Hepatic Cytochrome P450 Enzyme Imprinting in Adult Rat by Neonatal Benzo[a]pyrene Administration

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ABSTRACT

The effect of neonatal exposure to benzo[a]pyrene (BaP) on the hepatic cytochrome P450 of male and female adult rats has been examined. Newborn rats (<24 h old) were injected with a single dose of BaP (1 mg/rat, s.c.) and killed after 110 days. In both sexes, body and liver weight, microsomal protein content, and total cytochrome P450 were unchanged. Cytochrome P450 1A2 protein content and 7-ethoxyresorufin *O*-deethylase activity were significantly decreased ($p < 0.05$) in males, whereas these were unaltered in females. Male-specific cytochrome P450 2C11 of male rats was significantly increased as shown by Western blot and increased testosterone 2 α - and 16 α -hydroxylase activities by 29% ($p < 0.01$) and 22% ($p < 0.05$), respectively. Female-specific cytochrome P450 2C12 protein content was unaltered in females. In addition, the level of free hepatic glucocorticoid receptor in adult males was elevated by 35% after

BaP exposure, whereas it was unchanged in adult females. These results indicate, for the first time, that neonatal BaP exposure results in gender-specific lasting effects on hepatic cytochrome P450 1A2, cytochrome P450 2C11, and glucocorticoid receptors in adult male rats, whereas these parameters are unchanged in adult female rats. (*Pediatr Res* 37: 646–651, 1995)

Abbreviations

P450, cytochrome P450
CYP1A1, cytochrome P450 1A1
CYP2C11, cytochrome P450 2C11
BaP, benzo[a]pyrene
EROD, 7-ethoxyresorufin *O*-deethylase
PAH, polycyclic aromatic hydrocarbon
***p*-NPH**, *p*-nitrophenol hydroxylation

The rat hepatic P450 monooxygenase system is involved in the oxidative metabolism of a wide variety of xenobiotics and endogenous substrates including steroid hormones such as testosterone and androsterone. Sex-related differences in P450-mediated xenobiotic metabolism have been reported for several species, with the most striking differences observed in the rat (1). Sex-specific P450 isozymes for the male rat include CYP2C11, 2C13, 3A2, and 2A2 and for the females, CYP2C12 and 2A1 (2, 3). In male rats the predominant male-specific P450 is CYP2C11, which constitutes about 45% of total P450 and catalyzes 2 α and 16 α hydroxylation of testosterone, whereas in the females CYP2C12 constitutes about 35% of adult female hepatic P450 and catalyzes 15 β hydroxylation of sulfotestosterone (3). Some of the sex differences in steroid metabolism in rats are induced at puberty by the presence or absence of increased amounts of circulating androgens or estrogens. Other sexual differences, although not apparent until puberty, are "imprinted" during the neonatal period by the

presence or absence of an androgen surge (2). Furthermore, growth hormone plays a key role in the sexual differentiation and developmental regulation of several liver proteins including some isoforms of P450 (4–6). In the rat, both male- and female-specific hepatic P450 isoforms are subject to growth hormone regulation. The major rat hepatic P450 isoform 2C11, not expressed in immature rats, is induced at puberty in males but not females (7). The developmental induction of this male-specific P450 is imprinted by exposure to androgen during the neonatal period (3) and is ultimately regulated by the pulsatile pattern of pituitary growth hormone secretion that is characteristic of adult male rats (5, 8). By contrast, expression of adult female-specific CYP2C12 is dependent on the more continuous growth hormone secretory pattern exhibited by adult female rats (8, 9).

Exposure to xenobiotics has been shown to alter neonatal androgen levels during the critical neonatal period (10) and cause permanent changes in sex-specific P450 isozyme profiles in the grown animal (11). Long-term changes in the regulation of P450 levels resulting from chemical action and occurring only during critical stages of development have been termed imprinting (12). The sequence of events for such alterations is not well understood and may involve direct or indirect action

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of the xenobiotic on the hypothalamus, pituitary, or liver of the neonates. Neonatal exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin has been shown to result in decreased 2 α - and 16 α -testosterone hydroxylase activities (13) and demasculinize sexual behavior in male adult rats (14). Neonatal exposure to polychlorinated biphenyl, Aroclor 1254 (Monsanto, St. Louis, MO), has been reported to result in a permanent increase in the activities of 2 α - and 16 α -testosterone hydroxylase and androstenedione activities and decreased 7 α -testosterone hydroxylase activity (11), as well as decreased male reproductive function in rats (15).

