

MODULATION OF HEPATIC CYP2A1, CYP2C11, AND CYP3A9 EXPRESSION IN ADULT RATS BY NEONATAL ADMINISTRATION OF TAMOXIFEN

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ABSTRACT:

To examine the effect of neonatal administration of tamoxifen on adult expression of hepatic cytochrome P-450 (CYP) enzymes and steroid 5 α -reductase, male and female Sprague-Dawley rats were injected s.c. with tamoxifen (20 μ g) or peanut oil (control) once daily at days 1 to 5 of age and sacrificed at 3 months of age. Neonatal tamoxifen treatment did not affect b.wt. or liver weight of adult male and female rats, but decreased testicular weight by approximately 40% in adult male rats. Neonatal administration of tamoxifen decreased hepatic microsomal testosterone 6 β - and 7 α -hydroxylase activities in adult female rats whereas it did not alter steroid 5 α -reductase activity. The same treatment increased testosterone 7 α -hydroxylase activity, but did not affect testosterone 6 β -hydroxylase or steroid 5 α -reductase activity in adult male rats. Immunoblot analysis indicated that neonatal tamoxifen treat-

ment decreased CYP2C11 protein level by 26% and increased CYP2A1 protein content by 2.6-fold in adult male rats, whereas it had no effect on CYP3A or CYP2B protein expression. The reduction in the CYP3A-mediated testosterone 6 β -hydroxylase activity in adult female rats was accompanied by a decrease in CYP3A9 mRNA expression. Analysis of serum hormone levels indicated that neonatal exposure to tamoxifen resulted in a decrease in serum 17 β -estradiol concentration in adult female rats, whereas it did not alter serum testosterone concentration in adult male rats. In summary, treatment of neonatal rats with tamoxifen produced a long-lasting effect on hepatic CYP2A1, CYP2C11, and CYP3A9 expression in addition to testicular weight and serum 17 β -estradiol concentration.

Tamoxifen is used clinically as the first line endocrine therapy for all stages of breast cancer in both premenopausal and postmenopausal women (Buzdar and Hortobagyi, 1998). A major effort in tamoxifen research has been the investigation of this drug as a chemopreventive agent in healthy women who are at high risk for developing breast cancer (Jordan, 1997). Recently, the Breast Cancer Prevention Trial reported that the prophylactic use of tamoxifen resulted in a 49% reduction in the incidence of breast cancer (Fisher et al., 1998), but two smaller European clinical trials did not show an effect by tamoxifen (Powles et al., 1998; Veronesi et al., 1998). This drug is now approved in the U.S. for breast cancer prevention.

Although the prophylactic administration of tamoxifen is expected to be beneficial, there is potential risk associated with the long-term use of tamoxifen, such as increased incidence of endometrial cancer (Fornander et al., 1989) and in utero exposure. In adult rats, studies

have shown that chronic administration of this drug leads to development of liver tumors (Williams et al., 1993). It is now widely accepted that metabolic activation of tamoxifen to reactive intermediates that can form adducts with DNA (Han and Liehr, 1992) and protein (Mani and Kupfer, 1991) is required for the carcinogenic effects of tamoxifen (Dehal and Kupfer, 1995). The bioactivation of tamoxifen is catalyzed by cytochrome P-450 (CYP)¹ enzymes, such as CYP3A, in both rats and humans (Mani et al., 1994). However, tamoxifen can also modulate the expression of the CYP enzymes (e.g., CYP3A) involved in its bioactivation and metabolism (White et al., 1993). Moreover, tamoxifen is structurally similar to diethylstilbestrol, a compound which is known to result in altered CYP expression and procarcinogen activation in adult life when administered neonatally (Dieringer et al., 1980). Therefore, it is important to determine the long-term effects of tamoxifen administration on the expression of CYP enzymes.

Recent case reports have suggested an association between the use of tamoxifen by women during pregnancy and the incidence of birth defects (Cullins et al., 1994; Tewari et al., 1997). Rodent studies have shown that neonatal administration of tamoxifen results in reproductive tract lesions and sexual behavior deficits similar to those produced by diethylstilbestrol (Poulet et al., 1997). The sexual behavior

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¹ Abbreviations used are: CYP, cytochrome P-450; GH, growth hormone; 4-MA, 17 β -N,N-diethylcarbamoil-4-methyl-4-aza-5 α -androstan-3-one; RT-PCR, reverse transcription-polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

deficits observed in tamoxifen-treated rats appear to be due to neonatal neuronal loss and altered differentiation of the medial preoptic area (Vancutsem and Roessler, 1997). Interestingly, these areas of the hypothalamus control the secretion of growth hormone (GH) (Shirasu et al., 1990), which is a major regulator of the sex-dependent expression of drug- and steroid-metabolizing CYP enzymes and steroid 5 α -reductase (Waxman and Chang, 1995).

