Comparison on Metabolism of Estradiol and Its 17-Sulfate by Recombinant Human CYP Isoforms

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To identify the CYP isoforms involved in the production of 2-hydroxyestradiol 17-sulfate (2-OH-ES), which we assume to be an antioxidant in vivo, the 2-hydroxylation reaction of estradiol 17-sulfate (ES) by human liver microsome was investigated. As a result, it was estimated that CYP2C8 and 2C9 were largely involved in the production of 2-OH-ES. Therefore, the 2-hydroxylation kinetic analysis of ES was performed for both CYPs, and the metabolic clearance \( V_{\text{max}}/K_m \) (\( \mu \text{L/nmol CYP/min} \)) was determined. On comparing the results of ES with those of estradiol (E2), it was found that CYP2C8 was about 2.5 times higher and CYP2C9 was about 3 times higher, and ES was more likely to be a substrate for the 2-hydroxylation reaction by both CYPs. The CYP isoforms involved in A-ring hydroxylation of E2 and ES differed. From this, it was speculated that 2-OH-ES plays a different role to 2-hydroxyestradiol (2-OH-E2), which is recognized as an antioxidant in the body.

Key words  estradiol 17-sulfate; A-ring hydroxylation; antioxidant; CYP2C8; CYP2C9

INTRODUCTION

We previously reported that estradiol 17-sulfate (ES) is converted into 2-hydroxyestradiol 17-sulfate (2-OH-ES) by human placental microsomes (Ms), and that 2-OH-ES is a potential placental antioxidant of importance during pregnancy.1,2) Catechol estrogen (free form), which act as an endogenous antioxidant, has been considered one of the most likely candidates.3) However, its role is uncertain because of its low plasma concentration and rapid metabolic clearance rate in humans.4) Assuming that 2-OH-ES is an antioxidant that functions during pregnancy, it was thought that identifying the CYP isoforms involved in its production would clarify the relationship between preeclampsia and ES metabolism, leading to treatments to reduce the risk of serious diseases.

CYP isoforms are involved in the Phase I reaction of 17-position conjugated estrogen, and although the involvement of 2-OH-ES has been reported,5) it's significance has not been described. In the present study, to elucidate the physiological significance of the Phase I reaction of ES in humans, we conducted metabolic experiments with ES using human liver Ms and recombinant CYPs. We measured enzyme affinities and reaction rates and compared them with those of free-form estrogen, estradiol (E2).

MATERIALS AND METHODS

Chemicals and Human Pooled Liver Microsomes  ES, 6β-hydroxyestradiol 17-sulfate (6β-OH-ES), 2-OH-ES and 4-hydroxyestradiol 17-sulfate (4-OH-ES) were obtained by the same methods as described in a previous paper.6) All other steroids, human pooled liver microsomes (from 15 donors), recombinant CYPs, reagents and solvents were obtained from commercial sources.

Assay System for Metabolism of E2 and ES  The enzyme assay was carried out under the following conditions: Ice-cold reaction vessels contained microsomal protein (human liver Ms, 0.50 mg/mL, recombinant human CYP Ms, 2.570 pmol/mL), a reduced nicotinamide adenine dinucleotide phosphate-phosphate-generating system (nicotinamide adenine dinucleotide phosphate 0.5 mmol/L, G6P 5 mmol/L, glucose-6-phosphate dehydrogenase 0.6 units/mL, MgCl2 5 mmol/L), ethylenediaminetetraacetic acid (50 mol/L), ascorbic acid (AsA, 0.1 mmol/L) and substrate (0–100 μmol/L). The mixture was diluted with Tris–HCl buffer solution (50 mmol/L, pH 7.4) to 1 mL (for human liver Ms) or 0.5 mL (for recombinant human CYP Ms) as a final volume and incubated at 37 °C under aerobic conditions for 0–60 min. The reaction was stopped by heating reaction vessels in boiling water for 2 min, and the mixture was centrifuged at 1500 × g for 15 min.

The supernatant that included metabolites of E2 was extracted by ethyl acetate (1 mL × 3). The extracted fraction containing the internal standard (Δ5-estrene, 2.0 nmol) and AsA (25 nmol) as an antioxidant, was evaporated at 40 °C to produce the residue, which was dissolved in methanol (20 μL) and subjected to HPLC.

In the experiment on ES, after incubation the supernatant was passed through an Oasis WAX cartridge. After washing the cartridge with 3 mL of sodium acetate solution (25 mmol/L, pH 5.0), the steroid-containing fraction was obtained by elution with a mixture of ammonium (25%), methanol and acetonitrile (0.2:2:8, 3 mL), followed by addition of a precise amount (0.40 nmol) of internal standard (estriol, E3) and AsA (25 nmol) as an antioxidant. The elute was evaporated at 40 °C to produce the residue, which was dissolved in methanol (20 μL) and subjected to HPLC.

