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Role of cytochrome P450 in estradiol metabolism *in vitro* [1]

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KEY WORDS estradiol; cytochrome P-450 CYP1A2; cytochrome P-450 CYP3A4; 2-hydroxyestradiol

ABSTRACT

AIM: Catechol estrogens and 16α-hydroxy estrogen are important metabolites that cause carcinogenesis. This study was aimed to study the role of cytochrome P450 in estradiol metabolism. **METHODS**: The estradiol metabolites were determined with HPLC-ECD. Correlation of estradiol metabolites production between cytochrome P450 activity, the inhibitory effect of specific inhibitors and enzyme catalyzing kinetics were studied in cDNA-expressed P450 or human liver microsomes. **RESULT**: CYP1A2, CYP3A4, and CYP2C9 catalyze the estradiol 2-hydroxylation. CYP2C9, CYP2C19, and CYP2C8 have high activity in catalyzing 17β-hydroxy dehydrogenation in cDNA expressed P450, but CYP1A2 is the most important enzyme in catalyzing estradiol 2-hydroxylation. Using furafyllin and troleandomycin to inhibit CYP1A2 and CYP3A4 in liver microsomes, it was found that the 2-hydroxylation had been inhibited about the same amount. This result suggests that in human liver microsomes CYP1A2 and CYP3A4 play an important role in 2-hydroxy estradiol formation. At low substrate concentration, 17β-hydroxy dehydrogenation dominated the estradiol metabolism, but at high substrate concentration, 2-hydroxylation exceeded 17β-hydroxy dehydrogenation to become the important mechanism. **CONCLUSION**: CYP1A2 and CYP3A4 are two important enzymes catalyzing the main estradiol 2-hydroxylation metabolism pathway at high substrate concentrations. 17β-hydroxy dehydrogenation is the main metabolism pathway at low concentrations, and CYP2C9, CYP2C19, and CYP2C8 may have high catalyzing activity.

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INTRODUCTION

Estrogen carcinogenesis can be attributed to: (1) estrogen receptor mediated growth and proliferation derived from the hormone's ability to stimulate the expression of genes encoding for diverse growth factors (eg 16α-OHE_2 and 16α-OHE_1)[1]; and (2) DNA modification and formation of adducts derived from active metabolites (catechol estrogen) and free radicals formed during estrogen metabolism (hydroquinones or quinones)[2,3]. Administration of antiestrogens and modulators or inhibitors of estrogen metabolism apparently result in prevention of tumors in some cases[4]. Estradiol (E_2) is metabolized into estrone (E_1), 2-hydroxyestradiol (2-OHE_2), estriol (E_3), 16α-hydroxyestrone (16α-OHE_1) and many other metabolites[5] Studies in human liver microsomes found that CYP1A2 and CYP3A4 play important role in catalyzing E_1 and E_2 2-hydroxylation[6]. These studies are based on a high substrate concentration (about 100 μmol/L). In order to study the E_2 metabolism mechanism at physiological concentration, we have studied the metabolism of E_2 at different substrate concentrations, which include the very low concentration of 1 μmol/L, in cDNA-expressed P450 and human liver microsomes.

MATERIALS AND METHODS

Materials Estradiol, 16α-Hydroxyestrone, estriol, estrone, 2-hydroxyestradiol, phenacetin, acetaminophen, tolbutamide, (±)debrisoquine sulfate, d-glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PDH) and β-NADP were purchased from Sigma (St Louis, MO). Midazolam, 1-hydroxymidazolam, (±)mephenytoin, (±)-4-hydroxymephenytoin, (±)-4-hydroxydebrisoquin sulfate, hydroxytolbutamide were purchased from Ultrafine chemicals Ltd (Manchester Science Park, England). CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2C8 expressed from lymphoblast were purchased from Gentest Corporation (Massachusetts 01801, USA).

