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A CYTOCHROME P450 ISOZYME HAVING ALDEHYDE OXYGENASE ACTIVITY PLAYS A MAJOR ROLE IN METABOLIZING CANNABINOIDS BY MOUSE HEPATIC MICROSOMES

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Abstract—A cytochrome P450 (designated P450 MUT-2) which catalyses the oxidation of 11-oxo-Δ^8-tetrahydrocannabinol (11-oxo- Δ^8-THC) to Δ^8-THC-11-oic acid has been purified from hepatic microsomes of untreated male mice. Analysis of NH_2-terminal sequence suggests that the isozyme is a member of the P450 2C gene subfamily. P450 MUT-2 exhibited aldehyde oxygenase activity for 11-oxo-Δ^8-TH, 11-oxo-Δ^9-THC, 11-oxo-cannabinol (11-oxo-CBN) and 9-anthraldehyde together with high activity for the hydroxylation of cannabinoids at the 11-position. Antibody against P450 MUT-2 significantly inhibited the microsomal formation of Δ^8-THC-11-oic acid from 11-oxo-Δ^8-THC, but not that of 9-anthracene carboxylic acid from 9-anthraldehyde. Major metabolic reactions of Δ^8-THC, Δ^9-THC and CBN with mouse hepatic microsomes were the 11-hydroxylation (all cannabinoids), 7α-(Δ^8-THC) or 8 α-hydroxylation (Δ^9-THC) and epoxide formation (Δ^8- and Δ^9-THC). All these reactions except for 7 α-hydroxylation of Δ^8-THC and α-epoxide formation from Δ^9-THC were also markedly inhibited by the antibody. These results indicate that P450 MUT-2 is a major enzyme for metabolizing cannabinoids by mouse hepatic microsomes.

Extensive studies have revealed that the hepatic microsomes of animals contain multiple forms of cytochrome P450 (P450‖) [1–3], which constitute a superfamily of hemoprotein [4] and catalyse a wide variety of biological mono-oxygenations; alkanes to alcohols, alkenes to epoxides, arenes to phenols, sulfides to sulfoxides and sulfones or oxidative dealkylation of *N*-, *O*- and *S*-alkyl compounds [5].

Until recently, aldehyde dehydrogenase was generally considered to be responsible for the oxidation aldehydes in biological systems. Previous studies in our laboratory, however, have demonstrated for the first time that microsomal aldehyde oxygenase (MALDO) catalyses the oxidation of various aldehyde substrates to the corresponding carboxylic acids [6–8]. Recently, we reported the preliminary result that P450 MUT-2, a P450 isozyme, purified from mouse hepatic microsomes, which may belong to the 2C gene subfamily, catalysed the oxidation of 11-oxo-Δ^8-tetrahydrocannabinol (THC) to Δ^8-THC-11-oic acid [9]. The members of 2C subfamily of P450s are, in most cases, constitutively expressed, but in some cases, regulated developmentally or sexually [10–12]. The catalytic activity of the P450 for oxidizing aldehyde to carboxylic acid is not specific for a particular isozyme but rather common property. Many P450s purified from rat liver could catalyse the oxidation of 11-oxo-Δ^8-THC to Δ^8-THC-11-oic acid [13]. The present paper describes the purification and characterization of P450 MUT-2 having aldehyde oxygenase activity and playing a major role in the metabolism of cannabinoids in the mouse liver.

