

Neonatal Hyperoxia and Cytochrome P450 Imprinting in Adulthood

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ABSTRACT. We hypothesized that during a critical neonatal period hyperoxia may produce alterations of sex-specific cytochrome P450 isozymes in adulthood (enzyme imprinting). To test this, newborn rats were exposed to 24 or 72 h of hyperoxia ($O_2 > 95\%$) within 24 h after birth and killed at 120 d. In males, significant negative imprinting (decrease) was found in total cytochrome P450 content and male-specific CYP2C11 in the hyperoxia groups. Positive imprinting (increase) was noted in CYP1A2 and male-specific CYP3A2 in the 72-h hyperoxia group. These alterations were essentially similar when expressed on a per microsomal protein or per liver basis. In addition, the level of hepatic glucocorticoid receptor in adult male rats was elevated after neonatal hyperoxia. In females, there was a significant body and liver weight loss after hyperoxic exposure, which resulted in a negative imprinting of CYP1A2 and female-specific 2C12 in the 72-h hyperoxia group on a per liver basis, whereas the measured parameters were unaltered when expressed per microsome. In general, the changes were more marked with longer hyperoxic exposure, suggesting that more pronounced alterations may be induced with prolonged neonatal hyperoxia. Because hyperoxic exposure in premature neonates is a common clinical practice and decreased CYP2C11 in adult males is expected to result in feminization, we believe that the scope of this work should be expanded and eventually tested for its relevance in human subjects. (*Pediatr Res* 35: 255–258, 1994)

Abbreviations

P450, cytochrome P450
CYP, cytochrome P450 isozyme
Ah, aryl hydrocarbon
EROD, 7-ethoxyresorufin O-deethylase
GH, growth hormone
MSG, monosodium glutamate
PAH, polycyclic aromatic hydrocarbon

There are two lines of report that suggest that hyperoxia given during a critical neonatal period may produce permanent alterations of P450 of liver in adulthood, a condition known as enzyme imprinting (1). The first investigation involves similarity between hyperoxia and halogenated hydrocarbon or PAH administration. The demonstration from this laboratory (2), showing induction of rat hepatic CYP1A1 and 1A2 by hyperoxia, is unusual and suggests the formation of endogenous ligand(s) for

Ah receptor by hyperoxic exposure, because an induction of these isozymes has been principally associated with Ah receptor-ligand interaction with PAH (3). Several PAH have been shown to induce P450 enzyme imprinting. For example, 3-methylcholanthrene given during the neonatal period results in decreased P450 content in adult female rats at 200 d of age (4). The second line of investigation involves P450 enzyme imprinting produced by neonatal administration of MSG, which is apparently the result of altered GH secretion (5). Because hyperoxia of neonates inhibits glutamine synthetase (6), a situation prone to increase glutamate, it was reasoned that P450 enzyme imprinting may likewise occur after neonatal exposure to hyperoxia. This hypothesis was tested with male and female rats by evaluating sex-specific P450 (primarily CYP2C11 for male and CYP2C12 for female), hyperoxia-inducible P450 (CYP1A1 and 1A2), and glucocorticoid receptors.

MATERIALS AND METHODS

Animals and treatment. Newborn male and female pups (12 h old) with nursing mothers (Sprague-Dawley CD strain, viral pathogen-free) from nine litters were pooled and randomly assigned to a control, 24-h ($24-O_2$), or 72-h ($72-O_2$) hyperoxia group. Litter size was maintained at 10 pups [five males (mean weight 7.2 g) and five females (mean weight 6.7 g)] per dam. The pups were housed with a dam in a Plexiglas chamber at 24–26°C with a continuous flow of oxygen ($>97\% O_2$, $<0.05\% CO_2$) (2). Dams in the hyperoxia group were exchanged daily with new dams without a decrease of the O_2 concentration below 95%. The pups, which were kept on a 12-h light-dark cycle, were weaned from dams at 30 d, allowed rodent food and water *ad libitum*. The rats were killed after intraperitoneal sodium pentobarbital injection (70 mg/kg) at 120 d, in accordance with the guidelines of the University of California, Irvine. The livers were removed and weighed, and microsomes were isolated (5).

