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Phenobarbital Induction of Hepatic *CYP2B1* and *CYP2B2*: Pretranscriptional and Post-transcriptional Effects of Gender, Adult Age, and Phenobarbital Dose

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SUMMARY

Chemical induction of hepatic monooxygenases should not be viewed as an extracorporal process but rather as one that is liable to be influenced by numerous endogenous factors. In this regard, we examined the interactions of gender, adult age, and barbiturate dose on the course of phenobarbital induction of hepatic *CYP2B1* and *CYP2B2*. We observed that femaleness and youth were associated with the greatest inhibition, so that both the rate and initiation of *CYP2B1* and *CYP2B2* induction were suppressed most in the young adult (65 days of age) females, followed by the mature adult (150 days of age) females and then by the young adult males, with the mature adult males exhibiting the least suppression of phenobarbital induction. The differential expression rates of hepatic *CYP2B1* mRNA in the young and mature male and female rats, similarly reflected at the protein level, suggest that gender- and age-dependent suppression of *CYP2B1* occurs at a pretranscriptional or transcriptional level. In contrast, transcript levels of *CYP2B2* were unaffected by gender or age. However, accumulation of cytochrome P450 (P450) 2B2 protein was affected by the animal's age and gender, suggesting regulation of a post-transcriptional event. Highly selective (androstenedione 16β-hydroxylase) as well as nonspecific (total P450 and hexobarbital hydroxylase) P450 2B1- and 2B2-dependent catalytic activities were in agreement with protein levels. Determination of gender- and age-dependent circulating growth hormone profiles indicates that the continuous secretion of the hormone characteristic of the female is more suppressive of *CYP2B* induction than the episodic pattern of growth hormone secretion found in males. The considerably elevated growth hormone pulse amplitudes observed in the young rats of both genders seem to be an additional inhibitory signal antagonizing phenobarbital induction of *CYP2B1* and *CYP2B2*. Phenobarbital administration did not interfere with the normal gender- and age-dependent growth hormone secretory profiles. Last, although as little as 1 mg/kg phenobarbital increased *CYP2B1* mRNA concentrations by 100%, there was no translation into detectable levels of protein. In contrast, the same low dose of barbiturate inducing an equal percent increase in *CYP2B2* mRNA did result in an expression of protein. Unlike use of the 10 mg/kg dose, *CYP2B1* and *CYP2B2* induction by phenobarbital at 1 mg/kg was unaffected by age or gender.

In the rat, expression of the gender-dependent constitutive forms of hepatic P450 is regulated by the gender differences in the circulating profiles of growth hormone. Studies with rats have shown that the male pattern of pulsatile growth hormone release, characterized by episodic bursts every 3–4 hr with undetectable hormone levels between the pulses (1, 2), stimulates the expression of male-specific hepatic P450 form 2C11 and suppresses female-specific P450 form 2C12 (3, 4). In female rats, the hormone peaks are of lower magnitude than are those in males and occur more frequently and irregularly, whereas the levels of growth hormone in the troughs between peaks are elevated considerably compared with those seen in males (2, 5). It is this female pattern of growth hormone secretion, and the resultant continuous presence of the hormone in plasma, that stimulates the expression of P450 2C12 and fully suppresses P450 2C11 (for reviews, see Refs. 3, 4, and 6). These effects of growth hormone on *CYP 2C11* and *2C12* gene expression occur at the pretranscriptional level with the initiation of new mRNA synthesis and are reflected by similar changes in P450 2C proteins (3, 4). Although the constitutive expression levels of all the gender-dependent P450 forms are determined by the sexual profiles of plasma growth hormone, some of these P450s are optimally expressed in the absence of the hormone. That is, male-specific P450 2A2 and 3A2 are maximally expressed in the hypophysectomized rat, disappear when growth hormone is secreted constantly, but are only partially induced (7) relative to the high levels found in hypophysectomized rats under the influence of pulsatile growth hormone (7, 8).

Expression levels of the drug-inducible forms of P450 also

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ABBREVIATIONS: P450, cytochrome P450 protein; *CYP*, cytochrome P450 gene or gene transcript; MSG, monosodium glutamate.

seem to be affected by circulating growth hormone. Phenobarbital-, isosafrole-, and pregnenolone-16α-carbonitrile-induction levels of P450-dependent monooxygenases are suppressed by endogenous growth hormone (9–12), whereas 3-methylcholanthrene induction of P450 1A1-dependent monooxygenases is enhanced by plasma growth hormone (13). In the case of phenobarbital, studies with cultured hepatocytes have shown that growth hormone acts directly to antagonize phenobarbital induction of P450 2B1/2[1] (11, 14). Studies in animals have demonstrated that it is not the presence of growth hormone *per se* but rather its sexually dimorphic profiles that modulate phenobarbital induction levels of P450 2B1/2 protein; that is, the female pattern of continuous growth hormone secretion is far more suppressive than the male profile of episodic secretion (9, 12). In the current study, we examined the effects of gender, adult age, and phenobarbital dose on the induction of both *CYP2B1* and *CYP2B2* at transcriptional, translational, and catalytic levels.