Treatment of adult male and female rats with tamoxifen has been reported to cause a major suppression of GH pulse amplitude and nadir level that persists for at least 7 weeks after discontinuation of drug administration (Tannenbaum et al., 1992). This drug also decreases serum testosterone concentration in adult male rats (Bartke et al., 1978). Given that certain CYP enzymes and steroid 5 α -reductase are regulated by the sexually dimorphic pattern of GH secretion and are affected by androgens (Waxman and Chang, 1995), we hypothesize that neonatal exposure to tamoxifen has long-term consequences on the expression of these enzymes. It is important to study the developmental effects of tamoxifen because of the potential in utero exposure as a result of the use of this drug by pregnant women (Cullins et al., 1994; Tewari et al., 1997). A potential consequence of the in utero exposure to tamoxifen is a permanent, altered capacity to metabolize drugs, resulting in compromised therapeutic efficacy and enhanced systemic toxicity.

In the present study, we examined the effects of neonatal administration of tamoxifen on hepatic expression of CYP and steroid 5 α -reductase in adult male and female rats. Our findings indicate that neonatal exposure to tamoxifen results in a long-lasting and enzyme-selective modulation of CYP expression.

Materials and Methods

Chemicals and Reagents. Tamoxifen base and testosterone were bought from Sigma Chemical Co. (St. Louis, MO). Authentic 2 α -, 6 β -, 7 α -, and 11 β -hydroxytestosterone metabolite standards were bought from Steraloids, Inc. (Wilton, NH). NADPH and [4-¹⁴C]testosterone (58 mCi/mmol) were obtained from Boehringer Mannheim (Montreal, Quebec, Canada) and Amersham Canada Ltd. (Oakville, Ontario, Canada), respectively. 17 β -N,N-diethylcarbamoyl-4-methyl-4-aza-5 α -androstan-3-one (4-MA) was a gift from Merck Sharp and Dohme Research Laboratories (Rahway, NJ). Magnesium chloride, 10 \times PCR buffer II (100 mM Tris-HCl, pH 8.3, and 500 mM KCl), and AmpliTaq DNA polymerase were purchased from Perkin-Elmer Canada Ltd. (Rexdale, Ontario, Canada). TriZol, dithiothreitol, dNTP mix, oligo(dT)₁₂₋₁₆ primer, deoxyribonuclease I, and SuperScript II reverse transcriptase were bought from Canadian Life Technologies (Burlington, Ontario, Canada). Forward and reverse primers for CYP3A9 and cyclophilin were synthesized at the University of British Columbia Biotechnology Laboratory (Vancouver, British Columbia, Canada).

Animals. Timed pregnant Sprague-Dawley rats were obtained at postconception day 14 from Harlan Sprague-Dawley (Indianapolis, IN) and housed individually in a temperature-, light-, and humidity-controlled room with free access to water and laboratory rodent chow (PMI Feeds, Inc., Richmond, IN). Each pup was weighed on the day of birth, and sex of the animal was determined by measuring the anogenital distance.

Treatment of Animals. Pups were injected s.c. once daily with tamoxifen (20 μ g) at days 1 to 5 of age. A previous study has shown that this dosage of tamoxifen when administered during this neonatal period was effective in altering serum cholinesterase activity levels in adult rats (Lamartiniere et al., 1986). Control rats received an equal volume (50 μ l/pup/day) of the vehicle (peanut oil). Injection sites were immediately covered with Vetbond (3M Animal Care Products, St. Paul, MN) to prevent seepage. All rats were sacrificed at 3 months of age. To minimize any potential variations due to the stage of the estrus cycles, control female rats were sacrificed in estrus, proestrus, diestrus, and metestrus. Tamoxifen-treated female rats were in permanent estrus and, therefore, were sacrificed at the same stage.

Preparation of Liver Microsomes and Serum Samples. Rats were euthanized by decapitation. Livers were quickly excised, washed with ice-cold

saline, weighed, quick-frozen in liquid nitrogen, and subsequently stored at -80°C . Microsomes were prepared by differential ultracentrifugation as described previously (Shapiro et al., 1989). The final microsomal pellet was suspended in 50 mM Tris buffer (pH 7.4) containing 0.25 M sucrose. Aliquots were stored at -80°C until use. Blood was collected and allowed to clot. Serum was prepared by centrifugation and stored at -20°C until use.

Total CYP and Microsomal Protein Assays. Total CYP content was determined from the sodium dithionite-reduced carbon monoxide difference spectrum, using a molar extinction coefficient of 91 $\text{cm}^{-1}\text{mM}^{-1}$ (Omura and Sato, 1964). Microsomal protein concentration was determined using a Bio-Rad Protein Assay Kit.

Testosterone Hydroxylase Assay. Microsomal testosterone 2 α -, 6 β -, and 7 α -hydroxylase activities were measured by an HPLC method as described previously (Anderson et al., 1998), but with the following modifications. The incubation time was 5 min for microsomal samples from male rats and 15 min for samples from female rats, and 4-MA (2.5 μM final concentration) was added to incubations containing microsomes from female rats to inhibit 5 α -reduction of the testosterone substrate.

