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Administration of Xenobiotics With Anti-estrogenic Effects Results in mRNA Induction of Adult Male-Specific Cytochrome P450 Isozymes in the Livers of Adult Female Rats

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Abstract. Cytochrome P450 (CYP) catalyzes the oxidation of many endogenous and xenobiotic compounds. The expression of CYP isozymes are modulated by endogenous hormones and xenobiotics. We found that, although CYP2C11 and CYP3A2 are adult male-specific isozymes, they are also expressed in prepubertal female Sprague Dawley (SD) rats. However, the mRNA levels for these isozymes in prepubertal female SD rats decreased over time and became undetectable at 7 weeks of age. On the other hand, ovariectomy, administration of ICI182780, a specific estrogen antagonist, or administration of lindane, which is a widely used pesticide with anti-estrogenic effects, induced these adult male-specific CYP mRNAs in adult female SD rats. These results suggest that estrogen is involved in suppression of both CYP2C11 and CYP3A2 in adult female rats. The expression of these CYP isozymes in female rats, therefore, is affected by sexual maturity and by disrupting adult female hormonal homeostasis. We also performed a field survey to examine whether the induction of CYP2C11 or CYP3A2 differs between adult female roof rats in rural and metropolitan districts. RT-PCR showed that the mRNAs for CYP2C11 and CYP3A2 were expressed in half of the adult female roof rats captured in Osaka (as a metropolitan area district) but not in those captured in Hokkaido (as a rural district). Thus, induction of the adult male-specific CYP isozymes in adult female roof rats captured in Osaka might be caused by consumption of xenobiotics with anti-estrogenic effects.

Keywords: cytochrome P450 (CYP), CYP isozyme, biomarker, adult male-specific CYP, estrogen antagonist

Introduction

Cytochrome P450 (CYP), a superfamily of heme-containing microsomal monooxygenases, transforms a wide variety of xenobiotics and metabolizes numerous endogenous compounds including several steroid compounds (1). The expression of some hepatic CYPs depends on the levels of sex steroid hormones and is thus affected by age, gender, or both (2). Changes in the expression of CYP enzymes are also associated with alterations in the endocrine system in animals (3). CYP2C11 and CYP3A2, which are adult male-specific isozymes, have been isolated in the livers of adult male but not adult female rats (4), but they can be induced in female rats by environmental xenobiotics such as dichlorodiphenyltrichloroethane (DDT) (5). The induction mechanisms of these male-specific isozymes in adult female rats, however, remain unknown. In the present study, we examined how the expression of CYP2C11 and CYP3A2 in female rats is affected by sexual maturity and by antagonizing female hormone. Moreover, to see if the induction of CYP2C11 or CYP3A2 differs between adult roof rats in rural and metropolitan districts, we measured hepatic mRNA

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levels for the CYPs in those rats captured in Obihiro city in Hokkaido (as a rural district) and in Osaka and Sakai cities in Osaka prefecture (as a metropolitan district area) in Japan.

Materials and Methods

Animals and treatments

Wild roof rats, *Rattus rattus*, were trapped in the Obihiro city area of Hokkaido and in the Osaka and Sakai city areas of Osaka prefecture in Japan. The age of roof rats was estimated using eye lens weight as an age indicator (6, 7). Using this method, roof rats estimated to be 2 to 10 months of age were used as adults in the present study. Male and female Sprague Dawley (SD) rats were purchased from Japan SLC, Inc. (Shizuoka) The SD rats were maintained under controlled temperature and lighting conditions with a 12-h light / 12-h dark cycle (lights on at 06:00).

The SD rats were ovariectomized at 3 weeks of age. ICI182780 (Tocris, Ellisville, MO, USA) (500 μg/day), a specific estrogen antagonist, was dissolved in dimethyl sulfoxide and administered subcutaneously to the intact female SD rats for 3 days via an implanted ALZET mini-osmotic pump (Cupertino, CA, USA). Testosterone propionate was administered to the intact female SD rats for 7 days via a subcutaneously implanted, 1-cm-long silastic capsule (i.d., 1 mm; o.d., 2 mm) filled with crystalline testosterone propionate and sealed at both ends with silicon adhesive. Lindane (15 or 40 mg/kg) was dissolved in corn oil and then administered to the intact female SD rats once daily for 3 consecutive days by oral injection. Control rats were administered vehicle only.

