EFFECTS OF ENDOGENOUS STEROIDS ON CYP3A4-MEDIATED DRUG METABOLISM BY HUMAN LIVER MICROSOMES

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ABSTRACT:
In the present study, we investigated the effects of 14 endogenous steroids on the CYP3A4-mediated drug metabolism by human liver microsomes in vitro. Nevirapine (NVP) 2-, 12-hydroxylations, carbamazepine (CBZ) 10,11-epoxidation, triazolam (TZM) 1'-, 4-hydroxylations, erythromycin (EM) N-demethylation, and 2-sulphamoylacetylphenol (SMAP) formation from zonisamide (ZNS) were investigated. The activities of the NVP 2-, 12-hydroxylations, the CBZ 10,11-epoxidation, and the TZM 4-hydroxylation were activated by endogenous androgens, such as androstenedione (AND), testosterone, and dehydroepiandrosterone. However, these androgens inhibited EM N-demethylation, TZM 1'-hydroxylation, and SMAP formation. To understand the mechanisms of these effects of androgens on CYP3A4 activities, we performed a kinetic analysis of the metabolism of CBZ and ZNS in the presence or absence of AND using the modified two-site equation model. The addition of AND to the reaction mixture caused a drastic increase in the activity of CBZ 10,11-epoxide, especially at a low substrate concentration, and resulted in a change in the kinetics from the sigmoid to Michaelis-Menten type. On the other hand, the metabolism of ZNS was strongly inhibited by AND, although no allosteric change was observed in this case. These data demonstrate that endogenous steroids, especially androgens, strongly affect CYP3A4-mediated drug metabolism in vitro. The postulated mechanisms of the interactions between AND and CBZ or ZNS are discussed.

Cytochrome P450s (P450s) are comprised of a superfamily of enzymes that play important roles in drug metabolism. CYP3A4 is known to be a major form of P450 expressed in adult human livers (Shimada et al., 1994), and a majority of the drugs currently available on the market are metabolized by this isoform (Maurel, 1996). CYP3A4 is also responsible for the metabolism of endogenous compounds, such as steroid hormones. It has been reported that CYP3A4 is involved in the metabolism of cortisol (Abel and Back, 1993), testosterone (Kerr et al., 1994; Kerlan et al., 1992), and progesterone (Yamazaki and Shimada, 1997), all of which play important roles in various physiological actions.

Several reactions catalyzed by CYP3A4 display non-Michaelis-Menten kinetics, apparently due to an allosteric effect, which commonly yields a sigmoid velocity saturation curve. For example, a sigmoid kinetic character has been observed for the metabolism of CBZ (Kerr et al., 1994; Korzekwa et al., 1998), progesterone (Harlow and Halpert, 1998), and testosterone (Ueng et al., 1997; Harlow and Halpert, 1998) by CYP3A4. In addition, it is well known that α-naphthoflavone heterotropically stimulates the metabolism of progesterone (Schwab et al., 1988; Harlow and Halpert, 1998), and various other CYP3A substrates (Andersson et al., 1994), providing a change in the kinetic character to the Michaelis-Menten type. The active site in CYP3A4 is generally supposed to be spacious because CYP3A4 can metabolize relatively large molecules, such as cyclosporin (mol. wt., 1201). In contrast to the model for a single large active site, another model possessing two binding sites at the active site of a P450 has been suggested (Shou et al., 1994). Several kinetic analyses based on the latter hypothesis have been reported (Korzekwa et al., 1998; Shou et al., 2001).

Since many endogenous steroids are recognized to be substrates of CYP3A4, these endogenous steroids may competitively inhibit drug metabolism catalyzed by CYP3A4. However, a recent article has indicated that testosterone activates the metabolism of midazolam 4-hydroxylation by human liver microsomes (Maenpaa et al., 1998). Another study showed that testosterone activates the TZM 4-hydroxylation but inhibits 1'-hydroxylation by human liver microsomes (Schrag and Wienkers, 2001). Thus, the effects of testosterone on CYP3A4 activities seem to be complicated.