Materials and Methods

Animals were housed at the University of Pennsylvania Laboratory Animal Resources facility under the supervision of certified laboratory animal medicine veterinarians and were treated according to a research protocol approved by the university's Institutional Animal Care and Use Committee. At all times, animals were housed on hardwood bedding in plastic cages, with water and commercial rat chow supplied *ad libitum*. The animal quarters were air conditioned (20–23°) and had a light/dark cycle of 12 hr of light and 12 hr of darkness (light from 7:00 a.m. to 7:00 p.m.). Sprague-Dawley rats [Crl:CD(SD)BR] were bred and raised in our facility as previously described (2, 12). When the rats were 65 and 150 days of age, some of the animals were used to determine circulating growth hormone profiles (see details below); the remaining rats were treated with phenobarbital sodium (Sigma Chemical Co., St. Louis, MO). Male and female rats at both ages were injected daily intraperitoneally with either 1 mg or 10 mg/kg body weight of the barbiturate or an equivalent sodium concentration of NaCl diluent, pH 9.1. The animals received from one to six injections, administered once every 24 hr. Four or five rats in each treatment group were decapitated at 0.5, 3, 12, 64, and 136 hr after the first phenobarbital injection; the livers were quickly removed and perfused with ice-cold saline. Each liver was quickly minced; a portion reserved for mRNA determinations was plunged into liquid nitrogen and subsequently stored at −70°. The remaining minced liver was used for microsomal preparation.

Hepatic microsomes were isolated according to our reported method (15) and assayed by Western blotting for the presence of phenobarbital-inducible P450 2B1 and 2B2 (12). Ten micrograms of microsomal protein were electrophoresed on 0.75-mm-thick sodium dodecyl sulfate-polyacrylamide gels containing 7.5% polyacrylamide and electroblotted onto nitrocellulose filters. The blots were probed with monoclonal anti-rat P450 2B1 and 2B2 mouse IgG (Oxford Biomedical Research, Oxford, MI). The primary antibody was located with horseradish peroxidase conjugated to anti-mouse IgG and detected with minor modification (16) of an enhanced chemiluminescence kit (Amersham, Des Plaines, IL). Quantification of relative P450 levels was done by laser densitometry of the x-ray films.[2]

Total RNA was isolated from ~0.5 g of individual rat liver by a single-step guanidinium/thiocyanate method (19). RNA samples from individual livers were fractionated by electrophoresis under denaturing conditions in 1.2% agarose gels containing 1× 3-(*N*-morpholino)propanesulfonic acid buffer and 1.28% formaldehyde. The RNA was transferred to GeneScreen nylon membranes (DuPont-NEN, Boston, MA) by capillary transfer in 10× standard saline citrate (1× = 0.015 M sodium citrate, 0.15 M sodium chloride) and then fixed to the filters by UV cross-linking. Prehybridizations and hybridizations in Rapid-hyb buffer (Amersham) with a ^{32}P-labeled 2B1 oligonucleotide probe[3] were performed with high stringency washings. The washed blots were wrapped in clear plastic and exposed to x-ray films with two intensifying screens at −70° for 1–3 days. Northern blots were stripped and then reprobed with a selective ^{32}P-labeled 2B2 oligonucleotide probe. Evidence that RNA was equally loaded and transferred was obtained by equivalent intensity of ethidium bromide staining of 18*S* and 28*S* rRNA bands (11). Furthermore, the rat 18*S* rRNA oligonucleotide probe was used as a control to verify the consistency and integrity of RNA loading (21). Quantification of the mRNA was done with the use of laser densitometry of the x-ray films.

Hepatic microsomal hexobarbital hydroxylase was assayed by our modification (15) of the radioenzyme procedure of Kupfer and Rosenfeld (22). Total P450 was quantified by measuring the carbon monoxide difference spectra after reduction with dithionite (23), and cytochrome b_5 was determined spectrophotometrically as previously described (24). NADPH-P450 reductase activity was assayed by monitoring the rate of cytochrome *c* reduction at 550 nm in 0.3 M KPi, pH 7.7, at 25° in 1-ml incubations with 10 μg of microsomal protein (25). Androstenedione 16β-hydroxylation, a specific catalytic activity of P450 2B1 and 2B2 (17, 18), was measured with [4-^{14}C]androstenedione, and the hydroxylated products were resolved with thin layer chromatography and quantified with our previously reported method (26).

Repetitive blood samples (25 μl) were obtained at 15-min intervals from unrestrained, unstressed, and completely conscious, vehicle-treated 65- and 150-day-old male and female rats outfitted with our mobile catheterization apparatus (27, 28). Eight-hour plasma growth hormone profiles were determined with a homologous radioimmunoassay with a sensitivity of 2–3 μg/liter. Procedural details and statistical validation of the assay have been reported previously (2, 29).

The effect of phenobarbital treatment on growth hormone secretory profiles was examined in 150-day-old rats (three animals of each gender) that received 20 mg/kg intravenous phenobarbital every 12 hr for 3 days. Serial blood collections were obtained for 6 consecutive hr beginning 3 hr after the last injection of barbiturate.

The ultradian patterns in plasma growth hormone concentrations were analyzed with the aid of the Cluster analysis computer program (30), as we reported previously (29, 31). All data, including those obtained from the Cluster analysis program, were subjected to analysis of variance, and differences were determined with *t* statistics and the Bonferroni procedure for multiple comparison.

Results

To identify subtle as well as more apparent gender-dependent effects on phenobarbital induction of *CYP2B1* and *CYP2B2*, we planned to administer the barbiturate at threshold doses producing both qualitative and quantitative sexually dimorphic responses. Using our previous study (32) as a guide, we administered daily injections to the adult male and female rats (145–155 days of age) of either vehicle or 1, 3, or 10 mg phenobarbital/kg body weight for 6 consecutive days and then measured the highly sensitive but nonspecific phenobarbital-inducible hepatic microsomal hexobarbital hy-

[1] The "slash" in the nomenclature indicates that the study did not discriminate between P450 2B1 and P450 2B2 and often reflects the findings of 2B1 alone.

[2] Despite their high degree of amino acid sequence similarity, P450 2B1 and P450 2B2 can be distinguished on Western blots by their different rates of migration on sodium dodecyl sulfate-polyacrylamide gels (17, 18).