MATERIALS AND METHODS

Chemicals. Δ^9-THC and cannabinol (CBN) were purified from cannabis leaves as described previously [14]. Δ^8-THC [15], 11-hydroxy-Δ^8-THC (11-OH-Δ^8-THC) [16], 11-oxo-Δ^8-THC [16], 8α, 9α-epoxyhexahydrocannabinol (8α, 9α-EHHC) [17], 9α, 10α-EHHC [18], Δ^8-THC-11-oic acid [19], 11-OH-Δ^9-THC [20], 11-oxo-Δ^9-THC [20], Δ^9-THC-11-oic acid [20], 11-OH-CBN [21] and 11-oxo-CBN [16] were prepared by the previous methods. CBN-11-oic acid was prepared from 11-oxo-CBN by the method of Corey *et al.* [22]. NADP and glucose-6-phosphate were purchased from Boehringer-Mannheim GmbH (Darmstadt, F.R.G.); NADPH was from the Oriental Yeast Co. (Tokyo, Japan); 1,8-diaminooctane, 7-ethoxycoumarin, 9-anthraldehyde and 9-anthracene carboxylic acid were from the Aldrich Chemical Co. (Milwaukee, WI, U.S.A.); glucose-6-phosphate dehydrogenase (type V), dilauroylphosphatidylcholine and standard proteins for polyacrylamide gel electrophoresis were from the

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‖ Abbreviations: THC, tetrahydrocannabinol; CBN, cannabinol; P450, cytochrome P450; EHHC, epoxyhexahydrocannabinol; DTT, dithiothreitol; MALDO, microsomal aldehyde oxygenase.

Sigma Chemical Co. (St Louis, MO, U.S.A.); cholic acid and Freund's complete adjuvant were from Wako Pure Chemicals Ind. Ltd (Osaka, Japan); *N,O*-bis-trimethylsilyltrifluoroacetamide (BSTFA), trimethylsilylimidazole (TMSI), trimethylchlorosilane (TMCS) were from Tokyo Kasei Kogyo, Co. Ltd (Tokyo, Japan); Sepharose 4B, Protein A-Sepharose 4B, 2′,5′-ADP-Sepharose 4B and CM Sephadex C-50 were from Pharmacia Fine Chemicals (Uppsala, Sweden); hydroxylapatite was from Bio-Rad (Richmond, CA, U.S.A.); oxygen-18 gas (97 atom %) was from Amersham International plc (Bucks, U.K.). *d*-Benzphetamine hydrochloride and SKF 525-A were obtained from Upjohn (Kalamazoo, MI, U.S.A.) and Smith Kline and French Laboratories (Philadelphia, PA, U.S.A.), respectively. Emulgen 911 and 913 were gifts from Kao-Atlas, Co. Ltd (Tokyo, Japan).

Preparation and solubilization of hepatic microsomes. Male ddN mice (30–35 g) were used. The mice were fasted over night before being killed. The livers were removed and homogenized with 3 vol. of 1.15% KCl containing 1 mM EDTA. The homogenates were centrifuged at 9000 *g* for 20 min. The 9000 *g* supernatants were combined, and centrifuged at 105,000 *g* for 60 min. The pellets were resuspended in the 1.15% KCl containing 1 mM EDTA, and recentrifuged at 105,000 *g* for 60 min. The hepatic microsomes were solubilized with 0.6% sodium cholate in 100 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 1 mM EDTA and 0.1 mM dithiothreitol (DTT) (buffer A). The solubilized microsomes were centrifuged at 105,000 *g* for 60 min, and the supernatant fraction was used for the purification of P450.

