Arch. Pharm. Res. **15**(1), 78-86 (1992)

Sex-related Differences in Rat Hepatic Cytochromes P450 Expression Following Treatment with Phenobarbital or 3-Methylcholanthrene

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(Received February 19, 1992)

Abstract □ The induction of hepatic cytochromes P450 and metabolic effects have been examined in male and female Sprague-Dawley rats following treatment with either phenobarbital or 3-methylcholanthrene. Hepatic cytochrome P450 levels were higher in males than in females by ~40%. Treatment of male and female rats with phenobarbital or 3-methylcholanthrene resulted in an ~1.6- and 2-fold increase, respectively, in heptic microsomal cytochrome P450 levels in both sexes, relative to untreated animals. Immunoblot analyses were performed to compare sex-related changes in P450 levels. Hepatic P450IIB1 levels in males were greater than those in females following phenobarbital treatment. 3-Methylcholanthrene-induced male hepatic microsomes exhibited greater levels of P450 IA1 and IA2 than female microsomes, whereas uninduced microsomes from males or females failed to exhibit a band. Mab PCN 2-13-1 against P450IIIA recognized an intense band in uninduced hepatic microsomes from males whereas no band was recognized in uninduced microsomes from female rats. The levels of P450IIIA in males were increased 2 to 3-fold following treatment with phenobarbital, while the increase of IIIA levels in females by phenobarbital was minimal, as monitored by immunoblot analysis. Solid phase radioimmunoassay using monoclonal antibodies supported the results of immunoblot analysis. Phenobarbital treatment caused a 6.5-fold increase in the monoclonal antibody binding to IIB1 in males, whereas treatment of females with phenobarbital resulted in a 12-fold increase of IIB1 binding, relative to respective controls. The relative increase of IA levels by 3-methylcholanthrene was also greater in females than in males (10- vs. 8-fold) although the levels of induced IA were comparable in both sexes, as assessed by radioimmunoassay. Radioimmunoassay also showed that hepatic IIE1 level was 1.5-fold higher in males than in females and that either phenobarbital or 3-methylcholanthrene treatment caused 80% to 40% decrease in IIE1 levels, relative to control, in both sexes. Sex-related metabolic activities were examined in hepatic microsomes. Hexobarbital hydroxylase activity was 2- to 3-fold higher in uninduced microsomes from males than that from females. This hydroxylase activity was increased 2- and 3-fold in males and females, respectively, following phenobarbital treatment, as compared to controls. Addition of anti-P450IIB1 antibody to phenobarbital-induced hepatic microsomes from males and females produced 64% and 84% inhibition of hexobarbital oxidation, respectively. Aryl hydrocarbon hydroxylase activity was increased ~12- and 26-fold in males and females, respectively, following 3-methylcholanthrene treatment relative to controls. The anti-P450IA antibody inhibitable rate of aryl hydrocarbon hydroxylase activity was comparable in both sexes following 3-methylcholanthrene treatmet (~70%). These results demonstrate that levels of hepatic P450IIB1 or P450IA are greater in male than in female for untreated, phenobarbital- or 3-methylcholanthrene treated rats. In addition, the relative increase of P450IIB1 or IA by phenobarbital or 3-methylcholanthrene is more significant in females.

Keywords □ Hepatic cytochrome P450, sex differences, phenobarbital, 3-methylcholanthrene, monoclonal antibodies, hexobarbital hydroxylase, aryl hydrocarbon hydroxylase.

Sex-related change in the metabolism of drugs and toxicants is primarily associated with the differences in the proportion of cytochomes P450 and other Phase II metabolizing enzymes[1]. Differences in the frequency of tumor development following administration of carcinogens to animals of different sex may relate to the capacity to produce activated carcinogens. Thus, sex-specific P450 have been somewhat extensively studied[2]. Expression of sex-specific P450s are known to be developmentally regulated and sex- and developmental regulation of P450 expression is associated with the levels of sex and growth hormones. For example, P450h is expressed only in adult male rats, which is dependent on androgen exposure during the neonatal period and adulthood[3]. P450g and rat IIA2 are also male specific, whereas P450i and testosterone 16α-hydroxylase in the IIB subfamily have been characterized as female specific P450g[4-6].