Measurements of P450 enzymes and activities. P450 enzymes and activities were measured as described (7). Testosterone hydroxylation was assayed as described (8), using nonsaturating substrate concentration to retain the specificity of the microsomal hydroxylation pathways (6). Separation of testosterone metabolites was carried out by HPLC (Perkin-Elmer, Norwalk, CT) with a Supelcosil LC-18 reverse-phase C_{18} column (5 μm , 150 \times 4.6 mm) preceded by a Supelcosil LC-18 guard column (Supelco Inc., Bellefonte, PA) and a LC-235 diode array detector. After addition of methyltestosterone, the samples were extracted in dichloromethane, dried, injected, and eluted at a flow rate of 1 mL/min with column temperature at 40°C. A linear gradient of 100% solvent A (H_2O :methanol:acetonitril, 64:35:1) to 55% A and 45% solvent B (H_2O :methanol:acetonitril, 18:80:2) was used from 0 to 29 min, followed by a linear gradient of 45% to 100% B from 28 to 29 min, which was held there for a total of 10 min, and finally a linear gradient of 0% to 100% A was used from 30 to 40 min followed by reequilibration with 100% A for 40 to 60 min. The column eluant was monitored for radioactivity with an HP-Flo One radiochemical detector (Radiomatic Instruments

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Table 1. Neonatal hyperoxia on microsomal enzymes of adult rat liver*

	Male			Female		
	Control	24-O ₂	72-O ₂	Control	24-O ₂	72-O ₂
P450	1.166 ± 0.081	1.111 ± 0.036	1.051 ± 0.051†	0.838 ± 0.037	0.865 ± 0.087	0.801 ± 0.090
b _s	0.569 ± 0.033	0.564 ± 0.026	0.581 ± 0.028	0.616 ± 0.087	0.587 ± 0.049	0.598 ± 0.035

* Values are mean nmol/mg microsomal protein ± SD. *n* = 5 for all groups.

† Significantly different from the control group. *p* < 0.05.

Table 2. Neonatal hyperoxia on testosterone hydroxylation in adult rat liver*

	Control	24-O ₂	72-O ₂
2α	1141.5 ± 300.3	776.1 ± 123.7†	759.1 ± 142.5†
16α	1793.0 ± 469.2	1145.5 ± 178.3†	1176.7 ± 258.4†
6β	229.0 ± 40.4	260.7 ± 46.2	426.4 ± 10.8‡

* Values are mean pmol metabolite formed/mg protein/min ± SD.

Control, *n* = 4; 24-O₂, *n* = 5; 72-O₂, *n* = 4.

† Significantly different from the control group. *p* < 0.05.

‡ Significantly different from the control group. *p* < 0.01.

Inc., Tampa, FL) attached in tandem with HPLC and testosterone metabolites were identified with authentic standards.

Western blot analysis. Polyclonal anti-CYP1A1/1A2 serum (Oxford Biochemical Research Inc., Oxford, MI), MAb 1-7-1 (anti-CYP1A1/1A2) and HyHel-9 (anti-chicken lysozyme, Dr. Park, NIH), polyclonal anti-CYP2C11 and 2C12 (Dr. Imaoka, Osaka, Japan), and alkaline phosphatase-conjugated IgG (Sigma, St. Louis, MO) were used as described previously (7).

Glucocorticoid receptor binding study. The cytosol of homogenates was treated with dextran-coated charcoal for 2–4 min at 4°C to remove endogenous steroids. Free and total receptor assays were carried as described (9).

Statistical analysis. Results are expressed as mean ± SD and plotted. Statistical significance of the difference between control and O₂-exposed groups was determined by one-way analysis of variance and the Dunnett's *t* test.