Human liver sample collection and microsomes preparation Adult human liver tissue from re-

nal transplant donors without known liver disease and patients who had undergone partial hepatectomy were collected in our liver bank. The collection and utilization of human liver tissues were approved by the Ethics Committee of Hunan Medical University. Candidate patients for liver sample collection were those who did not suffer from acute or chronic hepatitis or cirrhosis, and took no medications known to induce or inhibit cytochrome P450 activity. Portions of surgical liver "waste tissue" distant from disease-affected regions and which appeared visually normal was collected. After removal, the liver sample was immediately cut into small pieces, washed with ice-cold isotonic saline, rapidly frozen in liquid nitrogen for 30 min, and was then stored at −80 ℃. Prior to use, all samples were confirmed as being normal histologically.

Washed microsomes were prepared by differential centrifugation[7] and stored at −80 ℃ until required. Microsomal protein concentrations were determined by the method of Lowry *et al*[8].

Metabolism of estradiol with P450s Fifty pmol of expressed P450 subtype, 5 μmol of magnesium chloride, 2.5 μmol of β-NADP, and 5 μmol of G-6-P in 470 μL of 50 mmol/L of K_2HPO_4-KH_2PO_4 buffer (pH 7.4) formed the incubation mixture. After addition of 20 μL of estradiol (25, 250, 2500 μmol/L), the mixture was preincubated for 5 min at 37 ℃ in a shaking water bath, and the reaction was initiated by addition of 0.5 IU of G-6-PDH (10 μL) and incubated for 30 min. The mixture was cooled in icy water and 2 mL of chloroform was added to terminate the reaction.

Correlation between individual P450 activity and estradiol metabolism The relationship between the rates of formation of metabolites (2-OHE_2 and E_1) and individual P450 activities in 13 liver microsomes were studied. One hundred μmol/L of midazolam (CYP3A4), 100 μmol/L of phenacetin (CYP1A2), 250 μmol/L of (±)mephenytoin (CYP2C19), 250 μmol/L of tolbutamide (CYP2C9), and 250 μmol/L of (±)debrisoquine (CYP2D6) were incubated in 490 μL of K_2HPO_4-KH_2PO_4 buffer 50 mmol/L (pH 7.4) mixture, which contained 0.5 mg of microsomal protein, 5 μmol of magnesium chloride, 2.5 μmol of β-NADP and 5 μmol of G-6-P. The mixture was preincubated for 5 min at 37 ℃ in a shaking water bath, and the reaction was initiated by addition of 0.5 IU of G-6-PDH (10 μL) and incubate for 30, 30, 60, 60, and 90 min, respectively. The reactions were terminated by cooling in icy water. The midazolam metabolites were extracted with 2 mL of ethyl ether. The (±)debrisoquine reaction mixture was applied to HPLC after depositing the microsomes protein by addition of 20 μL perchloric acid. Other incubated mixtures were extracted with 2 mL of ethyl acetate. Estradiol (1, 10, 100 μmol/L) was incubated in the same incubation system and incubate for 30 min. Two mL of chloroform were used to extract the metabolites.

Inhibition of estradiol metabolism in individual P450 Studied the inhibit effects of furafyllin (100 μmol/L, CYP1A2), troleandomycin (100 μmol/L, CYP3A4), sulphaphenazol (100 μmol/L, CYP2C9) and quinidine (100 μmol/L, CYP2D6) on different concentrations of estradiol metabolism in two individual microsomes, and the inhibitory effects of different concentrations of inhibitor on estradiol (100 μmol/L) metabolism in 4 individual microsomes. The incubation systems and methods used are the same as described above.

Individual P450 catalysing kinetics Different concentrations (1, 5, 10, 12.5, 20, 25, 40, 50, 75, 100, 200 μmol/L) of estradiol were incubated in 5 individual microsomes to study the kinetics of producing the two main metabolites (estrone and 2-hydroxyestradiol). The incubation systems and methods used are the same as described above.

