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MOUSE RENAL CYTOCHROME P450IIE1*: IMMUNOCYTOCHEMICAL LOCALIZATION, SEX-RELATED DIFFERENCE AND REGULATION BY TESTOSTERONE

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Abstract—Cytochrome P450IIE1 is responsible for the metabolic activation of *N*-nitrosodimethylamine and a variety of other chemicals. Renal P450IIE1 was shown previously to be regulated by testosterone in C3H/HeJ and BALB/c mice. The present study investigated the distribution of cytochrome P450IIE1 in the kidneys of C3H/HeJ and BALB/c mice. The amount of P450IIE1 was immunotitrated by immunohistochemistry using polyclonal antibodies against rat P450IIE1. Strong immunoreactivity was identified mainly in the cortical tubules, including proximal tubules and some distal tubules. Weak immunoreactivity was also observed in the outer medulla when higher concentrations of antibodies were used. Much higher immunostaining was observed in male mice than in female mice when identical antibody dilutions were used. The renal P450IIE1 level in females was elevated to the same level as that in males 24 hr after administration of testosterone. The results showed a specific cellular localization of cytochrome P450IIE1 in mouse kidney. The findings may lead to a better understanding of the site-specific renal toxicity and carcinogenesis due to the activation of chemicals by cytochrome P450IIE1.

Previous studies have shown that hepatic cytochrome P450IIE1 is responsible for the metabolic activation of a potent carcinogen, *N*-nitrosodimethylamine (NDMA) [1–3], a variety of environmental chemicals [4–6] and drugs [7, 8]. Extrahepatic tissues, such as lung and kidney, also have the ability to metabolize these compounds. This metabolic activity may be closely related to the toxicity and carcinogenicity in these target organs. Mouse kidney is one of the target organs for NDMA-induced carcinogenesis, but renal neoplasm occurs only in male mice [9]. This phenomenon is probably closely related to the fact that the renal P450IIE1 level is much higher in male than female mice. A key factor involved in the sex-related differences in response to some toxins and chemical carcinogens is the different rates of metabolic activation of the compounds by the cytochrome P450 system.

Metabolism of NDMA in mouse kidney has been studied extensively, and a sex-related difference in renal P450IIE1 has been demonstrated in four mouse strains, C57/BL, DBA, BALB/c, and C3H/HeJ [10–12]. The female renal NDMA demethylase activity in C3H/HeJ mice increases 17-fold after testosterone treatment. Further study also demonstrated much higher levels of P450IIE1 protein content and mRNA in male or testosterone-treated female mice in comparison to untreated female mice. Renal P450IIE1 in certain mouse strains was suggested to be regulated by testosterone [10, 12] and mediated by androgen receptor [13]. Earlier studies have also shown that administration of testosterone to adult female and immature mice could stimulate NDMA-induced mutagenesis by kidney microsomes [14, 15].

In this report, we investigated the localization of renal P450IIE1 in mouse kidney by immunocytochemistry using polyclonal antibodies against rat P450IIE1. Sex-related differences and induction of P450IIE1 in female mice by testosterone were also studied.

MATERIALS AND METHODS

Chemicals. Testosterone was obtained from the Sigma Chemical Co. (St. Louis, MO). Anti-rabbit IgG, biotinylated species-specific $F(ab')_2$ fragment, and streptavidin horseradish peroxidase were purchased from Amersham Inc. (Arlington Heights, IL).

Animals and treatment. C3H/HeJ and BALB/c mice (10 weeks old) were obtained from Jackson Laboratories (Bar Harbor, ME) and Taconic Farms (Germantown, NY) respectively. They were allowed food and water *ad lib.* For testosterone treatment, female mice were given one s.c. injection of testosterone (1.45 mmol/kg) in 0.1 mL olive oil, and the control female mice were given 0.1 mL olive oil. Animals were killed 24 hr after the treatment. In a different experiment, male mice were subjected to fasting for 48 hr before being killed. Kidneys were isolated and cut into two halves, fixed in Bouin's solution overnight, and processed through routine paraffin methods for immunocytochemical studies.

Preparation of antibodies. Cytochrome P450IIE1

* The enzyme studied herein is closed related to cytochrome P450IIE1 immunocheically and catalytically using *N*-nitrosodimethylamine as a substrate. For convenience, it is referred to as mouse kidney P450IIE1, realizing that additional information is needed to substantiate this tentative nomenclature.

