Effective NADH-Dependent Oxidation of 7β-Hydroxy-Δ⁸-tetrahydrocannabinol to the Corresponding Ketone by Japanese Monkey Hepatic Microsomes

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The NADH-dependent activity by hepatic microsomes of Japanese monkeys for 7-oxo-Δ⁸-tetrahydrocannabinol (7-oxo-Δ⁸-THC) formation from 7β-hydroxy-Δ⁸-THC exhibited about 70% of the NADPH-dependent activity (100%) at the substrate concentration of 72.7 μM, although NADPH was an obligatory cofactor for maximal activity. Both NADH- and NADPH-dependent activities were significantly inhibited by the typical P450 inhibitors, such as SKF525-A and metyrapone. Both activities were almost completely inhibited by the NADPH-P450 reductase inhibitor diphenyliodonium chloride. The ratio of NADH- and NADPH-dependent activities varied significantly according to the substrate concentration. Interestingly, the NADH-dependent activity was higher than that of NADPH at low substrate concentrations of 13—50 μM. The ratio was also affected by the cofactor concentration. In the reconstituted system of CYP3A8 purified from hepatic microsomes of Japanese monkeys as a major enzyme responsible for the NADPH-dependent oxidation, NADH as well as NADPH could sustain the oxidation of 7β-hydroxy-Δ⁸-THC to the corresponding ketone. The NADH-dependent oxidation of 7β-hydroxy-Δ⁸-THC by monkey livers is mainly catalyzed by CYP3A8 as well as the NADPH-dependent oxidation. These results indicate that NADH as a cofactor may be also useful for the oxidation of 7β-hydroxy-Δ⁸-THC, and that the cofactor requirement for the reaction is varied by the concentrations of substrate and/or cofactor.

Key words 7-hydroxy-Δ⁸-tetrahydrocannabinol; CYP3A8; monkey; NADH; P450; microsomal alcohol oxygenase

Tetrahydrocannabinol (THC) is a major psychoactive constituent of marijuana, Cannabis sativa L. It is well known that THC is oxidized to a number of metabolites in the liver of mammals.1) Recent studies have clarified that P450 plays a major role in the oxidation of THC.2—4) We have previously reported that a hepatic microsomal enzyme, named microsomal alcohol oxygenase (MALCO), is able to oxidize 7α- and 7β-hydroxy-Δ⁸-THC to 7-oxo-Δ⁸-THC in the presence of NADPH and molecular oxygen.5,6) We have purified CYP3A8 as a major enzyme of MALCO from hepatic microsomes of monkey.7) The activity was stereoselective and the rate of conversion of 7β-hydroxy-Δ⁸-THC to 7-oxo-Δ⁸-THC was higher than that from 7α-hydroxy-Δ⁸-THC.7,8)

In general, the P450-dependent monoxygenase system has an absolute requirement for NADPH. A few studies have indicated that NADH can also accelerate the oxidative metabolism of p-nitrophenetole9) and p-nitroanisole,10,11) and the bioactivation of 2-acetylaminofluorene12) and nitrosoamines,13,14) During our studies on the microsomal oxidative metabolism of Δ⁸-THC, we observed that 7β-hydroxy-Δ⁸-THC was effectively biotransformed to 7-oxo-Δ⁸-THC by using NADH as a cofactor, without NADPH (Fig. 1).

The present study was undertaken to define the basic characteristics of the microsomal NADH-mediated oxidation of 7β-hydroxy-Δ⁸-THC to 7-oxo-Δ⁸-THC in the liver of monkeys and compare it to the NADPH-dependent systems.

**MATERIALS AND METHODS**

**Materials** NADH, NAD and NADP were purchased from Boehringer-Mannheim GmbH (Darmstadt, Germany); NADPH was from Oriental Yeast Co., Ltd. (Tokyo, Japan); metyrapone and 5α-cholestane were from Sigma Chemical Co. (St Louis, MO, U.S.A.); and diphenyliodonium chloride (DPIC), potassium cyanide and cholic acid were obtained from Wako Pure Chemicals (Osaka, Japan). SKF525-A and Emulgen 911 were kindly provided by Smith, Kline and French Laboratories (Philadelphia, PA) and Kao-Atlas Co. (Tokyo, Japan), respectively. 7β-Hydroxy-Δ⁸-THC,15) 7-oxo-Δ⁸-THC16) and 5-nor-Δ⁸-THC-4'-oic acid17) were prepared by the methods previously reported. The purity of the cannabinoiids was verified to be more than 98% by gas chromatography. Microsomal lipids were extracted from hepatic microsomes with chloroform–methanol (2 : 1) and the solvent was evaporated to dryness in vacuo. Other chemicals and solvents used were of the highest quality commercially available.