HPLC-ECD assay of the estradiol metabolites After cooling the incubated system in ice water and adding 50 μL of internal standard (0.98 mg/L of stibestrol in methanol), 2 mL of cool chloroform were added to the mixture. Mixed the microsomes in a vortex mixer for 1 min, and centrifuged in 2500 × *g* for 10 min. The organic layer was separated and dried under high purity N_2 flow below 35 ℃. After dissolving the residues with 100 μL of mobile phase, 20 μL was applied to the HPLC system. With an analytical column of C_{18}(HP ODS Hypersil 5 μm, 250 mm × 4 mm, 799260D-584), a column temperature of 40 ℃, a mobile phase of acetic acid buffer-acetonitrile (50∶50, V/V, pH 4.5), a flow rate of 1.0 mL/min, and a potential of +0.7 V *vs* Ag/AgCl, the metabolites and internal standard were well separated and sensitively detected. The retention time of E_3, 16α-OHE_1, 2-OHE_2, E_2, E_1, and stibestrol were 3.4, 4.5, 5.7, 9.0, 11.9, and 15.6 min, respectively.

Statistical analysis The statistical software package SPSS (version 9.0, SPSS Inc, Chicago, USA) was used in the statistical analysis. Two-tailed *t*-test was used to analyze the significance of Correlation coefficients. Data of relative inhibitory ratio and K_m were expressed as $\bar{x} \pm s$. Duplicate incubations were employed through out the

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present study unless otherwise indicated. All data in the fig are the average of the duplicate incubation.

RESULTS

Fifty pmol of CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2C8 were used to study the E_2 metabolism at 1, 10, and 100 μmol/L concentrations. CYP2C9, CYP2C19, and CYP2C8 have high activity in catalyzing E_2 17β-hydroxy dehydrogenation to produce E_1, and lower activity in catalyzing E_2 2-hydroxylation to produce 2-OHE_2. CYP1A2 and CYP3A4 have relatively higher E_2 2-hydroxylation catalyzing activity than CYP2C9, CYP2C19, and CYP2C8. CYP1A2 have higher activity than CYP3A4 (Fig 1).

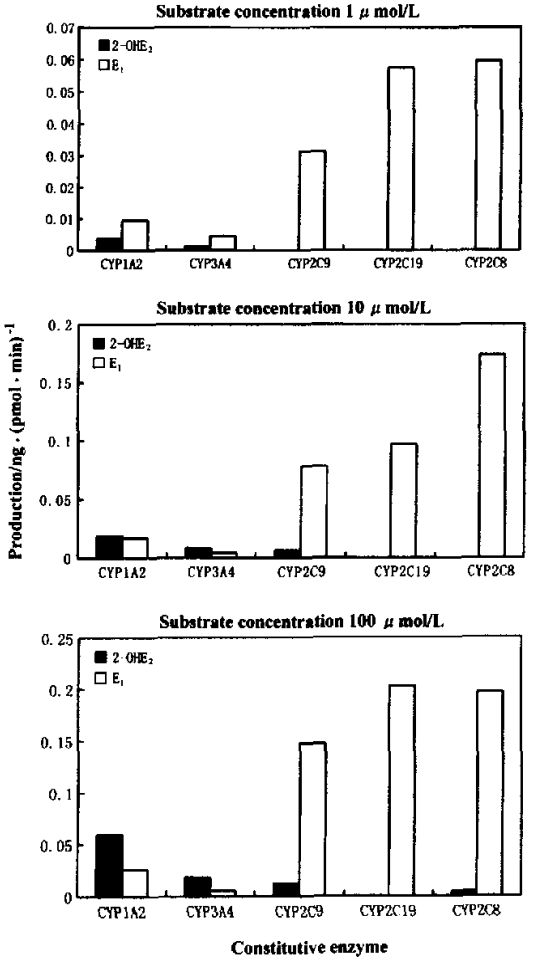

Fig 1. Different concentrations of estradiol metabolized in cDNA-expressed P450s.