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was purified from acetone-induced rat liver microsomes [3]. Polyclonal antibodies against the purified protein were raised in rabbits. The properties of the antibodies have been characterized previously [16]. For absorption control, anti-P450IIE1 antibodies (8.36 mg/mL) were incubated with an equal volume of acetone-induced rat liver microsomes (25 mg/mL) at room temperature for 60 min. Serial dilutions were prepared from either the antiserum or the preabsorbed antiserum in 1% sheep serum, 2% bovine serum albumin–Tris-buffered saline (SA–TBS).

Immunocytochemistry. Tissue sections (6 μm) were mounted on glass slides coated with poly-L-lysine, deparaffinized, and rehydrated. Then the tissue sections were washed in phosphate-buffered saline (PBS), soaked in 3% H_2O_2 in PBS for 10 min, and rinsed in PBS. Slides were allowed to shake in SA–TBS for 30 min. Serially diluted anti-P450IIE1 IgG was applied to the tissue sections and incubated overnight at 4° in a moist chamber. After the sections were washed with SA–TBS, 10 μL of anti-rabbit γ-globulin conjugated to biotin (1:50) was applied, and sections were incubated in a moist chamber for about 30 min. After a SA–TBS wash, the sections were treated with 10 μL of streptavidin conjugated to peroxidase (1:50) in SA–TBS, and incubated in a moist chamber for 15 min. After washing in TBS (pH 7.6), the bound peroxidase was demonstrated by the presence of brown precipitate after treating the section with 0.02% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and hydrogen peroxide (0.03%) in TBS. A constant reaction time for slides to be compared was used in each experiment (total time required 3–5 min). The slides were then rinsed with TBS, nuclei were counterstained with hematoxylin, and sections were processed through to mounting. The intensity of the immunostaining was blindly graded under light microscopic examination.

RESULTS

Localization of cytochrome P450IIE1. Immunocytochemical stains of serial sections of kidneys were examined carefully. In BALB/c control male mouse kidney, positive stain was observed mostly in cortical tubules, epithelial cells of the proximal tubules (Fig. 1). Weak staining was observed in the outer medulla. By comparing the P450IIE1-immunostained kidney section with a positive control stained for calcium binding protein [17], which only concentrated in distal tubules, some of the immunoreactivity of P450IIE1 was confirmed in distal tubules (results not shown). However, the staining of the outer medulla and some distal tubules was not observed when less concentrated antibodies were used. The weak staining in distal tubules and outer medulla may be due to non-specific reactivity, cross-reacting cytochromes, and/or lower levels of P450IIE1. These possibilities remain to be determined. No specific staining was observed in glomeruli and collecting ducts. To demonstrate the specificity of the antibody interaction, serial sections were either incubated with the antibody preparation or the antibody preparation preabsorbed with acetone-induced rat liver microsomes, which were enriched in cytochrome P450IIE1. In C3H/HeJ control male mouse kidney, the section incubated with preabsorbed antibodies was devoid of immunostaining (Fig. 2). The lack of staining in preabsorbed control sections provided evidence that the antibody staining is specific for P450IIE1 enzyme.

Sex-related difference and effect of testosterone. The stain intensities of a number of sections were compared under light microscopic examination. At the same antibody dilution (1:800, 105 ng protein in 10 μL SA–TBS), stain intensity was much higher in control males compared to females (data not shown). After testosterone treatment, the intensity of the staining in females was much higher than that in the control female kidney (Fig. 3).

Immunonitration of enzyme expression and reproducibility. By using serial dilutions of the P450IIE1 antibodies in the staining procedure, the expression of cytochrome P450IIE1 protein in kidney sections under light microscopic examination could be divided into five groups according to their stain intensity: strong, moderate, weak, partially positive, and without specific staining. The present data clearly demonstrated that the P450IIE1 level was much higher in control males than females, and that testosterone elevated the level of P450IIE1 in female mice to a level that was similar to control male (Table 1). Such an induction occurred in both C3H/HeJ and BALB/c mice. However, the P450IIE1 content in the BALB/c mouse strain was lower than that of the C3H/HeJ judged by the stain intensity at the same antibody dilution. Nevertheless, the immunotitration method was not sensitive enough to identify the induction of P450IIE1 by fasting in male mouse which was demonstrated previously by enzyme activity assay and immunoblot analysis [10].

