Biochemical Pharmacology, Vol. 42, No. 5, pp. 1093–1097, 1991.
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

MODULATION OF RAT HEPATIC CYTOCHROMES P450 BY CHRONIC METHAPYRILENE TREATMENT

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(*Received* 19 *February* 1991; *accepted* 15 *April* 1991)

Abstract—The antihistaminic compound methapyrilene (MP) when chronically administered has been shown to be a rat-specific hepatocarcinogen. To examine the effects of chronic MP treatment on the hepatic microsomal cytochromes P450, Fischer 344 rats were gavaged for 10 weeks (5 days on, 2 days off) with either vehicle or 50, 100, or 150 mg MP/kg body weight. Chronic MP treatment was found to have a significant effect on several microsomal enzymatic activities. Small (17–28%) but significant ($P < 0.05$) decreases were observed for total P450 levels and the activities of erythromycin *N*-demethylase (catalyzed by P450IIIA), *N*-nitrosodimethylamine demethylase (catalyzed by P450IIE1) and pentoxyresorufin *O*-dealkylase (catalyzed by P450IIB1). In addition, a relatively large decrease (approximately 80%) was observed for the activity of benzphetamine *N*-demethylase (representative of P450IIC11) and an induction of about 40% was observed for ethoxyresorufin *O*-dealkylase (catalyzed by P450IA). The metabolism of testosterone by microsomes isolated from the rats chronically treated with MP indicated that several reactions were compromized. Specifically, testosterone 2α-hydroxylase, indicative of P450IIC11, was reduced greatly (86%), whereas testosterone 6β-hydroxylase, reflecting P450IIIA, and testosterone 7α-hydroxylase, indicative of P450IIA1, were affected only slightly by MP treatment (approximately 25%). Immunoblot analyses of the various microsomal samples were performed to determine if chronic MP treatment had direct effects on the level of expression of the cytochromes P450. Decreases in the levels of P450IIIA, IIE1, and IIC11, determined by immunoblot analyses, closely paralleled those observed for their marker catalytic activities. Further studies will be required to determine the mechanism by which MP affects the levels of the cytochromes P450 (i.e. increased degradation or decreased synthesis).

Methapyrilene (MP) is an antihistaminic compound which was used widely in sleep-aids and cold medications. In 1979, the therapeutic use of MP ended when it was found to be a very potent hepatocarcinogen when administered chronically to rats [1]. However, MP appears to be an atypical carcinogen in that it has not been found to be mutagenic in the Ames test [2], does not cause sister-chromatid exchange [3], and does not induce tumors in other experimental species [4]. Recent studies suggest that MP is a relatively poor initiator in rats [5], although a metabolite of MP binds exogenous DNA *in vitro* [6]. Thus, MP appears to be a species-specific carcinogen and in that species, the rat, it acts as a weak initiator and a highly efficient promoter.

The cytochromes P450 are a superfamily of hepatic hemoproteins that are responsible for the metabolic activation of some chemical carcinogens. Studies on the metabolism of MP by hepatic microsomes indicate that at least seventeen metabolites can be formed from MP [7] and that the metabolism of MP by rats is different than that by rabbits and guinea pigs [7]. In addition, acute treatment of rats with MP has been shown to reduce the level of total microsomal cytochrome P450 [8]. On the other hand, chronic treatment of rats with MP did not alter the levels of total microsomal cytochrome P450 levels [9]. However, microsomes isolated from the livers of rats chronically treated with MP demonstrated a significant reduction in their ability to metabolize MP. This finding suggests that chronic MP treatment may alter the expression and/or activity of the specific form(s) of cytochrome P450 responsible for MP metabolism [9].

Since chronic MP treatment of rats results in a reduction in the cytochrome P450-mediated metabolism of MP, studies were undertaken to determine the effects of chronic MP treatment on the catalytic activities and expression of specific forms of rat hepatic cytochrome P450. Unlike a previous report on the effects of chronic MP treatment on rat microsomal cytochrome P450 levels [9], we observed a small but significant decrease in total cytochrome P450 levels which was associated with alterations in the activities and levels of several forms of hepatic microsomal cytochrome P450.