On studying the correlation between individual P450 activity and estradiol metabolism in 13 individual microsomes, it was found that CYP1A2 and CYP3A4 had moderate correlation between their activities and E_2 2-hydroxylation at a substrate concentration of 100 μmol/L ($r = 0.60$, $P < 0.05$, $r = 0.59$, $P < 0.05$, Fig 2), and poor correlation at substrate concentration of 1 μmol/L and 10 μmol/L. E_2 17β-hydroxy dehydrogenation had poor correlation with CYP1A2, CYP3A4, CYP2C9, CYP2C19, and CYP2D6 at substrate concentration of 10 μmol/L and 100 μmol/L, but had moderate correlation with CYP2D6 activity in substrate concentration of 1 μmol/L.

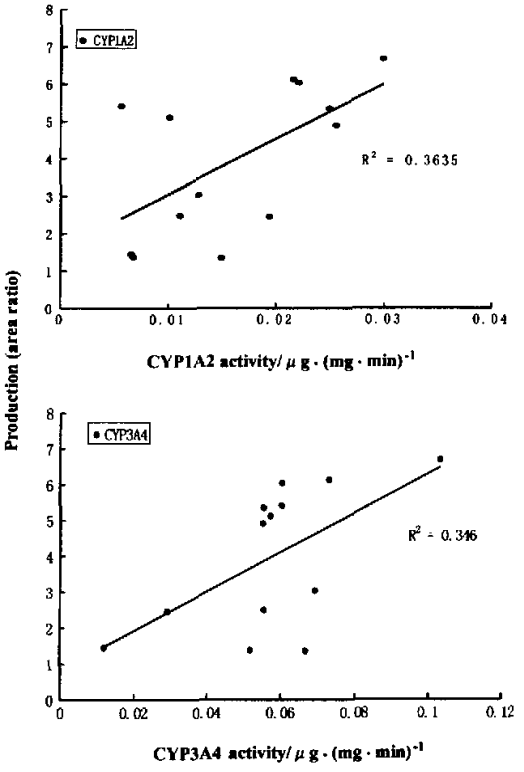

Fig 2. Correlation between 2-hydroxy estradiol formation and P450 activity in 13 human liver microsomes. The activity of CYP3A4 and CYP1A2 are correlation to 2-hydroxy estradiol formation at 100 μmol/L of substrate concentration ($n = 13$).

Inhibitory studies showed that furafyllin, troleandomycin, sulphaphenazol, and quinidine had poor inhibitory effect on E_2 17β-hydroxy dehydrogenation at any studied inhibitor concentration (25, 50, 100 μmol/L).

All the studied inhibitors can inhibit E_2 2-hydroxylation at concentrations of 25, 50, 100 μmol/L (Fig 3). The inhibitory effect of furafyllin and troleandomycin was relatively more stable in all microsomes. The relative inhibitory rate of furafyllin, troleandomycin, sulphaphenazol, and quinidine were 23 % ± 11 %, 8 % ± 13 %, 11 % ± 14 %, and 3 % ± 7 % at 25 μmol/L inhibitory concentration respectively, 34 % ± 12.8 %, 39 % ± 23 %, 29 % ± 4 %, and 22 % ± 24 % at 50 μmol/L inhibitory concentration respectively, and 45 % ± 9 %, 50 % ± 21 %, 32 % ± 13 %, and 34 % ± 20 % at 100 μmol/L inhibitory concentration respectively (Fig 4).

The catalyzing kinetics of the two main metabolites (E_1 and 2-OHE_2) was studied in 5 individual microsomes. The K_m of 17β-hydroxy dehydrogenation and 2-hydroxylation were 34 ± 16 and 96 ± 26, respectively (Fig 5). It suggests that E_1 is the main product at low substrate concentration, and 2-hydroxyestradiol is the main product at high substrate concentration.