Steroid 5 α -Reductase Assay. Microsomal steroid 5 α -reductase activity was determined by the reduction of [4-¹⁴C]testosterone to 5 α -[4-¹⁴C]dihydrotestosterone. This assay was performed according to a thin-layer chromatographic method as described previously (Chang et al., 1996).

Purified CYP Standards. Purified rat CYP2A1, CYP2B1, CYP2C11, and CYP3A1 were included as standards in the immunoblot assays. CYP2A1 was purified from Long Evans rats and was provided by Dr. A. Parkinson (University of Kansas Medical Center, Kansas City, KS). CYP2B1, CYP2C11, and CYP3A1 were purified as described previously (Anderson et al., 1998).

Preparation of Antibodies. Sheep anti-rat CYP2A1 IgG was provided by Dr. P. E. Thomas (Rutgers University, Piscataway, NJ) and mouse anti-rat CYP2C12 monoclonal IgG (F22) was provided by Dr. E. T. Morgan (Emory University, Atlanta, GA). The anti-CYP2A1 IgG reacts primarily with CYP2A1, but also recognizes CYP2A2. However, these two proteins can be resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The preparation of rabbit anti-rat CYP2B polyclonal IgG, monospecific rabbit anti-rat CYP2C11 polyclonal IgG, and rabbit anti-rat CYP3A1 polyclonal IgG have been described previously (Anderson et al., 1998). The anti-CYP 3A1 IgG cross-reacts with CYP3A2 and may also recognize other CYP3A proteins that are unresolved from CYP3A1 on SDS-PAGE gels.

SDS-PAGE and Immunoblot Assay. SDS-PAGE, electrophoretic transfer, and densitometric quantification were performed as described previously (Anderson et al., 1998). The anti-CYP2A1 IgG (10 $\mu\text{g}/\text{ml}$), anti-CYP2B IgG (10 $\mu\text{g}/\text{ml}$), anti-CYP2C11 IgG (15 $\mu\text{g}/\text{ml}$), anti-CYP2C12 IgG (2.5 $\mu\text{g}/\text{ml}$), and anti-CYP3A1 IgG (50 $\mu\text{g}/\text{ml}$) were used at the concentrations listed. Assay conditions were optimized to ensure that color development did not proceed beyond the linear response range of the phosphatase reaction. Staining intensities of the bands were measured with a pdi 420 oe scanning densitometer connected to an IBM-type personal computer using Quantity One^R Version 3.0 software (pdi Inc., Huntington Station, NY).

Isolation of RNA. Total liver RNA was isolated using the TriZol reagent according to the manufacturer's protocol. Briefly, approximately 100 mg of frozen liver tissue was homogenized in 500 μl of TriZol and the suspension was extracted with 100 μl chloroform/isoamyl alcohol (49:1, v/v). The aqueous phase was transferred to a new tube and RNA was precipitated by the addition of 250 μl of ice-cold isopropanol and subsequently washed with 200 μl ice-cold 70% ethanol. The RNA pellet was air dried, suspended in 50 μl sterile 10 mM Tris buffer (pH 8) containing 1 mM EDTA, and stored at -70°C until use.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assay. cDNA synthesis was carried by incubating isolated liver RNA (2 μg) with 0.5 μg oligo(dT)₁₂₋₁₈ primer and diethylpyrocarbonate-treated water in a volume of 9 μl at 65 $^{\circ}\text{C}$ for 10 min. The mixture was then placed on ice. After the addition of 2 μl of 10 \times PCR buffer II, 4 μl of 25 mM MgCl₂, 1 μl of 10 mM dNTP, 1 μl of 0.1 M dithiothreitol, and 2 U of deoxyribonuclease I, each tube was incubated at 37 $^{\circ}\text{C}$ for 30 min, 75 $^{\circ}\text{C}$ for 5 min, and then cooled on ice. Reverse transcription was initiated by the addition of 200 U of SuperScript II reverse transcriptase. The mixture was then incubated at 42 $^{\circ}\text{C}$ for 20 min. A

TABLE 1

Body, liver, and testicular weights of adult rats treated neonatally with tamoxifen

Female and male rats were injected s.c. once daily with tamoxifen (20 μ g) or an equal volume (50 μ l) of peanut oil (vehicle) at days 1 to 5 of age and killed at 3 months of age. Results are expressed as mean \pm S.E. for eight individual rats per treatment group.

	Female		Male	
	Control	Tamoxifen	Control	Tamoxifen
Body weight (g)	257 \pm 2	253 \pm 5	379 \pm 5	354 \pm 5
Absolute liver weight (g)	8.70 \pm 0.07	8.50 \pm 0.36	13.60 \pm 0.35	13.50 \pm 0.41
Relative liver weight (% b.wt.)	3.38 \pm 0.04	3.36 \pm 0.11	3.59 \pm 0.06	3.81 \pm 0.10
Absolute testicular weight (g)	N/A ^a	N/A	3.86 \pm 0.08	2.22 \pm 0.06 ^b
Relative testicular weight (% b.wt.)	N/A	N/A	1.02 \pm 0.02	0.63 \pm 0.01 ^b

^a Not applicable.

^b Significantly different from the male control group ($p < .05$).