DISCUSSION

In the present work, mouse renal cytochrome P450IIE1 was localized mainly in the cortex, epithelial cells of proximal tubules, and some distal tubules. No specific immunoreactivity was observed in glomeruli and collecting ducts. The level of P450IIE1 in males was much higher than in females. Treatment with testosterone induced P450IIE1 content in females to a level similar to that in males. The sex-related difference in the intensity of staining correlated with the differences in NDMA demethylase activity and P450IIE1 content in our previous study [10].

The properties of the presently used polyclonal antibodies against P450IIE1 have been described previously [16]. Further characterization has shown that the IgG fraction of the antiserum does not cross-react with P450IIA1, P450IIB1, P450IIC6 and P450IIC12 by immunoblot analysis. However, some cross-reactivity with P450IIC11 and P450IIIA, which have intensities only approximately 5–10% of an equimolar P450IIE1 [18], was observed. Such a cross-reactivity is believed not to affect the conclusions of this work. The appropriate controls have been used to verify the specificity of the staining: (i) use of two negative controls, without primary antibodies and with preabsorbed antibodies, (ii) use of a positive control, calcium binding protein

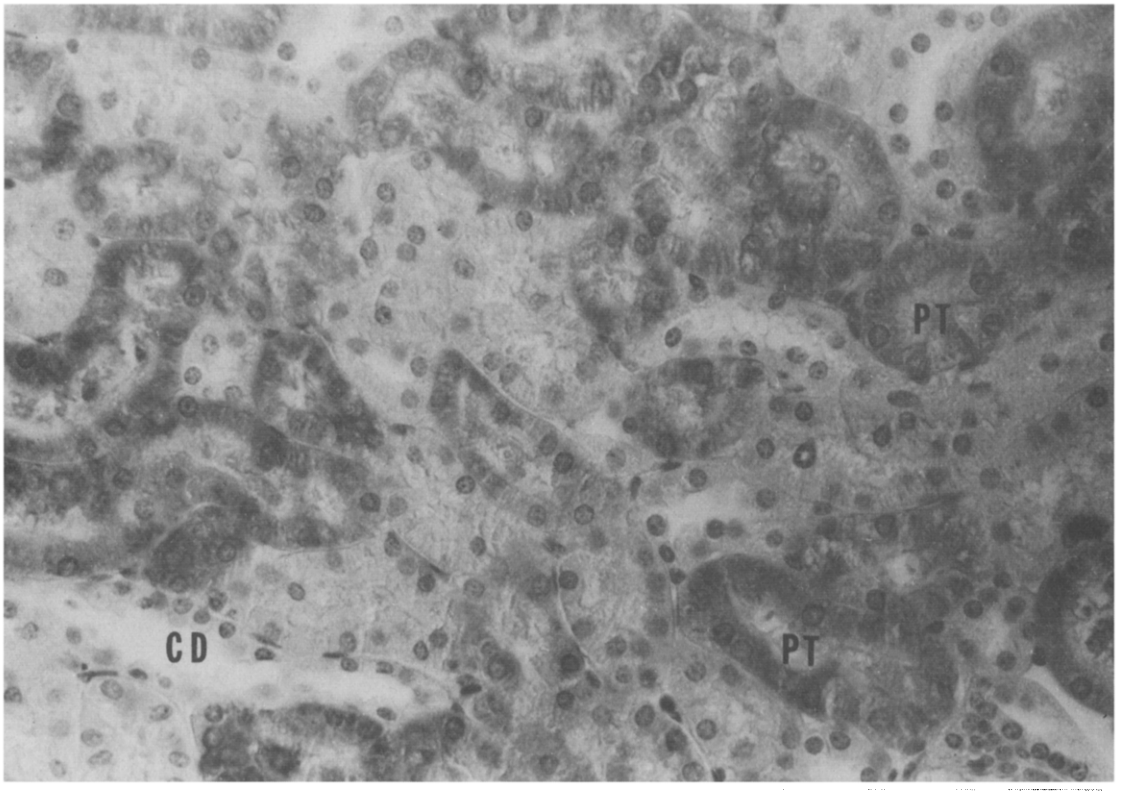

Fig. 1. Immunocytochemical localization of P450IIE1 in BALB/c control male mouse kidney. Specific staining of P450IIE1 in the proximal tubules (PT) was conducted at a 1:600 dilution of antibodies. Negative staining was observed in collecting ducts (CD). The immunostained section was counterstained with hematoxylin. Magnification, ×100.