METHODS

Materials. Methapyrilene, *N*-nitrosodimethylamine, testosterone, pentoxyresorufin and ethoxyresorufin were purchased from the Sigma Chemical

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Co. (St. Louis, MO). Aldrich (Milwaukee, WI) was the source of the erythromycin. Benzphetamine was synthesized at Eli Lilly and Company. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) reagents, secondary antibodies and nitrocellulose were obtained from Bio-Rad (Richmond, CA). [^{3}H]Styrene oxide was supplied by Dr. Thomas Guenthner (University of Illinois, Chicago, IL).

Animal treatment and microsome preparation. Male, Fischer 344 rats were purchased from Charles River and were 8 weeks old at the start of the study. Animals were gavaged daily with methapyrilene–HCl in water at dosages of 50, 100 and 150 mg/kg for 10 weeks excluding weekends. Controls were gavaged with water. Livers were excised 72 hr after the last dose, frozen in liquid nitrogen, and stored at −75°. Liver microsomes were prepared by differential centrifugation [10].

General assays. Protein concentrations of the microsomal samples were determined colorimetrically [11]. Total P450 concentrations were determined by the method of Omura and Sato [12], based on an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$. SDS–PAGE (10%, w/v) was performed by the method of Laemmli [13], and immunoblot analyses developed with murine monoclonal anti-P450IIIA IgG, rabbit anti-P450IA IgG or rabbit anti-P450IIE1 IgG were carried out as described elsewhere [14]. Murine monoclonal anti-P450IIC11 IgG was characterized as previously described [15].

Enzymatic assays. The N-demethylation of benzphetamine (3 mM final concentration), erythromycin (1 mM final concentration) and *N*-nitrosodimethylamine (1 mM final concentration) was determined by the method of Prough and Zeigler [16] with 1 mg/mL microsomal protein concentration, a 0.5 mL incubation volume, 66 mM Tris (pH 7.4) and 1 mM NADPH. The reactions were stopped with 250 μL of 25% trichloroacetic acid. Pentoxyresorufin *O*-dealkylase and ethoxyresorufin *O*-deethylase activities were measured by the method of Lubet *et al.* [17] in a 3.0-mL incubation containing 100 mM Tris–HCl (pH 7.4) and 1 mM NADPH. Testosterone hydroxylation was determined as previously described [18]. Microsomal epoxide hydrolase activity was determined by the method of Guenthner *et al.* [19].

RESULTS

Liver morphology. Chronic MP treatment of rats produced a spectrum of histopathologic lesions in the livers including biliary hyperplasia, hypertrophy of periportal hepatocytes, necrosis of single hepatocytes, and development of foci of cellular alteration. The observed foci were of the eosinophilic type. The hypertrophied hepatocytes had enlarged nuclei with prominent nucleoli. The occurrence of these lesions was influenced by the dose of MP with the high dose having an increased incidence in the number of animals expressing the lesions and increased severity of the individual lesions. These morphologic changes are consistent with a previous report on the morphology of lesions produced by comparable dietary doses of MP with approximately the same duration of exposure [20].

Hepatic enzyme activities. In a previous study [9], chronic treatment of rats with MP was shown to have little effect on the level of total hepatic microsomal cytochrome P450 content. However, in a study in which rats were treated acutely with MP, total cytochrome P450 levels were demonstrated to be reduced by 30–50% [8]. As shown in Table 1, chronic MP treatment resulted in a small (17%) but statistically significant ($P < 0.05$) decrease in hepatic microsomal total cytochrome P450 levels. In addition, unlike the 400% increase observed after acute treatment [8], microsomal epoxide hydrolase activity was increased only slightly by the chronic treatment of rats with MP and then only at the lowest dose (Table 1).