PAH such as BaP are ubiquitous environmental pollutants produced by incomplete combustion of fossil fuels, automobile exhaust, charbroiled food, and tobacco smoke (16). Exposure to tobacco smoke or to other pollutants containing BaP often begins at birth or *in utero*. Neonatal exposure of rats to BaP results in an increase in total content of P450 and aminopyrine demethylase activity in adult male rats (17), although the precise mechanism(s) for these changes are still unknown. Herd and Greene (18) have shown that perinatal exposure of rats to BaP alters not only the basal levels of aryl hydrocarbon hydroxylase and induction response to BaP, but also the production of metabolites capable of binding to DNA, suggesting that such exposure may affect the biologic activity of BaP. Neonatal exposure to tobacco smoke has been shown to cause a permanent increase of serum prolactin levels in adult male rats (19), suggesting the possibility that BaP may produce a hypogonadal state and sex-specific P450 isozyme alterations in adult rats. These studies, however, did not address changes of specific P450 isozymes.

The broad interest of this laboratory has been to characterize potential changes which may occur in hypothalamus, pituitary, gonadal organs, and liver after short-term as well as long-term exposure of neonatal and adult rats to xenobiotics. As a part of this project, we have reported earlier that exposure of adult rats to hyperoxia results in induction of mRNAs, proteins, and corresponding monooxygenase activities of CYP1A1 and 1A2 in the liver and CYP1A1 in the lung (20). Although the precise mechanism of the induction of CYP1A1 and 1A2 by hyperoxia is still unknown, it has been suggested that this induction may involve production of endogenous ligand(s) of Ah receptor by reactive oxygen species generated by hyperoxia followed by Ah receptor-mediated transcriptional activation of CYP1A1/1A2 genes (20). The present study was designed to investigate the changes of sex-specific hepatic cytochrome P450 isozymes in adult male and female rats after neonatal exposure to BaP. In addition, the BaP-inducible CYP1A subfamily was also investigated, inasmuch as we have demonstrated that neonatal hyperoxic exposure of rats in imprinting of CYP1A2 as well as sex-specific P450 isozymes (21).

METHODS

Newborn male and female pups (<12 h old) with nursing mothers (Sprague-Dawley CD strain, viral pathogen-free) were obtained from Harlan Sprague-Dawley (San Diego, CA). Pups were divided into BaP and hyperoxia groups. The results of hyperoxic exposure on P450 isozymes have been published

elsewhere (21). Pups from six litters were pooled to minimize differences in individual litters and randomly assigned to a control or experimental group. Litter size was maintained at 10 (five males, five females) pups per dam. The mean body weights of pups were 7.1 ± 0.5 g for the male and 6.6 ± 0.4 g for the females. Within 24 h after birth, experimental pups were given a single subcutaneous injection of BaP [1 mg/pup (17)] dissolved in 0.1 ml corn oil. The control animals were injected with corn oil alone. The control or BaP-treated pups, with dams, were kept on a 12-h light-dark cycle. The pups were weaned from dams at 30 d, and allowed food and water *ad libitum*. The rats were killed at 110 d for this study.

Animals were anesthetized with pentobarbital sodium (70 mg/kg, intraperitoneally), in accordance with guidelines of the University of California, Irvine. After exsanguination by transection of the abdominal aorta, the livers were removed and weighed, and microsomes were isolated as described elsewhere (22). The washed microsomal pellet was resuspended in 1.15% KCl and stored at -80°C .