RNA extraction and competitive RT-PCR

Total RNA isolated from rat livers using TRIZOL Reagent (Invitrogen, Tokyo) was quantified by measuring the absorbance at 260 nm, and its integrity was confirmed by denaturing agarose gel electrophoresis. The mRNAs for the adult male-specific CYP isozymes, CYP2C11 and CYP3A2, were measured by competitive RT-PCR using a rat cytochrome P450 competitive RT-PCR set and a Takara RNA LA PCR^{TM} Kit (AMV) v.1.1 (Takara Shuzo Co., Kyoto) according to the manufacturer's instructions. Using this assay system, the quantification of CYP mRNAs has been successfully conducted (8). The PCR reaction was carried out in a Bio-Rad I cycler (Bio-Rad, Tokyo). The mRNA levels of the rat liver CYPs were compared using competitor RNA diluted with 25 ng of total RNA and 1 pmol of sense and antisense primers for CYP2C11, CYP3A2, and cyclophilin. The levels of CYP2C11 and CYP3A2 mRNAs were quantified using competitor RNA diluted to 5.0×10^5, 2.0×10^6, 8.0×10^6, and 3.2×10^7 copies/μl. Variations in RNA recovery from the liver samples were corrected and constitutive levels of gene transcription were confirmed using the housekeeping gene cyclophilin.

Analysis of CYP cDNAs

Amplified cDNAs were separated on 3.0% agarose gels stained with SYBR Green (Takara Shuzo Co.) and then quantified using an Epi-Light UV FA500 analyzer (Aishin Seiki, Tokyo) and NIH imaging software. CYP mRNA levels were determined as the ratio of the fluorescence intensity of the CYP cDNA to that of competitor cDNA, and the data for each rat liver sample were normalized by comparison with the amounts of cyclophilin cDNA products.

Statistical methods

In the experiment to investigate the rate of induction of adult male specific CYPs in livers of adult female roof rats captured in Hokkaido and Osaka prefecture, data were analyzed by Fisher's exact probability test. In other experiments, statistical significance was determined using Student's unpaired *t*-test.

Animal care and ethical standards

All procedures for the care and use of experimental animals were approved by the Animal Research Committee at Obihiro University of Agriculture and Veterinary Medicine and were conducted according to the Guidelines for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine and the Guiding Principles for the Use of Animals in Toxicology adopted by the Society of Toxicology in 1989. The animals were humanely killed by an overdose of anesthetic ether at the end of the experiment.

Results

Levels of CYP2C11 and CYP3A2 mRNA expression in female SD rats at various ages and in ovariectomized adult female SD rats

Although both CYP2C11 and CYP3A2 are adult male-specific isoforms of CYP, they were also expressed in prepubertal female rats (Fig. 1: A and B). However, the mRNA levels for both CYP2C11 and CYP3A2 decreased over time and became undetectable at 7 weeks of age. On the other hand, both CYP2C11 and CYP3A2 were expressed in adult female rats that had been ovariectomized at 3 weeks of age (Fig. 1: A and B). Thus, the expression of both CYP2C11 and CYP3A2 in female rats was dependent on the presence of ovaries and seemed to be affected by sexual maturity.