[3] Use of the highly discriminating 18′-mer oligomeric probes complementary to *CYP2B1* and *CYP2B2* described by Omiecinski *et al.* (20) enabled us to assess the expression levels of the individual mRNAs.

droxylase and total P450 (data not shown). At the lowest dose of phenobarbital (1 mg/kg), only the males exhibited an elevation in total P450 and hexobarbital hydroxylase. At 3 mg/kg, the hepatic enzymes were increased to borderline significance levels ($p \simeq 0.05$) in the females. When phenobarbital was administered at a dose of 10 mg/kg, we observed a highly significant increase in total P450 and hexobarbital hydroxylase in both sexes, with the magnitude of the response greater in the males. Having identified phenobarbital doses that produced both qualitative (1 mg/kg) and quantitative (10 mg/kg) sexually dimorphic inductive responses on multi-P450-dependent enzymes, we were prepared to examine the gender effects of these doses on the course of *CYP2B1* and *CYP2B2* expression.

***CYP2B1* and *CYP2B2* mRNA.** When phenobarbital was administered at 1 mg/kg, we observed a significant ($p < 0.05$) increase in hepatic *CYP2B1* mRNA 16 hr after the third injection (or 64 hr after the first injection administered at 0 hr) that resulted in a >100% increase in mRNA after six daily injections of the barbiturate (when measured at 136 hr) (Fig. 1). There were no gender or age statistical differences in this response of *CYP2B1* mRNA to the 1-mg dose of phenobarbital. When the barbiturate was injected in males at the higher dose (10 mg/kg), we found a significant elevation in *CYP2B1* mRNA within 3 hr of administration. The concentration of hepatic *CYP2B1* mRNA continued to rise sharply during 64 hr of treatment, after which it plateaued in both age groups of males. In general, the same 10-mg/kg dose of phenobarbital induced significantly higher levels of *CYP2B1* mRNA in the mature (150-day-old) males compared with the younger (65-day-old) males. Females treated with 10 mg/kg of phenobarbital did not exhibit a significant elevation in *CYP2B1* mRNA until 12 hr after the first injection, with the concentration continuing to rise for 64 hr and plateauing thereafter. Compared with the males, the phenobarbital-induced concentrations of *CYP2B1* mRNA were lower in the females at all time points measured. However, in contrast to the males, there were no age effects with the 10-mg/kg dose in the females.

The effect of the low dose (1 mg/kg) of phenobarbital on *CYP2B2* mRNA expression was somewhat similar to that observed for *CYP2B1* mRNA; that is, significant above-baseline concentrations of *CYP2B1* mRNA were induced in the males within 12 hr of treatment and after 64 hr of treatment in the females. Although the livers of the males seemed to have higher phenobarbital-inducible levels of *CYP2B2* mRNA than the females, this simply reflected a pretreatment sexual dimorphism (male > female). After six injections at 1 mg/kg, both males and females had a 100–150% increase in their levels of the mRNA. There were no age effects at this dose. The effect of the high dose (10 mg/kg) of the barbiturate on *CYP2B2* mRNA induction was very different from that found for *CYP2B1* mRNA. The concentrations of hepatic *CYP2B2* mRNA increased above constitutive levels 12 hr after the first injection of phenobarbital, continued to rise through 64 hr, and plateaued thereafter. In contrast to the inductive effects on *CYP2B1* mRNA, 10 mg/kg phenobarbital exhibited no gender- or age-dependent effects on *CYP2B2* mRNA expression. At all time points measured, mature and young males and females had the same induced levels of *CYP2B2* mRNA.

P450 2B1 and 2B2. Although *CYP2B1* and *CYP2B2* mRNA responded quite differently to phenobarbital at 10 mg/kg), this dose of the barbiturate induced a similar pattern of response on protein (i.e., P450 2B1 and 2B2) levels (Fig. 2), which tended to resemble the induction pattern for *CYP2B1* mRNA (Fig. 1). Detectable levels of P450 2B1 and 2B2 proteins were found in livers of the males within 12 hr of the first 10 mg/kg injection of phenobarbital but were observed at 64 hr in the livers of the females. Although the concentrations of P450 2B1 and 2B2 in the livers of the males of both age groups increased at all time points measured, the older males consistently had higher levels of both proteins, resulting in an almost 100% differential after six injections of 10 mg/kg phenobarbital. In contrast, hepatic P450 2B1 and 2B2 concentrations peaked in the females after 64 hr of treatment, and at all time points were significantly lower than those found in the 65-day-old males. Moreover, greater levels of 2B1 protein were induced in the mature females than in the young females (no such age effect was observed in females for