The induction of the P450IA or P450IIB subfamilies is not primarily associated with the levels of sex hormones. However, it is also regulated during development. The effect of 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin on the developmental expression of P450IA1 and IA2 have been examined[7] and hepatic induction of the rat IIB1 and IIB2 mRNAs by phenobarbital as a function of age has been studied[8].

Studies in this laboratory have shown that administration of phenytoin or piperine resulted in substantial increase in P450IIB levels in rats and mice[9,10]. In the present study, the induction of hepatic cytochromes P450 and metabolic effects were compared in Sprague-Dawley rats of different sex following treatment with either phenobarbital or 3-methylcholanthrene for the purpose of examining the relative levels of major forms of P450 present in male and female hepatic microsomes and the sex-related difference in the expression of P450 by the inducers. In this study, phenotyping of major forms of P450 was accomplished using monoclonal antibodies, as assessed by both immunoblot analysis and radioimmunoassay. Finally, sex-related difference in expression of P450 was examined in metabolic activities using hexobarbital and aryl hydrocarbon hydroxylase assays in conjunction with immunochemical inhibition. This study demonstrates that absolute levels of P450IIB1 or P450IA are greater in male than in female for uninduced animals or the animals treated with PB or 3-MC, and that the relative increases of P450IIB1 or IA by PB or 3-MC, respectively, however, are more significant in females.

Abbreviations: HPLC, High pressure liquid chromatography; PB, phenobarbital; 3-MC, 3-methylcholanthrene; PCN, pregnenolone 16α-carbonitrile; P450, cytochrome P450; Mab, monoclonal antibody; IA, IIB and IIE, cytochromes P450IA, IIB and IIE (genes CYPIA, CYP2B and CYP2E) are the nomenclature recommended for members of these subfamilies which include P450IA1 and IA2, IIB1 and IIB2, and IIE1 in rats (Nebert *et al.*, 1991, DNA and cell biology) 10, 1-14.

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EXPERIMENTAL METHODS

Chemicals

Phenobarbital, 3-methylcholanthrene, NADPH and other standard reagents were purchased from Sigma Chemical Company (St. Louis, OH). Prestained protein molecular weight standards were obtained from BRL (Gaithersburg, MD) and goat antibody to mouse IgG and BCIP/NBT were purchased from Kirkegaard and Perry Lab. The monoclonal antibodies MC 1-7-1, PB 2-66-3, EtOH 2-13-1 and PCN 2-13-1 which recognized P450IA, IIB, IIE1 and IIIA were used in this study.

Animals and treatment

Male and female Sprague-Dawley rats at 7 weeks of age were used in this series of experiments. Groups of animals were treated with phenobarbital sodium (80 mg/kg/day, i. p.) dissolved in normal saline solution and 3-MC (25 mg/kg/day, i. p.) in corn oil for 3 days. Animals were starved for 16 h prior to sacrifice for preparation of microsomes. Livers used for the preparation of microsomes were perfused with isotonic saline.

Isolation of microsomal proteins

Hepatic microsomes, isolated by differential centrifugation, were washed in pyrophosphate buffer and stored in 50 mM Tris acetate buffer (pH 7.4), containing 1 mM EDTA and 20% glycerol, and stored at −70℃ until used. Protein was assayed by the method of Lowry *et al.*[11].

Total P450 content

Total content was measured as described by Omura and Sato[12].

Immunoblot analysis

Microsomal proteins were separated by 10% SDS-PAGE and electrophoretically transferred to nitrocellulose paper[13]. Following a one hour incubation in blocking solution (PBS with 5% nonfat powdered milk) at 37℃, the nitrocellulose membrane was incubated with monoclonal antibody. Alkaline phosphatase-conjugated goat anti-mouse IgG was empolyed as the secondary antibody and this was followed by incubation with BCIP/NBT substrates, as described previously[9].

Solid-phase radioimmunoassay

Radioimmunoassay was performed as described by *et al.*[14]. The wells of microtiter plates (rigid polystyrene, Dynatech) were precoated with mouse hepatic microsomes by incubating with 0.1 m*l* of 1 mg/m*l* of microsomal suspension for 2 h at 37℃. The remaining nonspecific sites were blocked with bovine serum albumin. After washing, the proteins in the wells were incubated with 50 μ*l* of 500 μg/m*l* monoclonal antibodies. The monoclonal antibodies in the wells were incubated with 35-S labeled anti-mouse IgG(k) (50,000 cpm/well) for 2 h. The individual wells were placed in 8 m*l* of Aquazol (NEN) and radioactivity was measured.