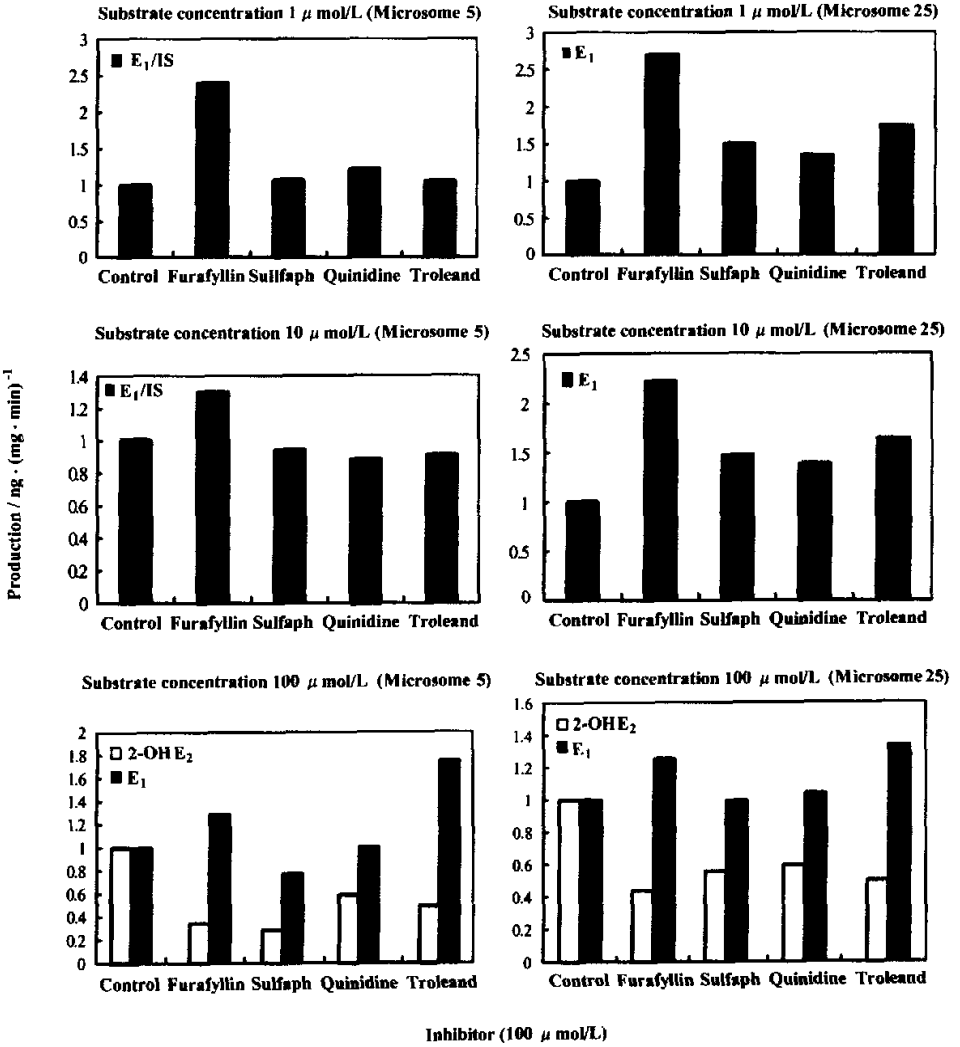

Fig 3. The effect of 100 μmol/L of different P450 inhibitors on estradiol 2-hydroxylation and 17β-hydroxy dehydrogenation at different substrate concentrations in two human liver microsomes.