**Animals and Preparation of Microsomes** Liver samples from Japanese monkeys were provided by the Primate Research Institute, Kyoto University (Inuyama, Japan). Liver tissue homogenized using a Teflon-homogenizer was centrifuged to obtain the microsomes as described previously.18) The microsomal pellets obtained were suspended in 100 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and 5 mM EDTA.

**Enzyme Assays** In the study of the microsomal fraction,
the substrate was incubated with microsomes, 1 mM NAD, NADP, NADH and/or NADPH and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 0.5 ml. In the reconstitution studies of the complete system, each substrate was incubated with CYP3A8 (30 pmol), 0.33 units of NADPH-cytochrome c (P450) reductase, 30 pmol of cytochrome b₅, 50 μg of microsomal lipids, 100 μg of sodium cholate, 1 mM NADPH or NADH, and 100 mM potassium phosphate buffer (pH 7.4) to make a final volume of 0.5 ml. The reaction was allowed to proceed for 20 min at 37 °C. The metabolites were determined using previously reported methods.

**Other Methods** CYP3A8 was purified from hepatic microsomes of Japanese female monkeys as previously reported.⁷) NADPH-cytochrome c (P450) reductase and cytochrome b₅ were purified from hepatic microsomes of ddY male mice (Hokuriku Experimental Animals Lab., Kanazawa, Japan) by the methods of Yasukochi and Masters,¹⁹) and Funae and Imaoka, ²⁰) respectively. One unit of the reductase was defined as the amount of reductase catalyzing the reduction of 1 μmol of cytochrome c per minute. The detergent was removed using a hydroxylapatite column (φ5 mm × 10 mm).

Protein concentration was estimated by the method of Lowry et al.,²¹) using bovine serum albumin as a standard. P450 and cytochrome b₅ contents were determined by the methods of Omura and Sato,²²) and Omura and Takesue,²³) respectively.

**RESULTS**

**Cofactor Requirements and Optimum pH in Oxidation of 7β-Hydroxy-Δ⁹-THC to 7-Oxo-Δ⁹-THC**  The cofactor requirement for 7-oxo-Δ⁹-THC formation from 7β-hydroxy-Δ⁹-THC with hepatic microsomes of male Japanese monkeys was examined (Table 1). NADPH was an obligatory cofactor to show the maximal activity, while NAD and NADP, cofactors of dehydrogenase, were virtually inactive. The NADH-dependent activity exhibited about 70% of the NADPH-dependent activity at the substrate concentration of 72.7 μM. Figure 2 shows the effect of pH on 7-oxo-Δ⁹-THC formation. The optimum pH in the NADH system was 7.5 and was the same as that in the NADPH system.

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**Table 1. Cofactor Requirement for 7-Oxo-Δ⁹-THC Formation from 7β-Hydroxy-Δ⁹-THC with Liver Microsomes of Male Japanese Monkeys**

<table>
<thead>
<tr>
<th>Monkey</th>
<th>Year-old</th>
<th>Activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NAD</td>
</tr>
<tr>
<td>71Mf</td>
<td>6</td>
<td>0.04</td>
</tr>
<tr>
<td>73Mf</td>
<td>2</td>
<td>0.07</td>
</tr>
<tr>
<td>74Mf</td>
<td>2</td>
<td>0.02</td>
</tr>
</tbody>
</table>

The data are expressed as the mean of two experiments. 7β-Hydroxy-Δ⁹-THC (72.7 μM) was incubated with the microsomes for 20 min at 37 °C. Each cofactor was added to the incubation mixture at 1 mM.
Effects of Inhibitors on 7-Oxo-Δ⁸-THC Formation