It is interesting to note that a dose–response relationship was not observed for the effect of chronic MP treatment on total cytochrome P450 levels (Table 1). A possible explanation for this observation is that the various treatment levels inactivated and/or induced different forms of cytochrome P450. To assess the possibility that chronic MP treatment affected individual forms of cytochrome P450 in different ways, the metabolic capabilities of hepatic microsomes isolated from the MP- and vehicle-treated rats were determined. The N-demethylation of erythromycin has been shown to be catalyzed in the rat specifically by members of the P450IIIA subfamily [21]. As shown in Table 1, chronic MP treatment decreased microsomal erythromycin *N*-demethylase activity in a dose-dependent manner that plateaued at about 25% inhibition with the 100 mg/kg dose.

P450IIE1 has been identified as the high-affinity *N*-nitrosodimethylamine *N*-demethylase in rat hepatic microsomes [22]. Chronic MP treatment of rats resulted in a small dose-dependent decrease in microsomal *N*-nitrosodimethylamine demethylase activity reaching a maximum (28%) in this study at the 150 mg/kg dose level (Table 1).

Pentoxyresorufin O-dealkylation has been shown to be catalyzed in the rat specifically by the members of the phenobarbital-inducible P450IIB subfamily [17]. Microsomal pentoxyresorufin *O*-dealkylase activity was also found to be slightly decreased (25%) after chronic MP treatment (Table 1).

Several cytochromes P450 have been shown to contribute to the N-demethylation of benzphetamine [23]. However, in the untreated rat, P450IIC11 appears to catalyze the majority of the N-demethylation of benzphetamine [23]. Chronic MP treatment of rats resulted in a dramatic dose-dependent decrease in the microsomal benzphetamine *N*-demethylase activity (Table 1). This activity was decreased by 78% at the highest MP dose examined (Table 1).

Ethoxyresorufin O-deethylation has been demonstrated to be specifically catalyzed by P450IA1 and IA2 in the rat [17, 23]. Microsomal ethoxyresorufin *O*-deethylase activity was the only oxidative activity found to be induced in the rat by chronic MP treatment (Table 1). Specifically, this activity was increased in a dose-dependent manner to a maximum

Table 1. Effect of methapyrilene on the activities of epoxide hydrolase and the cytochromes P450

Dose of MP (mg/kg)	Total P450 (pmol/mg protein)	Epoxide hydrolase*	Erythromycin *N*-demethylase*	*N*-Nitroso-dimethylamine demethylase*	Pentoxyresorufin *O*-dealkylase*	Benzphetamine *N*-demethylase*	Ethoxyresorufin *O*-dealkylase*
0	228 ± 24†	97 ± 16	480 ± 70	1140 ± 110	15.2 ± 0.2	3170 ± 480	69.1 ± 16.5
50	190 ± 28‡	124 ± 22‡	420 ± 110	120 ± 170	11.8 ± 0.2	1900 ± 600‡	73.5 ± 10.7
100	188 ± 13‡	112 ± 12	350 ± 50‡	890 ± 30‡	11.0 ± 0.2‡	1140 ± 170‡	87.3 ± 6.6‡
150	190 ± 21‡	102 ± 13	358 ± 60‡	830 ± 160‡	12.2 ± 0.2	1010 ± 80‡	96.6 ± 4.9‡

* Units: pmol product per mg of microsomal protein per min.
† Values are means ± SD of five determinations.
‡ $P < 0.05$, determined using Dunnett's test.

Table 2. Effect of chronic methapyrilene treatment of rats on liver microsomal testosterone 2α-, 6β- and 7α-hydroxylation

Dose of MP (mg/kg)	Testosterone metabolism* 2α	6β	7α
0	674 ± 59†	1957 ± 218	176 ± 27
50	331 ± 57‡	1929 ± 317	147 ± 20
100	95 ± 34‡	1431 ± 196‡	133 ± 29‡
150	113 ± 40‡	1444 ± 53‡	135 ± 22‡

* Units: pmol product per min per mg microsomal protein.
† Values are means ± SD of five determinations.
‡ $P < 0.05$, determined using Dunnett's test.

of 140% of the activity found with the vehicle-treated controls.

