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NATURE OF CYTOCHROMES P450 INVOLVED IN THE 2-/4-HYDROXYLATIONS OF ESTRADIOL IN HUMAN LIVER MICROSOMES

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Abstract—Kinetics of the 2- and 4-hydroxylations of estradiol (E2) by human liver microsomal samples were studied to determine the major P450 isoform involved in these endogenous reactions. Thirty human liver microsomal samples were analysed. Metabolism of 25 μM [^{14}C]E2 produced 2-hydroxy and 4-hydroxy derivatives with a ratio of 3.2 ± 1.5 and a great inter-individual variation. Kinetic analysis of the 2- and 4-hydroxylations of E2 exhibited a curvilinear double reciprocal plot with an apparent K_m of 15 μM. Further experiments demonstrated that α-naphthoflavone, testosterone and progesterone increased the 2-hydroxylation activity, suggesting the involvement of a substrate activation mechanism. These two hydroxylations of E2 were shown to be catalysed by cytochrome P450 with an apparent dissociation constant K_s of 0.8 μM. These 2- and 4-hydroxylations inter-correlated significantly ($r = 0.93$; N = 30). The 2-hydroxylation of E2 correlated with four monooxygenase activities known to be supported by P450 3A4/3A5, namely nifedipine oxidation ($r = 0.78$; N = 29); erythromycin N-demethylation ($r = 0.69$; N = 27), testosterone 6β-hydroxylation ($r = 0.66$; N = 25) and tamoxifen N-demethylation ($r = 0.64$; N = 29). On the other hand, E2-hydroxylations did not correlate with activities supported by P450 1A2 and P450 2E1. Furthermore, drugs as cyclosporin, diltiazem, triacetyl-oleandomycin and 17α-ethynylestradiol inhibited more than 90% of the E2-hydroxylations at concentrations <250 μM, while weak inhibition was shown with 500 μM cimetidine and no significant inhibition with caffeine, phenacetin and omeprazole. Finally, 2- and 4-hydroxylations of E2 correlated significantly with the content of P450 3A4/3A5 immunodetected by a monoclonal antibody anti-human P450-nifedipine ($r = 0.84$; N = 28). E2-hydroxylation activities were inhibited by more than 80% with polyclonal anti-human anti-P450-nifedipine. Preincubation of human liver microsomes with 100 μM gestodene (a suicide substrate of P450 3A4) inactivated this P450 isoform and accordingly allowed evaluation of the contribution of other P450 isoforms to the E2 metabolism to about 21% (± 17%, N = 29). All these results taken together suggest that P450 3A4/3A5 are the major forms involved in the formation of catecholestrogens in the human liver microsomes.

The predominant route of oxidative metabolism of the endogeneous steroid hormone estradiol (E2‖) in mammals consists of the ortho-hydroxylation of the phenol ring at the C-atoms 2 and 4. Although these 2- and 4-hydroxyestrogens are regarded as inactivated compounds, they are probably of physiological relevance as catecholestrogens [1, 2]. These A-ring hydroxylations of estradiol are mainly catalyzed by hepatic cytochromes P450 (EC 1.14.14.1) in rat [3, 4]. Although metabolic pathways of E2 are the same in rat and man, i.e. 2- and 4-hydroxylations, there is some direct evidence, at least in the rat, for the involvement of P450s of four gene families: P450 1A, P450 2B, P450 2C and P450 3A [5] while in humans the situation seems to be different.

In human liver microsomes, Guengerich *et al.* [6] described the immuno-inhibition of E2 2- and 4-hydroxylase activities by an antibody anti-P450-nifedipine (P450 3A4, according to the recommended nomenclature [7]). With the direct demonstration of E2-hydroxylase activity of P450 3A4 [8] and the suggestive increase of this activity by rifampicin known to induce this enzyme [9] the potential involvement of other enzymes has not been explored thoroughly. However recently, Ball *et al.* [5] demonstrated by correlation and immuno-inhibition studies that the 2-hydroxylation of E2 was mainly produced by P450 1A2, P450 2E1 and P450 2C8/10 while the relative contribution of P450 3A still remained to be clearly established. In opposite, Aoyama *et al.* [8] demonstrated a major contribution of P450 3A4 and a minor contribution of P450 1A2. Such a discrepancy merits further attention.