Contents of P450 and cytochrome *b*₅, and the EROD activity were measured as described previously (23, 24). *p*-NPH was measured by the method of Reinke and Moyers (25). Microsomal protein concentrations were determined as described by Lowry *et al.* (26) with bovine serum albumin as the standard.

Testosterone hydroxylation was assayed, using nonsaturating substrate concentration to retain the specificity of the microsomal hydroxylation pathways (27). Separation of testosterone metabolites was carried out by HPLC (Perkin-Elmer, Norwalk, CT), equipped with a Supelcosil LC-18 reverse-phase C₁₈ column (5 μm , 150×4.6 mm) preceded by a Supelcosil LC-18 guard column and an LC-235 Diode Array Detector as described in our earlier communication (21). The column eluant was monitored for radioactivity with HP-Flo One radiochemical detector (Radiomatic Inst. Inc., Tampa, FL) attached in tandem and testosterone metabolites were identified with authentic standards.

Polyclonal anti-CYP1A1/1A2 antibody (Oxford Biochemical Research, Inc. Oxford, MI), polyclonal anti-CYP2C11 and 2C12 antibodies (Dr. Imaoka, Osaka City Medical College, Japan), and alkaline phosphatase-conjugated IgG (Sigma, St. Louis, MO) were used for Western blot analyses as described in our earlier communications (23, 24).

For glucocorticoid receptor assays, the cytosolic fractions were treated with dextran-coated charcoal to remove endogenous hormones. Free and total receptor assays were carried out as previously described (28).

p-Nitrophenol, 4-nitrocatechol, dexamethasone, sodium molybdate, testosterone, and androstenedione were purchased from Sigma. Ethoxyresorufin and resorufin were purchased from Pierce (Rockford, IL). Activated charcoal (Norit A) was from Fisher Scientific (Pittsburgh, PA). 16 α -Hydroxy testosterone, 2 α -hydroxy testosterone, 11 α -, 11 β -hydroxy testosterone, 7 α -hydroxy testosterone, 6 β -hydroxy testosterone, 16 β -hydroxy testosterone, and methyl testosterone were from Steraloids, Inc. (Wilton, NH). [6,7-³H(N)]Dexamethasone (43.9 Ci/mmol) and [4-¹⁴C]testosterone (57.3 mCi/mmol) were purchased from DuPont NEN (Boston, MA). All other chemicals were of analytical grade.

Table 1. Effect of neonatal BaP treatment on growth parameters and P450 levels in 110-d-old rats

Group	BW (g)	Liver weight (g)	Microsomal protein	P450	Cytochrome <i>b</i> ₅
Male					
Oil	385 ± 32	13.4 ± 1.7	197 ± 32*	1.00 ± 0.03†	0.52 ± 0.05†
BaP	370 ± 37	13.2 ± 1.7	198 ± 39	1.00 ± 0.09	0.52 ± 0.05
Female					
Oil	245 ± 15	7.8 ± 1.0	94 ± 17	0.82 ± 0.06	0.61 ± 0.03
BaP	235 ± 11	7.7 ± 0.2	100 ± 11	0.81 ± 0.03	0.59 ± 0.05

Each value represents the mean ± SD (*n* = 5).

* mg/liver.

† nmol/mg microsomal protein.

Results are expressed as mean values ± SD (*n* = 5). Data were analyzed using the two-tailed *t* test at a significance level of *p* < 0.05.

RESULTS

Body and liver weight and total P450. Neonatal BaP treatment had no effect on body weight, liver weight, and microsomal protein content in both the male and female rats at 110 d of age (Table 1). There was no change in total P450 and cytochrome *b*₅ contents between control and BaP groups in both the male and females when values were expressed as nmol/mg microsomal protein (Table 1) or as nmol/liver.

CYP1A1/1A2. EROD activity, an indicator for the activity of CYP1A1 and 1A2, was decreased by 20% (*p* < 0.05) in the males, whereas it was unaffected in the females (Table 2). Taking into consideration that in adult animals the CYP1A1/1A2 inducing action of PAH lasts only about 72 h, these results suggest that a single neonatal intraperitoneal dose of BaP caused radical changes that persisted for several months, resulting in decreased EROD activity in adulthood. The 110-d interval between BaP exposure and the measured effects is consistent with "lifelong" changes in the regulation of these enzymes, that is enzyme imprinting (12). Similar results have been reported with the neonatal administration of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin that showed 35% decrease of the activity in 120-d-old male rats (14).