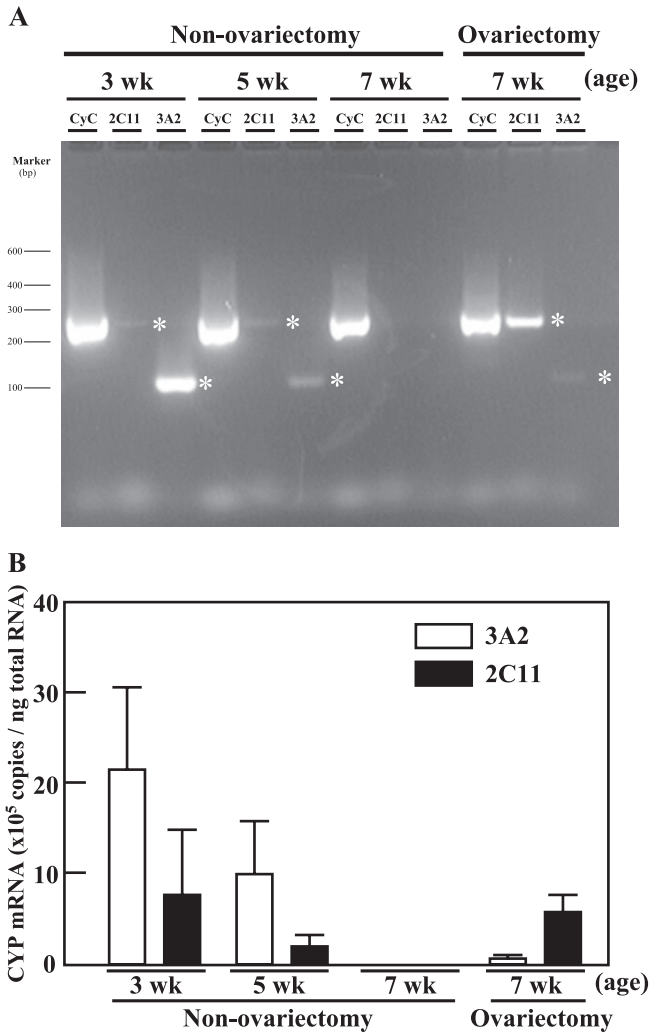

Fig. 1. The mRNA levels of CYP2C11 and CYP3A2 in female SD rats at different ages and in ovariectomized adult female SD rats. A: The levels of CYP2C11 and CYP3A2 mRNAs were determined by RT-PCR. Adult female SD rats were ovariectomized at 3 weeks of age and maintained until 7 weeks. Total RNA was isolated from 3- (n = 3), 5- (n = 3), and 7-week-old SD rats (n = 3) and from 7-week-old ovariectomized female SD rats (n = 3). Total RNA (25 ng) was reverse-transcribed using an oligo (dT) primer and AMV reverse transcriptase. First-strand cDNA products were amplified using primers for rat CYPs 2C11 and 3A2 and for cyclophilin. Agarose gels stained with SYBR Green show cDNA fragments generated from CYP2C11, CYP3A2, and cyclophilin mRNAs. Asterisks, cDNA fragments of CYP2C11 and CYP3A2; CyC, cyclophilin. B: The levels of CYP2C11 and CYP3A2 mRNA expression in livers of the female SD rats at different ages and the ovariectomized adult female SD rats were examined by competitive RT-PCR. Both CYP2C11 and CYP3A2 mRNA levels were normalized to those of cyclophilin (CyC) and are expressed as the number of copies of mRNA per ng total RNA. Results represent the means ± S.D. (n = 3).

Effect of lindane on the expression of CYP2C11 and CYP3A2 mRNA in adult female SD rats

Lindane, which is a widely used pesticide with anti-estrogenic effects, is known to affect the expression

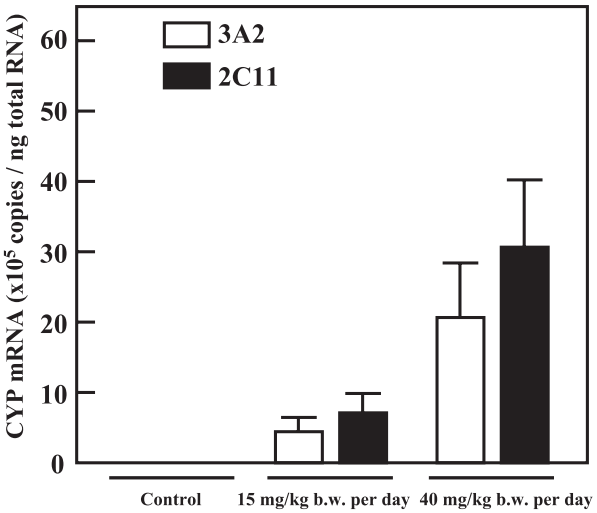

Fig. 2. Measurement of CYP2C11 and CYP3A2 mRNA expression in livers of adult female SD rats treated with lindane (15 and 40 mg/kg b.w.) for 3 days by competitive RT-PCR. Both CYP2C11 and CYP3A2 mRNA levels were normalized to those of CYC and are expressed as the number of copies of mRNA per ng total RNA. Results represent the means ± S.D. (n = 3).

level of CYP1A1, CYP2B1/2B2, CYP2C11, and CYP3A2 proteins (9). We therefore investigated how lindane affects the expression of CYP2C11 and CYP3A2 mRNA in adult female SD rats. Competitive RT-PCR showed that administration of lindane (15 and 40 mg/kg b.w. once daily for 3 days) to 8-week-old female SD rats induced the expression of CYP2C11 and CYP3A2 mRNA in a dose-dependent manner (Fig. 2). In contrast, the mRNAs for both CYP2C11 and CYP3A2 could not be detected in the control female SD rats. Thus, lindane can induce these adult male-specific CYP isozymes in adult female rats.