Because endogenous steroids always exist in vivo, it is of interest to clarify the effects of endogenous steroids on drug metabolism mediated by CYP3A4. If endogenous steroids substantially affect CYP3A4 activities, it may be insufficient to estimate drug metabolism by CYP3A4 without considering the effects of endogenous steroids. In this study, we focused on the effects of endogenous steroids on
CYP3A4-mediated drug metabolic events, such as CBZ 10,11-epoxidation, NVP 2-, 12-hydroxylation, TZM 1-, 4-hydroxylation, EM N-demethylation, and SMAP formation by human liver microsomes. The effects of 14 endogenous steroids (pregnenolone, pregnenolone-sulfate, progesterone, aldosterone, 17α-hydroxyprogrenolone, 17α-hydroxypregnenolone-sulfate, 17α-hydroxyprogesterone, cortisol, DHEA (5-androsten-3β-ol-17-one), DHEA-sulfate, AND (4-androsten-3,17-dione), testosterone, estrone, and estradiol-17β) on the above reactions were investigated in vitro.

**Experimental Procedures**

**Materials.** NVP, 2-hydroxy-NVP, and 12-hydroxy-NVP were provided by Boehringer Ingelheim Pharma Co. (Ingelheim, Germany). CBZ and CBZ 10,11-epoxide were obtained from Novartis Pharma Co. (Tokyo, Japan). TZM, 1’-hydroxy-TZM, and 4-hydroxy-TZM were donated by Kyowa Hakko Kogyo Co. (Tokyo, Japan). AND and 6β-hydroxy-AND were purchased from Sigma Chemical Co. (St. Louis, MO) and Steraloids Co. (Wilton, NH), respectively. ZNS and its metabolite named SMAP were provided by Dainippon Pharmaceutical Co. (Osaka, Japan). Pregnenolone (5-pregnen-3β-ol-20-one), pregnenolone-sulfate (5-pregnen-3β-ol-20-one sulfate), progesterone (4-pregnen-3,20-dione), aldosterone (4-pregnen-11β,21-diol-3, 20-dione), cortisol (11β,17α,21-trihydroxypregnen-4-ene-3,20-dione), DHEA, DHEA-sulfate, AND, testosterone (4-androsten-17β-ol-3-one), estrone (1,3,5(10)-estratrien-3-01), and β-estradiol (1,3,5(10)-estratrien-3,17β-diol) were purchased from Sigma Chemical Co. 17α-Hydroxypregnenolone (5-pregnen-3β, 17β-diol-20-one), 17α-hydroxyprogrenolone-sulfate (5-pregnen-3β, 17β-diol-20-one-sulfate), and 17α-hydroxyprogesterone (4-pregnen-3β-ol-3, 20-dione) were purchased from Steraroids Co. All other chemicals and solvents used were of the highest grade or analytical grade commercially available.

**Specimens and the Preparation of Human Liver Microsomes.** An adult liver sample from a Japanese was obtained at autopsy from the Department of Legal Medicine (School of Medicine, Chiba University) under the approval of the ethics committee of Chiba University. The donor was a 57-year-old male with no known drug history who had frozen to death. Liver specimens were stored at −80°C until use. Liver microsomes were prepared by differential centrifugation, as described previously (Ohmori et al., 1993). Total P450 content was measured by the method of Omura and Sato (1964) in the presence of 20% glycerol and 0.2% Emulgen 911 (Kao, Tokyo, Japan). Protein was determined, as described by Lowry et al. (1951), using bovine serum albumin as a standard.