TABLE 2

Testosterone hydroxylase and steroid 5 α -reductase activities in hepatic microsomes from adult rats treated neonatally with tamoxifen

Female and male rats were treated neonatally with tamoxifen as described in Table 1. Rats were killed at 3 months of age. Hepatic microsomes were prepared and testosterone hydroxylase and steroid 5 α -reductase activities were determined as described in *Materials and Methods*. Results are expressed as mean \pm S.E. for eight individual rats per treatment group.

	Female		Male	
	Control	Tamoxifen	Control	Tamoxifen
Testosterone 2 α -hydroxylase activity ^a	none detected	none detected	1.25 \pm 0.20	0.80 \pm 0.12
Testosterone 6 β -hydroxylase activity ^a	0.037 \pm 0.007	0.012 \pm 0.004 ^c	0.58 \pm 0.08	0.65 \pm 0.17
Testosterone 7 α -hydroxylase activity ^a	1.25 \pm 0.08	0.96 \pm 0.07 ^c	0.16 \pm 0.01	0.41 \pm 0.02 ^c
Steroid 5 α -reductase activity ^b	2.33 \pm 0.16	1.96 \pm 0.05	0.13 \pm 0.03	0.16 \pm 0.01

^a Activity expressed as nmol/min/nmol total CYP.

^b Activity expressed as nmol/min/mg microsomal protein.

^c Significantly different from the same sex control group ($p < .05$).

negative control incubation was carried out in the same manner, but with the exclusion of the reverse transcriptase enzyme. Reaction was stopped by heating at 95°C for 5 min. The synthesized cDNA was stored at -20°C until use.

PCR coamplification of CYP3A9 and cyclophilin (internal control) cDNAs was performed based on a modification of a published method (Mahnke et al., 1997). Sequences for the forward (5'-GGA-CGA-TTC-TTG-CTT-ACA-GG-3') and reverse (5'-ATG-CTG-GTG-GGC-TTG-CCT-TC-3') primers for CYP3A9 (Mahnke et al., 1997) and those for the forward (5'-CTT-CGA-CAT-CAC-GGC-TGA-TGG-3') and reverse (5'-CAG-GAC-CTG-TAT-GCT-TCA-GG-3') primers for cyclophilin (Morris and Davila, 1996) were obtained from published studies. Each 25- μ l reaction mixture contained 1 \times PCR buffer II [10 mM Tris (pH 8.3), 50 mM KCl], 2 mM MgCl₂, 2 μ l cDNA, 400 μ M dNTP mix, 25 pmol each of the forward and reverse primers for CYP3A9 and cyclophilin, and 4 U of AmpliTaq DNA polymerase. PCR amplification was initiated by heating at 95°C for 1 min; this was followed by 28 cycles of the following: 30 s for denaturation at 94°C, 60 s for annealing at 60°C, and 60 s for extension at 72°C. A final incubation was carried out for 10 min at 72°C. Negative control incubations were carried out in the same manner, but with the exclusion of either cDNA or primers.

PCR amplification was carried out in a Peltier Model PTC-150 DNA thermal cycler (MJ Research, Inc., Watertown, MA). Preliminary experiments indicated that 28 cycles of PCR amplification were below the plateau phase of amplification for CYP3A9 and cyclophilin. To visualize the PCR products, 12 μ l of each preparation was subjected to agarose (1.7%) gel electrophoresis in the presence of ethidium bromide (0.08 μ g/ml). The gel was photographed using Polaroid 665 film and the intensity of each band was quantified using the Quantity One^R Version 3.0 software (pdi Inc., Huntington Station, NY). Each assay was performed in triplicate. Results are expressed as a ratio of the optical intensity of the CYP3A9 band to that of the cyclophilin band. This approach allows for the semiquantitation of CYP3A9 gene expression.

Serum Steroid Hormone Assays. Serum testosterone (Bahr et al., 1983) and 17 β -estradiol (Bahr and Ben-Jonathan, 1981) concentrations were measured according to procedures described previously.

Statistics. The significance of difference between the means of two groups was assessed by the independent *t* test. The level of significance was set a priori at $p < .05$.

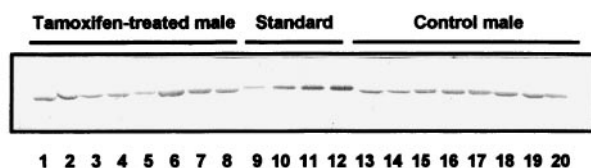
Results

Body Weight, Liver Weight, and Testicular Weight. Neonatal administration of tamoxifen did not affect the body weight or liver weight of adult female or male rats (Table 1). In contrast, neonatal tamoxifen treatment decreased both the absolute (42%) and relative (38%) testicular weight in adult male rats.