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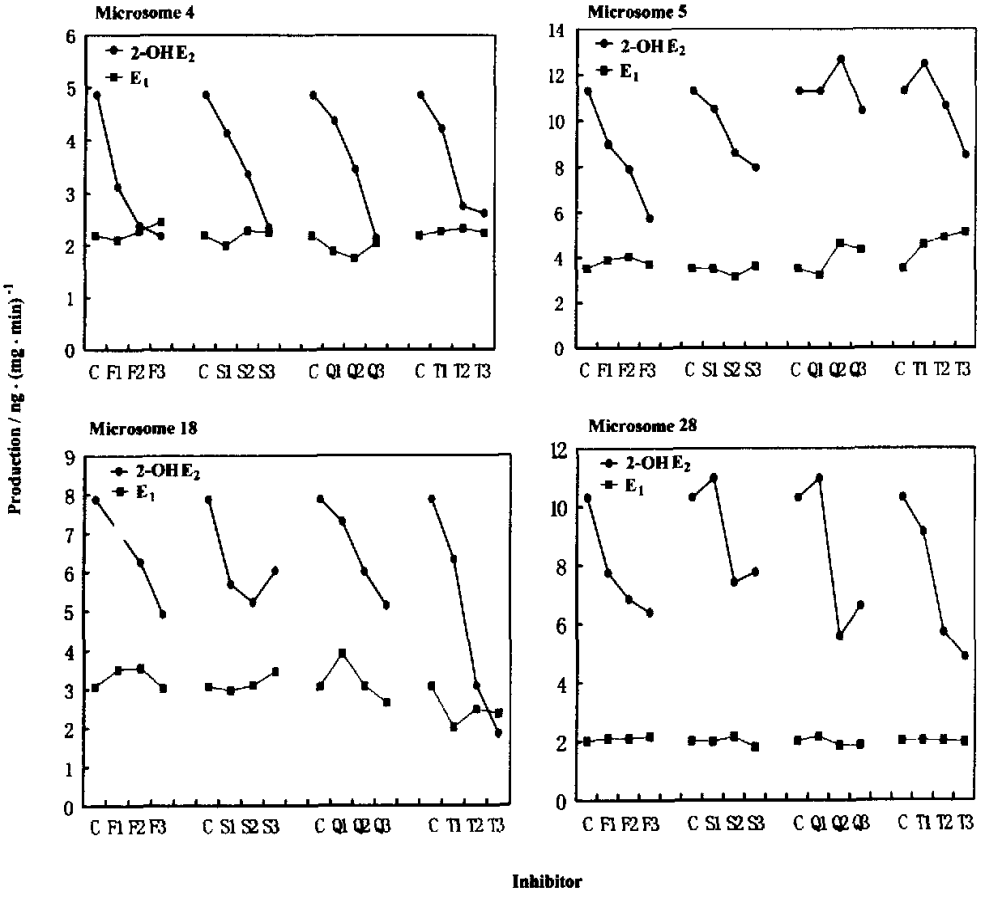

Fig 4. The inhibitory effect of different concentrations of inhibitors on estradiol 2-hydroxylation and 17β-hydroxy dehydrogenation at 100 μmol/L of substrate concentration in 4 microsomes. C, F, S, Q, and T represent control, furafyllin, sulfaphenazol quinidine and troleandomycin, respectively. 1, 2, and 3 represent 25 μmol/L, 50 μmol/L, and 100 μmol/L of inhibitor concentrations, respectively.

DISCUSSION

Estrogens are important hormones, which possess many biological functions. Some of the estrogen biological functions are believed to proceed through the formation of 16α-hydroxy estrogens and catochol estrogens. The tumorigenicity of catochol estrogens may be due to the direct DNA damage and DNA adduct formation effect of their reactive intermediates, such as semiquinone, quinone and their free radicals. 16α-hydroxy estrogens are the active metabolites, which related to the estrogen carcinogenicity in target organs[4].

Estradiol (E_2) is metabolized into estrone (E_1), 2-hydroxyestradiol (2-OHE_2), estriol (E_3), 16α-hydroxyestrone (16α-OHE_1) and many other metabolites[5]. Studies in human liver microsomes found that CYP1A2 and CYP3A4 play an important role in catalyzing E_1 and E_2 2-hydroxylation[9,10]. All these studies were based on a high substrate concentration (about 100 μmol/L).