The 2- and 4-hydroxylation of E2 was studied in 30 hepatic human microsomal samples to evaluate which P450 isozyme (P450 1A2 or P450 3A4) mainly catalyses these metabolic reactions.

MATERIALS AND METHODS

Chemicals

E2 was obtained from Merck (Darmstadt,

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‖ Abbreviations: E2, estradiol [1,3,5,(10)-estratrien-3,17β-diol]; 2-OH-E2, 2-hydroxy-estradiol; 4-OH-E2, 4-hydroxy-estradiol; P450, microsomal cytochrome P450 enzyme [EC 1.14.14.1]; PBS, phosphate buffer saline; β-NF, β-naphthoflavone (5,6-benzoflavone); α-NF, α-naphthoflavone (7,8-benzoflavone); TAO, triacetyl-oleandomycin; IgG, immunoglobulin G; EROD, ethoxyresorufin *O*-dealkylase; MROD, methoxyresorufin *O*-dealkylase.

Table 1. Cytochrome P450-dependent activities in microsomal samples from 30 human livers

Subject	Sex	Age	P450*	2-OH-E2†	4-OH-E2†	P4503A‡	P4501A‡
FH 1	M	18Y	269	26	9	ND	17.3
FH 2A	M	41Y	527	395	46	42.6	34.2
FH 3	M	47Y	470	213	45	ND	11.2
Br015	M	43Y	414	25	13	17.8	12.2
Br016	M	44Y	369	85	15	29.4	20.9
Br017	M	26Y	593	83	64	16.0	33.0
Br018	F	45Y	334	32	13	26.3	8.1
Br019A	M	45Y	241	52	18	18.9	6.1
Br021	M	49Y	257	52	16	25.2	8.1
Br022	M	23Y	286	236	54	49.8	3.8
Br023	M	5M	228	208	61	ND	1.0
Br024	F	15Y	505	385	103	54.6	3.5
Br025	M	2Y	110	77	23	30.7	5.0
Br027	M	21Y	99	37	18	27.5	8.1
Br028	M	27Y	165	250	68	45.4	14.6
Br029	M	36Y	77	49	21	18.4	2.1
Br031	M	23Y	154	96	28	10.8	3.4
Br032	M	36Y	286	459	121	56.5	2.5
Br033	M	44Y	363	196	66	41.6	15.8
Br034	M	32Y	286	93	25	25.0	6.6
Br035	M	33Y	252	566	156	50.6	3.0
Br036	M	56Y	153	195	50	38.0	3.3
Br037	M	56Y	230	17	22	16.4	1.0
Br038	F	51Y	120	10	12	12.7	1.0
Br039	F	46Y	538	124	51	31.6	7.2
Br040	M	60Y	264	41	23	38.1	ND
Br041	M	2D	55	17	20	23.7	ND
Br042	M	47Y	143	367	85	60.9	ND
Br043	M	60Y	198	370	80	39.6	ND
Br046	M	40Y	433	530	145	ND	ND
Mean		38	280	176	49	32.6	9.3
±SD		15§	147	167	40	14.5	9.1

* pmol/mg microsomal protein.
† pmol/min/mg microsomal protein.
‡ Arbitrary relative units.
§ Br023, Br025 and Br041 not included.
Y, year; D, day; M, month.
ND, not determined.

Germany) and its metabolites from Steraloids (Wilton, NH, U.S.A.). [4-^{14}C]E2, sp. act. 56 mCi/mmol, was from Amersham (Amersham U.K.); it was purified by TLC on silicalgel F-254 with two migrations by chloroform/ethanol (9:1, v/v) and ethylacetate/cyclohexane (1:1, v/v) mixtures. Caffeine, cimetidine, α-naphthoflavone (α-NF), phenacetin, erythromycin, troleandomycin, cyproterone acetate, ascorbic acid, tamoxifen and testosterone were from the Sigma Chemical Co. (St. Louis, MO. U.S.A.). Cyclosporin was supplied by Sandoz (Rueil-Malmaison, France), diltiazem by Lers-Synthelabo (Meudon-La-Forêt, France), dextromethorphan by Norgan-Labs (Paris, France) and nifedipine by Bayer Pharma (Sens, France). Gestodene was provided from Dr H. Kuhl, (J. W. Goethe-University, Frankfurt, Germany).