Purification of P450 MUT-2. The procedure for purification of P450 MUT-2 has been briefly reported [9]. The supernatant fraction of the cholate-solubilized hepatic microsomes from 147 male ddN mice was applied to an ω-aminooctyl-Sepharose 4B column (4 × 20 cm) equilibrated with the buffer A containing 0.5% sodium cholate. The column was washed with the same buffer (1000 mL), and P450 was eluted with buffer A containing 0.4% sodium cholate and 0.2% Emulgen 913. The fractions (O.D. > 0.3 at 417 nm) were pooled, and concentrated by ultrafiltration (UK-50, Toyo Roshi, Tokyo, Japan), and dialysed against 10 mM Tris–acetate buffer (pH 7.5) containing 20% glycerol and 0.4% Emulgen 911. The dialysed solution was subjected to a preparative DEAE-5PW column (2.15 × 15 cm, Toso, Tokyo, Japan) chromatography on a Hitachi 655A-11 liquid chromatograph equipped with a L-3000 photodiode array detector as described by Funae and Imaoka [23]. The pass-through fraction that had the major portion of aldehyde oxygenase activity for 11-oxo-Δ^8-THC was concentrated and applied to HPLC with a SP-5PW column (2.15 × 15 cm, Toso, Tokyo, Japan). The elution of P450 was monitored at 417 nm. The fractions containing P450 having the oxidation activity of 11-oxo-Δ^8-THC were combined and concentrated by ultrafiltration, and then dialysed against buffer A containing 10 mM potassium phosphate buffer (pH 7.25) and 0.4% Emulgen 911. The dialysed sample was applied to a CM-Sephadex C-50 column, previously equilibrated with the same buffer as used in the dialysis. P450s were eluted with 300 mL each of buffer A containing 40, 80 and 160 mM potassium phosphate buffer. P450 fractions eluted with 80 mM buffer A were combined, concentrated by the ultrafiltration and dialysed against 5 mM buffer A containing 0.4% Emulgen 911. The sample was applied to a hydroxylapatite column. An aliquot of each fraction was analysed by SDS–PAGE for examining the purity. The fractions containing a P450 as a homogenous band were combined. To remove the detergent, the combined fraction was diluted with 4 vol. of 5 mM buffer A, and applied to a small hydroxylapatite column. P450 was eluted with 350 mM sodium phosphate buffer (pH 7.4) containing 20% glycerol, 1 mM EDTA-2Na, 0.1 mM DTT and 0.05% sodium cholate after washing enough with a washing buffer. The purified P450 was stored at −80°.

Preparation of antiserum. Antiserum against purified P450 MUT-2 was raised in female New Zealand white rabbits as described previously [24], except that about 100 μg of enzyme protein was immunized once a month.

Enzyme assays. Metabolic activities for aniline [25], *p*-nitroanisole [26], ethoxycoumarin [27], benzphetamine [28], 9-anthraldehyde [29] and testosterone [30] were determined by the previous methods. Reconstituted systems were prepared by mixing 10–20 pmol of P450 MUT-2 with 0.33 U of NADPH-cytochrome *c* reductase and 15 μg of dilauroylphosphatidylcholine in 100 mM sodium–potassium phosphate buffer (pH 7.4) to make a final volume of 0.45 mL. The reaction was initiated by addition of NADPH (10 mM) after addition of appropriate amounts of substrates. For the assay for cannabinoids, the substrate (each 10 μg) was incubated at 37° for 30 min under the above conditions. The metabolites formed were extracted with 4 mL of ethyl acetate after the addition of 0.5–1.0 μg of 5′-nor-Δ^8-THC-4′-oic acid methyl ester as the internal standard. The metabolites were determined by GC–MS described previously [31, 32].

Hepatic microsomal metabolism of the cannabinoids was assayed under the conditions described previously [8, 31]. In some cases, incubation atmosphere was replaced by oxygen-18 gas and oxygenation mechanism in the formation of metabolites was analysed by GC–MS as described previously [6, 7, 9]. The inhibitory effect of the antibody raised to the P450 was determined in the presence of 0.34–11.5 mg of antibody/mg of microsomal protein. Microsomes were preincubated with the antibody at 37° for 30 min, and then incubated with the cannabinoids in the presence of an NADPH-generating system. The metabolites formed were determined as described above. Protein concentration was determined by the method of Lowry *et al.* [33] with bovine serum albumin as the standard. The concentration of P450 was assayed by the method of Omura and Sato [34] using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$. Cytochrome *c* reductase was purified from mouse hepatic microsomes by the method of Shephard *et al.* [35].