We studied the estradiol metabolism mechanism at different substrate concentrations in cDNA-expressed P450 and human liver microsomes. We found that CYP1A2, CYP3A4, and CYP2C9 catalyze the E_2 2-hydroxylation. CYP2C9, CYP2C19, and CYP2C8 have high activity in catalyzing 17β-hydroxy dehydrogenation. CYP1A2 is the most important enzyme in catalyzing E_2 2-hydroxylation in cDNA expressed P450, but in human liver microsomes both CYP1A2 and CYP3A4 plays an important role in 2-hydroxy estradiol formation. Using furafyllin and

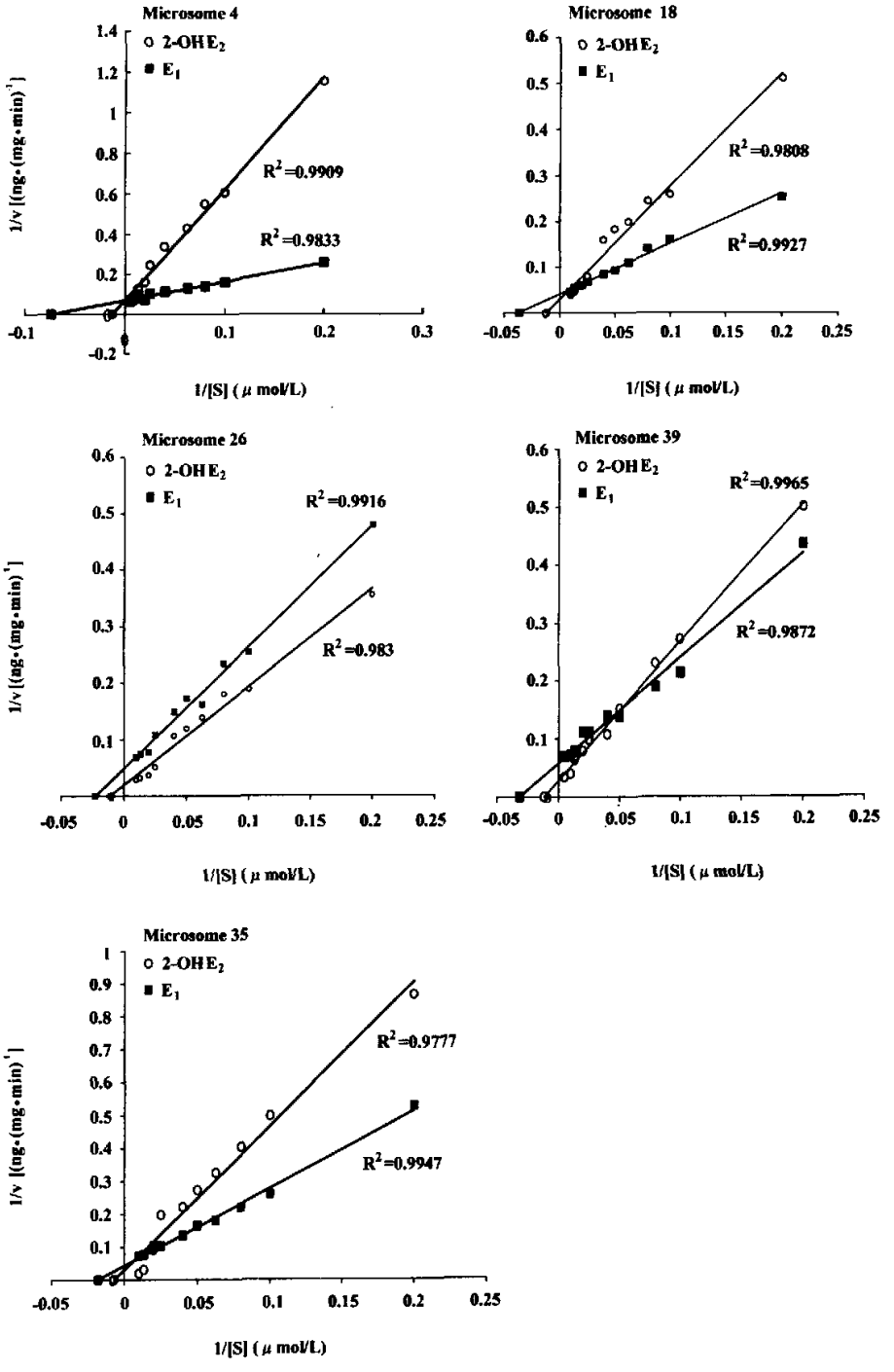

Fig 5. Catalyzing kinetics of estradiol 2-hydroxylation and 17β-hydroxy dehydrogenation in 5 liver microsomes. The mean K_m of 2-hydroxylation and 17β-hydroxy dehydrogenation are 34 ± 16 and 96 ± 26, respectively.

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troleandomycin to inhibit CYP1A2 and CYP3A4 in liver microsomes, it was found that the 2-hydroxylation had been inhibited to about the same degree. At low substrate concentrations, 17β-hydroxy dehydrogenation dominated the E_2 metabolism, but at high substrate concentration, 2-hydroxylation exceeded 17β-hydroxy dehydrogenation to become the important mechanism. These results explain why CYP1A2 and CYP3A4 have poor correlation between their activities and E_2 2-hydroxylation at low substrate concentrations (1 μmol/L and 10 μmol/L). It suggested that E_2 was mainly metabolized into E_1 and then to 2-OHE_1 and other metabolites *in vivo*.