Hexobarbital hydroxylase activity

Microsomal hexobarbital hydroxylase activity was measured using HPLC. The reaction mixture contained 0.1 M phosphate buffer (pH 7.4), 7 mg hexobarbital sodium, 0.5 mg microsomal suspension, 0.1 M magnesium chloride and 5 mg NADPH in a total volume of 1.0 m*l*. Reactions were initiated by NADPH addition after a 2 min preincubation period and the incubation was continued at 37℃ for 15 min. Reactions were stopped by the addition of 1.5 m*l* acetonitrile and the precipitated protein was removed by centrifugation. The hexobarbital remained in the supernatant was separated from oxygenated metabolites on a C-18 reverse phase column (5 m*l*, 15×0.46 cm, NOVAPACK™, Waters) isocratically and quantitated spectrophotometrically at 238 nm with a variable wavelength detector (Hitachi, Japan). The mobile phase consisted of 3.5 mM sodium phosphate (pH 2.7) buffer and 33% acetonitrile and the flow rate was 0.6 m*l*/min.

Table I. Effect of PB and MC on hepatic microsomal cytochrome P450 contents in male and female rats

Treatment (microsomes)	Total P450 (nmol/mg of protein)	Percent of Control
Male		
Uninduced	1.29	100
PB-induced	2.15	167
3-MC-induced	2.55	198
Female		
Uninduced	0.91	100
PB-induced	1.50	164
3-MC-induced	1.82	200

Aryl hydrocarbon hydroxylase activity

Aryl hydrocarbon hydroxylase activity was determined by measuring 3-hydroxybenzopyrene produced from benzopyrene, as described by Nebert and Gelboin[15].

Immunochemical inhibition

The reaction mixture for immunoinhibition of hexobarbital hydroxylase and aryl hydrocarbon hydroxylase activities contained 100 mM phosphate buffer (pH 7.4), 1 mM NADPH, 0.5 mg of hepatic microsomal protein, 1.5 mg of monoclonal antibody and each substrate in a volumn of 0.5 m*l*. Antibodies were added to the microsomes and incubated at 4℃ for 30 min with gentle agitation. Metabolic activities were assayed as described previously.

RESULTS

Total P450 content

Total P450 contents of male and female rats at 7 weeks of age are summarized in Table I. Males had higher level of P450 in hepatic tissue than females with the values of 1.29 and 0.91 nmol/mg protein, respectively (~140%). Treatment of male and female rats with PB resulted in a ~1.6-fold increase in hepatic microsomal cytochrome P450 levels in both sexes relative to untreated animals

(2.15 vs. 1.50 nmol/mg protein). Microsomes isolated from 3-MC-exposed male and female rats contained 1.82 and 2.55 nmol cytochrome P450 per mg protein, respectively, exhibiting a 2-fold increase relative to controls. These data showed that males have higher concentrations of P450 than females and that the relative increase of P450 by PB or 3-MC is approximately the same in both sexes.

Immunoblot analysis

Western immunoblot analyses of hepatic microsomes from utreated, PB- and 3-MC-treated male and female rats were performed using monoclonal antibodies including PB 2-66-3, MC 1-7-1, PCN 2-13-1 and EtOH 1-98-2, which recognized P450IIB1, IA1 and IA2, IIIA, and IIE1, respectively. Immunoblot analysis using monoclonal anti-rat P450IIB1 antibody showed that hepatic P450IIB1 levels in males were greater than those in females following PB treatment (Fig. 1(a)). 3-MC-induced male hepatic microsomes exhibited greater levels of P450IA1 and IA2 (upper and lower bands, respectively) than 3-MC-induced female microsomes, whereas uninduced microsomes failed to exhibit a band in both sexes (Fig. 1(b)).

Although the levels of P450IIB1, and IA1 and IA2 are not detectable in either male or female uninduced microsomes, male uninduced microsomes exhibited an intense band of P450IIIA, as detected by immunoblot analysis using Mab PCN 2-13-1, whereas uninduced microsomes from females failed to show a band (Fig. 1(c)). P450IIIA levels were increased 2 to 3-fold in males following PB treatment, as compared to untreated animals. A faint band was detectable in female PB-induced microsomes. No significant change in the levels of IIIA was observed following treatment of animals with 3-MC in either sex, relative to that in untreated animals.