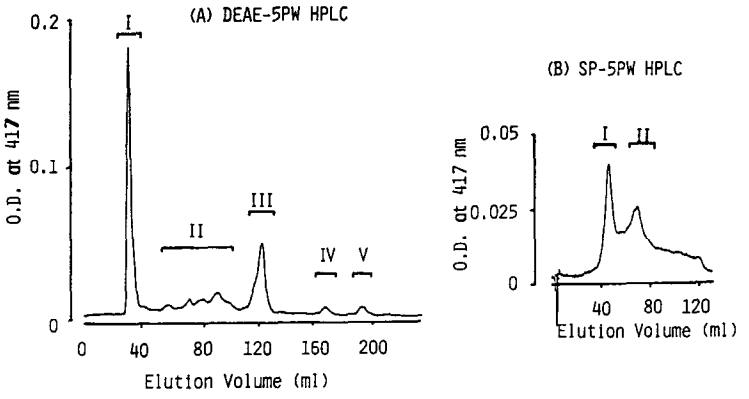

Fig. 1. Elution profiles of mouse hepatic microsomal cytochrome P450s from DEAE-5PW and SP-5PW columns.

RESULTS

Purification of P450 MUT-2

Figure 1A illustrates a typical elution profile of P450s from a DEAE-5PW column. Assay for MALDO activity toward 11-oxo-Δ^8-THC indicated that most of the activity was eluted in the fraction (I), in which the enzymatic activity was 12.2 nmol Δ^8-THC-11-oic acid formed/min/nmol P450 under the conditions reconstituted with NADPH-cytochrome *c* reductase and dilauroylphosphatidylcholine as described in the Materials and Methods. The activity in the other fractions (II–V) was less than 0.2 nmol/min/nmol P450. The fraction (I) was then applied to a SP-5PW column. Figure 1B shows a typical elution profile of P450 indicating that the cytochromes were mainly separated into two fractions (SP-I and -II). The MALDO activities toward 11-oxo-Δ^8-THC in the fractions SP-I and -II were 16.3 and 3.5 nmol/min/nmol P450, respectively. P450 MUT-2 was purified from the SP-I fraction by CM Sephadex C-50 and hydroxylapatite column chromatographies as described in the Materials and Methods. SDS–PAGE of the purified enzyme revealed a single band which showed an apparent molecular mass of 50,000. The purified enzyme showed spectral peaks at 417 and 451 nm, respectively, on the oxidized and reduced CO-complex forms. The NH_2-terminal amino acid sequence of P450 MUT-2 was as follows: M-D-L-V-V-F-L-A-L-T-L-S-X-L-I-L-L-S-L-W-R-Q-S-X-G-R-G-K-X-P. The sequence up to the twentieth residue had been reported already [9].

Catalytic activities

Table 1 summarizes catalytic activity of P450 MUT-2 toward well established substrates for P450. The P450 catalysed the hydroxylation of aniline and the demethylation of *p*-nitroanisole and benzphetamine, although these activities were almost the same as those in microsomes. Ethoxycoumarin *O*-deethylase activity of the P450 was relatively low and no metabolites were detectable by HPLC for the assay of testosterone hydroxylation. P450 MUT-2 exhibited the catalytic activity for converting aldehyde metabolites of cannabinoids to their corresponding carboxylic acids, although the turn over number was different in the substrates (Table 2). The P450 showed the highest catalytic activity toward 11-oxo-Δ^8-THC, followed by 11-oxo-CBN oxidation and the activity toward 11-oxo-Δ^9-THC was about a twentieth of 11-oxo-Δ^8-THC. GC–MS analyses confirmed the oxygenation mechanism for the oxidation of 11-oxo-Δ^9-THC and 11-oxo-CBN to their corresponding carboxylic acids formed under $^{18}O_2$ atmosphere by P450 MUT-2, since the trimethylsilylated and methylated derivative of the carboxylic acid metabolites of 11-oxo-Δ^9-THC and 11-oxo-CBN showed their molecular ions at *m/z* 432 and 428, respectively, while these molecular ions in the metabolites formed under air were *m/z* 430 and 426, respectively. P450 MUT-2 also had high activities for hydroxylations of the cannabinoids at the 11-position. Turn over numbers (nmol/min/nmol P450) with the P450 for Δ^8-THC, Δ^9-THC and CBN were 29.0, 23.7 and 26.8, respectively, which are comparable to the catalytic activity of the P450 in the MALDO activity for 11-oxo-Δ^8-THC. P450 MUT-2 showed the catalytic activities for the 8α, 9α-epoxidation of Δ^8-THC and the 8α-hydroxylation of Δ^9-THC. Furthermore, as Table 2 indicates P450 MUT-2 has a catalytic activity for converting the methyl group of the cannabinoids to carboxylic acid in which three consecutive oxidations are involved.