Human liver samples and microsome preparation

Human liver samples were obtained from 27 adult kidney donors (23 males, four females, mean age: 38 ± 15 years old) who died after traffic accidents and three children Br023, Br025 and Br041 (see Table 1). Sampling was made in accordance with French legal considerations. Ethical committee approval was obtained prior to this study. At brain death, the liver was removed and was frozen immediately and stored in liquid nitrogen until use for preparation of microsomes. Although pre-death dietary habits and exposure to environmental chemicals were not known, P450 specific content in human liver microsomes (see Table 1) was in agreement with data published previously [10]. Microsomal fractions were prepared as described previously [11] and stored at −80° until use. The enzyme activity was shown to be stable during storage.

Determination of monooxygenase activities

Protein content of microsomal preparations was determined by the Bio-Rad protein microassay (Bio-Rad, Munich, Germany). Total cytochrome P450 levels were measured according to Omura and Sato [12] with the method slightly modified, i.e. CO was bubbled in both sample and reference cuvettes containing 2 mg/mL of microsomal proteins before sodium dithionite addition only to the sample cuvette.

E2 incubation. The 1 mL standard incubation mixture contained 100 mM potassium phosphate buffer pH 7.4, 1 mM ascorbic acid, 25 μM [4-^{14}C]E2 (sp. act. 3.25 mCi/mmol) in 10 μL methanol and 0.2 mg microsomal proteins. After 5 min preincubation at 37°, the reaction was started by addition of 1 mM NADPH. After 20 min shaking at 37°, the reaction was stopped by addition of 5 mL of chilled methylene chloride. The organic phase was taken to dryness under nitrogen stream at 45°. To the dry extract were added 50 μL of HPLC mobile phase containing the cold hydroxylated metabolites of E2. Control incubations were run as described except that microsomal proteins or NADPH were omitted. Values reported represent the mean of at least duplicate experiments. Some microsomal samples were incubated with the addition of 5 μM α-NF added in 5 μL methanol. Kinetics studies were carried out in the 10–250 μM range by using FH-2A or Br028 and Br046 samples.

E2 metabolites were analysed by HPLC equipped with a Nucleosil C-18, 5 μm, 250 × 4 mm, from Interchim (Montluçon, France). The mobile phase consisted of water, acetic acid and acetonitrile (62:1:38, by vol.) mixture at a flow rate of 1 mL/min. Eluates were detected by UV at 280 nm with a 0.02 AUFS sensitivity. Peaks were identified by their retention times and spectral characteristics in comparison with standard compounds. HPLC peaks were collected and their radioactivity counted by liquid scintillation spectrometry. Biotransformation of E2 was expressed as percentage of substrate transformed into known metabolites relative to the total radioactivity contained in the organic extract.

Other monooxygenase activities. In order to correlate the 2-/4-hydroxylations of E2 with different monooxygenase activities known to be specific of P450 families, different activities were measured in the microsomal preparations. For the P450 1A family, ethoxy- (EROD), methoxy- (MROD) resorufin *O*-dealkylase, phenacetin *O*-deethylase and acetanilide 4-hydroxylase were determined according to procedures described previously [13]. For the P450 3A family, the following monooxygenase activities were carried out: tamoxifen N-demethylation, testosterone 6β-hydroxylation and erythromycin demethylation, all according to Jacolot *et al.* [14]; nifedipine oxidation according to Ref. 6 and dextromethorphan N-demethylation according to [15]. For the P450 2E1 subfamily, nitrosodimethylamine demethylation, *n*-butanol oxidation and chlorzoxazone 6-hydroxylation were quantified according to Refs 16 and 17, respectively.

Inhibition of E2-hydroxylation by different compounds

Incubations were performed as described above with 45 μM E2 (sp. act. 2.15 mCi/mmol) added in 5 μL methanol. Compounds such as diltiazem, tamoxifen, troleandomycin, cimetidine, phenacetin, caffeine, omeprazole, cyproterone acetate and cyclosporin were previously added in 5 μL methanol at concentrations ranging between 100 and 1000 μM. Control experiments were conducted with the same amounts of organic solvent. The Br035, Br028 or Br046 liver samples were used for these experiments; they were chosen on the basis of their high E2 metabolizing activities.