No band was recognized by Mab EtOH 1-98-2 in these microsomal preparations under the immunoblotting conditions employed.

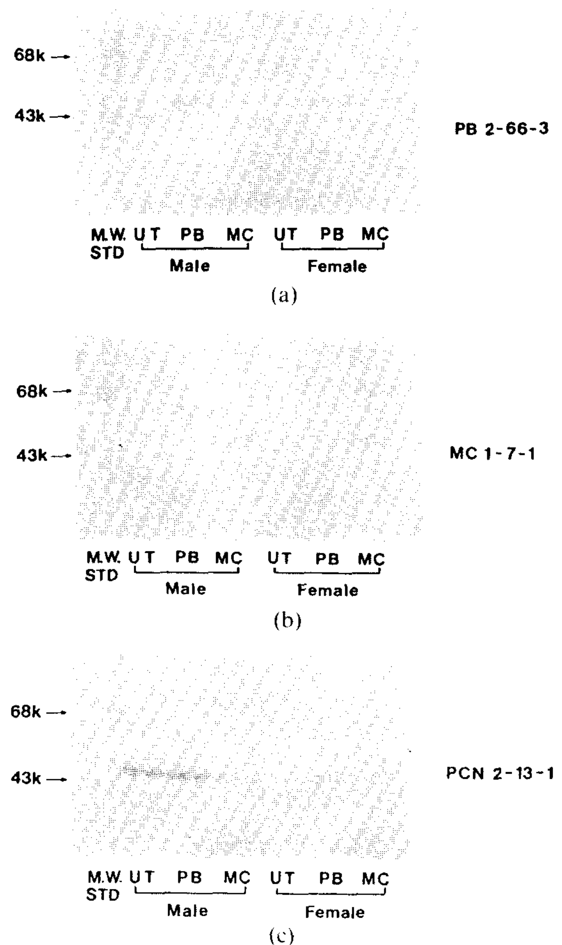

Fig. 1. (a) Western blot analysis of the levels of P450IIB 1. Lanes contained 10 μg of microsomal protein from untreated, PB- and 3-MC-treated male and female rats, respectively. (b) Immunoblot analysis of the levels of P450IA1 and IA2. An identical replicate blot employed in (a) was immunoblotted with monoclonal anti-rat P450IA antibody. (c) Western blot analysis of the levels of IIIA using Mab PCN 2-13-1. A replicate blot was immunoblotted with monoclonal anti-rat P450IIIA antibody.

Radioimmunoassay

In order to further examine the relative levels of major forms of P450 in uninduced, PB- and 3-MC-induced hepatic microsomes from males and females, solid phase radioimmunoassay was employed. Although this assay had some drawbacks (e.g. high intrinsic background due to nonspecific binding of primary antibody to the similar forms of P450, and nonspecific binding of radiolabeled secondary antibody to wells and microsomes), this assay had sufficient sensitivity to detect changes of IIB1 or IA levels between male and female uninduced microsomes. These changes were not detectable by immu-