HPLC Apparatus  HPLC was carried out using a model CCPS pump equipped with an EC-8020 electrochemical detector ( Tosoh, Tokyo, Japan). The suppression voltage of the detector was fixed at 0.9 V vs. Hg/HgCl2 reference electrode.

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A reversed-phase column packed with Mightysil RP-18GP (3.0 mm i.d. × 250 mm, Kanto, Tokyo, Japan) was used at 40 °C. The following solvent systems were used for the mobile phase at a flow rate of 0.5 mL/min for the analyses of conjugated and free steroids, respectively: system A was 0.5% ammonium phosphate buffered solution (pH 3.0) with methanol (65/35 (v/v)), and system B was 0.5% ammonium phosphate buffered solution (pH 3.0) with methanol (50/50 (v/v)).

RESULTS

Metabolites of E2 and ES by Human Pooled Liver Ms

When the metabolites of ES obtained in the experiment using human pooled liver Ms were identified by HPLC, 2-OH-ES was found to be the main metabolite. Furthermore, the production of 4-OH-ES and 6β-OH-ES was confirmed (Fig. 1A). In addition, when E2 was used as a substrate, 2-OH-E2 was confirmed as the main metabolite, and 4-hydroxyestradiol (4-OH-E2) and E3 were also produced (Fig. 1B); 6β-OH-ES and E3 were confirmed via the increased HPLC sensitivity.

E2 and ES 2- and 4-Hydroxylation by Human Pooled Liver Ms

The amounts (nmol/assay) of A-ring hydroxylated metabolites for each substrate obtained in the experiments performed using human pooled liver Ms were compared. Regardless of which substrate was used, the amounts of 2-hydroxylated metabolites were higher than those of 4-hydroxylated metabolites (Table 1).

When the amount of 2- and 4-hydroxylated metabolites obtained from E2 and ES were compared, ES produced greater amounts of both hydroxylated metabolites than E2. However, when comparing the ratio of the amount of 2-hydroxylated metabolite to that of 4-hydroxylated metabolite (2-OH-E2/4-OH-E2, 2-OH-ES/4-OH-ES), the ratio was 10 for the free form and approximately 9 for the conjugate. The choice of substrate did not result in a difference in the ratio of hydroxide site of A ring.

To investigate the substrate specificity for 2- and 4-hydroxylation, the ratios of the amounts of hydroxylated metabolite generated by E2 and ES (2-OH-ES/2-OH-E2, 4-OH-ES/4-OH-E2) were calculated. Consequently, the ratio of 2-OH-ES and 2-OH-E2 was found to be 1.5, and that of 4-OH-ES and 4-OH-E2 was 1.7. It was determined that the amounts of hydroxylated metabolites produced by ES were higher than the hydroxylated metabolites by E2. These results suggested that ES is a superior substrate for hydroxylase in A-ring hydroxylation by human liver Ms.

E2 and ES 2- and 4-Hydroxylation by Recombinant CyPs

To identify the CYP isoforms involved in A-ring hydroxylation of ES, seven CYP isoforms that are abundant in the liver and estrogen-target tissues are selected and incubated with their recombinant CYPs under certain conditions to generate hydroxides. The amounts were measured, and the results are shown in Table 2. The numerical values are the averages of the two cases.

Table 2 showed that there was a large difference in the amount of A-ring hydroxylated metabolite generated by the E2 and ES reactions, depending on the CYP isoforms. The ratio of the amounts of hydroxylated metabolite generated by E2 and ES was determined to evaluate the substrate selectivity.

Comparing 2-hydroxylation, the amounts of 2-OH-E2 produced with CYP1A1 and 1A2 were approximately 20 times higher than the amounts of 2-OH-ES, and the amount of 2-OH-E2 produced with CYP3A4 was 280 times higher than the amount of 2-OH-ES. However, when CYP2C8 and 2C9 were used, 2-OH-ES production was higher than 2-OH-E2 production (by 4 and 25 times, respectively). In the experiment with CYP2E1, only 2-OH-ES was produced.

Regarding 4-hydroxylation, 4-OH-E2 formation was 60

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Table 1. The Amounts of 2- and 4-Hydroxylated Metabolites Generated by E2 and ES from Human Pooled Liver Ms

<table>
<thead>
<tr>
<th>Substrate</th>
<th>2-OH-E2 (nmol/assay)</th>
<th>4-OH-E2 (nmol/assay)</th>
<th>2-OH-ES (nmol/assay)</th>
<th>4-OH-ES (nmol/assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>1.0 ± 0.1</td>
<td>0.10 ± 0.01</td>
<td>10</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>ES</td>
<td>1.0 ± 0.1</td>
<td>0.10 ± 0.01</td>
<td>10</td>
<td>1.5 ± 0.2</td>
</tr>
</tbody>
</table>

Substrate: 50 µmol/L, human pooled liver microsomes: 0.5 mg protein, incubation time; 30 min. n = 4.
times higher than 4-OH-ES formation with CYP3A4, but 4-OH-ES formation was about 4 times higher than that of 4-OH-E2 with CYP2C9. With CYP2E1, only 4-OH-ES production was confirmed. In the experiments with CYP1A1, 1A2 and 2C8, no differences were observed in the production of either catechol.