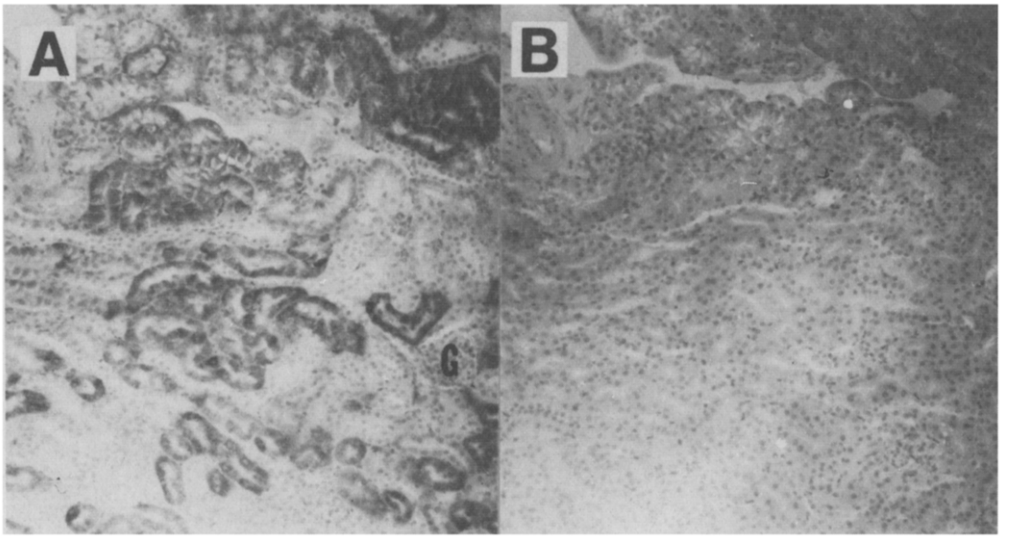

Fig. 2. Specificity of the immunocytochemical staining of cytochrome P450IIE1. In adjacent sections from C3H/HeJ control male mouse kidney one section (A) was incubated with antibodies to P450IIE1, while the section shown in panel B was incubated with the preabsorbed immune serum as described. The antibody dilution used was 1:800. Magnification, ×25.2.

[17], and (iii) use of a serial dilution for the primary antibodies for each preparation. Resultant immunostained slides are believed to be specific for cytochrome P450IIE1.

Testosterone and other androgenic steroids stimulate the synthesis of several proteins in the proximal tubules of the female mouse kidney [19], suggesting the localization of androgen receptors on