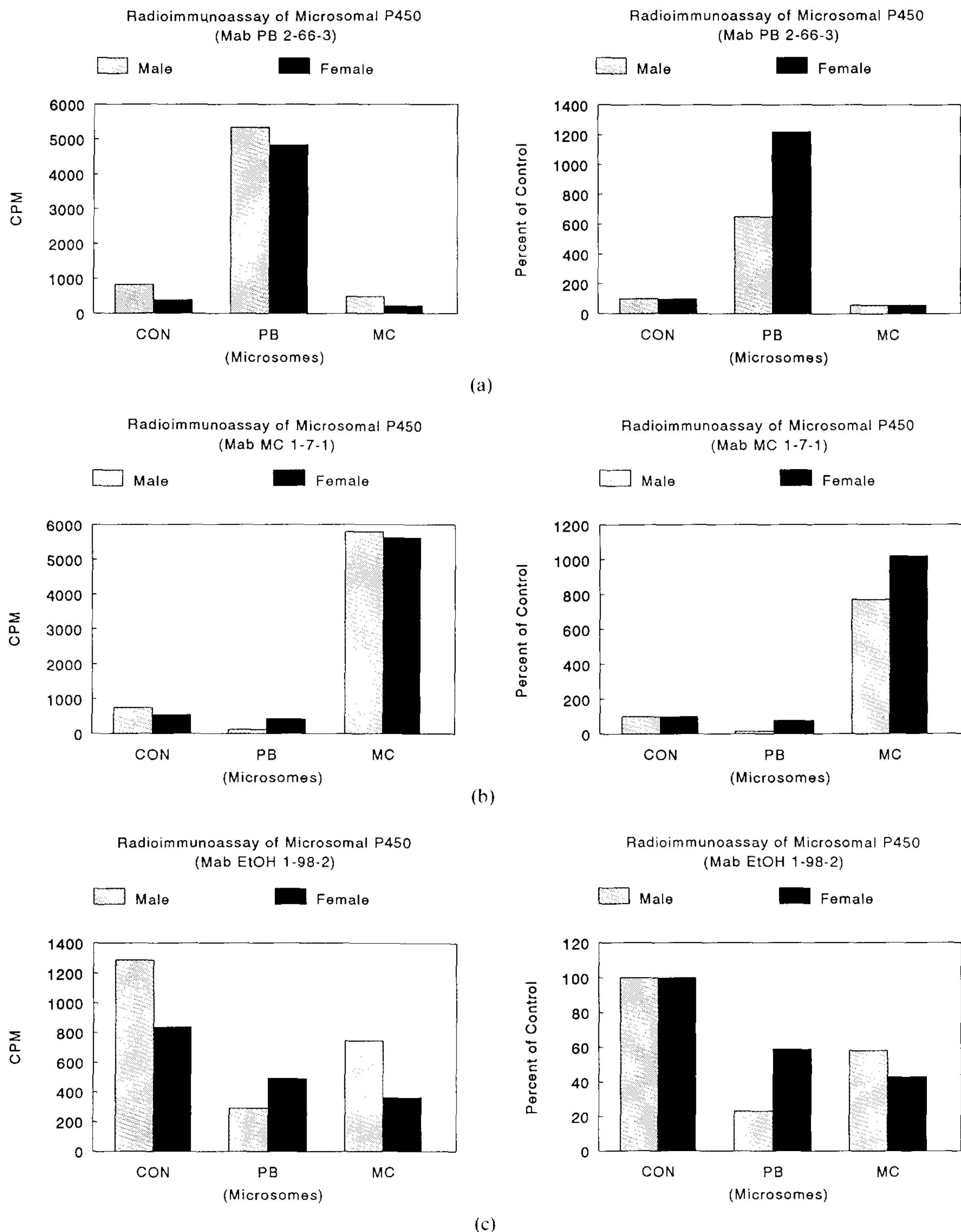

Fig. 2. Solid phase radioimmunoassay of hepatic microsomes from untreated, PB- and 3-MC-treated male and female rats using monoclonal antibodies including PB 2-66-3 (a), MC 1-7-1 (b), and EtOH 2-13-1 (c), which recognized P450IIB1, IA1 and IA2, and IIE1, respectively.

noblot analysis under the assay conditions employed.

In order to minimize the background, paralleled experiments were performed with monoclonal antibodies against normal spleen cell as controls and the values of non-specific binding (~600 cpm) were subtracted from the values of respective monoclonal antibody binding. Whereas PB-induced microsomes from males exhibited an ~6.5-fold increase of IIB1 levels relative to uninduced microsomes, treatment of female rats with PB resulted in a 12-fold increase of IIB1 relative to control. This difference in the relative increase was due to lower IIB1 levels in untreated females than those in males (395 vs. 828) (Fig.2(a)). The levels of IA recognized by Mab MC 1-7-1 in male hepatic microsomes were higher than those in female hepatic microsomes were higher than those in female hepatic microsomes by ~37%. Accordingly, the relative increase of hepatic IA levels by 3-MC was greater in females than in males (10- vs. 8-fold) although the binding of Mab MC 1-7-1 to uninduced hepatic microsomes from both sexes was comparable (Fig. 2(b)). Binding of PCN 2-13-1 to male PB-induced microsomes was significantly higher than that to untreated microsomes (713 vs 1435 cpm, values without background substraction). 3-MC treatment resulted in a slight decrease of the antibody binding. The values in female microsomes were below the background levels (data not shown). Radioimmunoassay using Mab 1-98-2 revealed that the hepatic IIE1 level was 1.5-fold higher in males than in females. PB treatment caused ~80% and 40% decreases in IIE1 levels in males and females, respectively, relative to control. Administration of 3-MC resulted in similar changes in IIE1 levels (Fig. 2(c)).