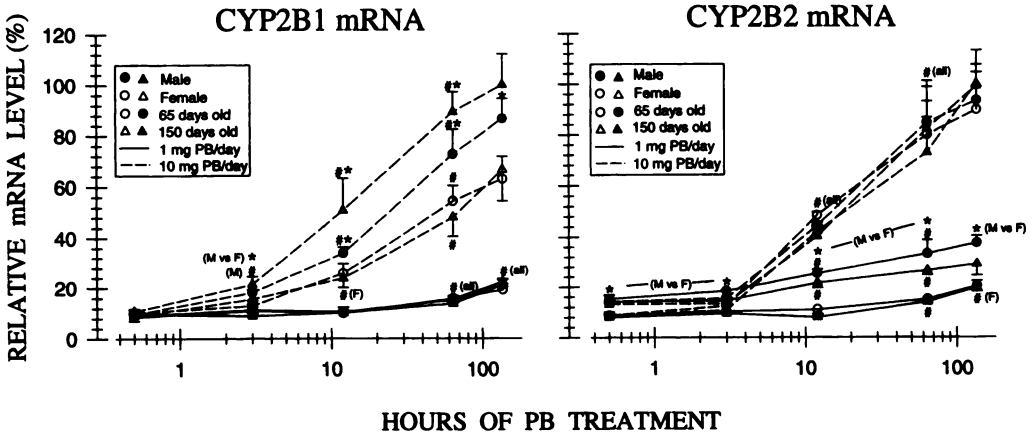

Fig. 1. Transcriptional levels of hepatic *CYP2B1* and *CYP2B2* of 65- and 150-day-old male and female rats measured at various times during treatment with either 1 mg or 10 mg/kg phenobarbital/day for 6 consecutive days. Procedural details are described in Experimental Procedures. *Data points*, mean ± standard deviation for four or more rats; expressed as a percentage of the mean value of the group expressing the greatest induction (i.e., 10 mg/kg phenobarbital-treated mature males at 136 hr of treatment). #, $p < 0.05$, compared with the preceding time period in the same treatment group. *, $p < 0.05$, compared with the smaller mean values of treatment groups receiving the same phenobarbital dose and measured at the same time during barbiturate treatment. *Parentheses*, a more encompassing comparison; *M*, males; *F*, females; *all*, both males and females.

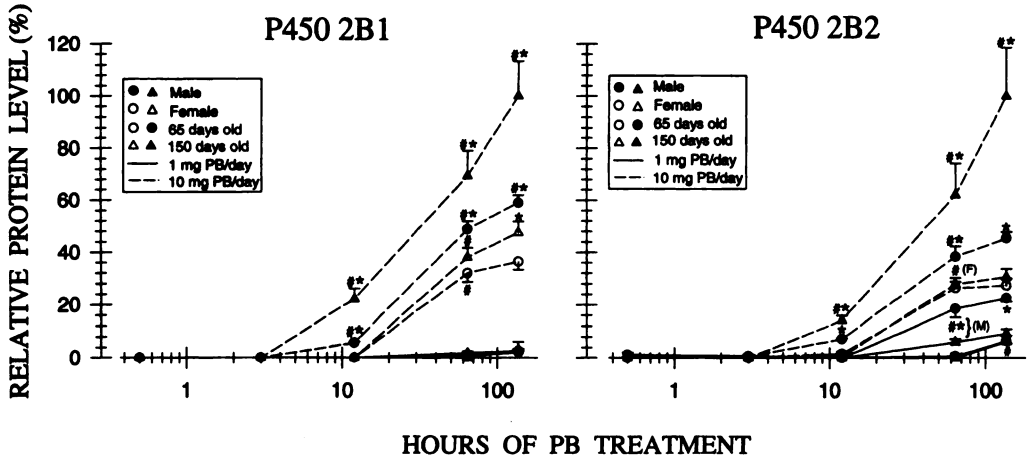

Fig. 2. Translational levels of hepatic microsomal P450 2B1 and 2B2 of 65- and 150-day-old male and female rats measured at various times during treatment with either 1 mg or 10 mg/kg phenobarbital/day for 6 consecutive days. Procedural details are described in Experimental Procedures. *Data points*, mean ± standard deviation for four or more rats; expressed as a percentage of the mean value of the group expressing the greatest induction (i.e., 10 mg/kg phenobarbital-treated mature males at 136 hr of treatment). #, $p < 0.05$, compared with the preceding time period in the same treatment group. *, $p < 0.05$, compared with the smaller mean values of treatment groups receiving the same phenobarbital dose and measured at the same time during barbiturate treatment. *Parentheses*, a more encompassing comparison; *M*, males; *F*, females.

P450 2B2). Although the threshold dose of 1 mg/kg phenobarbital had no inductive effect on 2B1 protein levels in any of the groups studied, it did induce measurable concentrations of P450 2B2: 65-day-old males > 150-day-old males > 150-day-old females = 65-day-old females.

Androstenedione 16β-hydroxylase. In all groups, the low baseline level of microsomal androstenedione 16β-hydroxylase observed in uninduced livers [which is not P450 2B1- or 2B2-dependent (33)] remained unchanged after six daily injections of 1 mg/kg phenobarbital (Fig. 3). At the 10-mg/kg dose, we observed a significant increase in 16β-hydroxylase activity in livers of males within 12 hr of the first barbiturate injection, whereas measurable induction in the females was observed at 64 hr of treatment. Although androstenedione 16β-hydroxylase levels consistently increased with each injection of 10 mg/kg phenobarbital in all treatment groups, there were clear gender- and age-dependent effects. The same dose of phenobarbital (10 mg/kg) induced the highest concentration of androstenedione 16β-hydroxylase in the livers of the mature (150-day-old) males, followed by the young (65-day-old) males and then by the mature females, with the lowest induction of the catalytic enzyme in the young females.

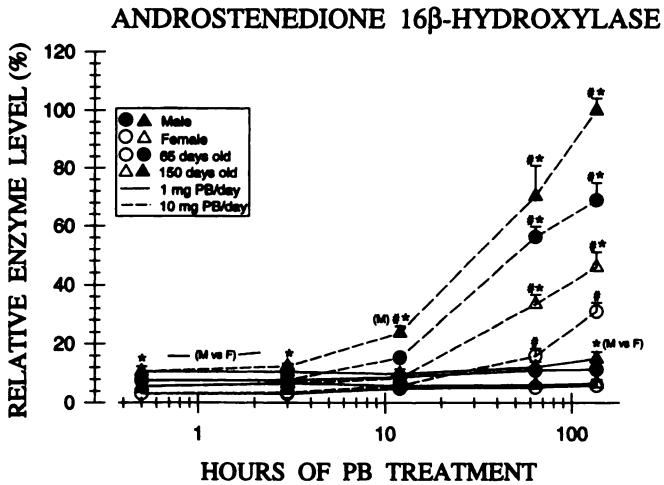

Fig. 3. Catalytic levels of hepatic microsomal P450 2B1/2-dependent androstenedione 16β-hydroxylase of 65- and 150-day-old male and female rats measured at various times during treatment with either 1 mg or 10 mg/kg phenobarbital/day for 6 consecutive days. Procedural details are described in Experimental Procedures. *Data points*, mean ± standard deviation for four or more rats; expressed as a percentage of the mean value of the group expressing the greatest induction (i.e., 10 mg/kg phenobarbital-treated mature males at 136 hr of treatment). #, $p < 0.05$, compared with the preceding time period in the same treatment group. *, $p < 0.05$, compared with the smaller mean values of treatment groups receiving the same phenobarbital dose and measured at the same time during barbiturate treatment. *Parentheses*, a more encompassing comparison; *M*, males; *F*, females.