Michnovicz *et al* have studied the inhibitory effects on E_2 2-Hydroxylation in women[11]. They found that after 1-month course of cimetidine (800 mg, bid oral), the serum estradiol level significantly increased, and bone metabolism related biochemical indexes also changed beneficially.

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体外研究人细胞色素 P450 在雌二醇代谢中的作用[1]

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关键词 雌二醇；细胞色素 P450 CYP1A2；细胞色素 P450 CYP3A4；2-羟基雌二醇

目的：研究雌二醇在 cDNA 表达的 P450 和人肝微粒体中的代谢机制，为在体内研究细胞色素 P450 活性与肿瘤发生的关系提供依据. **方法**：用 HPLC-ECD 法测定雌二醇的代谢产物. 通过雌二醇在不同 cDNA表达的 P450 中代谢，13 例人肝微粒体中相关性研究，抑制剂对代谢的影响以及微粒体中 17β-羟基脱氢化和 2-羟基化代谢的催化动力学的研究来推断雌二醇的代谢机理. **结果**：在 cDNA 表达的 P450 中，催化 2-羟基化代谢的 P450 按活性排列依次为 CYP1A2、CYP3A4、CYP2C9. CYP2C9、CYP2C19 和 CYP2C8 均具有较高的催化 17β-羟基脱氢化活性. 抑制 CYP1A2 与抑制 CYP3A4 对 2-羟基化代谢产物生成的影响相似，可认为 CYP1A2 和 CYP3A4 在人肝微粒体中催化 2-羟基化代谢的作用相近. 雌二醇代谢的途径与底物浓度有关，低浓度时(1，10 μmol/L) 17β-羟基脱氢化为主要代谢途径；高浓度时(100 μmol/L)，2-羟基化成为主要代谢途径. **结论**：高底物浓度时，雌二醇主要由 CYP1A2 和 CYP3A4 催化代谢为 2-羟基化产物. 低底物浓度时，主要由 CYP2C9、CYP2C19 和 CYP2C8 催化生成 17β-羟基去氢化产物.

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