Hexobarbital hydroxylase activity and immunochemical inhibition

In subsequent research, hexobarbital hydroxylase activity was measured in uninduced microsomes or in the microsomes isolated following PB or 3-MC treatment (Fig. 3). Hexobarbital hydroxylase activity was 3-fold higher in uninduced male hepatic microsomes than in uninduced female microsomes (1.5 vs. 0.47 nmol/mg protein/min).

The microsomal hexobarbital hydroxylase activity was increased >2-fold in males following PB treatment, resulting in ~3.13 nmol/mg protein/min. PB-induced female microsomes exhibited an ~3-fold increase of hexobarbital hydroxylase activity relative to control with the catalytic rate of 1.35 nmol/mg protein/min. Anti-P450IIB1 antibody addition produced 64% and 84% inhibition of hexobarbital oxidation in PB-induced hepatic microsomes from males and females, respectively.

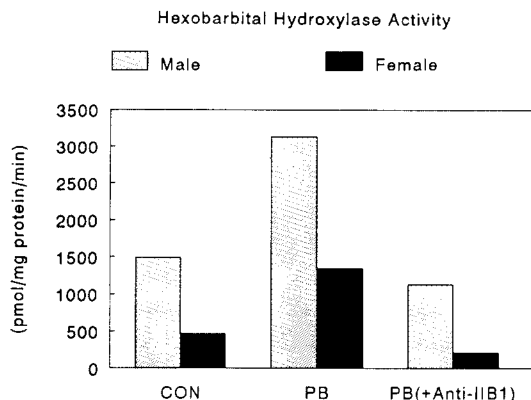

Fig. 3. Increase in microsomal hexobarbital hydroxylase activity following treatment with PB and immunochemical inhibition by monoclonal anti-P450IIB1 antibody. The anti-P450IIB1 inhibitable rate of hexobarbital hydroxylase activity in PB-induced male and female microsomes were 64 and 84%, respectively.

Aryl hydrocarbon hydroxylase activity

Aryl hydrocarbon hydroxylase activity was examined in 3-MC-induced hepatic microsomes from males and females, resulting in a 12- and 26-fold increase in the production of 3-hydroxybenzopyrene from benzopyrene relative to that in respective uninduced hepatic microsomes (Fig. 4). The effect of anti-P450IA antibody on aryl hydrocarbon hydroxylase activity in 3-MC-induced microsomes was examined. Anti-P450IA antibody inhibited aryl hydrocarbon hydroxylase activity in 3-MC-induced microsomes from male or female rats by ~70%. These data showed that relative increase in aryl hydrocar bon hydroxylase activity is more significant in females than in males following 3-MC treatment and that the elevated aryl hydrocarbon hydroxylase activity represents the increase of P450IA forms (i. e. IA1 and IA2), as evidenced by immunoinhibition.

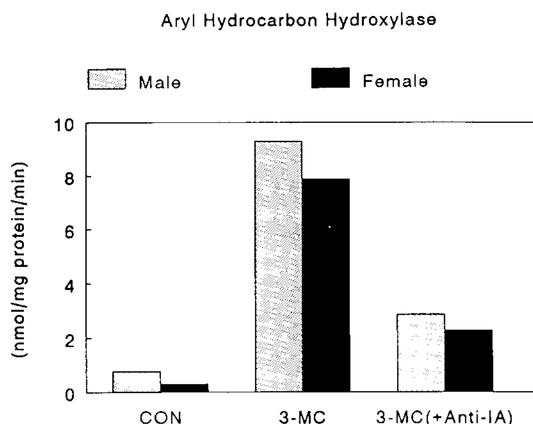

Fig. 4. Rates of microsomal aryl hydrocarbon hydroxylase activity following treatment of animals with 3-MC and immunochemical inhibition by monoclonal anti-P450IA antibody. The anti-P450IA inhibitable rate of aryl hydrocarbon hydroxylase activity in 3-MC-induced hepatic microsomes was comparable between males and females (~70%).