**Assay of NVP 2- and 12-Hydroxylation Activities.** Analysis of NVP 2- and 12-hydroxylation was carried out according to the method described previously (Nakasa et al., 1993) with modification of the substrate concentration. The reaction mixture contained 100 mM potassium phosphate, pH 7.4, 0.1 mM EDTA, 1 mg/ml of human liver microsomal protein, an NADPH-generating system (0.33 mM NADPH, 0.1 U of glucose-6-phosphate dehydrogenase, 8 mM glucose 6-phosphate, and 6 mM MgCl₂), a methanolic solution of substrate (100 mM N,N-dimethyl zonisamide in chloroform) was added. Then, the metabolite was extracted with 5 ml of chloroform/ethanol (10:1, v/v). After centrifugation (3000 rpm, 10 min), the organic phase was evaporated at 40°C. The residue was dissolved in 120 μl of HPLC mobile phase, and 50 μl was injected into the HPLC. The HPLC system used was the same as that for the assay of NVP metabolites except that a Purecil column (5 μm, 4.6 × 250 mm; Waters, Milford, MA) was used, and the metabolite was detected at 235 nm. The mobile phase, which consisted of methanol/acetonitrile/1% acetic acid (3:1:7, v/v/v), was delivered at a flow rate of 1 ml/min at 35°C. Under these conditions, the retention times of CBZ 10,11-epoxide and the internal standard were 7.5 and 10.5 min, respectively.

**Assay of CBZ 10,11-Epoxidase Activity.** The composition of the reaction mixture was the same as that for the assay of NVP metabolism described above except that 100 μM CBZ was used as the substrate. The reaction mixtures were incubated for 20 min at 37°C. The reactions were stopped on ice, and 10 μl of internal standard (20 μg/ml N,N-dimethyl zonisamide in chloroform) was added. Then, the metabolite was extracted with 5 ml of chloroform/ethanol (10:1, v/v). After centrifugation (3000 rpm, 10 min), the organic phase was evaporated at 40°C. The residue was dissolved in 120 μl of HPLC mobile phase, and 50 μl was injected into the HPLC. The HPLC system used was the same as that for the assay of NVP metabolites except that a Purecil column (5 μm, 4.6 × 250 mm; Waters, Milford, MA) was used, and the metabolite was detected at 235 nm. The mobile phase, which consisted of methanol/acetonitrile/1% acetic acid (3:1:7, v/v/v), was delivered at a flow rate of 1 ml/min at 35°C. Under these conditions, the retention times of CBZ 10,11-epoxide and the internal standard were 7.5 and 10.5 min, respectively.

**Assay of TZM 1’- and 4-Hydroxylation Activities.** The same reaction mixture used for the assay of NVP metabolism was prepared except that 100 μM TZM was used as the substrate and the concentration of microsomal protein was 0.2 mg/ml. The reactions were carried out for 7 min at 37°C. One hundred microliters of internal standard (5 μg/ml lorazepam in methanol) was added to the reaction mixture. The same HPLC system used for the NVP assay was used. Elution of the metabolites was monitored at 220 nm. The mobile phase, which consisted of water/acetonitrile/methanol (7:3:1, v/v/v), was delivered at a flow rate of 1 ml/min at 40°C. Under these conditions, the retention times of 1’-hydroxy-TZM, 4-hydroxy-TZM, the internal standard, and TZM were 19.5, 20.8, 25.7, and 30.8 min, respectively.

**Assay of EM N-Demethylation Activity.** The EM N-demethylation activity was assayed by measuring formaldehyde according to the method described previously (Nash, 1953) with minor modifications. The same reaction mixture used for the assay of NVP metabolism was prepared except that 100 μM EM was used as the substrate and the concentration of microsomal protein was 0.5 mg/ml. The substrate and the steroid were dissolved in acetone. The final concentration of acetone in the reaction mixture was 2%. The reactions were carried out for 15 min at 37°C and stopped by adding of ice-cold 10% trichloroacetic acid. After centrifugation (3000 rpm, 10 min) to precipitate denatured proteins, the supernatant was mixed with Nash reagent and incubated for 30 min at 37°C with shaking. The fluorescence intensity of the product was measured at 410 nm (excitation wavelength) and 510 nm (emission wavelength) by a fluorescence spectrophotometer (Hitachi F-2000; Tokyo, Japan).