RESULTS

Body, liver weight, and P450 content. In male rats, hyperoxia groups showed a slight tendency for body weight loss. Female rats in the 72-O₂ group showed an 11% decrease in body weight (265.4 ± 18.3 versus 273.2 ± 14.9 g, *p* < 0.05, *n* = 5) and a 16% decrease in liver weight (9.02 ± 0.96 versus 7.55 ± 0.79 g, *p* < 0.05, *n* = 5). Likewise, the microsomal protein content per liver was decreased by 22% in the female 72-O₂ group (114.08 ± 19.43 versus 88.42 ± 15.11 mg, *p* < 0.05, *n* = 5), although the values per gram of liver were not altered among the three groups including both males and females.

P450 content as expressed per mg microsomal protein of the male 72-O₂ group showed slight (10%) but significant depression (Table 1, *p* < 0.05), whereas in females there was no change of P450.

CYP2C11. The activity of 2α testosterone hydroxylase was decreased by 32% and 33% in the 24-O₂ and the 72-O₂ groups, respectively. The activity of 16α testosterone hydroxylase was reduced by 36% and 34% in the 24-O₂ and the 72-O₂ groups, respectively (Table 2).

Figure 1A shows Western blot analyses of microsomal P450 protein from the three groups of male rats using anti-CYP2C11 antibody, which cross-reacts with CYP2C12, 2C13, and 2C6. Lane 0 is from female control rat and the main band at 49 kD appears to represent cross-reacting CYP2C12. Lanes 1 through 9 are from male rats and are composed of dense bands of CYP2C11 (and probably 2C6) at 50 kD and light bands of CYP2C13 at 48kD. The densities of the 24-O₂ and the 72-O₂ groups were 90.7% and 75.5% of the control values, respectively (Fig. 1C), results consistent with those obtained for 2α and 16α testosterone hydroxylase activity, indicating a 25–35% decrease of CYP2C11 in the 72-O₂ group. Male-specific CYP2C123 was unchanged among the three groups. In female rats from the three groups, there were no changes (data not shown).

CYP2C12. Because the substrate for 15β-hydroxylase specific for CYP2C12 is not available, we used immunoblot to characterize the changes of female-specific CYP2C12 in female rats. Anti-CYP2C12 cross-reacts with CYP2C6, 2C11, and 2C13. CYP2C12 is shown as dense bands at 49 kD in lanes 1 through 9, which appeared to be unaltered among control, 24-O₂, and 72-O₂ groups (Fig. 1B). Densitometric measurements showed a slight tendency to decrease (Fig. 1C). Because this measurement