Effect of testosterone and ICI182780 on CYP2C11 and CYP3A2 mRNA expression in female SD rats

Because both CYP2C11 and CYP3A2 mRNAs in female rats were induced by ovariectomy and lindane, we suspected that estrogen is involved in their suppression in adult female rats. We therefore examined the effect of ICI182780, a specific estrogen antagonist, on the expression of CYP2C11 and CYP3A2 mRNAs. Figure 3 shows that ICI182780 induced the expression of mRNAs for both CYP2C11 ($2.9 \pm 1.1 \times 10^5$ copies / ng total RNA) and CYP3A2 ($0.7 \pm 0.3 \times 10^5$ copies / ng total RNA) in adult female rats. Thus, estrogen is critical for the suppression of adult male-specific CYP isozymes in adult female rats. On the other hand, chronic testosterone administration stimulated the mRNA expression for CYP2C11 ($0.3 \pm 0.1 \times 10^5$ copies / ng total RNA) but not CYP3A2 (Fig. 3).

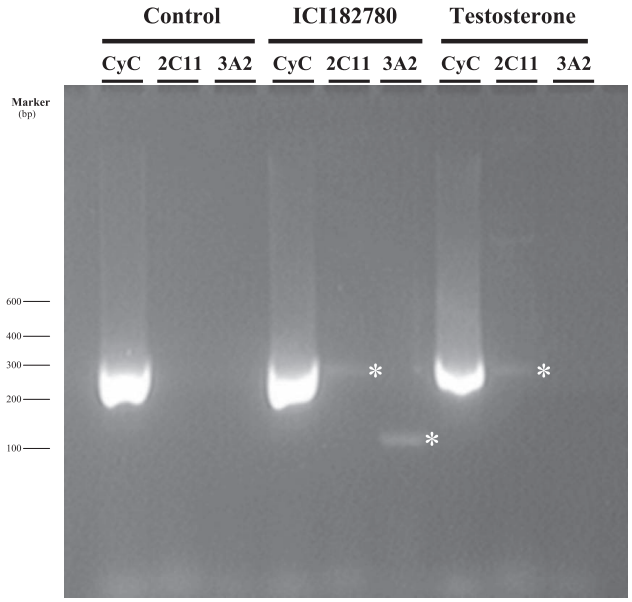

Fig. 3. Effect of testosterone and ICI182780 on the expression of CYP2C11 and CYP3A2 mRNA in female SD rats. ICI182780 (500 μg/day), a specific estrogen antagonist, was administered subcutaneously to rats (n = 3) for 3 days. Testosterone propionate was administered subcutaneously to rats (n = 3) for 7 days and then the levels of CYP2C11 and CYP3A2 mRNA were determined by RT-PCR as described in Fig. 1. Agarose gels stained with SYBR Green show cDNA fragments derived from CYP2C11, CYP3A2, and cyclophilin (CyC) mRNAs. Asterisk, cDNA fragments of CYP2C11 and CYP3A2.

Although anti-estrogenic agents such as lindane and ICI182780 can induce the expression of mRNAs for both CYP2C11 and CYP3A2 in adult female rats, testosterone only induced the mRNA expression of CYP2C11. These results suggest that the suppression of CYP2C11 in the liver of adult female rat can be controlled by at least two different mechanisms in the transcriptional regulation, that is, one is common to CYP3A2 (high level of estrogen) and another is different from it (low level of testosterone).

Analysis of mRNA expression for CYP2C11 and CYP3A2 in the livers of adult female Hokkaido and Osaka rats

We compared the mRNA expression levels of adult male-specific CYP isozymes in the livers of adult female roof rats captured in Hokkaido and in Osaka prefecture. CYP2C11 and CYP3A2 mRNAs were expressed in half of the female Osaka rats but not in any of the Hokkaido rats (Figs. 4 and 5, Table 1). The mRNA levels for CYP2C11 and CYP3A2 in the female Osaka rats were a little higher in the adult female SD rats treated with lindane (Figs. 2 and 5). Moreover, the CYP mRNAs were always coexpressed (Table 1). In contrast, induction of these CYP isozymes did not significantly differ between adult male Osaka and Hokkaido rats (Fig. 5).