**Assay of SMAP Formation from ZNS under Anaerobic Conditions.** SMAP formation by the reductive metabolism of ZNS under anaerobic conditions was measured according to the method developed in our laboratory (Nakasa et al., 1993) with modification of the substrate concentration. In this study, we used 100 μM ZNS as the substrate. The reactions were carried out for 20 min at 37°C in the presence of an oxygen-consuming system (Mallet et al., 1982) in sealed tubes in which the head-space gas was replaced with argon. The same HPLC system used for the analysis of the NVP metabolites was used except that an Inertsil ODS-40A column (5 μm, 4.6 × 250 mm) was used, and detection was at 260 nm. The mobile phase consisted of 0.1 M potassium phosphate, pH 4.0/acetonitrile/2-propanol (75:15:2, v/v/v) delivered at a flow rate of 0.9 ml/min at 35°C. Under these conditions, the retention times of SMAP, the internal standard (phenobarbital), and ZNS were 10.3, 12.6, and 26.2 min, respectively.

**Assay of AND 6β-Hydroxylation Activity.** The assay conditions for AND 6β-hydroxylation were the same as those for the CBZ 10,11-epoxidation assay. 6β-Hydroxy-AND eluted at 14.0 min under the same HPLC conditions used to determine CBZ 10,11-epoxide. All assays were performed within the linear range for incubation time and protein concentration.

**Mathematical Derivation and Analysis.** Kinetic parameters for CBZ 10,11-epoxidation and AND 6β-hydroxylation were determined by the modified two-site equation (Vmax = 0) (Korzekwa et al., 1998; Domanski et al., 2000): \[ V = \left( V_{max} S/K_{m1} + S/K_{m2} \right) / \left( 1 + S/K_{m1} + S/K_{m2} \right) \]. Parameters for SMAP formation were determined by the Michaelis-Menten equation. The values were adjusted by iteration of the calculation until the best data fit was obtained using the Levenberg-Marquardt (Marquardt, 1963) nonlinear least-
Results

Effects of Various Endogenous Steroids on Drug Metabolism

To investigate the effects of endogenous steroids on drug metabolism catalyzed by CYP3A4 in human liver microsomes, endogenous steroids or α-naphthoflavone as a reference effector were added to reaction mixtures. As shown in Fig. 1, A and B, aldosterone, 17α-hydroxyprogesterone, DHEA, AND, and testosterone activated NVP 2-, 12-hydroxylations and CBZ 10,11-epoxidation by more than 2-fold. In particular, the activity of CBZ 10,11-epoxidase was activated about 6-fold by the addition of AND. Several endogenous steroids inhibited the TZM 1', 4-hydroxylations, and stronger inhibition was observed in the presence of DHEA and testosterone. The concentration of endogenous steroids, α-naphthoflavone, and drugs was 100 μM. Basal activities of NVP 2-, 12-hydroxylations, CBZ 10,11-epoxidation, TZM 1', 4-hydroxylations, EM N-demethylation, and SMAP formation were 0.028, 0.016, 0.155, 0.892, 2.893, 1.412, and 0.378 nmol/mg/min, respectively.
The concentration of CBZ and ZNS was 100 μM. O, aldosterone; △, AND; ●, DHEA; ▲, testosterone.

The effects of steroid concentration on CBZ 10,11-epoxidation and SMAP formation were investigated. These activities were selected because a drastic change in the rate of metabolism was observed when androgens were added. Figure 2 shows the changes in CBZ 10,11-epoxidation and SMAP formation activities with increasing concentrations of four steroids (aldosterone, DHEA, AND, and testosterone). The activity of CBZ 10,11-epoxidase increased in a concentration-dependent manner for all four steroids. The greatest increase (4.8-fold) in the formation of the CBZ 10,11-epoxide by AND was observed at a low AND concentration (25 μM). In contrast, SMAP formation decreased in a concentration-dependent manner for DHEA, AND, and testosterone. These three androgens decreased the control activity to 30 to 50%, even at a low steroid concentration (25 μM). The effects of aldosterone on CBZ 10,11-epoxidation and SMAP formation were much weaker than the other three androgens.