Inhibition with antibody

The effects of antibody against purified P450 MUT-2 on the oxidation of 11-oxo-Δ^8-THC and 9-anthraldehyde are shown in Fig. 2. The microsomal formation of Δ^8-THC-11-oic acid was suppressed with increasing amounts of the antibody. The reaction was inhibited by 67% at a concentration ratio of 4.1 mg immunoglobulin G (IgG)/mg microsomal protein, while IgG from control rabbit did not affect significantly the activity (Fig. 2A). Unlike Δ^8-THC-11-oic acid formation, MALDO activity for 9-anthraldehyde was suppressed only by

Table 1. Catalytic activity of cytochrome P450 MUT-2 and hepatic microsomes

Enzyme activity	Specific activity*	
	Microsomes	P450 MUT-2
Aniline hydroxylase	1.67	5.45
p-Nitroanisole *O*-demethylase	1.78	2.18
Ethoxycoumarin *O*-deethylase	0.01	0.02
Benzphetamine *N*-demethylase	4.55	4.42
9-Anthraldehyde oxygenase	2.75	6.34

Enzyme activities were assayed as described in Materials and Methods.
* nmol/min/nmol P450.

Table 2. Catalytic activity of cytochrome P450 MUT-2 in oxidizing cannabinoids and their metabolites

Substrate	Metabolites formed (nmol/min/nmol P450)			
	11-OH	EHHC	11-Oxo	11-Oic acid
Δ^8-THC	29.0	3.1	6.2	1.0
11-OH-Δ^8-THC	—	—	16.2	7.8
11-oxo-Δ^8-THC	—	—	—	25.7
	11-OH	8α-OH	11-Oxo	11-Oic acid
Δ^9-THC	23.7	23.1	1.2	0.5
11-OH-Δ^9-THC	—	—	4.5	2.4
11-oxo-Δ^9-THC	—	—	—	1.5
CBN	26.8	—	3.2	0.4
11-OH-CBN	—	—	1.7	ND*
11-oxo-CBN	—	—	—	5.6

Data are means of duplicate incubations.
* ND, not detected.

16% at a concentration ratio of 9.6 mg serum protein/mg microsomal protein.

Table 3 summarizes the inhibitory effect of the antibody against P450 MUT-2 on the formation of major metabolites of the cannabinoids with mouse hepatic microsomes. Mouse hepatic microsomes catalysed metabolic reactions of the cannabinoids at the 11-position (all cannabinoids examined), 7α-(Δ^8-THC) or 8α-hydroxylation (Δ^9-THC) and α-epoxide formation (Δ^8- and Δ^9-THC). All the 11-hydroxylations of the cannabinoids were almost completely suppressed, and 8α-hydroxylation of Δ^9-THC was inhibited to approximately 20% of control value. Epoxide formation from the THC isomers was differently affected by addition of the antibody. 8α, 9α-EHHC formation from Δ^8-THC was significantly suppressed to 5% of control level, whereas 9α, 10α-EHHC formation from Δ^9-THC was markedly stimulated to approximately 500% of control. 7α-Hydroxylation activity for Δ^8-THC was also enhanced 2-fold by addition of the antibody.