Discussion

In the present study, we demonstrated that estrogen is involved in suppression of the adult male-specific CYP isozymes in the livers of adult female rats. The adult male-specific CYP isozymes, CYP2C11 and CYP3A2, are present in the livers of adult male but not adult female rats (4). Although these CYP isozymes were also expressed in prepubertal female SD rats, the mRNA levels for these isozymes in these rats decreased over time and became undetectable at 7 weeks of age. Thus, the expression of both CYP2C11 and CYP3A2 in female rats seems to be affected by sexual maturity. On the other hand, ovariectomy and administration of lindane, a widespread pesticide, induced these adult male-specific CYP mRNAs in adult female SD rats. Chadwick et al. (10) suggested that lindane has an anti-estrogenic activity in female rats. Induction of both CYP2C11 and CYP3A2 in female rats caused by administration of lindane, therefore, seems to be caused by antagonizing female hormone.

To determine whether lindane-induced induction of CYP2C11 and CYP3A2 is caused by its anti-estrogenic activity and whether estrogen is involved in the suppression of these CYPs mRNAs in adult female rats, we examined the effect of ICI182780, a specific estrogen antagonist, on the expression of CYP2C11 and CYP3A2 mRNAs. ICI182780 induced the induction of these isozymes in adult female rats, indicating that estrogen is importantly involved in their suppression in these animals. These results suggest that the anti-estrogenic activity of lindane is involved in the mechanism by which lindane induces the expression of CYP2C11 and CYP3A2 in adult female rats. On the other hand, chronic testosterone administration stimulated the expression of CYP2C11 but not CYP3A2 mRNA. These results suggest that, in female rats, expression of the gene for CYP2C11 but not for CYP3A2 can be enhanced by testosterone. Thus, although estrogen is involved in the suppression of both CYP2C11 and CYP3A2 in female rats, testosterone can antagonize the estrogen-induced suppression of CYP2C11 but not CYP3A2. Expression of sex-specific CYP genes in rat liver is known to be regulated at the level of transcription initiation varied in response to sexually dimorphic plasma growth hormone (GH) profiles. Continuous exposure to GH, mimicking the adult female plasma GH profile, induces the expression of female-specific CYPs and concomitantly inhibits the expression of male-specific CYPs such as CYP2C11 (11). In contrast, intermittent plasma GH