**Kinetic Analysis of the Effects of AND on CBZ 10,11-Epoxidation and SMAP Formation.** To elucidate the mechanisms for the steroid-induced stimulation or suppression of CYP3A4 activities, kinetic studies were conducted on CBZ 10,11-epoxidation and SMAP formation. The substrate-velocity curve and corresponding Eadie-Hofstee plots for CBZ 10,11-epoxidation and SMAP formation are shown in Fig. 3, A and B, respectively. Since there was a possibility that CYP3A4-mediated steroid metabolism might be conversely affected by drugs, the kinetics of the 6β-hydroxylation of AND was also examined (Fig. 3C). Eadie-Hofstee plots in Fig. 3A demonstrate that the kinetic character of CBZ 10,11-epoxidation is sigmoid in the absence of steroid, indicating that multiple substrate-binding sites may be involved in the metabolism. The sigmoid curve changed to a hyperbolic curve upon the addition of AND. Moreover, AND caused a marked increase in the rate of metabolism, and this stimulation was more remarkable at low substrate concentrations. On the other hand, ZNS did not affect CBZ 10,11-epoxidation at all.

The rate of SMAP formation was reduced strongly by AND and slightly by CBZ (Fig. 3B, left panel). Although SMAP formation showed irregular kinetics at a low substrate concentration, as seen in Eadie-Hofstee plots, the kinetic parameters could be calculated by the Michaelis-Menten equation regardless of the absence or presence of steroid.

The kinetics of AND 6β-hydroxylation also showed sigmoid character, as evidenced by Eadie-Hofstee plots (Fig. 3C, right panel). It was observed that the rate of AND 6β-hydroxylation decreased upon the addition of CBZ at higher substrate concentrations. However, the sigmoid kinetics were not altered by the addition of CBZ (Fig. 3C, right panel). ZNS had no substantial effect on AND 6β-hydroxylation.

**Discussion**

Endogenous steroids always exist in vivo, and most of them are the substrate of CYP3A4. Considerable amounts of endogenous steroids are continuously metabolized by CYP3A4 expressed in the liver, where drugs are mainly metabolized. Thus, drug-endogenous steroid interactions should be considered to predict the metabolism of drugs by CYP3A4 in vivo.

In this study, we found that the NVP 2-, 12-hydroxylations and CBZ 10,11-epoxidation activities in the human liver microsomes are strongly activated by many endogenous steroids, especially androgens such as AND, testosterone, and DHEA. However, these androgens inhibit EM N-demethylation and SMAP formation. The activation of CBZ 10,11-epoxidation and the inhibition of SMAP formation by these androgens occur in a concentration-dependent manner and are observed even at low concentrations. Therefore, these androgens may affect drug metabolism catalyzed by CYP3A4 in vivo. Several previous articles have suggested that females have a greater capacity for CYP3A4-mediated drug metabolism than males (Gilmore et al., 1992; Hulst et al., 1994; Schwartz et al., 1994). Watkins et al. (1992) and Watkins (1994) analyzed EM N-demethylation in a large number of patients and healthy volunteers by an EM breath test. Although there was marked interindividual variability, females had a statistically greater rate of 14CO2 release than males. The reasons for this difference might be related to gender differences in steroid hormone levels. Supporting this idea, the inhibitory effects of androgens on EM N-demethylation were found to be larger than those of estrogens in the present study (Fig. 1D).
To elucidate the activation and inhibition mechanisms of CYP3A4-catalyzed drug metabolism by AND, we performed a kinetic analysis of CBZ 10,11-epoxidation and SMAP formation from ZNS. As reported previously (Kerr et al., 1994; Korzekwa et al., 1998), CBZ 10,11-epoxidation showed sigmoid kinetics, and the kinetics changed to the Michaelis-Menten type as the activity was activated by the addition of \( \beta \)-naphthoflavone (Ueng et al., 1997). In this study, AND caused an activation of CBZ 10,11-epoxidation and induced the same kinetic change. Interestingly, the degree of stimulation by AND was larger than that by \( \beta \)-naphthoflavone. It has been reported that the high protein concentration in the assay mixture provides unusual kinetic profile (Obach, 1997). In this study, however, based on the results of these analyses using the modified two-site equation, we propose the following hypotheses about the mechanisms for the changes in the kinetics of CBZ 10,11-epoxidation. Table 1 summarizes the kinetic parameters obtained from the data in Fig. 3. Namely, in the absence of AND, the binding of CBZ to site 1 facilitates substrate binding to site 2, the sole site responsible for product formation following a conformational change in the enzyme (homo-tropic cooperativity). In the presence of AND, however, AND binds preferentially to site 1 due to its lower \( K_m \) value (5.7 \( \mu \)M) compared with that of CBZ (71.6 \( \mu \)M). This causes an increase in the affinity of
Kinetic parameters of CBZ 10,11-epoxidation, SMAP formation, and AND 6β-hydroxylation by human liver microsomes