Hexobarbital hydroxylase. Although age had no effect on the constitutive levels of hexobarbital hydroxylase, there was a profound sexual dimorphism, with males having 4–5 times the enzyme activity of females (Fig. 4). This sexual difference in baseline hexobarbital hydroxylase levels made it difficult to assess the effects of gender on phenobarbital induction; that is, the percent increase in hexobarbital hydroxylase produced by 10 mg/kg phenobarbital was greater in females, but the magnitude of this increase was much greater in males. Nevertheless, a comparison of the effects of age alone clearly demonstrated that phenobarbital (at 10 mg/kg) induced significantly higher hexobarbital hydroxylase levels in the older males and females than in their younger cohorts. In this regard, the 150-day-old males were the only animals to respond to the 1-mg/kg dose of the barbiturate with a significant elevation in hepatic microsomal hexobarbital hydroxylase activity.

Total P450. Although 65- and 150-day-old rats of the same gender had similar levels of uninduced hepatic total P450, there was a significant sexual dimorphism in that livers of the males contained higher concentrations of total P450 than did livers of the females (Fig. 5). Similar to the hexobarbital hydroxylase results, the 1-mg/kg dose of phenobarbital induced an increase in total P450 in only the 150-day-old males. When administered at 10 mg/kg, phenobarbital induced the greatest increase in hepatic total P450 (percentage as well as magnitude) in the mature males, followed by the young males and by the mature females, with the least induction occurring in the young females.

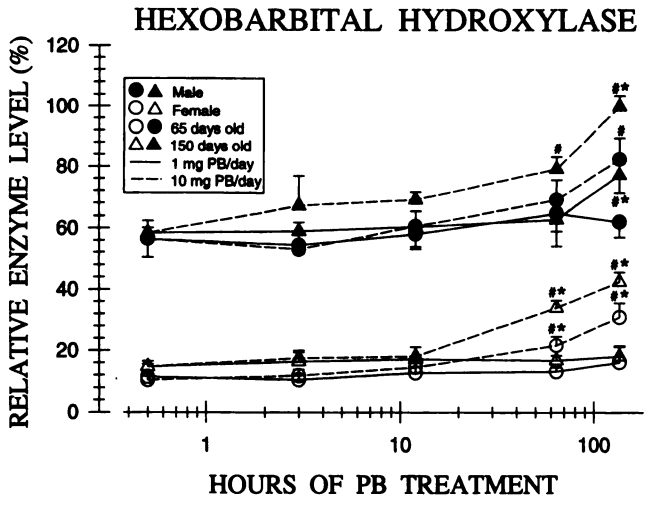

Fig. 4. Catalytic levels of multi-P450-dependent hepatic microsomal hexobarbital hydroxylase of 65- and 150-day-old male and female rats measured at various times during treatment with either 1 mg or 10 mg/kg phenobarbital/day for 6 consecutive days. Procedural details are described in Experimental Procedures. *Data points*, mean ± standard deviation for four or more rats; expressed as a percentage of the mean value of the group expressing the greatest induction (i.e., 10 mg/kg phenobarbital-treated mature males at 136 hr of treatment). #, $p < 0.05$, compared with the preceding time period in the same treatment group. *, $p < 0.05$, compared with the smaller mean values of treatment groups receiving the same phenobarbital dose and measured at the same time during barbiturate treatment. Statistical comparisons were not made between sexes.

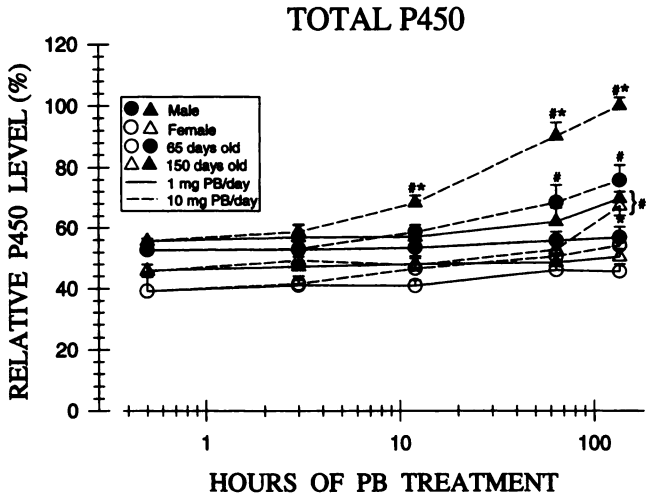

Fig. 5. Catalytic levels of hepatic microsomal total P450 of 65- and 150-day-old male and female rats measured at various times during treatment with either 1 mg or 10 mg/kg phenobarbital/day for 6 consecutive days. Procedural details are described in Experimental Procedures. *Data points*, mean ± standard deviation for four or more rats; expressed as a percentage of the mean value of the group expressing the greatest induction (i.e., 10 mg/kg phenobarbital-treated mature males at 136 hr of treatment). #, $p < 0.05$, compared with the preceding time period in the same treatment group. *, $p < 0.05$, compared with the smaller mean values of treatment groups receiving the same phenobarbital dose and measured at the same time during barbiturate treatment. Statistical comparisons were not made between sexes.

