Characterization of Microsomal Alcohol Oxygenase Catalyzing the Oxidation of 7-Hydroxy-Δ⁸-tetrahydrocannabinol to 7-Oxo-Δ⁸-tetrahydrocannabinol in Rat Liver

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The formation of 7-oxo-Δ⁸-tetrahydrocannabinol (7-oxo-Δ⁸-THC) from 7β-hydroxy-Δ⁸-THC was found in hepatic microsomes of rats. The activity was stereoselective and about 3-fold higher than that from 7α-hydroxy-Δ⁸-THC. The oxidative activity of 7α- and 7β-hydroxy-Δ⁸-THC to 7-oxo-Δ⁸-THC was significantly higher in male than in female, and significantly enhanced by both dexamethasone and phenobarbital, and then inhibited up to about 20% of the control value by antibody against P450GFP-B, presumably a member of the 3A subfamily, a major enzyme responsible for the formation of 7-oxo-Δ⁸-THC in guinea pigs. This antibody also inhibited the formation of 7α- and 7β-hydroxy-Δ⁸-THC, and 7-oxo-Δ⁸-THC from Δ⁸-THC by hepatic microsomes of rats. These results indicate that there is a sex-related difference in the oxidation of 7-oxo-Δ⁸-THC to 7-oxo-Δ⁸-THC and the reaction is mainly catalyzed by P450 enzyme(s) belonging to the 3A subfamily as major enzyme(s) of microsomal alcohol oxygenase in rats.

Key words 7-hydroxy-Δ⁸-tetrahydrocannabinol; microsomal alcohol oxygenase; CYP3A; 7-oxo-Δ⁸-tetrahydrocannabinol; cytochrome P450; sex-related difference

Tetrahydrocannabinol (THC) is the major psychoactive constituent of marijuana. It is well known that Δ⁸-THC is oxidized to a number of metabolites in the liver of mammals. 1 We have previously reported that guinea pig hepatic microsomal enzyme, named microsomal alcohol oxygenase (MALCO), is able to oxidize 7-hydroxy-Δ⁸-THC to 7-oxo-Δ⁸-THC, 2 which is one of active metabolites of Δ⁸-THC. 3 Bornheim et al. 4,5 have reported that the formation of 8-oxo-Δ⁸-THC from Δ⁸-THC with hepatic microsomes of mouse and human was markedly suppressed by antibody against CYP3A, whereas the contribution of the 3A enzyme to the formation of 8-oxo-Δ⁸-THC has not been directly demonstrated. We have recently purified two P450 enzymes, named P450GFP-B and P450MDX-B, which are the major enzymes responsible for the formation of 7-oxo-Δ⁸-THC in hepatic microsomes of guinea pig 6 and mouse, 7 respectively. Both of these enzymes are believed to be members of the 3A subfamily from the NH₂-terminal amino acid sequences and their catalytic properties. CYP3A is a highly expressed subfamily in both rodents and humans, and it has been demonstrated to have a prominent role in the metabolism of many clinically important drugs in animals. 8 The compounds bound to the active site of CYP3A may not be constrained to the same defined substrate structure–activity relationship, since CYP3A shows no apparent selectivity for substrates. 9 CYP3A, however, shows selectivity for chemical substituents. 10 For instance, the allylic positions are the major site of oxidation catalyzed by CYP3A. 11–14 The metabolism of chemicals has often been shown to differ quantitative or qualitative between male and female animals. In particular, the microsomal metabolism of drugs catalyzed by P450 is known to be sexually different in rat liver. 15–17 These large sex-related difference reflect the contributions of sex-specific P450 enzymes such as CYP2A1, 18 CYP2C11, 19 CYP2C12 20 and CYP3A2 21–23 to overall microsomal metabolism.

In the present study, we characterized MALCO which catalyzed the formation of 7-oxo-Δ⁸-THC from 7α- and 7β-hydroxy-Δ⁸-THC in the hepatic microsomes of rats and clarified the involvement of the enzyme in the metabolism of the allylic position in Δ⁸-THC.