The results on the Western blot analyses reported in Figure 1A show that anti-CYP1A1/1A2 antibody detected upper CYP1A1 and lower CYP1A2 bands in the hepatic microsomes of rats injected with 3-methylcholanthrene used as a positive control (lane 0). Control males (Fig. 1A, lanes 1–3) showed CYP1A2 (53 kD) as identified in our previous study (24),

indicating constitutive expression of this isozyme in the rat liver. The light bands below CYP1A2 protein are probably a degradative product of CYP1A2 (29). In the males of BaP group, densitometric measurements of CYP1A2 bands (Fig. 1A, lanes 4–6) showed a 30% decrease of CYP1A2 protein compared to the controls. In the females, CYP1A2 protein of rats in the BaP group (Fig. 1A, lane 8) was unaltered compared with the control group (Fig. 1A, lane 7). These data from the Western blot study are consistent with the observed EROD activity of the control and BaP-treated groups.

CYP2E1. *p*-NPH was measured as the indicator for CYP2E1. Table 2 shows that *p*-NPH activity was 54% higher in the females compared with the males in the control group. In

Figure 1. Western blot analysis of rat liver microsomes with CYP1A1/1A2 (A), CYP2C11 (B), and CYP2C12 (C) antibodies. A, Lane 0 shows 3-methylcholanthrene-induced induction of CYP1A1 (upper band) and 1A2 (lower band) used as control. BaP-treated male rats (lanes 4–6) show diminished CYP1A2 bands as compared with the oil controls (lanes 1–3). BaP-treated female rats (lane 8) shows no change compared to the oil controls (lane 7). B, The major bands at 50 kD (2C11) in BaP-treated male rats (lanes 4–6) are denser than those of controls (lanes 1–3). The light bands below 2C11 bands are CYP2C13 (48 kD) and show no changes. In the female rats CYP2C6 (50 kD) and 2C12 (49 kD) do not appear altered (lanes 7 and 8). C, In the control females (lanes 1–3), anti-CYP2C12 antibody detected a dense band of CYP2C12 at 49 kD and a light band of CYP2C6 at 50 kD, which appeared to be of equal density to those of the BaP group (lanes 4–6). In the control males (lane 7), anti-CYP2C12 antibody detected a dense band of a combination of CYP2C6 and 2C11 at 50 kD and a light band of CYP2C13 at 48 kD, which were not altered in BaP group (lane 8).

Table 2. Effect of neonatal BaP treatment on microsomal enzyme activities in adulthood

Group	EROD	<i>p</i> -NPH
Male		
Oil	14.10 ± 1.91*	0.410 ± 0.046†
BaP	11.35 ± 1.66‡	0.402 ± 0.091
Female		
Oil	19.88 ± 1.79	0.633 ± 0.124
BaP	19.15 ± 4.18	0.765 ± 0.099

Each value represents the mean ± SD (*n* = 5).

* pmol/mg microsomal protein/min.

† nmol/mg microsomal protein/min.

‡ Significantly different from oil control (*p* < 0.05).

Table 3. Effect of BaP treatment on testosterone hydroxylation in male rats

Treatment	Testosterone hydroxylation				
	2 α	16 α	6 β	7 α	Androstenedione
Oil	0.733 $\pm$ 0.072	1.03 $\pm$ 0.14	0.337 $\pm$ 0.023	0.322 $\pm$ 0.077	0.230 $\pm$ 0.051
BaP	0.944 $\pm$ 0.097*	1.25 $\pm$ 0.14†	0.306 $\pm$ 0.089	0.353 $\pm$ 0.100	0.311 $\pm$ 0.024†

Each value represents mean nmol metabolite formed/mg microsomal protein/min $\pm$ SD ($n = 5$).

* Significantly different from oil control, $p < 0.01$.