NADPH-P450 reductase and cytochrome b_5. NADPH-P450 reductase and cytochrome b_5 are required for optimal P450 2B1/2-dependent androstenedione 16β-hydroxylase activity (34). In general, only the 10-mg/kg dose of phenobarbital induced an increase in NADPH-P450 reductase and cytochrome b_5 (data not shown). Because neither age nor gender affected the induction of these enzymes, it seems reasonable to conclude that the observed alterations in 16β-hydroxylase levels simply reflected changes in P450 2B1 and 2B2 concentrations.

Plasma growth hormone profiles. Growth hormone measurements of serial blood samples obtained from vehicle-treated, 150-day-old rats revealed the established sexual dimorphism in the ultradian patterns of plasma growth hormone (Table 1). In male rats, growth hormone was released in pulses every 3–3.5 hr, resulting in peaks of ~200 μg/liter and followed by ~2 hr of virtually undetectable nadir levels (<3 μg/liter). In contrast, growth hormone was released in female rats in a more continuous pattern. Frequent lower-amplitude pulses of the hormone resulted in peaks of ~100 μg/liter, followed by short-lived 30-min troughs that were always measurable and averaged ~20 μg/liter.

A comparison of the circulating growth hormone profiles in the 65- and 150-day-old control rats indicated a greater secretory rate in the younger animals. In the case of males, plasma peak heights were 2 times as great, and the duration of the peaks was 2.5 times as long in the young males, which resulted in plasma growth hormone pulses containing 400% more hormone than that found in the mature adults. To maintain the same periodicity of growth hormone peaks characteristic of both the young and mature male rats (i.e., 3–3.5 hr), the interpeak valley widths were significantly reduced in the 65-day-old males. Common to both age groups of males were interpeak nadir concentrations of growth hormone that were below detectability.

Like the males, the basic difference in the ultradian patterns of plasma growth hormone in the vehicle-treated 65- and 150-day-old females was the higher mean concentration of the hormone in the younger animals. The elevated mean growth hormone concentration in the young females resulted from higher peak amplitudes (~200%) with a concomitant 250% increase in the peak areas. In contrast, the time intervals between peaks, duration of the peaks and interpeak valleys, and growth hormone concentrations in the nadirs were similar in young and mature adult female rats.

To determine whether the sexually dimorphic induction of *CYP2B1* and *CYP2B2* by phenobarbital was a direct result of barbiturate-induced alterations in the gender-dependent growth hormone-secretory profiles, we measured circulating hormone levels in 150-day-old male and female rats administered twice the dose (i.e., 20 mg/kg) of phenobarbital at twice the frequency (i.e., every 12 hr) of our highest dosed (i.e., 10 mg/kg/day) rats. Under this treatment protocol, phenobarbital had no measurable effect on any of the gender-dependent parameters of the growth hormone-secretory profiles identified in Table 1 (data not shown).

Discussion

The results of the current study demonstrate that gender and adult age, individually or in combination, can antagonize phenobarbital induction of hepatic monooxygenase activities. We observed that femaleness and youth resulted in the greatest inhibition, so that enzyme induction was suppressed most in the young adult (65 days of age) females, followed by the mature adult (150 days of age) females and then by the young adult males, with the mature adult males exhibiting the least

TABLE 1

Analysis of circulating growth hormone profiles in young and mature adult male and female rats

At 65 or 150 days of age, rats fitted with chronic indwelling right atrial catheters had serial blood samples collected over 8 hr at 15-min intervals assayed for rat growth hormone (27, 28). Data were analyzed with the aid of the Cluster analysis program for hormonal pulse detection (30).

Sex	Age	Mean[a] concentration	Peak Interval[b]	Peak			Valley	
				Width	Height	Area	Width	Nadir
	Days	*μg/liter*	*min*	*min*	*μg/liter*	*mg · min · l⁻¹*	*min*	*μg/liter*
Male	65	86 ± 17[c]	201 ± 27	101 ± 19	396 ± 39	13.2 ± 2.1	86 ± 16	n.d.[f]
	150	36 ± 7[d]	190 ± 21	41 ± 8[d]	202 ± 31[d]	3.3 ± 0.6[d]	130 ± 28[d]	n.d.
Female	65	75 ± 15	70 ± 12[e]	45 ± 10[e]	187 ± 31[e]	2.7 ± 0.4[e]	31 ± 8[e]	26 ± 7[e]
	150	42 ± 9[d]	76 ± 15[e]	37 ± 6	98 ± 18[d,e]	1.1 ± 0.1[d,e]	33 ± 10[e]	22 ± 5[e]

[a] Mean concentration is calculated for entire 8-hr collection period.
[b] Peak interval indicates time period between peaks; width, duration of growth, hormone peaks or interpulse valleys; height, amplitude of hormone peaks; area, integrated area under growth hormone peaks (concentration × duration); nadir, mean baseline growth hormone concentration.
[c] Values are mean ± standard deviation for five or more rats per group.
[d] $p < 0.01$ compared with young rats of the same gender.
[e] $p < 0.01$ compared with rats of the same age but opposite gender.
[f] Not detected (n.d.); below the sensitivity of the assay (i.e., 2–3 μg/liter).

suppression of phenobarbital induction. Moreover, when we examined the inductive effects of the barbiturate on *CYP2B1* and *CYP2B2* expression [the primary phenobarbital-inducible P450 genes (17, 18)], we found that gender, adult age, and even inductive dose could differentially modulate the inductive events leading to the expression of the 2B proteins.