MATERIALS AND METHODS

Materials Δ⁸-THC, 24 7α- and 7β-hydroxy-Δ⁸-THC, 25 7-oxo-Δ⁸-THC 26 and 5’-nor-Δ⁸-THC 4'-oic acid 27 were prepared by the methods previously reported. Dexamethasone, sodium phenobarbital and 3-methylcholanthrene were purchased from Wako Pure Chemicals (Osaka, Japan). Other chemicals and solvents used were of the highest quality commercially available.

Animal Treatment and Preparation of Microsomes Male and female rats of Sprague-Dawley strain (4- and 8-week-old, Hokuriku Experimental Animals Lab., Kanazawa, Japan) were used in the experiments. Phenobarbital sodium (100 mg/kg in saline) and 3-methylcholanthrene (40 mg/kg in salad oil) were administered i.p. every 24 h for 2 d. Dexamethasone (500 mg/kg in salad oil) was injected i.p. at a single dose. Acetone was given in 5% (v/v) solution in drinking water for 10 d until sacrificed. Following fasting for 12 h, the animals were sacrificed by decapitation 48 h after the first injection of phenobarbital, 3-methylcholanthrene and dexamethasone. Hepatic microsomal pellet was suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 5 mM EDTA. 28

Preparation of Antibodies Polyclonal antibody against P450GFP-B was raised in female New Zealand White rabbits as described previously. 29 The IgG fraction from the rabbit serum was obtained by the previously reported method. 28

Measurement of Oxidative Activity The formation of 7-oxo-Δ⁸-THC was measured essentially as previously described. 30 7-Hydroxy-Δ⁸-THC (12 µg) was incubated with...
hepatic microsomes (0.5—1 mg protein) of rats, an NADPH-generating system (0.5 mM NADP, 10 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase, 10 mM magnesium chloride), and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 0.5 ml. The mixture was incubated at 37°C for 20 min. Metabolites were extracted with 2.5 ml of ethyl acetate after addition of 5'-nor-Δ4-THC-4'-oic acid as an internal standard (2.5 μg). A portion of the extract was evaporated to dryness. 7-Oxo-Δ9-THC formed was derivatized to heptafluorobutyrate and determined by electron capture detector-gas chromatography. Formations of 11-, 7α- and 7β-hydroxy-Δ9-THC, and 7-oxo-Δ9-THC from Δ9-THC was quantified by GC/MS.

Oxidative metabolism of testosterone was determined as described previously.

Inhibition of MALCO Activity by Antibody The antibody was added to medium containing microsomes and 100 mM potassium phosphate buffer (pH 7.4), and preincubated at 37°C for 30 min. The NADPH-generating system described above was added to the medium, and adding substrate then started the reaction. After incubation at 37°C for 20 min, the metabolites were assayed by the same methods described above.

Other Methods Protein concentration was estimated by the method of Lowry et al., using bovine serum albumin as a standard. P450 content was determined by the methods of Omura and Sato. The statistical significance of differences was determined using Student’s t-test.

RESULTS

7-Hydroxy-Δ9-THC MALCO Activity in Male and Female Rats Figure 1A, B shows the enzyme activities for the formation of 7-oxo-Δ9-THC from 7α- (A) and 7β-hydroxy-Δ9-THC (B) in hepatic microsomes of 4- and 8-week-old male or female rats. The activity was stereoselective and the rate of conversion of 7-oxo-Δ9-THC from 7β-hydroxy-Δ9-THC in both sexes was about 3-fold higher than that from 7α-hydroxy-Δ9-THC. The 7α- and 7β-hydroxy-Δ9-THC MALCO activities in 4-week-old female rats were approximately 50% of those of 4-week-old male rats. The activities in the female significantly decreased with aging, although the activities in the male did not change. The 7α- and 7β-hydroxy-Δ9-THC MALCO activities in 8-week-old female rats were 41 and 36%, respectively, of those of 4-week-old female rats. The activities were more than 5-fold higher in male than female at 8 weeks of age.