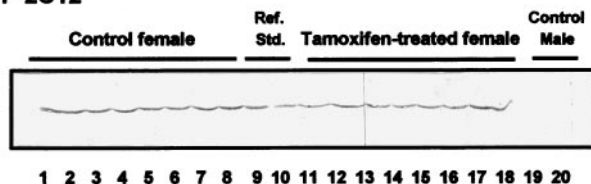
Hepatic Microsomal Testosterone Hydroxylase and Steroid 5 α -Reductase Activities. Microsomal testosterone 2 α -, 6 β -, and 7 α -hydroxylase activities are selective for hepatic CYP2C11, CYP3A, and CYP2A1, respectively (Waxman and Chang, 1995). Therefore, we used these activities as enzyme-selective catalytic markers. As shown in Table 2, neonatal administration of tamoxifen decreased testosterone 6 β -hydroxylase activity and testosterone 7 α -hydroxylase activity by 68 and 23%, respectively, relative to the vehicle-treated control group, whereas it did not affect steroid 5 α -reductase activity in adult female rats. Neonatal tamoxifen treatment increased testosterone 7 α -hydroxylase by 2.6-fold, but it had no effect on testosterone 6 β -hydroxylase or steroid 5 α -reductase activity in adult male rats. In the same microsomal samples, testosterone 2 α -hydroxylase activity was decreased by 36%, although this effect was not statistically significant ($p = .07$).

Immunoblot Analysis of Hepatic CYP2C11, CYP2C12, CYP2A1, and CYP3A Protein Levels. The results obtained with the testosterone hydroxylase assay (Table 2) suggested that neonatal tamoxifen treatment affected the adult expression of sex-dependent CYP enzymes. Therefore, immunoblot assays were performed to determine the levels of CYP2C11 (Fig. 1A), CYP2C12 (Fig. 1B), CYP2A1 (Fig. 1C), and CYP3A proteins (Fig. 1D) in adult male and female rats administered tamoxifen at days 1 to 5 of age. Densitometric analysis (Fig. 2, A-D) of the immunoblots showed that neonatal tamoxifen treatment decreased CYP2C11 protein content by 26%, increased CYP2A1 protein level by 58%, and had no effect on CYP3A protein expression in adult male rats. By comparison, neo-

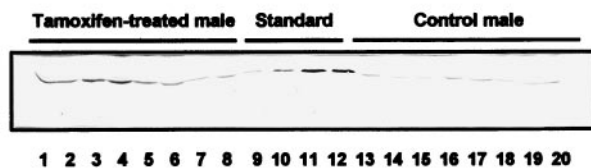
A. CYP 2C11



B. CYP 2C12



C. CYP 2A1



D. CYP 3A

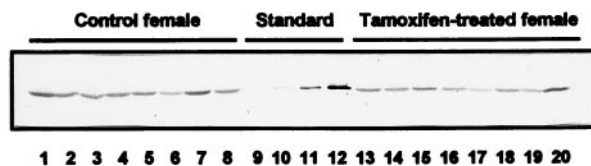


FIG. 1. Immunoblots of CYP2C11, CYP2C12, CYP2A1, and CYP3A proteins in hepatic microsomes isolated from adult rats treated neonatally with tamoxifen.

Female and male rats were treated with tamoxifen at days 1 to 5 of age as described in Table 1. Hepatic microsomes (4 pmol total CYP per lane for CYP2C11, 10 pmol total CYP per lane for CYP2A1 and CYP3A, and 20 pmol total CYP per lane for CYP2C12) and purified CYP standards were subjected to SDS-PAGE. Proteins were transferred electrophoretically onto nitrocellulose membrane. The immunoblot was probed with anti-CYP2C11 IgG (15 $\mu\text{g}/\text{ml}$, A), anti-CYP2C12 (2.5 $\mu\text{g}/\text{ml}$, B), anti-CYP2A1 IgG (10 $\mu\text{g}/\text{ml}$, C), or anti-CYP3A IgG (50 $\mu\text{g}/\text{ml}$, D) followed by alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (for CYP2C11 and CYP3A, 1:3000 dilution), anti-mouse secondary antibody (for CYP2C12, 1:3000 dilution), or anti-sheep secondary antibody (for CYP2A1, 1:250 dilution). Immunoreactive CYP proteins were detected as described in *Materials and Methods*. A: lanes 1 to 8, tamoxifen-treated males; lanes 9 to 12, purified CYP2C11 standards at 0.25, 0.5, 0.75, and 1 pmol/lane, respectively; and lanes 13 to 20, control males. B: lanes 1 to 8, control females; lanes 9 and 10, pooled microsomes from untreated, adult females; lanes 11 to 18, tamoxifen-treated female; and lanes 19 and 20, control males. C: lanes 1 to 8, tamoxifen-treated males; lanes 9 to 12, purified CYP2A1 standards at 0.125, 0.25, 0.375, and 0.5 pmol/lane, respectively; and lanes 13 to 20, control males. D: lanes 1 to 8, control females; lanes 9 to 12, purified CYP3A1 standards at 0.0025, 0.125, 0.2, and 0.5 pmol/lane, respectively; and lanes 13 to 20, tamoxifen-treated females.

natal tamoxifen treatment had no effect on CYP2C12, CYP2A1, or CYP3A in adult female rats. Although the mean hepatic CYP3A protein level was decreased by 28% in adult female rats treated with tamoxifen neonatally, this change was not statistically significant ($p = .09$).