For studying the effects of α-NF on E2 metabolism, 5 μL of methanol containing α-NF at different concentrations were added to medium previously to 25 μM E2 (sp. act. 3.25 mCi/mmol) addition. The same procedure was used when testosterone was added to incubation medium of four microsomal preparations (FH-2A, Br032, Br035 and Br043).

Inhibition of E2-hydroxylations by gestodene was carried out as described by Guengerich [18]. In outline, the initial incubation was performed with 0.2 mg of microsomal proteins, 100 μM gestodene and 5 mM NADPH for 20 min at 37°. The 50 μL incubation medium was then diluted 20-fold in 0.1 M potassium phosphate buffer pH 7.4 containing 25 μM E2 (sp. act. 3.25 mCi/mmol), 1 mM ascorbic acid and 1 mM NADPH. After 20 min of incubation, the hydroxylated metabolites were measured by HPLC after extraction by organic solvent.

Immunoblot analysis

Protein samples (20 μg for P450 3A and P450 2E1; 40 μg for P450 1A) were separated by electrophoresis on 9% SDS–polyacrylamide gel according to Ref. 19, and transferred electrophoretically to a nitrocellulose sheet [20]. After blocking incubation at 37° for 30 min by 3% bovine serum albumin and 10% newborn calf serum in phosphate-buffered serum (PBS), the nitrocellulose sheet was treated overnight at 4° either with monoclonal anti-human P450 3A4 [21] or polyclonal anti-rat P450 1A1 (β-naphthoflavone-B according to Ref. 22) or polyclonal anti-rat P450 2E1 (Oxygene, Dallas, TX, U.S.A.) according to the P450 isoenzyme, respectively. After washings with PBS, the sheet was treated with PBS containing either rabbit anti-mouse immunoglobulins conjugated to peroxidase (Dako, Versailles, France), or anti-rabbit IgG and horseradish peroxidase anti-peroxidase complex raised against rabbit immunoglobulins (ICN Biomedicals, Bucks, U.K.) or anti-rabbit IgG conjugated to peroxidase (Dako, Versailles), respectively for P450 3A4, P450 1A2 and P450 2E1. Finally, the sheet was washed with PBS, and the peroxidase activity was detected with 4-chloronaphthol and H_2O_2. The quantification of blots was performed by densitometry and the integrated peak area of the various microsomal preparations was expressed as arbitrary units relatively to the amount of proteins.

Inhibition of E2 metabolism by anti-P450 3A antibody

Diluted microsomes from FH-2A and Br035 samples, 120 and 50 pmol P450 respectively in 100 mM potassium phosphate buffer (pH 7.4) containing 45 μM E2 (sp. act. 2.15 mCi/mmol), were incubated at room temperature for 20 min in the presence of increasing amounts (1–10 mg IgG/nmol P450) of polyclonal P450-NF antibody [21] or non-immune rabbit IgG. Reaction was started by NADPH addition and proceeded as indicated above.

Data analysis

The values of 2-/4-hydroxylations are means ± SD from 30 human livers including the Br023, Br025 and Br041 children samples. Correlation coefficients