Table 2 summarizes the effects of various inhibitors on 7-oxo-Δ⁸-THC formation in the presence of NADH or NADPH. SKF525-A and metyrapone inhibitors of P450 inhibited the formation of 7-oxo-Δ⁸-THC from 7β-hydroxy-Δ⁸-THC in the NADH system by 45%. DPIC, which is an inhibitor of NADPH-P450 reductase, 24) almost completely inhibited the activity. These inhibitors also inhibited to an extent similar to that in the NADPH system. On the other hand, potassium cyanide, an inhibitor of a terminal oxidase in fatty acid desaturase, had no significant effect on the formation of 7-oxo-Δ⁸-THC.

Effects of Substrate and Cofactor Concentration on the Formation of 7-Oxo-Δ⁸-THC

Figure 3 shows the effect of substrate concentration (13.3—150 μM) on 7-oxo-Δ⁸-THC forming activity in the presence of NADH or NADPH, at a final concentration of 1 mM. In the NADH system, the activity increased slowly compared with the NADPH system until the substrate concentration reached 40 μM, however, marked increases in activity were observed when the substrate concentration increased to more than 40 μM. As the result, the NADH-dependent activity was higher than that of NADPH at substrate concentrations of 13—50 μM (Fig. 3). When both NADH and NADPH are used as cofactors at lower concentrations of the substrate (below 40 μM), 7-oxo-Δ⁸-THC forming activity was almost the same as the activity when using NADH alone, whereas at a higher concentration (above 55 μM) it was the same as NADPH alone (Fig. 4).

Effects of Substrate and Cofactor Concentration on the Formation of 7-Oxo-Δ⁸-THC

Figure 5 shows the effect of cofactor concentration (0.01—2 mM) on the oxidative activity. When NADPH was used as the cofactor, maximum activity at the substrate concentrations of 40 and 80 μM was achieved at the cofactor concentrations of 0.2 and 1 mM, respectively, and then declined. In contrast, when NADPH was replaced with NADH for any substrate concentration, the activity was increased according to the cofactor concentration up to 2 mM.

7-Oxo-Δ⁸-THC Formation by CYP3A8 in a Reconstituted Enzyme System

CYP3A8 was purified from hepatic microsomes of Japanese monkeys as a major enzyme responsible for the NADPH-dependent oxidation of 7β-hydroxy-Δ⁸-THC. 7β-Hydroxy-Δ⁸-THC (13.3 to 150 μM) was incubated with the microsomes for 20 min at 37 °C in the presence of 1 mM NADH plus NADPH. The data are expressed as the mean of two experiments.

Table 2. Effects of Inhibitors on NADH- or NADPH-Dependent Oxidative Activity of 7β-Hydroxy-Δ⁸-THC in Monkey Hepatic Microsomes

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration</th>
<th>Activity (nmol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NADH</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>0.29±0.03 (100)</td>
</tr>
<tr>
<td>SKF525-A</td>
<td>1.0 mM</td>
<td>0.13±0.02 (45)</td>
</tr>
<tr>
<td>Metyrapone</td>
<td>1.0 mM</td>
<td>0.13±0.01 (45)</td>
</tr>
<tr>
<td>Potassium cyanide</td>
<td>0.1 mM</td>
<td>0.31±0.03 (105)</td>
</tr>
<tr>
<td></td>
<td>1.0 mM</td>
<td>0.31±0.03 (106)</td>
</tr>
</tbody>
</table>

The data are expressed as the mean±S.E. of three experiments. Numbers in parentheses represent the relative activities. 7β-Hydroxy-Δ⁸-THC (72.7 μM) was incubated with the microsomes for 20 min at 37 °C in the presence of various inhibitors. Each cofactor was added to the incubation mixture at 1 mM.
NADPH was used as the cofactor, the oxidative activity of 7β-hydroxy-Δ⁸-THC to 7-oxo-Δ⁸-THC by CYP3A8 at the substrate concentrations of 40 and 72.7 μM was 4.56 and 8.03 nmol/min/nmol P450, respectively (Table 3). NADH alone could also sustain the oxidation of 7β-hydroxy-Δ⁸-THC in the reconstituted system as indicated the microsomal fraction, although the activity was lower than the NADPH-dependent activity. However, the activity could not be detected in the absence of CYP3A8 or NADPH-cytochrome c (P450) reductase. Cytochrome b₅ was an absolute requirement, especially in the NADPH-dependent reaction, because the activity in the absence of cytochrome b₅ was decreased to about 10% of the activity in its presence.