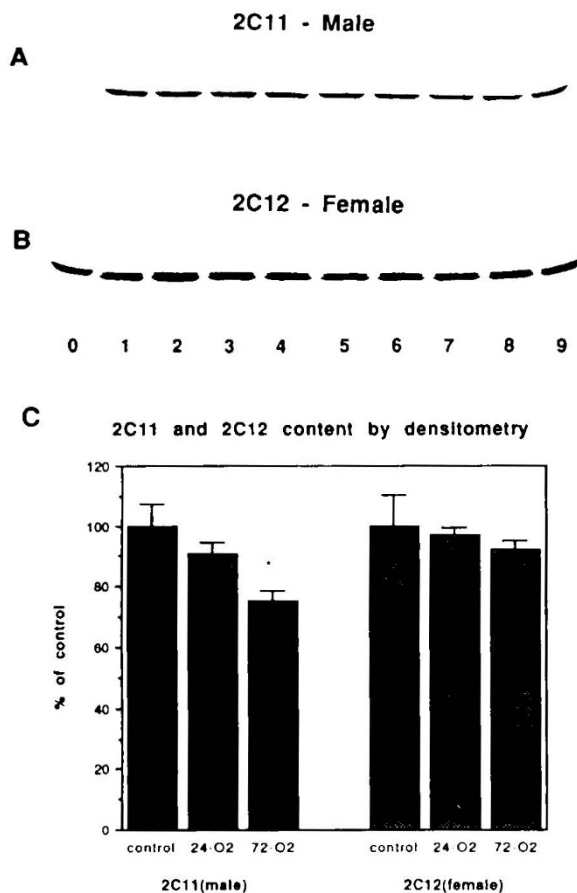


Fig. 1. Western blot analysis with anti-2C11 and anti-2C12 antibodies. A, Male rats (120 d old) that received 24 or 72 h of hyperoxia immediately after birth. Each lane represents one animal. Western blot with anti-2C11 antibody shows decreased 2C11 for the 72-h group (lanes 7–9). B, Female rats (120 d old) with anti-2C12 antibody showing a slight tendency for decreased 2C12 in hyperoxic groups. C, In males, the densitometric measurements of anti-2C11 for the 24-O₂ and the 72-O₂ groups were decreased (100 ± 7.3%, 90.7 ± 3.8%, and 75.5 ± 3% in control, 24-O₂, and 72-O₂, respectively; *, *p* < 0.01). In females, the densities of anti-2C12 showed a slight tendency to decrease (100 ± 10.4%, 97.2 ± 2.0%, and 92.3 ± 2.6% in control, 24-O₂, and 72-O₂, respectively). On a per liver basis, there was a 30% decrease in the female 72-O₂ group (100.0 ± 5.0 vs 70.1 ± 2.3%, *p* < 0.01).

Table 3. Neonatal hyperoxia on total glucocorticoid receptor of adult rat liver*

Male			Female		
Control (n = 5)	24-O ₂ (n = 4)	72-O ₂ (n = 4)	Control (n = 5)	24-O ₂ (n = 5)	72-O ₂ (n = 5)
0.40 ± 0.03	0.44 ± 0.01†	0.45 ± 0.03	0.35 ± 0.04	0.40 ± 0.05	0.39 ± 0.05

* Values are mean pmol ³H-dexamethasone bound/mg protein ± SD.

† Significantly different from the control group, *p* < 0.05.

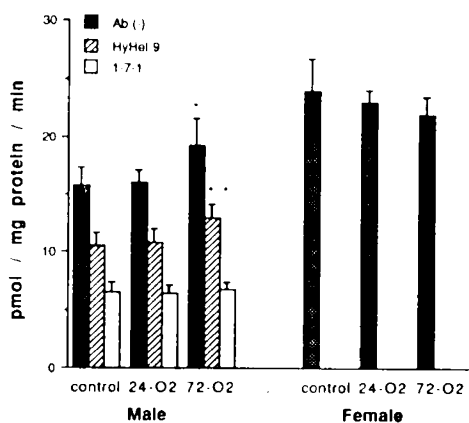


Fig. 2. Neonatal hyperoxia and EROD activity in the adult. *Left panel.* In males, EROD was significantly increased with (**, *p* < 0.01, HyHel1-9; 10.57 ± 1.18 vs 13.05 ± 1.13 pmol/mg of microsomal protein) or without [**p* < 0.05, Ab(-)] nonspecific antibody in the 72-O₂ group. The 1-7-1 antibody abolished a significant difference in the EROD activity of control and 72-O₂ groups. *Right panel.* In females, there was a slight tendency for EROD activity per microsome to decrease in hyperoxia groups. There was, however, a significant decrease in EROD activity per liver (1935 ± 320 vs 2731 ± 466 pmol, *p* < 0.01).

is based on microsomal protein, we calculated the values per liver, which showed a decrease of 30% in the 72-O₂ group (*p* < 0.01). The light bands above CYP2C12 at 50 kD probably represent CYP2C6. In males, this band represents a combination of 2C6 and 2C11, shown in lane 0. Thus, these studies demonstrate that CYP2C12 decreased in female rats on a per liver basis after neonatal exposure to hyperoxia. In male rats from three groups, the results were identical with that shown in lane 0 of Figure 1B and showed no changes among the three groups (data not shown).