Depending on the isoform of *CYP2B* examined, each factor (i.e., age, gender, and dose) could affect a different molecular event in the induction process; that is, phenobarbital at 10 mg/kg induced almost twice as much hepatic *CYP2B1* mRNA in the 150-day-old males than in females of the same age. Moreover, induced transcript levels of *CYP2B1* were significantly higher in young males than in either young or mature females. This age and sexual dimorphism is a result of both a greater and an earlier accumulation of *CYP2B1* mRNA in the males (within 3 hr of phenobarbital treatment) compared with the females (within 12 hr of phenobarbital treatment). Thus, in the case of phenobarbital induction of *CYP2B1*, it seems that the gender and age of the animal regulates some pretranscriptional or transcriptional event or events that result in the different levels of *CYP2B1* mRNA observed in young and mature male and female rats. In this regard, the gender- and age-dependent effects on P450 2B1 protein levels reflected the transcriptional kinetics; phenobarbital (10 mg/kg) induced twice as much P450 2B1 protein in mature (150 days of age) males than in young (65 days of age) males, which in turn had higher levels of the hepatic protein than mature females, with young females having the lowest concentration of P450 2B1. Like transcriptional events, age and gender effects on *CYP2B1* translation could be explained by the greater and earlier accumulation of the protein in males (within 12 hr of phenobarbital exposure) compared with females (within 64 hr of barbiturate treatment).

In contrast to our findings with *CYP2B1*, phenobarbital-induced transcription of *CYP2B2* was unaffected by age or gender. However, despite the fact that all of the 65- and 150-day-old males and females had the same amount of *CYP2B2* mRNA during the entire 136 hr of induction, the mature males produced more than twice as much P450 2B2 than the younger males, which had more of the protein than the females (there was no age effect in the females). Similar to the translational kinetics for P450 2B1, age and gender effects on phenobarbital induction of P450 2B2 could be accounted for by the greater and earlier accumulation of P450 2B2 protein in the males compared with the females. Thus, in the case of phenobarbital induction of *CYP2B2*, it seems that the gender and age of the animal affect some post-transcriptional event or events that result in the different levels of P450 2B2 observed in young and mature male and female rats.

The age- and gender-dependent effects on phenobarbital induction of P450 2B1 and 2B2 were clearly reflected in the catalytic activities of the proteins. Despite the sexual dimorphism in constitutive multi-P450-dependent hexobarbital hydroxylase and total P450, results of our studies with these enzymes, combined with the highly selective P450 2B1/2-dependent androstenedione 16β-hydroxylase findings, clearly show that the elevation in hepatic drug-metabolizing capacity induced by phenobarbital is suppressed most in young adult females, less so in mature females, and followed by young adult males, with the greatest enzyme induction occurring in the mature males.[4]

The effects of gender that we report can be best explained by our (12) earlier findings and those of Yamazoe *et al.* (9) (limited to protein measurements) that growth hormone *per se* inhibits phenobarbital induction of P450 2B1/2 but that the female pattern of continuous growth hormone secretion is more suppressive than the male profile of episodic hormone release. The possibility, however, that other hormones may alter phenobarbital induction cannot be excluded. In this regard, although growth hormone depletion in the globally hormone-deficient hypophysectomized and selective growth hormone-deficient MSG-treated rat allows for greatly enhanced phenobarbital induction of hepatic P450 2B, the magnitude of response is generally somewhat greater in the hypophysectomized rats (12). This discrepancy may be explained by the presence of subdetectable (<2–3 ng/ml) concentrations of plasma growth hormone in the MSG-treated rats (7). However, a more likely explanation may involve thyroid hormone, which, like growth hormone, can suppress phenobarbital induction of P450 2B (35, 36). The thyrothrophin-depleted hypophysectomized rat is profoundly hypothyroid, whereas the MSG-treated rat is basically eu-

[4] Androstenedione 16β-hydroxylase activities may be in somewhat closer agreement with P450 2B1 levels than with P450 2B2 levels because the former isoform seems to express higher 16β-hydroxylase activity than the latter form (17, 18).

thyroid (37, 38), with sufficient circulating concentrations of thyroxine and triiodothyronine to possibly inhibit, to some degree, phenobarbital induction of P450 2B. Moreover, the possible involvement of nonhormonal, endogenous regulators of phenobarbital induction have been suggested (12). The fact that a gender-dependent response to phenobarbital induction persists in hypophysectomized rats despite the elimination of a half-dozen of circulating anterior pituitary hormones and their dependent gonadal, adrenal, and thyroid hormones suggests the involvement of nonhormonal, but inherent or hormonally imprinted, gender-dependent regulatory factors (12).

If the gender-dependent patterns of growth hormone secretion are the primary factors responsible for the gender effects on phenobarbital induction of *CYP2B1* and *CYP2B2*, then why would younger rats exhibit a greater degree of suppression than more mature animals? Reproductive organ weights (e.g., gonads, seminal vesicles, prostate, and uterus) were the same in the 65- and 150-day-old rats (data not shown). Moreover, the young rats are reproductively competent and are usually bred by vendors at ~65 days of age (39). Indeed, the literature is replete with studies with 60–70-day-old rats, and even 50-day-old rats, described as adults. However, we observed a ~2-fold increase in the concentration of plasma growth hormone in young male and female rats compared with gender-matched mature adults [which, in the case of the males, may be a result of a concurrent elevation in pulsatile testosterone secretion (40)]. The increased growth hormone levels in the young rats were due to an increase in the amount of growth hormone secreted per burst (i.e., peak) without any change in the pulse frequency or interpulse hormone concentration. Similar alterations in growth hormone profiles have been found in humans during late puberty (41). The significance of this elevated growth hormone secretion in the 65-day-old rats can be explained by our earlier observation that it is the height of the growth hormone pulse, and not necessarily its frequency or the interpulse trough periods, that signals the suppressive effects of the hormone on phenobarbital induction of P450 2B1/2 (12). The corollary is that as the pulse amplitudes decline, so does the inhibitory effect of growth hormone on P450 2B1/2 expression. Because the growth hormone secretory peak heights are greater in the 65-day-old rats of both sexes, it is not surprising that phenobarbital induction of *CYP2B1* and *CYP2B2* is suppressed more in the young than in the mature rats of both sexes.[5]