**DISCUSSION**

Shigematsu et al. described the NADPH-dependent O-dealkylation of p-nitroanisole by rabbit hepatic microsomes and concluded that the reaction is probably P450-independent because it was not inhibited by carbon monoxide. On the other hand, Kamatoki et al. reported that the antibody against P450 inhibited the NADPH-dependent O-demethylation of p-nitroanisole; thus it would appear that the reaction is in fact mediated by a species of P450 even though it is atypical in not being inhibited by carbon monoxide. Kuwahara and Manering concluded that NADH- and NADPH-dependent O-deethylation of p-nitrophenetole involve different P450 enzymes and separate electron transfer systems. Murray and Butler also reported that NADH, as well as NADPH, is able to support the efficient biotransformation of parathion to paraoxon and 4-nitrophenol *in vitro* and that P450 is involved in parathion oxidation mediated by both cofactors.

In a previous study, we have found that 7β-hydroxy-Δ⁸-THC is NADPH- and O₂-dependently oxidized to 7-oxo-Δ⁸-THC by hepatic microsomes and clarified that CYP3A is a major enzyme responsible for the NADPH-dependent oxidation in various animals. Murray and Butler also reported that NADH, as well as NADPH, is able to support the efficient biotransformation of parathion to paraoxon and 4-nitrophenol *in vitro*. Therefore, it can be suggested that P450-dependent monoxygenase systems preferentially require NADPH as a source of reducing equivalents; the NADPH-P450 reductase transfers two electrons from this nucleotide to the P450. In the presence of NADPH, however, NADH can function as the source of the second electron, transferred to the P450 through cytochrome b₅ reductase, following incorporation of the oxygen to the enzyme-substrate complex. Furthermore, some P450 monoxygenase reactions are supported by NADH at only 10—20% of that supported by NADPH. Actually, when NADPH is the cofactor, this process is less efficient. A report demonstrated an approximate 4000-fold greater Michaelis constant for the reduction of cytochrome c by NADH than for the NADPH-mediated reaction. However, the study did not evaluate whether inclusion of P450 and its substrates may enhance the rate of reduction supported by NADH. On the other hand, extensive NADPH-dependent oxidation of 7β-hydroxy-Δ⁸-THC was observed in hepatic microsomes of monkeys (Table 1). Indeed, the oxidative activity of 7β-hydroxy-Δ⁸-THC in the presence of NADH was about 70% of that of NADPH. Moreover, in the low-substrate concentration system or high-cofactor concentration system, the NADH was more effective, under the study conditions, than NADPH in supporting the oxidation of 7β-hydroxy-Δ⁸-THC to 7-oxo-

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**Table 3. Component Requirements and Effect of Substrate Concentration on Oxidative Activity of 7β-Hydroxy-Δ⁸-THC in Reconstituted System of CYP3A8**

<table>
<thead>
<tr>
<th>Activity (nmol/min/nmol P450)</th>
<th>NADH</th>
<th>NADPH</th>
<th>Ratio (NADH/NADPH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete (Substrate 40 μM)</td>
<td>2.13</td>
<td>4.56</td>
<td>0.47</td>
</tr>
<tr>
<td>Complete (Substrate 72.7 μM)</td>
<td>2.54</td>
<td>8.03</td>
<td>0.32</td>
</tr>
<tr>
<td>−P450</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>–</td>
</tr>
<tr>
<td>−NADPH-P450 reductase</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>–</td>
</tr>
<tr>
<td>−b₅</td>
<td>0.28</td>
<td>2.79</td>
<td>0.10</td>
</tr>
</tbody>
</table>