RT-PCR Analysis of Hepatic CYP3A9 Gene Expression. The observation that neonatal tamoxifen treatment decreased hepatic microsomal testosterone 6 β -hydroxylase activity in adult female rats but not in adult male rats (Table 1) suggested that this drug altered the level of a CYP3A enzyme(s) expressed in female rats. It is now known that the rat CYP3A subfamily consists of several genes. Of the CYP3A forms identified to date, only CYP3A9 is female predominant

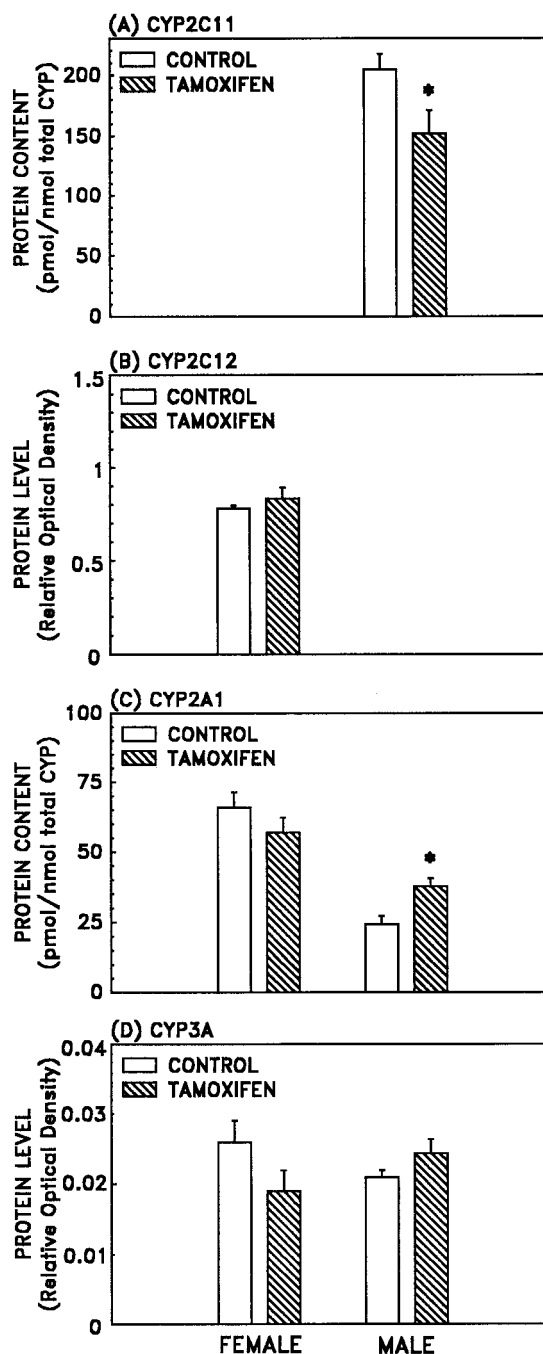


FIG. 2. Effect of neonatal administration of tamoxifen on hepatic CYP2C11, CYP2C12, CYP2A1, and CYP3A protein levels in adult rats.

Female and male rats were injected s.c. once daily with tamoxifen at days 1 to 5 of age as described in Table 1. Hepatic microsomes were isolated from 3-month-old rats as described in *Materials and Methods*. Results are expressed as mean \pm S.E. for eight individual rats per treatment group. *, significantly different from the same sex control group ($p < .05$). A: CYP2C11; B: CYP2C12; C: CYP2A1; D: CYP3A. Determination of CYP3A and CYP2C12 content was not possible because purified CYP3A enzymes other than CYP3A1 and purified CYP2C12 were not available for use as calibration standards for quantification. Thus, CYP3A and CYP2C12 protein levels are expressed as the optical density (OD \times mm) of the stained band relative to the optical density of the internal standard.

(Mahnke et al., 1997; Wang and Strobel, 1997; Robertson et al., 1998). Therefore, RT-PCR analysis (Fig. 3) was performed to assess hepatic CYP3A9 gene expression in adult female rats that were treated neonatally with tamoxifen. Densitometric analysis indicated

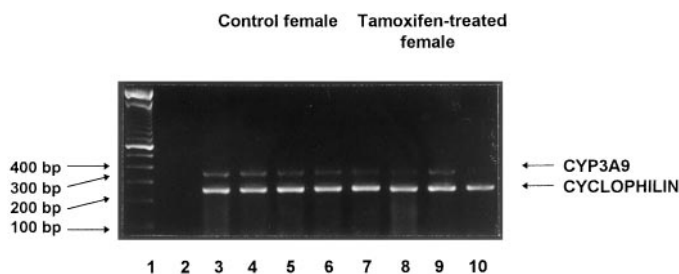


FIG. 3. RT-PCR analysis of hepatic CYP3A9 gene expression in adult female rats treated neonatally with tamoxifen.