Testosterone metabolism. The metabolism of testosterone by certain rat liver cytochromes P450 is stereospecific and regioselective [18, 24]. Thus, the hydroxylation of testosterone by rat liver microsomes provides a method to examine simultaneously the metabolic capabilities of several forms of rat cytochrome P450. Testosterone 6β-hydroxylase activity has been shown to reflect the level of the rat P450IIIA subfamily. Chronic MP treatment of rats resulted in approximately a 25% decrease in this P450IIIA activity (Table 2). The hydroxylation of testosterone at the 2α position has been shown to be indicative of the level of P450IIC11. As shown in Table 2, testosterone 2α-hydroxylase was decreased dramatically (86%) by chronic MP treatment. Finally, testosterone 7α-hydroxylase has been shown to reflect the level of P450IIA1, and chronic MP treatment of rats was found to have only a slight effect (24% decrease) on this activity (Table 2).

Quantification of immunoreactive cytochromes P450. The effects of chronic MP treatment on the catalytic activities indicative of various rat microsomal cytochromes P450 suggested that MP may be modulating the level of expression and/or the metabolic activities of the cytochromes P450. To examine the effects of chronic MP treatment on the levels of expression of the various cytochromes P450, quantitative immunoblot analyses were performed, and the results are summarized in Table 3. As was indicated by the microsomal erythromycin *N*-demethylase and testosterone 6β-hydroxylase activity studies, the level of immunoreactive P450IIIA was decreased significantly (up to 50%) by chronic MP treatment (Table 3). A small decrease was observed, although it was not statistically significant, in the level of immunoreactive P450IIE1 (Table 3) as was suggested by the decrease in the ability of the microsomes to *N*-demethylate *N*-nitrosodimethylamine. The level of immunoreactive P450IIC11 in the hepatic microsomes obtained from the rats chronically treated with MP was found to be only 25% of the level of P450IIC11 in the vehicle-treated rats (Table 3). As previously observed [14], the levels of immunoreactive P450IIB were below the limit of detection in all the microsomal samples.

Table 3. Effect of methapyrilene on levels of cytochromes P450*

Dose of MP (mg/kg)	P450IIIA1 and 2 (P450p and p2)	P450IIC11 (P450h)	P450IIE1 (P450j)	P450IA2 (P450d)
0	107 ± 32	103 ± 10	89 ± 22	98 ± 21
50	74 ± 37	69 ± 28†	89 ± 14	134 ± 71
100	63 ± 19†	35 ± 21†	76 ± 18	84 ± 13
150	52 ± 10†	26 ± 4†	66 ± 21	126 ± 92

* Values represent arbitrary densitometer units and are the means ± SD of five microsomal samples from each treatment group.
† $P < 0.05$, determined using Dunnett's test.

The level of immunoreactive microsomal epoxide hydrolase and NADPH–cytochrome P450 reductase were not affected by the chronic MP treatment (data not shown). Finally, the levels of immunoreactive P450IA2 were found to vary greatly within a treatment group (Table 3) as has been observed previously [14]. However, when examined *in toto*, an upward trend was observed as a result of chronic MP treatment in the levels of the expression of P450IA2 (Table 3).

DISCUSSION

Lampe and Kammerer [9] have demonstrated previously that hepatic microsomes isolated from rats chronically treated with MP have a reduced capacity to metabolize MP. These authors speculated that chronic MP treatment of rats resulted in either an inactivation of the MP-metabolizing enzyme(s) or the formation of a metabolite of MP that inhibits the enzyme(s). In addition, others have shown that the acute treatment of rats with MP decreases the levels of total microsomal cytochrome P450 and the metabolism of a limited number of substrates of the cytochromes P450 [8]. The results presented here characterize the effects of chronic MP treatment of rats on the hepatic mixed-function oxidase system. Specifically, chronic MP treatment of rats was found to decrease total microsomal cytochrome P450 levels, to alter to differing extents the metabolic activities and levels of several cytochromes P450, and to have little effect on microsomal epoxide hydrolase and NADPH–cytochrome P450 reductase.