The data are expressed as the mean of two experiments. The complete system contained purified CYP3A8 (30 pmol), NADPH-cytochrome c (P450) reductase (0.33 units), cytochrome b₅ (30 pmol), microsomal lipids (50 μg), sodium cholate (100 μg), NADPH or NADH (1 mM) and potassium phosphate buffer (pH 7.4, 100 mM) to make a final volume of 0.5 mL. The reaction was allowed to proceed for 20 min at 37°C. The metabolites were determined by previously reported methods.
The typical P450 inhibitors SKF 525-A and metyrapone decreased the rates of NADH-dependent 7β-hydroxy-Δ⁸-THC oxidation. These inhibitors also inhibited to an extent similar to that with NADPH. The NADPH-P450 reductase inhibitor DPIC almost completely inhibited 7β-hydroxy-Δ⁸-THC metabolism mediated by both NADH and NADPH. These results indicate the involvement of P450 in the NADH-dependent oxidation.

It is not still unknown why the NADPH-dependent activity, especially at low substrate concentration, is dramatically decreased depending on the cofactor concentration (Fig. 5A). It is known that the NADPH-dependent aldo-keto reductase was present in the microsomal fraction. There is a possibility that the resulting 7-oxo-Δ⁸-THC is reduced to 7-hydroxy-Δ⁸-THC by aldo-keto reductase. However, we could not detect the reduced metabolites such as 7α- or 7β-hydroxy-Δ⁸-THC when 7-oxo-Δ⁸-THC was incubated with hepatic microsomes in the presence of NADPH (data not shown). THC and many of the metabolites are highly lipophilic compounds. The n-octanol/water partition ratio of THC at neutral pH is in the order of 6000. It is well known that the primary and secondary hydroxylations occur sequentially. The 7-oxo-Δ⁸-THC that resulted might be further metabolized by CYP enzymes preferentially that used NADPH as an electron donor. Further experiments may be required to clarify the above mechanisms.

Certain hepatic microsomal P450 reactions may be enhanced by NADH, which appears to involve electron transfer via cytochrome b₅. There was no synergism in the reaction in the presence of either NADH or NADPH. When both NADH and NADPH are used as cofactors, 7-oxo-Δ⁸-THC forming activity was almost the same as the activity when using NADH alone at lower concentrations of the substrate, whereas it was the same as NADPH alone at higher concentrations (Fig. 4). These results suggest that NADH is utilized as a cofactor in preference to NADPH in the low 7β-hydroxy-Δ⁸-THC concentration system (Fig. 6).

In the reconstituted system, the oxidation activity of 7β-hydroxy-Δ⁸-THC in the NADH-dependent system at a low substrate concentration (40 μM) was about 50% of that in NADPH, although it was not sufficiently reflected in the results of the microsomal fractions (Table 3). CYP3A8 is a major enzyme responsible for testosterone 6β-hydroxylation in monkey liver microsomes. In the reconstituted system, however, the rate of testosterone 6β-hydroxylation supported by NADH was only 10—20% of that by NADPH (data not shown). This result was consistent with the data from monkey liver microsomes (data not shown). It is possible that the NADH-dependent oxidation of 7β-hydroxy-Δ⁸-THC is also catalyzed by enzyme(s) other than CYP3A8. Yamazaki et al. have reported, however, that NADH-dependent testosterone 6β-hydroxylation and nifedipine oxidation were about one-eighth and one-third, respectively, of the levels obtained in the NADPH-supported system in human liver microsomes. The cofactor requirements of CYP3A8 may be changed by the substrates.

These observations support the possibility that NADPH may be replaced by NADH as a cofactor for the microsomal oxidation of 7β-hydroxy-Δ⁸-THC, especially at a low substrate concentration or high cofactor concentration (Fig. 6). It has been suggested that NADH can transfer electrons directly to the NADPH-P450 reductase and P450, because sufficient activity was detected in the absence of NADH-cytochrome b₅ reductase. The NADH-dependent reaction may be a significant pathway of 7β-hydroxy-Δ⁸-THC metabolism since NAD(H) is the predominant pyridine nucleotide in liver. Further extensive studies including kinetic experiments are required to clarify the NADH-dependent oxidation mechanisms of 7β-hydroxy-Δ⁸-THC in monkey liver microsomes.

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