Female rats were injected with tamoxifen at days 1 to 5 of age as described in Table 1. Total liver RNA was isolated and reverse transcribed as described in *Materials and Methods*. CYP3A9 and cyclophilin (internal control) cDNAs were coamplified for 28 cycles and the PCR products were subjected to agarose (1.7%) gel electrophoresis. The assay was performed in triplicate for four individual rats per treatment group. Shown is an ethidium bromide-stained agarose gel. The upper bands in lanes 3 to 10 (372-bp PCR products) correspond to CYP3A9 mRNA, whereas the lower bands (265-bp PCR products) correspond to cyclophilin mRNA. Lane 1: DNA ladder; lane 2, negative control (no primers); lanes 3 to 6, control females; and lanes 7 to 10, tamoxifen-treated females.

that CYP3A9 gene expression, measured as a ratio of the optical density of the CYP3A9 band to that of the cyclophilin band (internal control), was decreased by 57% in tamoxifen-treated female rats compared with control female rats (0.12 ± 0.03 versus 0.28 ± 0.04 , $p = .01$).

Hepatic CYP2B Protein Content. Previous studies have reported that acute treatment of adult rats with tamoxifen induces hepatic CYP2B (White et al., 1993). Therefore, an immunoblot assay was performed to determine CYP2B1 and CYP2B2 protein levels in adult rats treated neonatally with tamoxifen. These two proteins were resolved by the SDS-PAGE conditions used in the present study. However, CYP2B1 protein was not quantifiable in rats from any of the groups (data not shown). In contrast, CYP2B2 protein level was quantifiable, but neonatal administration of tamoxifen did not alter the hepatic level of this CYP enzyme significantly in adult male or female rats (Fig. 4).

Serum Steroid Hormone Levels. Steroid hormone levels were measured in adult rats treated neonatally with tamoxifen. Serum testosterone concentration was not significantly different between the vehicle-treated control group (2.4 ± 0.4 ng/ml, mean \pm S.E., $n = 9$) and the tamoxifen-treated male rats (2.5 ± 0.4 ng/ml, $n = 8$). By comparison, the group mean serum 17β -estradiol concentration was reduced by 64% ($p < .05$) in tamoxifen-treated female rats (9 ± 3 pg/ml, $n = 9$) when compared with the vehicle-treated control rats (25 ± 2 pg/ml, $n = 19$).

Discussion

Previous animal studies have shown that exposure to certain compounds, such as diethylstilbestrol (Dieringer et al., 1980), during the neonatal period can permanently alter the adult expression of specific hepatic CYP enzymes. This altered CYP expression impacts on pro-carcinogen activation and DNA adduct formation, and it may play a role in determining the susceptibility of an individual to the effects of chemical carcinogens (Faris and Campbell, 1981). Treatment of adult rats with tamoxifen has been shown to increase the hepatic level of several inducible CYP enzymes, such as CYP2B and CYP3A (White et al., 1993). The present study provides the first demonstration that neonatal treatment of rats with tamoxifen results in a long-lasting alteration in the hepatic expression of CYP2A1, CYP2C11, and CYP3A9, but it has no effect on CYP2C12, steroid 5α -reductase, or CYP2B2.

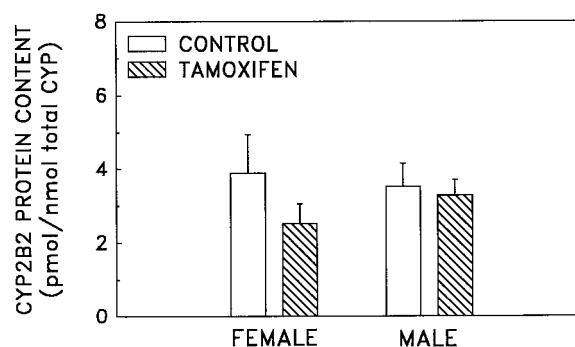


FIG. 4. Hepatic CYP2B2 protein level in adult rats treated neonatally with tamoxifen.

Female and male rats were injected s.c. once daily with tamoxifen at days 1 to 5 of age as described in Table 1. Hepatic microsomes were isolated from 3-month-old rats as described in *Materials and Methods*. Results are expressed as mean \pm S.E. for eight individual rats per treatment group.

Treatment of adult male and female rats with tamoxifen has been reported to cause a major suppression of GH pulse amplitude and nadir levels that persists for at least 7 weeks after discontinuation of drug administration (Tannenbaum et al., 1992). Long-term suppression of plasma GH levels is also observed after neonatal administration of monosodium glutamate (Waxman and Chang, 1995), which selectively destroys neurons in the arcuate nucleus so that levels of GH-releasing factors are reduced (Millard et al., 1982). Similar to the findings with neonatal administration of monosodium glutamate (Yamazoe et al., 1988; Waxman et al., 1990; Pampori and Shapiro, 1994), neonatal tamoxifen treatment increased hepatic CYP2A1 expression in adult male rats, whereas it did not alter the expression of CYP2C12, CYP2A1, or steroid 5α -reductase in adult female rats. However, these two neonatal treatments produce notable differences in CYP2C11, CYP2C12, and steroid 5α -reductase in adult male rats. As shown in the present study, neonatal administration at doses that produced systemic toxicity, as evidenced by testicular hypoplasia, was associated with a modest decrease in hepatic CYP2C11 expression, whereas the same treatment did not induce CYP2C12 or steroid 5α -reductase. In contrast, neonatal administration of monosodium glutamate (4 mg/g) results in a major suppression of CYP2C11 expression (Shapiro et al., 1989) and increased CYP2C12 protein content (Shapiro et al., 1989) and steroid 5α -reductase activity (Pampori et al., 1991). Therefore, with respect to adult expression of sex-dependent hepatic CYP enzymes, the overall pattern of response produced by neonatal administration of tamoxifen is different from that observed after neonatal administration of monosodium glutamate, suggesting that the effects of tamoxifen are, at least in part, GH independent.