Substrate concentrations used were 15 to 500 μM for CBZ, 15 to 1000 μM for ZNS, and 15 to 300 μM for AND. The reactions were performed in the absence or presence of 100 μM ebastin. Units of the constants are as follows: (apparent) micromolars; and V_max, nanomoles per milligram per minute.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CBZ 10,11-Epoxidation</th>
<th>SMAP Formation from ZNS</th>
<th>AND 6β-Hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_m1</td>
<td>71.6</td>
<td>63.0</td>
<td>63.0</td>
</tr>
<tr>
<td>K_m2</td>
<td>1384.4</td>
<td>1789.5</td>
<td>1789.5</td>
</tr>
<tr>
<td>V_max1</td>
<td>4.2</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>V_max2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>a</em></td>
<td>Analyzed by the modified two-site equation (V_max = 0)</td>
<td>V = (V_max1 S/K_m1 K_m2) (1 + S/K_m1 + S/K_m2 K_m2) (Korezekwa et al., 1998)</td>
<td><em>b</em> Analyzed by Michaelis-Menten equation V = V_max / (1 + (K_m/S))</td>
</tr>
</tbody>
</table>

Only Plus AND Plus CBZ

ZNS only

Plus CBZ

Plus ZNS

**TABLE 1** Kinetic parameters of CBZ 10,11-epoxidation, SMAP formation, and AND 6β-hydroxylation by human liver microsomes

It was successful in determining kinetic parameters for the SMAP formation with or without AND by the Michaelis-Menten equation. Site 1 probably participates in the formation of SMAP because the activity was inhibited by either CBZ or AND (Fig. 3B), both of which have a high affinity for site 1. Consistently, the kinetics of both CBZ and AND metabolism did not change in the presence of ZNS (Fig. 3, A and C), suggesting that ZNS cannot remove CBZ or AND bound to site 1. Figure 4 shows a kinetic scheme for the activation of CBZ 10,11-epoxidation and the inhibition of SMAP formation. Since we used human liver microsomes as an enzyme source in these studies, unusual kinetics may be due to the contribution of multiple enzymes. However, from the preliminary examination, it was shown that expressed CYP3A4 gave a similar result that was obtained using human liver microsomes (data not shown). Therefore, we considered that the changes of the kinetic curve in the nature of CBZ are predominantly due to contribution by CYP3A4 enzyme.

CBZ for site 2, probably due to allosteric effects. This heterotropic cooperativity stimulates the production of the CBZ metabolite together with the loss of sigmoid kinetics (Fig. 3A).

In conclusion, endogenous steroids could cause a marked modification of CYP3A4-mediated drug metabolism in vitro. We demonstrate in the present study that the effects depend on the combination of drugs and steroids. Further studies are necessary to clarify whether these phenomena occur in vivo. It may be of value to investigate whether there is any association between our findings and the previously reported gender differences in CYP3A4-mediated drug metabolism. To establish a system to extrapolate the situation in vivo from the in vitro data, further detailed investigations of the effects of endogenous steroids on drug metabolism catalyzed by CYP3A4 are required.

**References**