CYP3A2. Testosterone 6β hydroxylase activity, representing male-specific CYP3A2, increased almost 2-fold in male rats of the 72-O₂ group (Table 2).

Glucocorticoid receptor. The total number of glucocorticoid receptors increased by 10% in the 24-O₂ group in the males (*p* < 0.05) and increased further (12.5%) in the 72-O₂ group compared with the controls, the latter showing large individual variations (Table 3). In the females, there was a tendency for increase in the hyperoxia groups. These results are similar to those of hypophysectomy in males and females.

CYP1A1/1A2. In males, EROD activity was significantly increased in the 72-O₂ group (*p* < 0.01, Fig. 2), which was similar to HyHel-9 (nonspecific antibody control). MA b 1-7-1 abolished a significant difference in the EROD activity of control and 72-O₂ groups in male rats, indicating that the increased EROD activity was likely due to an increase in CYP1A1/1A2. Although in female rats there was no significant difference between control and hyperoxia groups per mg microsomal protein, there was a significant decrease of EROD activity per liver (2731 ± 466 versus 1935 ± 320 pmol, *p* < 0.01).

CYP1A2 protein (53 kD), as identified in our previous study (7), was seen as the major band for control, 24-O₂, and 72-O₂ groups (Fig. 3A and B). Light bands below CYP1A2 protein are probably a degradative product of 1A2 (10). In males, CYP1A2

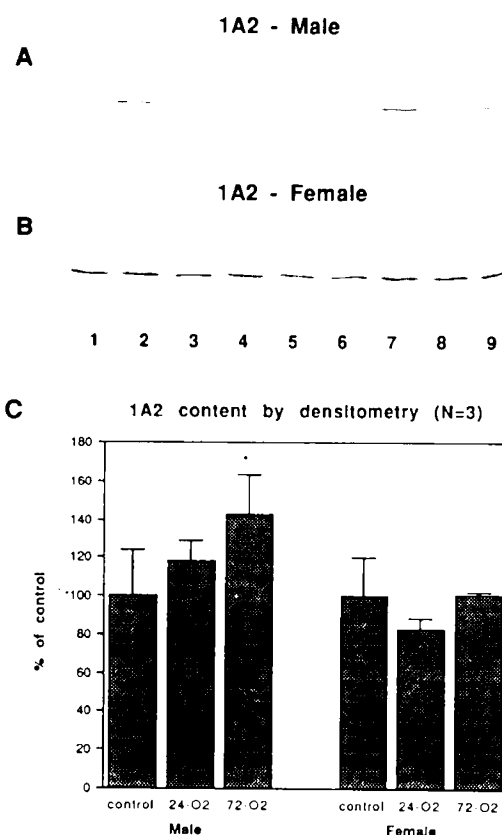


Fig. 3. Western blot analysis with anti-1A2 antibody. *A.* In males with polyclonal anti-1A1/1A2 antibody showing 1A2 protein, the major bands are denser in the 72-O₂ group (lanes 7-9) compared with those of controls (lanes 1-3). *B.* In females, 1A2 proteins were unaltered among the three groups. *C.* In males, the densitometric measurements for the 72-O₂ group showed a 42% increase over the control value (100.0 ± 23.8% vs 142.2 ± 21.3%; *, *p* < 0.05). In females, there were no changes.

protein bands were denser in the 72-O₂ group compared with those in the controls and showed a 42% increase over the control value by densitometric measurements (*p* < 0.01, Fig. 3C). A small increment in 1A1 protein could have been missed in this study because of the very low constitutive level of CYP1A1. In females, CYP1A2 proteins were unaltered among the three groups (Fig. 3B and C). These results are consistent with the values of EROD activity shown in Figure 2 and show increased CYP1A2 in males after neonatal hyperoxia, the only documented enzyme imprinting involving this subfamily of P450 to date.