Thus, it can be concluded that exposure to the plasma growth hormone profile of continuous secretion (female pattern) has a greater inhibitory effect on phenobarbital induction of *CYP2B1* and *CYP2B2* than exposure to episodic secretion (male pattern). An elevation in the amplitudes of the growth hormone-secretory pulses in either gender (as found in the 65-day-old rats) increases the suppressive effect of the hormone on phenobarbital induction. Moreover, our results, in agreement with findings with hepatocytes in culture (11, 14), indicate that growth hormone acts by directly antagonizing phenobarbital induction of *CYP2B1* and *CYP2B2*, and not the reverse; that is, phenobarbital does not interfere with the normal secretory profiles of growth hormone, which in turn might alter expression of *CYP2B1* and *CYP2B2*.

Although our research has identified some of the signaling elements in the sexually dimorphic growth hormone profiles regulating expression of P450s,[5] little is known about how the hepatocytes recognize, discriminate, and process the information in these signals. One possibility is that growth hormone could regulate P450 gene expression by altering the availability and activity of transcriptional factors, many of which are derived from signal transduction pathways. Also, growth hormone could regulate P450 gene expression by affecting transcript splicing, RNA transport and stability, mRNA translation, and the stability and activity of the translated enzyme. In this regard, we measured the accumulation of *CYP2B1* and *CYP2B2* mRNAs and proteins, which reflects both synthetic and degradative processes. Although phenobarbital induces *CYP2B1* and *CYP2B2* by transcription initiation (18, 45), the actual site of growth hormone suppression of phenobarbital induction is unknown, and it is possible that the hormone acts by regulating either or both the synthesis and stability of *CYP2B* transcripts and proteins. Because gender suppresses phenobarbital induction of *CYP2B1* at the mRNA level and *CYP2B2* at the protein level, it seems reasonable to characterize the former as a pretranscriptional or transcriptional effect and the latter as a post-transcriptional effect.

Consistent with the many- and often unrelated cellular functions of growth hormone are reports of various growth hormone-activated signal transduction pathways (46), each of which, it could be hypothesized, is regulated by a different signaling element in the circulating growth hormone profile. For example, female-specific *CYP2C12* expression by continuous growth hormone signaling may involve phospholipase A_2-dependent pathways (47), whereas episodic growth hormone may induce male-specific *CYP2C11* by triggering tyrosine phosphorylation-dependent nuclear translocation of a hepatic signal transducer and activator of transcription 5 (Stat 5)-related DNA binding protein (48). Because phenobarbital induction of *CYP2B1* and *CYP2B2* requires new protein synthesis (49) and may involve inhibition by cAMP (50) and protein kinase C (51) signal transduction pathways, gender- and age-dependent growth hormone signals could pretranscriptionally and post-transcriptionally regulate *CYP2B* expression by activating different signal transduction pathways.

In the present study, phenobarbital was administered at doses that are considerably below the generally used maximally effective dose of 75–85 mg/kg (52, 53). Unfortunately, the use of these high doses can mask subtle, but important, sexual dimorphisms in the induction mechanism (54). Instead, we administered two low doses of phenobarbital that produced different qualitative and/or quantitative gender-dependent responses. In contrast to mature males, hepatic hexobarbital hydroxylase and total P450 in females were completely unresponsive to the inductive effects of 1 mg of phenobarbital [which, incidentally, could correspond to insidious environmental exposure levels and explain, in part, gender differences in the incidence of certain cancers and drug toxicities (55, 56)]. The gender- and age-selective responsive-

[5] Although the pulse height in the growth hormone profile signals the hormone's suppressive effect on *CYP2B1* and *CYP2B2* expression, it is the duration of the interpulse period (42), not the pulse amplitude (43), that signals the expression of male-specific CYP2C11, and it is the absence of the interpulse trough periods in the presence of low levels of continuously released hormone that signals the expression of female-specific CYP2C12 (44). Clearly, different genes are regulated by different "signals" in the circulating, sexually dimorphic growth hormone profiles.

ness of the 150-day-old males to 1 mg/kg phenobarbital agreed with the observation that this was the only treatment group to exhibit a substantial induction of P450 2B2 (P450 2B1 was uninduced in all groups). The absence of a concomitant increase in androstenedione 16β-hydroxylase could be explained by the poor 16β-hydroxylase capacity of P450 2B2 (17, 18) or, perhaps, instead by having a substantial hexobarbital hydroxylase activity. Of course, one cannot rule out the possibility that other phenobarbital-inducible isoforms, e.g., P450 2C6, 2C7, 3A1, and 3A2 (18), could have contributed to the elevated multi-P450-dependent hexobarbital hydroxylase and total P450 levels.

There seems to be a minimum threshold level of transcript, characteristic of each P450 2B isoform, required for the initiation of translation. In the case of *CYP2B1*, a 100% increase in mRNA induced by phenobarbital (1 mg/kg) in all treatment groups resulted in no measurable accumulation of protein. In contrast, similar percentage increases in *CYP2B2* mRNA did translate into elevated protein levels. Whether mRNA threshold levels are a mechanism for differentially regulating the translation of P450s is unknown.

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