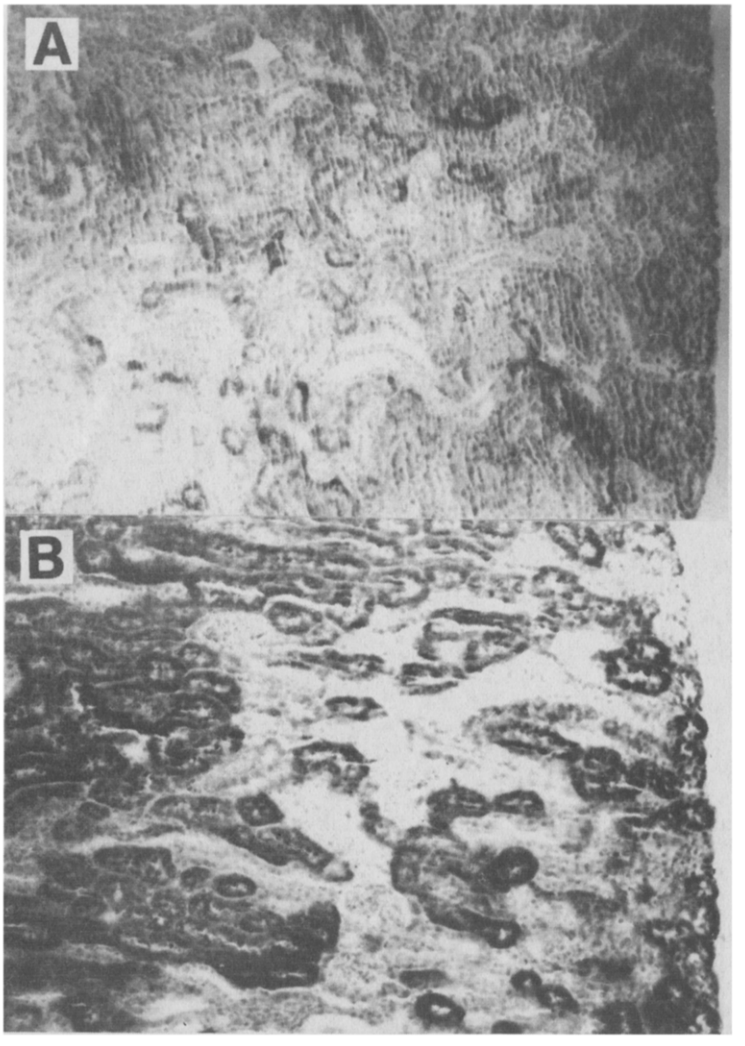

Fig. 3. Induction of P450IIE1 by testosterone. Sections were from (A) a C3H/HeJ female mouse treated with 0.1 mL olive oil as control, and (B) a C3H/HeJ female mouse treated with testosterone (1.45 mmol/kg) dissolved in 0.1 mL olive oil. Immunostained sections were counterstained with hematoxylin. Both sections were incubated with primary antibodies at a dilution of 1:800. Magnification, ×40.

Table 1. Immunotitration of the binding of cytochrome P450IIE1 antibodies to kidney

		Intensity of staining				
		Antibody dilution (μg/mL)				
Sex	Treatment	1:400 (20.9)	1:600 (13.9)	1:800 (10.5)	1:1000 (8.4)	1:1200 (7.0)
(A) C3H/HeJ						
F	Control	++	+/−	−	−	−
F	Olive oil	+	+	+/−	−	−
F	Testosterone	+++	+++	++	++	+
M	Control	+++	++	++	++	+
M	Fasting	+++	++	++	++	+
(B) BALB/c						
F	Control	+/−	−	−	−	−
F	Olive oil	+/−	−	−	−	−
F	Testosterone	++	++	+	+/−	+/−
M	Control	++	++	+	+/−	+/−
M	Fasting	++	++	+	+/−	+/−

Key: (+++) strong staining, (++) moderate staining, (+) weak staining, (+/−) some positive staining, and (−) no specific staining.

these sites. Studies with several hormones have shown that mouse renal NDMA demethylase activity is regulated specifically by androgenic hormones [13]. In the present study, P450IIE1 was located mainly in the proximal tubules, which was probably closely related to the localization of androgen receptors. However, this point remains to be substantiated by immunolocalization of the androgen receptors in mouse kidney. The localization of P450IIE1 in epithelial cells of proximal tubules suggests that these calls can be used in culture for studying the expression of P450IIE1.

Cytochrome P450IIE1 is involved in the metabolism of a number of xenobiotics, many of which cause nephrotoxicity and/or carcinogenesis in the mouse. For example, the originating site for NDMA-induced renal cell tumors in rat is the proximal tubule [20]. In rats, O^6-methylguanine in DNA induced by NDMA also localizes in kidney cortex, mainly proximal tubules, while the glomeruli and renal medulla are negative [21]. These phenomena could be attributed to the metabolic activation of NDMA by P450IIE1 at the specific region of the kidney. The presence of P450IIE1 in the renal cortex may make the epithelial cells of the cortical tubules susceptible to carcinogenesis.

Sex-related differences between males and females in response to environmental toxins, drugs and carcinogens have been studied extensively in the mouse kidney [10, 22, 23]. Such a sexual dimorphism has been suggested to be determined by testosterone [19]. The molecular mechanism of such a difference is not fully understood. Several recent studies have provided evidence that male-specific hormones may modulate the cytochrome P450 system in mouse kidney, which consequently affects the metabolism of xenobiotics [10, 13, 22]. Most probably, this difference contributes to the increased chemically-induced nephrotoxicity and carcinogenesis in the males. Epidemiological studies also show that the incidence of human renal carcinoma is twice as high in males as in females [24]. It is not known whether this is related to a possible difference between males and females in the capacity to activate carcinogens or due to other factors.

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