† Significantly different from oil control, $p < 0.05$.

both the male and female rats, p -NPH activity showed no alteration after neonatal BaP administration.

Sex-specific P450 isozymes. In the rat, P450 exhibits a high degree of sex specificity (1). The activities of male-specific CYP2C11 are represented by those of testosterone 2 α - and 16 α -hydroxylases and androstenedione formation which were increased by 29% ($p < 0.01$), 22% ($p < 0.05$), and 35% ($p < 0.05$), respectively, in the BaP-treated male rats (Table 3).

Figure 1B shows Western blot analyses of microsomal protein using anti-CYP2C11 antibody, which is known to react with 2C11 (50 kD), 2C12 (49 kD), 2C13 (48 kD), and 2C6 (50 kD) (30). In the control males, anti-CYP2C11 antibody detected a dense band of CYP2C11 (and 2C6) at 50 kD and a light band of CYP2C13 at 48 kD (lanes 1–3). Densitometric analyses showed that the density of the 50-kD band of the BaP group (lanes 4–6) was 120% of the control value, consistent with the increased values obtained for 2 α - and 16 α -hydroxylase activity. CYP2C13 bands showed no alterations after neonatal BaP administration. In the females, anti-CYP2C11 antibody cross-reacted weakly with CYP2C6 (50 kD) and 2C12 (49 kD) for both the control (lane 7) and the BaP groups (lane 8) and showed no demonstrable difference between the two groups.

Table 3 demonstrates that in adult male rats testosterone 6 β -hydroxylase, representing male-specific CYP3A2, showed no significant changes although there was some decreasing tendency of this isozyme in adult male rats. Testosterone 7 α -hydroxylase, representing female-specific CYP2A1, similarly showed a tendency to increase.

Figure 1C shows the results of Western blot analyses of female rats, using anti-CYP2C12 antibody, which cross-reacts with CYP2C6, 2C11, and 2C13 (Imaoka S, personal communication). In the control females (lanes 1–3), anti-CYP2C12 antibody detected a dense band of CYP2C12 at 49 kD and a light band of CYP2C6 at 50 kD, both of which appeared to be of equal density to those of the BaP group (lanes 4–6). This was further confirmed by densitometric measurements. These observations indicate that neonatal BaP administration did not alter the content of CYP2C12 in adult females. In the control males (lane 7), anti-CYP2C12 antibody detected a dense band of combination of CYP2C6 plus 2C11 at 50 kD and a light band of CYP2C13 at 48 kD, which were not altered in the BaP group (lane 8).

Total and free glucocorticoid receptor levels. The number of total glucocorticoid receptors increased by 21% in the male BaP group compared to the controls ($p < 0.05$), while in the females it was not significantly changed (Table 4). The free receptor number was also increased by 35% in males ($p <$

Table 4. Effect of neonatal BaP treatment on glucocorticoid receptor in rat liver cytosol

Group	Glucocorticoid receptor	
	Free	Total
Male		
Oil	0.198 $\pm$ 0.036	0.231 $\pm$ 0.034
BaP	0.267 $\pm$ 0.011*	0.279 $\pm$ 0.056*
Female		
Oil	0.185 $\pm$ 0.039	0.218 $\pm$ 0.028
BaP	0.223 $\pm$ 0.025	0.233 $\pm$ 0.023

Each value represents mean pmol dexamethasone bound/mg cytosol protein $\pm$ SD ($n = 5$).

* Significantly different from oil control ($p < 0.05$).

0.05) and increased tendency was seen in the females. This degree of change is probably physiologically important since glucocorticoid suppression produced by total hypophysectomy of adult male rats has been shown to produce only 60% increase in the receptor number (31).

DISCUSSION

The results presented here show that neonatal BaP administration resulted in gender-specific changes of P450 isozymes. Adult male rats demonstrated decreased expression of CYP1A2 and increased expression of male-specific CYP2C11 along with decreasing and increasing tendency of male-specific CYP3A2 and female-specific CYP2A1, respectively. In the adult females there were no significant changes of P450 isozymes. Further, free glucocorticoid receptor was elevated in male rats. These results suggest that BaP given during neonatal period causes abnormality in the sexual differentiation of male rats.