Table 1. Effects of Pretreatment with Various Inducers on P450 Content, and 7α-OH- and 7β-OH-Δ9-THC MALCO Activities in Rat Liver Microsomes

<table>
<thead>
<tr>
<th>Treatment</th>
<th>P450 content (nmol/min/mg protein)</th>
<th>MALCO activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>7α-OH-Δ9-THC</td>
</tr>
<tr>
<td>Control (None)</td>
<td>0.72±0.05</td>
<td>0.65±0.06</td>
</tr>
<tr>
<td></td>
<td>(100)</td>
<td>(100)</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>1.45±0.03</td>
<td>5.20±0.12</td>
</tr>
<tr>
<td></td>
<td>(200)</td>
<td>(805)</td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>1.10±0.05</td>
<td>2.13±0.13</td>
</tr>
<tr>
<td></td>
<td>(152)</td>
<td>(329)</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>0.93±0.03</td>
<td>1.27±0.14</td>
</tr>
<tr>
<td></td>
<td>(128)</td>
<td>(196)</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.86±0.02</td>
<td>0.85±0.12</td>
</tr>
<tr>
<td></td>
<td>(118)</td>
<td>(131)</td>
</tr>
</tbody>
</table>

Male rats of Sprague-Dawley strain (8-week-old) were used in the experiments. The inducers were administered as described in Materials and Methods. Each value represents the mean±S.E. of three determinations. Numbers in parentheses are relative value (%) to the control. a) Significantly different from the control (p<0.05). b) Significantly different from the control (p<0.01).

EFFECTS OF INDUCTORS ON 7-HYDROXY-Δ9-THC MALCO ACTIVITY Table 1 shows the effect of pretreatment with dexamethasone, phenobarbital, 3-methylcholanthrene and acetone on 7α- and 7β-hydroxy-Δ9-THC MALCO activities in rat liver. 7α- and 7β-hydroxy-Δ9-THC MALCO activities were significaiently enhanced by both dexamethasone and phenobarbital. Especially, dexamethasone induced the MALCO activities more than 5-fold over untreated rats. On the other hand, MALCO activities were not significantly enhanced by pretreatment with 3-methylcholanthrene or acetone, except for 7α-hydroxy-Δ9-THC MALCO activity by 3-methylcholanthrene.

EFFECTS OF ANTIBODY AGAINST P450GFP-B ON 7-HYDROXY-Δ9-THC MALCO ACTIVITY AND MICROSONAL TESTOSTERONE OXIDATION Addition of the anti-P450GFP-B IgG fraction to the incubation medium caused a dose-dependent suppression of both activities of 7α- and 7β-hydroxy-Δ9-THC MALCO
Table 2. Effects of Antibody against P4500GPF-B on Δ^8-THC Metabolism with Male Rat Liver Microsomes

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Metabolites formed (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7α-OH-Δ^8-THC</td>
</tr>
<tr>
<td>Control</td>
<td>0.19</td>
</tr>
<tr>
<td>P450QPF-B</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>(20)</td>
</tr>
</tbody>
</table>

Male rats of Sprague-Dawley strain (8-week-old) were used in the experiments. Hepatic microsomes were preincubated with the IgG fraction (4.0 mg/mg of microsomal protein) for 30 min at 37°C, and then incubated with Δ^8-THC in the presence of an NADPH-generating system. Each value represents the mean of two determinations. Numbers in parentheses are relative values (%) to the control. ND, not detected.