In the CYP1B1 experiment, unlike the results of the other six CYPs, the formation of 4-hydroxylated metabolite exceeded that of 2-hydroxylated metabolite for both substrates. Furthermore, about 10 times as much 4-OH-E2 was produced as 4-OH-ES. From these outcomes, it was considered that CYP2C8 and 2C9 may be CYP isoforms with high selectivity for ES.

To investigate the regioselectivity of each CYP isoform for hydroxylation for the different substrates, the ratio of the amount of 2- and 4-hydroxylated metabolite when each substrate was used was calculated.

When ES was used, 2-hydroxylation by CYP2C8 was 37 times higher than 4-hydroxylation, and 2-hydroxylation by CYP2C9 was about 7 times higher than 4-hydroxylation, indicating that both enzymes can easily catalyze 2-hydroxylation of ES. Since the ratios of 2-hydroxylated metabolite to 4-hydroxylated metabolite for CYP1A1, 1A2, 2E1 and 3A4 ranged from 1.0 to 1.8, the regioselectivity for hydroxylation by these CYP isoforms was assumed to be low.

For E2, hydroxylation by CYP1A1 and 1A2 had high 2-position selectivity, and 2-hydroxylation was also predominant with CYP2C8 and 3A4. With CYP1B1, 4-hydroxylation was dominant regardless of which substrate was used. From the above results, CYP2C8 and 2C9 were considered important CYP isoforms in 2-OH-ES production.

**Kinetic Analysis of 2-Hydroxylation of ES by Recombinant CYP2C8 and 2C9**

The CYP isoforms most likely to be involved in 2-hydroxylation of ES were CYP2C8 and 2C9. Therefore, we decided to perform kinetic analysis of 2-hydroxylation of ES using the recombinants CYP2C8 and 2C9 and compare them with the results of E2.

Enzymatic parameters were calculated from the Lineweaver–Burk plot under optimal conditions for 2-OH-ES and 2-OH-E2 production by CYP2C8 and 2C9, and the results were shown in Table 3.

On comparing the 2-hydroxylation clearance, ES was about 2.5 times superior to E2 with CYP2C8, and ES was about 3 times superior to E2 with CYP2C9. CYP2C8 and 2C9 were therefore considered important CYP isoforms in the production of 2-OH-ES.

### DISCUSSION

The CYP isoforms involved in A-ring hydroxylation of E2 were the same as those previously reported by many research groups. However, unlike for E2, the CYP isoforms involved in A-ring hydroxylation of ES were CYP2C8 and 2C9. The percentages of each CYP isoform in human liver microsomes vary among the published reports, but the total percentage of the CYP2C subfamily is 20–50%, of which the proportion of CYP2C8 and 2C9 is high, and the ratio of CYP2C9 is reportedly 2 to 3 times that of CYP2C8.

This study clarified the involvement of CYP2C8 and 2C9 in A-ring hydroxylation of ES. However, the amount of CYP2C9 in human liver microsomes and the higher degree of metabolic clearance of CYP2C9 than that of CYP2C8 suggest that CYP2C9 is mainly involved in A-ring hydroxylation of ES. CYP2C9 has been found to show genetic polymorphism, and steroid metabolism activity has been reported to change depending on the genotype.

We consider that 2-OH-ES is an important antioxidant during pregnancy, as urinary and blood 2-OH-ES levels of pregnant women with preeclampsia are lower than those of healthy pregnant women. It is also thought that the genetic polymorphism of CYP2C9 affects the production of 2-OH-ES during pregnancy and causes preeclampsia.

In addition, the conversion of arachidonic acid to epi-oxeycosatrienoic acid (EETs) is a known metabolic process involving CYP2C8 and 2C9. EETs have vasodilatory and anti-arteriosclerotic effects and are thought to be involved in maintaining cardiovascular homeostasis and function. Because hypertension is the main symptom of preeclampsia, it has been speculated that 2-OH-ES, which is also produced by CYP2C9, may be involved in the cardiovascular system as an antioxidant.

### Conflict of Interest

The authors declare no conflict of interest.

### REFERENCES


4) Kono S, Merriam GR, Brandon DD, Loriaux DL, Lipsett MB. 


