals treated for only 1 day (Fig. 5). Under the selected conditions, none of the other related cDNAs could be detected at any time investigated. These data clearly indicate that rHSTa was induced. With the primers designed for rHST20 and rHST60, strong signals were obtained when the cor-
responding cDNA was used as a template. However, no signal was obtained when the cDNA of a different SULT2A form was used as template, indicating specificity of the detection method. Only weak, approximately equal signals were found when RNA from the 1- and 7-day treatment groups was used as a template for the PCR analysis with the primers for rHST20 and rHST60, showing that expression of these forms was not significantly affected by the tamoxifen treatment. Likewise, no amplification products were detected with the primers designed for SMP-2.

**DISCUSSION**

Detection and characterization of DNA adducts is an informative way of investigating the pathways of metabolic activation of carcino- genic compounds. Of several methods available, 32P-postlabeling analysis (55–57) has the advantages of sensitivity and of providing both qualitative and quantitative data on adducts. In the case of tamoxifen, much of our current knowledge on the mechanism of genotoxicity of this drug has come from postlabeling studies. It is now clear that the principle pathway by which the drug is activated to DNA-binding products in rat liver cells involves hydroxylation at the α-position (18, 23–25, 51), followed by sulfation by SULT2A enzyme(s) (28, 29, 39, 40). Recent studies have indicated that a proportion of the adducts are also N-demethylated, while still being activated by the α-hydroxylation and sulfation pathway (30–32).

DHEA sulfotransferase activity is very low in the liver of newborn rats but increases in both sexes up to 21 days of age (42, 43). Thereafter, however, males and females differ significantly. In males, activity declines by 8 weeks of age to the low levels seen at birth, whereas in females, activity continues to rise until about 6 weeks of age, after which it also declines, but to a level that is 10-fold higher than in males (43). Singer et al. (42) have described differing sulfotransferase enzyme activities and protein patterns, using anion-exchange column chromatography, in male and female rats, and analogous sex differences have been observed in levels of SULT RNA expression (44). The present study shows that DHEA sulfotransferase activity and SULT2A protein levels are relatively constant in female rat liver through the 2 weeks of daily treatment of tamoxifen, whereas the levels in males are dramatically increased by treatment. A previous study showed that rHSTa (ST2A2) RNA levels in male rat liver were increased by tamoxifen treatment, whereas the levels in female liver were reduced (47). Here, we also show that rHSTa RNA increases in a time-dependent manner. In addition, we have expanded our studies on the effect of tamoxifen treatment to the other known forms of the SULT2A subfamily. We have found that rHSTa RNA is indeed induced exclusively among the SULT2A enzyme forms. Furthermore, we have shown that not only RNA but also protein and enzyme activity levels are increased. The significance of our finding is that the SULT form, which is specifically induced in male rats, is the form rHSTa, which is responsible for metabolic activation of tamoxifen. As a consequence, tamoxifen-DNA adducts in males increase rapidly with prolonged exposure to tamoxifen until, by 14 days, they are similar to those in females.

Tamoxifen has been demonstrated to induce unscheduled DNA synthesis in primary cultures of hepatocytes prepared from rats that had been treated with tamoxifen in vivo but not in hepatocytes from untreated ones (13). This implies that tamoxifen induces enzymes responsible for its activation and/or suppresses the activity of enzymes involved in its detoxification. Phase I enzymes induced by tamoxifen include CYP2B1, CYP2B2, CYP3A, and microsomal epoxide hydrolase (45, 46); the expression of CYP1A1 and CYP1A2 was unaffected. Phase II enzymes whose expressions were suppressed in rat liver by tamoxifen include forms of glutathione S-transferase (47). An interesting finding in the present study is the changing relative amounts of the desmethyltamoxifen- and tamoxifen-derived DNA adducts in liver after prolonged treatment with tamoxifen. Although this could be the result of slower DNA repair of the demethylated (i.e., monomethylated) adduct compared with the dimethylated (i.e., tamoxifen) one, a more likely explanation is that demethylation is induced by tamoxifen treatment.

The result of tamoxifen treatment is an increase in sulfotransferase activity in males, resulting from an increase in the amount of enzyme produced; because this is the enzyme that is responsible for the metabolic activation of α-hydroxytamoxifen to the ultimate DNA binding intermediate (28, 29, 39, 40), adduct levels also increase, and there is a close association between all three events. Therefore, the results show a close association between levels of DNA adduct formation and sulfotransferase activity. The induction in males to levels similar to those in females, and the concomitant rise in adducts to similar levels, correlates with the equal susceptibility of males and females to liver carcinogenesis by tamoxifen (6).

Thus a potential lack of correlation between DNA adduct formation and carcinogenicity is avoided. The male rat liver becomes feminized with regard to rHSTa expression, making male rats equal to females in their ability to form tamoxifen-DNA adducts with prolonged exposure to the drug and equally susceptible to liver tumour induction. Toremifene and idoxifene, structural analogues of tamoxifen, form few if any adducts in rat liver cells (8, 13, 58–61) and are not carcinogenic to rat liver (8, 58, 59). The fact that tamoxifen also causes mutations in the livers of rats treated for 1 day; Lanes 7–9, rats treated for 7 days; Lanes rHSTa, rHST20, and rHST60, primer specificity controls (plasmids with the cDNA inserted); Lane –, negative control. The size of the predicted PCR product from rHSTa cDNA is 258 bp.

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