DISCUSSION

The major isozyme in catalysing MALDO activity for 11-oxo-Δ^8-THC was purified from male ddN mice and characterized by its catalytic activity toward

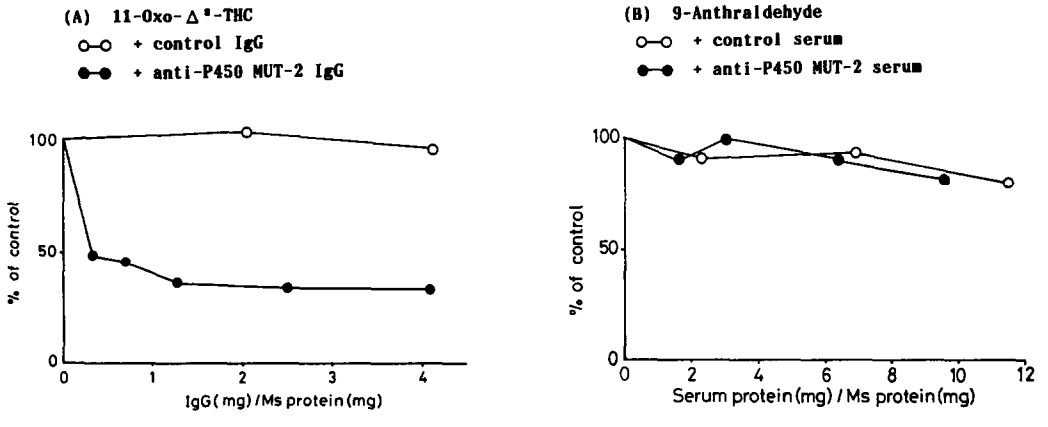

Fig. 2. Inhibitory effect of anti-P450 MUT-2 IgG and serum on MALDO activities for 11-oxo-Δ^8-THC and 9-anthraldehyde in mouse hepatic microsomes.

Table 3. Inhibitory effect of anti-P450 MUT-2 serum on metabolism of cannabinoids with mouse hepatic microsomes

Substrate	Additions	Metabolites formed (nmol/min/mg protein) 11-OH	7α-OH	EHHC
Δ^8-THC	Control*	1.93 (100)	1.25 (100)	0.30 (100)
	Anti-P450 MUT-2†	0.02 (1)‡	2.50 (200)	0.02 (7)
		11-OH	8α-OH	EHHC
Δ^9-THC	Control*	2.02 (100)	1.37 (100)	0.08 (100)
	Anti-P450 MUT-2†	0.02 (1)	0.29 (21)	0.41 (513)
			11-OH	
CBN	Control*		2.18 (100)	
	Anti-P450 MUT-2†		0.02 (1)	

Enzyme activities were assayed as described in Materials and Methods. Data are means of duplicate incubations.
* 4.3 mg serum protein/mg microsomal protein.
† 6.0 mg serum protein/mg microsomal protein.
‡ % of control.

various substrates. The purified enzyme, designated P450 MUT-2, having an apparent molecular mass of 50,000, appears to be a member of the P450 2C subfamily from its NH_2-terminal amino acid sequence. Two peptide fragments of P450 MUT-2 degradated by BrCN were highly homologous with a part of the amino acid sequence of P450 2C7 [10] (unpublished data). The NH_2-terminal amino acid sequence was very similar to those of P450 PBIII [36], P450 m51a [37] and $P450_{THC}$ [38], although some differences in the apparent molecular mass, spectral characterization and catalytic activity exist between these isozymes.