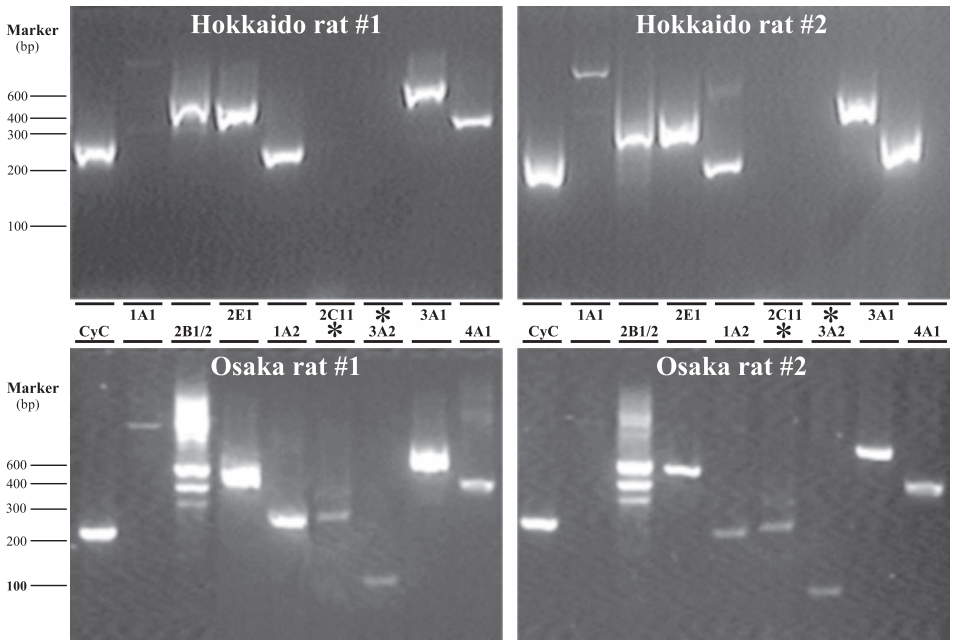

Fig. 4. Analysis of CYP mRNA expression in livers of adult female roof rats captured in Osaka prefecture (Osaka rats 1 and 2) and Hokkaido (Hokkaido rats 1 and 2) by RT-PCR. Total RNAs (25 ng) were reverse transcribed using oligo (dT) primer and AMV reverse transcriptase. First-strand cDNA products were amplified using primers for rat CYPs 1A1, 1A2, 2B1/2, 2C11, 2E1, 3A1, 3A2, and 4A1, and cyclophilin (CyC). Agarose gels stained with SYBR Green show cDNA fragments derived from CYP and cyclophilin mRNAs. Osaka rats 1 and 2, and Hokkaido rats 1 and 2 represent the profiles of two different rats in each group (Osaka rats: n = 12, Hokkaido rats: n = 11). Asterisk, adult male-specific CYPs.

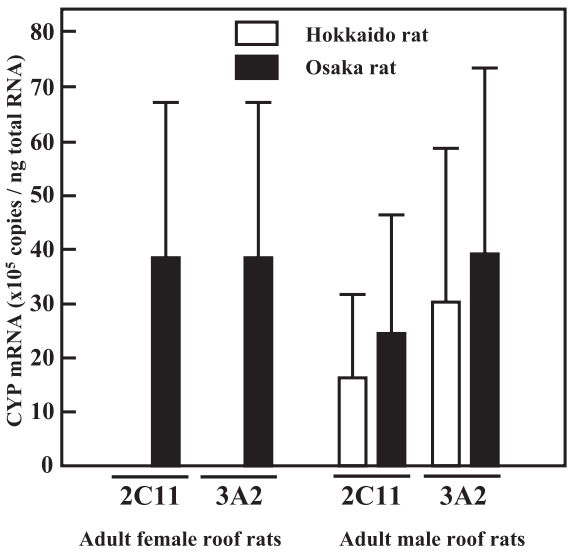

Fig. 5. Measurement of CYP2C11 and CYP3A2 mRNA expression in livers of the adult female and male roof rats captured in Osaka prefecture and Hokkaido by competitive RT-PCR. Both CYP2C11 and CYP3A2 mRNA levels were normalized to those of cyclophilin and are expressed as the number of copies of mRNA per ng total RNA. Results represent the means ± S.D. (n = 6).

Table 1. Induction of adult male-specific CYPs in livers of adult female roof rats

	Hokkaido rat female	Osaka rat female
CYP2C11 alone	0/11	0/12
CYP3A2 alone	0/11	0/12
Both CYP2C11 and CYP3A2	0/11	6/12**

The number of adult female roof rats expressing male-specific CYPs / the total number of captured adult female roof rats. Data were analyzed by Fisher's exact probability test. $^{**}P<0.01$ (compared with Hokkaido rat female).

pulses, characteristic of adult male rats, stimulate the expression of CYP2C11 in male rat liver (12). Thus, high level expression of CYP2C11 requires repeated stimulation by male plasma GH pulses. In contrast to CYP2C11, expression of CYP3A2 remains at a high level in hypophysectomized male rat liver and is induced to nearly normal male levels after hypophysectomy of females (13). Thus, hepatic expression of CYP3A2 is independent of plasma GH pulses. In the present study, we newly found that both CYP2C11 and CYP3A2 can be induced in the adult female rats treated with anti-estrogenic agents. Our results suggest that estrogen is involved in the suppression of both CYP2C11 and CYP3A2 in adult female rats.

Based on the present study, we thought that the induction of adult male-specific CYP isozymes in adult female roof rats might allow assessment of the level of exposure to environmental chemicals with anti-estrogenic effects. However, no information is available on the use of these isozymes for biological monitoring. We, therefore, performed a field survey to compare the levels of mRNA for CYP2C11 and CYP3A2 in the livers of the adult female roof rats in Hokkaido and Osaka prefecture. The present report is the first to investigate whether the induction of adult male-specific CYP2C11 and CYP3A2 in adult female roof rats differs in rural and metropolitan districts. We found that the mRNAs