The expression of and catalytic activities associated with several forms of rat microsomal cytochrome P450 were decreased by chronic MP treatment. Relatively small decreases were observed for P450IIIA and P450IIE1 and the catalytic activities associated with P450IIA1, testosterone 7α-hydroxylase, and P450IIB1, pentoxyresorufin *O*-dealkylase. In addition, a small induction of the P450IA family by chronic MP treatment was observed. However, rat P450IIC11 and the catalytic activities associated with it, benzphetamine *N*-demethylase and testosterone 2α-hydroxylase, were found to be reduced dramatically (>75%) by chronic MP treatment. The dramatic decrease in the level of immunochemically detected P450IIC11 indicates that chronic MP treatment increases the degradation and/or decreases the synthesis of P450IIC11. One possible explanation for the loss of P450IIC11 is that P450IIC11-mediated metabolism of MP results in the formation of a reactive metabolite(s) that destroys this P450. Furthermore, the loss of P450IIC11 documented here may also account for the 50% decrease in the ability of microsomes isolated from the livers of rats chronically treated with MP to metabolize MP *in vitro* as observed by Lampe and Kammerer [9]. It is also of interest that in the aforementioned study [9], the authors routinely recovered only 50% of the metabolites of MP formed with microsomes from untreated rats. The decrease they observed in MP metabolism with microsomes from rats chronically treated with MP was not due to a change in the metabolites that had been recovered and characterized but in those that were not identified. Therefore, it is tempting to speculate that P450IIC11 is responsible for the formation of the metabolites not found by Lampe and Kammerer [9] and that were not formed after chronic MP treatment. A possible reason that these P450IIC11 generated metabolites have not yet been identified is that they are highly reactive and bind to P450IIC11 and/or other microsomal nucleophiles. The *in vitro* metabolism of MP by P450IIC11 is the subject of future studies.

In adult rats, P450IIC11 has been shown to be expressed exclusively in males [23]. On the other hand, studies on the carcinogenic potential of chronic MP treatment indicate that MP is a potent carcinogen in both male and female rats [1]. Therefore, the loss of P450IIC11 that results from chronic MP treatment does not appear to be the causal factor in MP-initiated carcinogenesis. This is despite our belief that P450IIC11 catalyzes the formation of a reactive metabolite(s) of MP. However, it is also possible that a female-specific cytochrome P450 metabolizes MP in a fashion similar to P450IIC11 in male rats.

The diuretic drug, tienilic acid, induces an autoimmune hepatitis in about 1 in 10,000 patients which is characterized by the presence of antibodies in the patient's serum that recognize antigens in the endoplasmic reticulum of liver and kidney cells [25]. These antibodies are referred to as anti-LKM2 antibodies. One of the antigens recognized by the anti-LKM2 antibodies is the form of cytochrome P450, P450IIC9, responsible for the metabolism of tienilic acid in humans [25]. It appears that during the oxidative metabolism of tienilic acid by P450IIC9 a reactive metabolite is formed that binds to

P450IIC9 ultimately rendering it antigenic [25]. The reactive metabolite is believed to arise from the hydroxylation of the thiophene ring of tienilic acid at the 5-position [26]. In addition to recognizing P450IIC9 in human liver microsomes, anti-LKM2 antibodies recognize the related proteins P450IIC11, IIC6 and IIA1 in rat liver microsomes [27]. Like tienilic acid, MP contains a thiophene ring. Therefore, it is our working hypothesis that the yet to be identified reactive metabolite(s) of MP responsible for the destruction of rat P450IIC11 is a hydroxylation product(s) of the thiophene ring of MP. Studies are underway to test this hypothesis.

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