DISCUSSION

The results presented here show that neonatal hyperoxia of 24 or 72 h duration altered the profiles of P450 in adult rats in a gender-differentiated manner. The 120-d interval between oxygen exposure and the observed effects is consistent with lifelong changes in the regulation of these enzymes (enzyme imprinting). There was a general trend for the 72-h exposure group to show more pronounced alterations than those seen in the 24-h exposure group, indicating the need for further examination of dose-

response of the measured parameters with the hyperoxic exposure of varied concentration and duration.

In rats, P450 exhibit a high degree of sex specificity. Sex-specific P450 for males include CYP2C11, 2C13, 2A2, and 3A2 and for females, CYP2C12 and 2A1. Enzyme imprinting of P450 has been demonstrated primarily in sex-specific P450, as observed after exposure to estrogenic or androgenic compounds (1), neonatal castration (1), MSG administration (5), and exposure to xenobiotics (4). Many studies have demonstrated that the characteristic gender-specific pattern of GH secretion is of prime importance in the expression of male- or female-specific P450 isozymes (1), which may be one of the targets of androgen imprinting for sexual differentiation occurring during a critical neonatal period (1). Neonatal castration results in a decrease in CYP2C11 and an increase in CYP2C12 in adult male rats (1). By depleting GH-releasing factor through destruction of arcuate nucleus of hypothalamus, MSG treatment of neonates produced a decrease in male-specific CYP2C11, 3A2, and 2A2 and an increase in female-specific CYP2A1 in adult male rats (11). In both instances, the patterns of P450 isozyme changes are significantly different from the results of the current study. Hypophysectomy of adult male rats showed a decrease in CYP2C11, an increase in CYP3A2 (1, 11), and an increase in glucocorticoid receptor (12), a pattern similar to that seen in the male rats in current investigation. In female rats, hypophysectomy results in a decrease in CYP2C12 (11). Our results, showing a decrease of this isozyme expressed per microsome, a denominator used in other published studies, may ultimately become significant if hyperoxic exposure is prolonged. Thus, the altered profiles in both males and females in this study are similar to the acute effects of hypophysectomy. It remains to be seen that the changes of P450 isozymes seen in this study are indeed a manifestation of pituitary pathology. Similarly, the relationship between the current observation and a karyorrhexis of the pontosubicular region of brains seen after neonatal hyperoxic exposure (13) needs to be defined. The neonatal rats exposed to 100% oxygen for 2 h demonstrated decreased glutamine synthetase activity of brain (6), which is expected to cause an accumulation of glutamate, a situation similar to that with MSG administration. Such a mechanism may also contribute to the development of the observed effects in the current study. The pathogenetic mechanism, however, may be more complex than the hypothalamo-pituitary involvement: we showed that neonatal hyperoxia produced permanent up-regulation of CYP1A2 in the liver. Assuming that an induction of CYP1A1/1A2 by hyperoxia is mediated through Ah receptors as in PAH (3), the increased glucocorticoid receptor level seen in this study may be mediated through demonstrated structural homology between a xenobiotic-responsive element of CYP1A1 gene and a glucocorticoid-responsive element of the glucocorticoid receptor gene (14); both elements promote transcription of their respective gene products. Furthermore, changes in sex steroids occurring secondary to the altera-

tions of sex-specific P450 isozymes may likewise contribute to the ultimate outcome.

Whatever the mechanism may be, a long-lasting decrease in CYP2C11 may be expected to feminize the male rats because the level of estradiol may have been elevated in these rats for several reasons: androgens are normally deactivated by hydroxylation by CYP2C11, and decreased CYP2C11 and a subsequent reduction of hepatic clearance of androgens may produce excess estrogen through extrahepatic aromatization of surplus androgens (15). In addition, CYP2C11 plays an important role in the deactivation of estrogens via 16α -hydroxylation and catechol formation (15). Down-regulation of CYP2C11 is expected to facilitate the accumulation of estradiol. Furthermore, an increased level of estradiol may create a vicious cycle because it has been established that estradiol interacts with hypothalamic receptors and modifies the release of neuroendocrine factors including somatostatin and GH-releasing factor (15) and thus down-regulates CYP2C11.

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