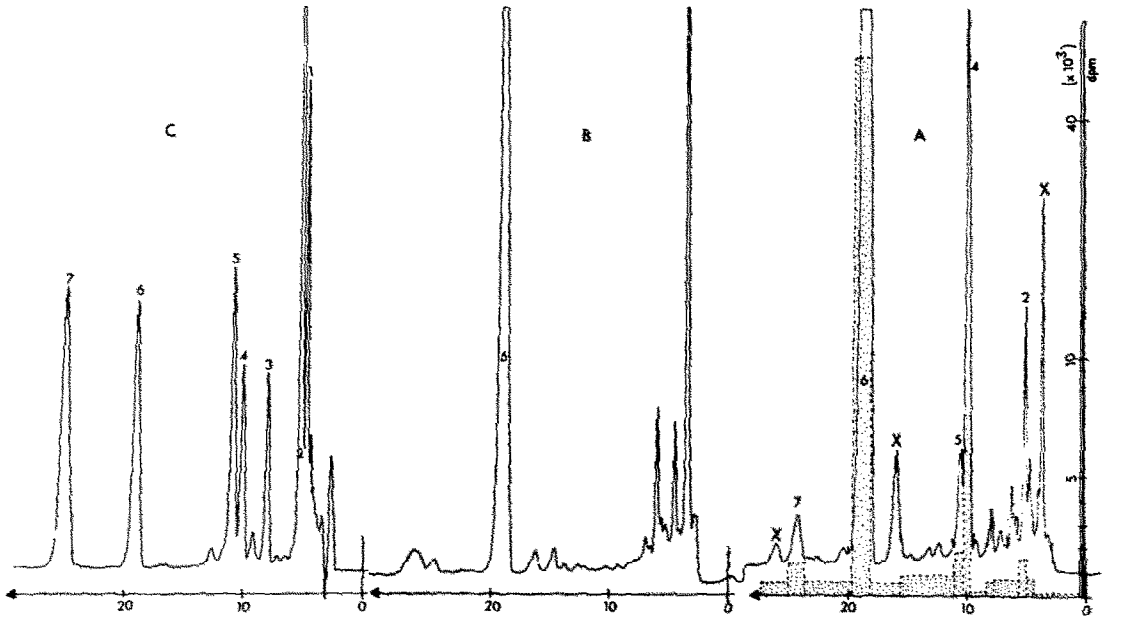

Fig. 1. HPLC chromatogram of incubation medium of 83 μM E2 with microsomal sample from FH-2A sample containing NADPH (A) and *not containing NADPH* (B, control); (C) HPLC profile of pure compounds. Peaks are identified: 1 = *16β-estriol (epiestriol)*; 2 = estriol; 3 = 6β-hydroxy-estradiol; 4 = 2-OH-E2; 5 = 4-OH-E2; 6 = E2; 7 = *estrone*; X = unknown compounds. UV response at 280 nm was recorded; the radioactivity recovered in chromatographic peak is represented by the hatched bars (A).

were calculated using an ANOVA table by the least squares regression analysis from the raw data. As a normal guassian distribution in the population was observed (skewness = 0.88 and 1.17 for the 2- and 4-hydroxylations; N = 30), correlation coefficients were determined by including all the samples.

RESULTS

Metabolites of E2 by human liver microsomes

By using the optimized HPLC conditions described above, seven metabolites of E2 were totally separated within 25 min. Two major metabolites were produced from estradiol:2-OH- and 4-OH-E2 (2-OH-E2 and 4-OH-E2) (Fig. 1). They were identified on the basis of their retention times and UV spectra (results not shown). Furthermore, these HPLC peaks were pure on the basis of their relative ratio of λ_{max} (233/290 nm) which were identical to those of standards. Two minor metabolites could be detected, probably *16α*-hydroxy-E2 or estriol and estrone. Approximately 91% (± 3.7) of incubated E2 remained lipophilic and was extracted by methylene chloride.

Cytochrome P450 dependence of 2-/4-hydroxylations of E2

The involvement of P450 in the 2-/4-hydroxylations of E2 by human liver microsomal samples was evidenced by the absolute dependence upon the presence of NADPH (Fig. 1B). Up to 60–80% of E2-hydroxylations were inhibited by bubbling a CO/O_2 (80:20, v/v) mixture into the medium reaction while estriol and estrone formations were not modified. Furthermore, the binding of E2 to P450 of human liver microsomes was determined by differential spectroscopy. Type I difference spectra with trough at 420 nm and maximum at 390 nm were obtained upon addition of increasing amounts of E2 in liver microsomes. The reciprocal plot of the absorbance change at 390 minus 420 nm against E2 concentration (Fig. 2) allowed to determine an apparent dissociation constant K_s of 0.8 μM.

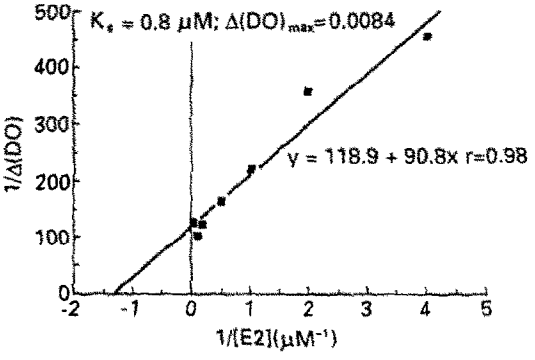

Fig. 2. Reciprocal plot of A (A_{390}–A_{420}) against E2 concentration. Microsomal sample from Br035 liver was diluted at 1 mg protein/mL or 0.25 nmol P450/mL. Increasing amounts of E2 were added to the sample cuvette and the same amounts of methanol to the reference cuvette. After zero recording, differential spectra were recorded.