for CYP2C11 and CYP3A2 were expressed in half of the female Osaka rats but not Hokkaido rats. In contrast to adult female roof rats, the induction of these CYP isozymes did not differ significantly between male Osaka and Hokkaido rats. Since the mRNAs for CYP2C11 and CYP3A2 were always co-expressed in half of the female Osaka rats, the induction of these isozymes might be caused by consumption of food and water that are contaminated by xenobiotics with anti-estrogenic effects such as lindane. A detailed mechanism, however, still remains unknown. CYP2C11 and CYP3A2 can be induced in female rats not only by lindane but also by the administration of environmental xenobiotics such as DDT (5) and dexamethasone (4, 14), which can affect female hormonal homeostasis. Moreover, these chemicals are released into the environment as industrial byproducts, and consequently, humans and animals can be exposed to them through contact with contaminated air, water, soil, and food (15, 16). The induction of adult male-specific CYP isozymes in adult female Osaka rats, therefore, might be due to environmental pollution by these chemicals or derivatives. Further studies are required to elucidate what environmental xenobiotics are involved in the induction of adult male-specific CYP2C11 and CYP3A2 in adult female Osaka rats.

Acknowledgments

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References

1 Waxman DJ. Interaction of hepatic cytochromes P-450 with steroid hormones: Regioselectivity and stereospecificity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. Biochem Pharmacol. 1988;37:71–84.

2 Kawai M, Bandiera SM, Chang TKH, Bellward GD. Growth hormone regulation and developmental expression of rat hepatic CYP3A18, CYP3A9, and CYP3A2. Biochem Pharmacol. 2000; 59:1277–1287.

3 Kato R, Kamataki T. Cytochrome P-450 as a determinant of sex difference of drug metabolism in the rat. Xenobiotica. 1982; 12:787–800.

4 Ghosal A, Sadrieh N, Reik L, Levin W, Thomas PE. Induction of the male-specific cytochrome P450 3A2 in female rats by phenytoin. Arch Biochem Biophys. 1996;332:153–162.

5 Sierra-Santoyo A, Hernandez M, Albores A, Cebrian ME. Sex-dependent regulation of hepatic cytochrome P-450 by DDT. Toxicol Sci. 2000;54:81–87.

6 Yabe T. Eye lens weight as an age indicator in the Norway rat. J Mamm Soc Japan. 1979;8:54–55.

7 Tanikawa T. An eye-lens weight curve for determining age in black rats, *Rattus rattus*. J Mamm Soc Japan. 1993;18:49–51.

8 Sugano S, Kobayashi T, Tanikawa T, Kawakami Y, Kojima H, Nakamura K, et al. Suppression of CYP3A2 mRNA expression in the warfarin-resistant roof rat, *Rattus rattus*: possible involvement of cytochrome P450 in the warfarin resistance mechanism. Xenobiotica. 2001;31:399–407.

9 Oropeza-Hernandez LF, Sierra-Santoyo A, Cebrian ME, Manno M, Albores A. Ovariectomy modulates the response of some cytochrome P450 isozymes to lindane in the rat. Toxicol Lett. 2001;124:91–99.

10 Chadwick RW, Cooper RL, Chang J, Rehnberg GL, McElroy WK. Possible antiestrogenic activity of lindane in female rats. J Biochem Toxicol. 1988;3:147–158.

11 Pampori NA, Shapiro BH. Gender differences in the responsiveness of the sex-dependent isoforms of hepatic P450 to the feminine plasma growth hormone profile. Endocrinology. 1999;140:1245–1254.

12 Waxman DJ, Pampori NA, Ram PA, Agrawal AK, Shapiro BH. Interpulse interval in circulating growth hormone patterns regulates sexually dimorphic expression of hepatic cytochrome P450. Proc Natl Acad Sci USA. 1991;88:6868–6872.

13 Agrawal AK, Shapiro BH. Differential expression of gender-dependent hepatic isoforms of cytochrome P-450 by pulse signals in the circulating masculine episodic growth hormone profile of the rat. J Pharmacol Exp Ther. 2000;292:228–237.

14 Waskiewicz MJ, Choudhuri S, Vanderbeck SM, Zhang X, Thomas PE. Induction of "male-specific" cytochrome P450 isozymes in female rats by oxandrolone. Drug Metab Dispos. 1995;23:1291–1296.

15 Colborn T, Saal FS vom, Soto AM. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environ Health Perspect. 1993;101:378–384.

16 Walsh LP, Stocco DM. Effects of lindane on steroidogenesis and steroidogenic acute regulatory protein expression. Biol Reprod. 2000;63:1024–1033.