DISCUSSION

In the course of our metabolic studies on THC, we have found for the first time that hepatic microsomes of guinea pig are able to oxidize 7-hydroxy-Δ^8-THC to 7-oxo-Δ^8-THC.2) Recently, we purified two P450 enzymes belonging to the 3A subfamily as the major enzyme responsible for the oxidation of 7-hydroxy-Δ^8-THC to 7-oxo-Δ^8-THC in hepatic microsomes of guinea pig6) and mouse.7) The present study characterized the major P450 enzymes responsible for the oxidation of 7α- and 7β-hydroxy-Δ^8-THC to 7-oxo-Δ^8-THC in hepatic microsomes of rats. The activity was stereospecific and the rate of conversion of 7-oxo-Δ^8-THC from 7β-hydroxy-Δ^8-THC in both sexes was significantly higher than that from 7α-hydroxy-Δ^8-THC (Fig. 1). The activity, especially for 7β-hydroxy-Δ^8-THC, in 8-week-old female rats was significantly lower than that in 4-week-old female rats, although the male showed almost the same activity. The MALCO activities of the males were significantly higher than that of the females, especially in 8-week-old rats. The sex-related difference is recognized to be manifested by the presence of sex-specific forms of P450 in hepatic microsomes of rats.6) This means that the MALCO activity may be catalyzed by the male-dominantly expressed isoform in the adult. Waxman et al.17) have reported the age and sex dependence of P450 expression for eight distinct enzymes. They also present experiments designed to evaluate the responsiveness of each sex-specific P450 enzyme induction in rat liver. Among these sex-specific forms, CYP2C11 and CYP3A2 are known to be involved in male-dominant hydroxylations of testosterone and androstenedione in non-induced rat. 7α- and 7β-hydroxy-Δ^8-THC MALCO activities were significantly enhanced by treatments with dexamethasone or phenobarbital (Table 1). Especially, dexamethasone induced MALCO activities more than 5-fold over untreated rats. Dexamethasone and phenobarbital significantly induce CYP3A1 that shows the same enzymatic specificity with undetectable CYP3A2.19) Both enzymes possess testosterone 6β-hydroxylase activity, although the complete enzymatic roles of these enzymes in cellular metabolism are unclear.10,11,19) The microsomal testosterone 6β-hydroxylase activity and the content of CYP3A2 or its mRNA are detectable in neonate and reached
a maximal level in both sexes at 14 to 20 d of age, but was abruptly diminished at puberty only in female rats. The identities of P450GPF-B and P450MDX-B with CYP3A2 are 57 and 83%, when the first 23-amino-acid sequence in the NH₂-terminus was compared. Antibody against P450GPF-B showed an immunological cross reaction with rat hepatic microsomal protein believed to be CYP3A2 by Western blotting analysis (data not shown). Antibody against P450GPF-B dose-dependently suppressed 7α- and 7β-hydroxy-Δ⁴-THC (Fig. 4). The same antibody also inhibited the microsomal testosterone 6β-hydroxylase activity in hepatic microsomes of adult male rats. On the other hand, no significant effects were found with 16α-hydroxy-testosterone or androstenedione formation, which were known to be catalyzed by CYP2C11 in adult male rats. These results indicate that CYP3A2 is the major enzyme responsible for the formation of 7-oxo-Δ⁴-THC from 7α- and 7β-hydroxy-Δ⁴-THC in hepatic microsomes of untreated adult male rats.

The allylic positions are the major site of oxidation catalyzed by CYP3A in such structurally unrelated compounds as lovastatin, clofibrate, and quinidine. In addition to these compounds, the 6β-position in testosterone is allylic to the intracyclic double bond, and is equivalent to the 7- and 8-position in Δ⁴- and Δ¹₅-THC, respectively (Fig. 4). The catalytic properties of P450 enzymes in the same gene families were suggested to be generally similar among animal species examined.

The formation of 8α- and 8β-hydroxy-Δ⁸-THC from Δ⁸-THC with human hepatic microsomes was significantly suppressed by antibody against CYP3A. In mice, however, the formation of 8α-hydroxy-Δ⁸-THC from Δ⁸-THC in the microsomes was markedly suppressed by the antibody against CYP2C, and the 7α-hydroxylation of Δ⁸-THC was conversely stimulated by the antibody. Species-related differences exist in the catalytic properties of P450 enzymes belonging to the same family or subfamily, and it is difficult to elucidate the enzyme responsible for a particular reaction across species. In the present study, the formation of 7α- and 7β-hydroxy-Δ⁸-THC from Δ⁸-THC with liver microsomes of 8-week-old male rats was also strongly suppressed by antibody against P450GPF-B. On the other hand, the 11-hydroxylation of Δ⁸-THC in the microsomes was slightly stimulated by the antibody, although the 11-position in Δ⁸-THC is also allylic to the double bond. Our present study also indicates that the formation of 7α- and 7β-hydroxy-Δ⁸-THC as well as 7-oxo-Δ⁸-THC in hepatic microsomes of adult male rats are mainly mediated by CYP3A2, and there is a species-related differences in the catalytic properties of P450 enzymes belonging to the same family or subfamily.

Acknowledgements This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan, and by the Special Research Fund of Hokuriku University.

REFERENCES