Hepatic expression of CYP2C11, CYP2C12, CYP2A1, and steroid 5α -reductase is also influenced by gonadal steroids, which act on the hypothalamic-pituitary axis to affect the pattern of GH secretion (Jansson et al., 1985). Androgen is required for the normal basal expression of CYP2C11 and is suppressive toward CYP2A1 in adult male rats (Waxman and Chang, 1995). In the present study, neonatal administration of tamoxifen reduced CYP2C11 and increased CYP2A1 protein levels in 3-month-old male rats. The changes in hepatic expression of these two CYP proteins are not likely due to androgen because neonatal administration of tamoxifen did not alter serum testosterone concentrations in adult male rats. Neonatal administration of tamoxifen resulted in a decrease in serum 17β -estradiol concentration that was still observable in 3-month-old female rats. The reduced serum level of 17β -estradiol was not associated with alteration in hepatic expression of CYP2C12, CYP2A1, or steroid

5 α -reductase. These data are consistent with previous findings indicating that CYP2C12, CYP2A1, and steroid 5 α -reductase are expressed in gonadectomized female rats (Waxman and Chang, 1995).

The rat CYP3A subfamily consists of several genes, including CYP3A1, CYP3A2, CYP3A9, and CYP3A18 (Mahnke et al., 1997). The present study shows that neonatal administration of tamoxifen did not affect hepatic microsomal testosterone 6 β -hydroxylase activity or CYP3A protein content in adult male rats. CYP3A2 is a male-specific CYP3A that is active in testosterone 6 β -hydroxylation (Nagata et al., 1990). Therefore, our data suggest that neonatal tamoxifen treatment does not affect hepatic CYP3A2 expression. In contrast to the lack of an effect in adult male rats, neonatal administration of tamoxifen substantially reduced hepatic microsomal testosterone 6 β -hydroxylase activity in adult female rats. RT-PCR analysis indicated that the adult expression of CYP3A9 mRNA was decreased by neonatal tamoxifen treatment. Relatively little is known about the hormonal regulation of CYP3A9. However, it has been reported that continuous GH infusion to male rats increases hepatic CYP3A9 mRNA level, whereas androgen does not appear to have a suppressive influence in CYP3A9 expression because castration does not increase CYP3A9 mRNA level in male rats (Robertson et al., 1998). By comparison, estrogen appears to play a role in CYP3A9 expression in female rats because ovariectomy reduces hepatic CYP3A9 mRNA expression and this effect can be reversed by estrogen administration (Wang and Strobel, 1997). The postulate that estrogen plays a role in the control of hepatic CYP3A9 expression is strengthened by our findings that tamoxifen, an estrogen receptor antagonist, decreases the mRNA expression of this CYP in female rats.

Neonatal administration of tamoxifen did not influence hepatic CYP2B1 or CYP2B2 protein expression in adult male and female rats. By comparison, neonatal administration of monosodium glutamate, which depletes plasma GH levels, also does not affect the constitutive levels of these two CYP enzymes (Yamazoe et al., 1988). In contrast, it enhances the inducibility of both CYP2B1 and CYP2B2 in adult rats by phenobarbital, consistent with the proposal that GH has a suppressive effect on CYP2B1 and CYP2B2 inducibility (Yamazoe et al., 1987). It remains to be determined if neonatal tamoxifen administration has a similar effect on CYP2B1 and CYP2B2 inducibility.

In summary, neonatal administration of tamoxifen (20 μ g/rat/day at days 1–5 of age) increased hepatic CYP2A1 and decreased CYP2C11 protein level, whereas it had no effect on the expression or activity of CYP2B2, CYP2C12, CYP3A, or steroid 5 α -reductase in adult male rats. It also decreased testicular weight but had no effect on body weight or serum testosterone concentration. The same neonatal treatment decreased hepatic CYP3A9 gene expression in adult female rats and this was accompanied by a reduction in serum 17 β -estradiol concentration. In contrast, neonatal tamoxifen treatment had no effect on the adult expression or activity of CYP2A1, CYP2B2, CYP2C12, or steroid 5 α -reductase in female rats. Thus, treatment of neonatal rats with tamoxifen results in an enzyme-selective and long-lasting effect on hepatic CYP expression.

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