The Ah receptor shares many physicochemical properties in common with the glucocorticoid receptor (32), suggesting that both the Ah receptor and the glucocorticoid receptor are structurally and functionally closely related and may belong to the same superfamily of regulatory proteins as the steroid hormone receptor. An interesting aspect of the regulation of CYP1A subfamily is the presence of a glucocorticoid-responsive element within intron 1 of the CYP1A1 gene (33, 34). Mathis *et al.* (35) have reported that glucocorticoids act in an apparently multiplicative fashion to potentiate the induction of CYP1A1 by PAH, suggesting that the glucocorticoid receptor may interact with the CYP1A1 gene to enhance the induction of this protein. Dexamethasone has been found to potentiate by 2- to 3-fold the inducing effect of 3-methylcholanthrene on hepatic CYP1A1 and 1A2 after administration of these agents to rats *in vivo* (36). Liddle *et al.* (37) showed increased expression of

CYP2C11 mRNA and decreased 2C13 mRNA after dexamethasone treatment of liver cell culture. Furthermore, CYP1A and glucocorticoid receptor transcription may be simultaneously activated by BaP because of the structural homology of xenobiotic responsive element of CYP1A1 gene and the glucocorticoid responsive element of the glucocorticoid receptor gene (38).

The most intriguing observation in the present investigation is the positive imprinting (increase) of CYP2C11, one of the most predominant male-specific isoform that undergoes developmental and age-dependent regulation (3). Positive imprinting of CYP2C11 has been reported only by the use of Aroclor 1254, a commercial polychlorinated biphenyl mixture (4). On the other hand, negative imprinting of CYP2C11 has been shown with neonatal castration (39) and monosodium glutamate administration (40) with an accompanying change in the pattern of growth hormone secretion, as well as after neonatal hyperoxic exposure (21). The mechanisms for the negative or positive imprinting are not well understood except for the changes with monosodium glutamate administration in which the negative imprinting of CYP2C11 was induced by the altered pattern of growth hormone secretion induced by decreased growth hormone releasing hormone secondary to the necrosis of the arcuate nucleus (41). Although earlier studies have suggested that the metabolic activation of BaP and BaP diol is catalyzed largely by CYP1A1 (42), Hall *et al.* (43) have reported that rat cytochrome P450C2, 2C5, 2B1, 2B2, 1A1, 1A2, and 2A1, were capable of metabolizing BaP to the ultimate carcinogen, BaP-7,8-dihydrodiol-9,10-epoxide. Recently Todorovic *et al.* (44) have shown that MAb 1-68-11, which is a MAb raised to constitutive CYP2C11 from male Sprague-Dawley rat liver, inhibited most of the formation of BaP-7,8-dihydrodiol and BaP-9,10-dihydrodiol from BaP, indicating that this P450 isozyme is an important contributor to the catalytic formation of these metabolites. Herd and Greene (18) have shown that perinatal exposure of rats to BaP resulted in decreased levels of aryl hydrocarbon hydroxylase activity and DNA adduct formation, suggesting one or more of BaP metabolizing P450 isozymes were permanently altered in adult rats. Whether the changes affecting individual isozymes are similar to the changes we have shown here needs to be pursued as there are cases in which perinatal and postnatal xenobiotic exposure results in a differing pattern of changes of P450 isozymes. Thus, there appears to be a reasonable rationale for the detailed study of the interrelationship between BaP administration and changes of CYP2C11 as well as other P450 isozymes involved in BaP metabolism.

Likewise, how these interrelated observations among CYP1A, 2C11, and glucocorticoid receptor can be reconciled with the observed enzyme imprinting effects of BaP (down-regulation of CYP1A2, and up-regulation of CYP2C11 and glucocorticoid receptor) must await systematic and chronological study of estrogens, androgens, growth hormone, P450 isozymes, and steroid receptors from birth to adulthood after BaP administration.

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