DISCUSSION

Previous work in this laboratory has shown that administration of phenytoin elevates hepatic microsomal P450 content in rats and that the level of P450IIB1 is significantly increased in phenytoin-induced hepatic microsomes, as assessed by both radioimmunoassay and immunoblotting[16]. In the present research, the levels of several major forms of P450 present in uninduced male and female rats were compared and the effects of phenobarbital and 3-MC on the levels of those P450 including the P450IIB and IA subfamilies were examined in both sexes. Hepatic microsomes from adult male rats have been reported to exhibit higher metabolic activities than those from females[17-19]. Results of the present study confirmed the analysis that hepatic cytochrome P450 levels are greater in males than in females (~40%). Treatment of animals with either PB or 3-MC resulted in a 1.6 to 2-fold sex-related difference in hepatic microsomal cytochrome P450 levels. In this research, the changes in the levels of P450IA, IIB, IIE and IIIA forms were examined. Expression of the forms are known to be regulated primarily by chemical inducers, not by sex or growth hormones, although most of the forms are reported to be under developmental regulation[3]. Although the levels of P450IIB1 induced by PB were higher in males than females, inductive responsiveness of P450IIB1 (i.e. relative increase) was greater in females following PB treatment, as evidenced by both immunoblotting and radioimmunoassay. This is further supported by metabolic analyses: the anti-P450IIB1 inhibitable rate of hexobarbital hydroxylase activity was ~1.5-fold greater in males than females (~3.13 vs. 1.35 nmol/mg protein/min) whereas the relative change was more significant in females, as compared to control (6- vs. 3-fold).

3-MC also induced the P450IA subfamily in males to a somewhat greater extent than that in females, as evidenced by immunoblotting and radioimmunoassay. However, the relative increase of IA levels by 3-MC was again more significant in females than in males (10- vs. 8-fold). This sex-related difference was accentuated in microsomal aryl hydrocarbon hydroxylase activity, which was increased 12- and 26-fold in males and females following 3-MC treatment, respectively, relative to control. Thus, these results showed that levels of hepatic P450IIB1 or P450IA are greater in male than in female for untreated, phenobarbital- or 3-methylcholanthrene treated rats. In addition, the relative increase of P450IIB1 or IA by phenobarbital or 3-methylcholanthrene is more profound in females.

The results of this study also showed that levels of P450IIIA (PCN-inducible forms) were increased 2 to 3-fold following treatment of male rats with PB, while the increase of IIIA levels by PB was minimal in females. The levels of IIIA in uninduced animals were markedly different between two sexes: an intense band was immunochemically detected in uninduced male microsomes whereas no band was recognized in uninduced female microsomes. The data from solid phase radioimmunoassay failed to completely agree with the results of immunoblot analysis because of the lower monoclonal antibody binding with the P450 than backgrounds. Nonetheless, both analyses provided evidence that PCN-inducible forms are sex-specifically expressed in uninduced animals and the induction profile of P450IIIA by PB or 3-MC was entirely different between males and females.

Additional radioimmunoassay using monoclonal antibody against P450IIE1 revealed that the hepatic IIE1 level was 1.5-fold higher in males than in

females. This result is in contrast with the previous published observation[20]: the livers of female rats have been reported to contain approximately 1.5- to 2.0-fold higher levels than is present in the livers of male rats. This difference may be due to the analytical methods employed in different research laboratories. P450IIE1 band failed to be detected in uninduced hepatic microsomes by Western blotting with the experimental conditions selected in this study, although radioimmunoassay allowed us to monitor significant decrease of IIE1 levels from those in untreated animals after treatment of animals with inducers. Moreover, it was interesting that PB treatment caused ~80% decrease in IIE1 levels in males whereas ~40% decrease was noted in females, as compared to control. Thus, PB appeared to suppress the expression of P450IIE1 in both males and females. Administration of 3-MC resulted in similar changes in IIE1 levels.

In summary, these results demonstrate that the levels of P450IIB1, IA, IIE1 and IIIA are greater in male rats than in females, and that relative increases of IIB1 and IA by PB or 3-MC are more significant in female rats.

ACKNOWLEDGEMENTS

This work was supported by the research grant from the Ministry of Education, Republic of Korea in 1990.

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