P450 MUT-2 showed the metabolic activities for aniline, *p*-nitroanisole, benzphetamine and 9-anthraldehyde, which were 1.0- to 3.3-fold those measured in the microsomes. The result suggests that P450 MUT-2 may not be a major isozyme responsible for the metabolism of these substrates in mouse hepatic microsomes, since western blot analysis indicated that P450 MUT-2 consisted of 4–5% of total P450 in the mouse hepatic microsomes (unpublished data). P450 MUT-2 was purified from mouse hepatic microsomes by tracing the catalytic activity for the oxidation of 11-oxo-Δ^8-THC to Δ^8-THC-11-oic acid. Therefore, it is anticipated that this isozyme is a major P450 isozyme responsible for the MALDO activity toward 11-oxo-Δ^8-THC in the microsomes. Antibody raised against P450 MUT-2 markedly inhibited the microsomal formation of Δ^8-THC-11-oic acid from 11-oxo-Δ^8-THC, supporting that P450 MUT-2 or immunologically-related isozyme(s) is a major isozyme contributing to the MALDO activity in the mouse liver. The incomplete inhibition may, however, to some extent be due to the contribution of other isozymes for the MALDO activity. On the contrary, the MALDO activity for 9-anthraldehyde was not significantly inhibited by the anti-P450 MUT-2 antibody indicating that an immunologically different isozyme catalyses this reaction in mouse hepatic microsomes. Recently, we presented data suggesting that the major isozymes responsible for MALDO activities toward 11-oxo-Δ^8-THC and 9-anthraldehyde in the mouse hepatic microsomes may be different [39]. P450 MUT-2 showed the catalytic activities for 11-oxo-Δ^9-THC and 11-oxo-CBN, which are metabolic intermediates of Δ^9-THC and CBN, respectively, although these activities are not so high as that for 11-oxo-Δ^8-THC. This indicates some strict structural requirement for aldehyde oxidation by P450, since the three aldehyde metabolites of the cannabinoids have similar structure.

A major metabolic pathway of cannabinoids with mouse hepatic microsomes is known to be the oxidation at the 11-position [40, 41]. Extensive studies have been done on the oxidative metabolism of cannabinoids with hepatic microsomes, however, only limited information is available for the regioselectivity of a particular form of P450 in the metabolism of the cannabinoids. Recently, we have reported that P450 2C11 and 2C6 are major isozymes for oxidizing Δ^9-THC at the 11-position in the hepatic microsomes of male and female rats, respectively [42, 43]. Bornheim *et al.* [44] reported that Δ^9-THC was metabolized by P450 2C9, and the antibody against P450 2C9 inhibited most of the 11-hydroxylation of the cannabinoid by human hepatic microsomes. In the mouse, Bornheim and Correia [38] reported that $P450_{THC}$, which may be a member of 2C subfamily, is a major isozyme in catalysing the oxidation of Δ^9-THC at the 11-position. These data indicate that all of the major P450s responsible for the THC metabolism at the 11-position belong to 2C subfamily. P450 MUT-2 appears to be also a member of 2C subfamily from its NH_2-terminal amino acid sequence, which is identical to that of $P450_{THC}$. Although the reported data indicate that both isozymes have similar catalytic activity, it is difficult to draw a definitive conclusion from limited data whether two isozymes are the same form or not. Available data suggest that both isozymes may be different forms and some sequence of amino acids is lacking in $P450_{THC}$, since molecular masses are considerably different from each other (P450 MUT-2, 50,000; $P450_{THC}$, 47,000). P450 MUT-2 exhibited the catalytic activities for the 11-hydroxylation (Δ^8-THC, Δ^9-THC and CBN), 8α-hydroxylation (Δ^9-THC) and α-epoxidation (Δ^8-THC). These activities in the hepatic microsomes were markedly suppressed by the antibody raised against P450 MUT-2, whereas the 7α-hydroxylation of Δ^8-THC and α-epoxidation of Δ^9-THC in the microsomes were conversely stimulated by the antibody. These results suggest that P450 MUT-2 plays a major role in the metabolism of the cannabinoids at the 11-position, 8α-hydroxylation of Δ^9-THC and α-epoxidation of Δ^8-THC in the microsomes.

The present study demonstrated that P450 MUT-2 having catalytic activity of aldehyde oxygenase is the major isozyme responsible for the hepatic microsomal metabolism of the cannabinoids and catalyses three consecutive steps of the oxidation of the cannabinoids at the 11-position.

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