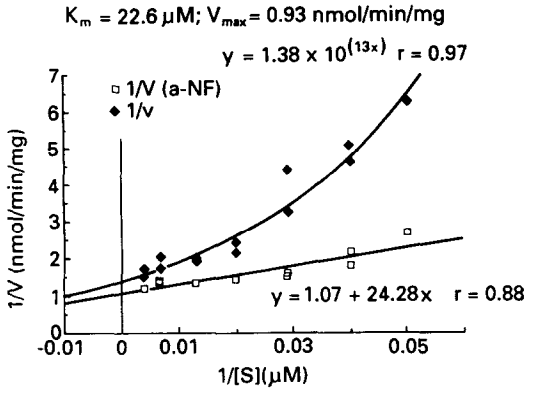

Fig. 3. Reciprocal plot of the E2 2-hydroxylation V (nmol/min/mg protein) against E2 concentration (μM) according to Lineweaver and Burk. Kinetics were performed in the absence (◆) or in the presence of 5 μM α-NF (□). Microsomal sample from FH-2A liver was used.

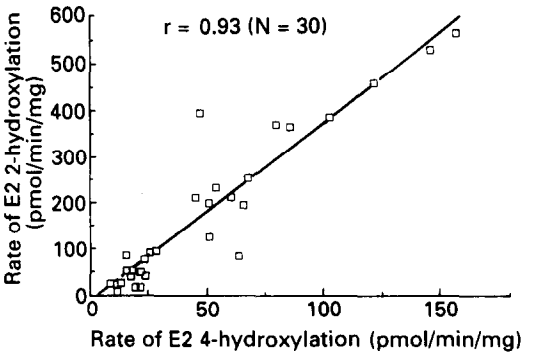

Fig. 4. Correlation between 2-hydroxylation and 4-hydroxylation of E2 in different human liver microsomal samples. The line was drawn by linear regression analysis and the correlation coefficient (r) was calculated by the least squares method.

Kinetic parameters

Double reciprocal plot for the dependence of the rates of E2 2-hydroxylation on the substrate concentration exhibited a pronounced curvature for hepatic microsomes prepared from FH-2A sample. In the presence of 5 μM α-NF, an essentially linear double reciprocal plot was obtained (Fig. 3). At high E2 concentrations, the velocities obtained in the presence or in the absence of α-NF were about the same. By this linearization procedure, K_m was 22 μM and V_{max} 0.93 nmol/min/mg, while by using the Eadie–Hofstee method K_m was 15 μM and V_{max} 0.83 nmol/min/mg protein. A similar curve was obtained for the 4-hydroxylation of E2 (not shown) and K_m was 11 μM and V_{max} 0.14 nmol/min/mg. Similar results were obtained with Br028 and Br046 microsomal preparations.

Rate of 2- and 4-*hydroxylations of E2 in different liver microsomal preparations*

Table 1 shows the rate of biotransformation of estradiol by 30 human liver microsomes. The 2-hydroxylation of E2 ranged from 10 to 566 pmol/min/mg microsomal protein with a mean of 176 (± 167) while the 4-hydroxylation ranged from 9 to 156 with a mean of 49 (± 40). The ratio of 2-hydroxylation to 4-hydroxylation was 3.2 (± 1.5) with a 1 to 11.2 inter-individual range.

The rates of hydroxylations of the aromatic carbons 2 and 4 of E2 inter-correlated significantly (Fig. 4), suggesting that they are mediated by the same P450 isozyme (r = 0.93, N = 30, $P < 0.001$).

Correlation of rates of E2-hydroxylations with different other monooxygenase activities

If two reactions are catalysed by the same enzyme, then the rates should be correlated to each other when compared in a series of microsomal preparations containing varying levels of the enzyme [23]. The 2-hydroxylation of E2 correlated significantly with two catalytic activities known to be supported by P450 3A4/3A5, i.e. tamoxifen N-demethylation and nifedipine oxidation (Fig. 5). Correlation coefficients between biotransformation rates measured with substrates specific of different human P450s were determined in the 30 liver microsomal preparations (Table 2). Four of the catalytic activities specific to P450 3A4/A5, namely testosterone 6β-hydroxylation, nifedipine oxidation, tamoxifen N-demethylation and erythromycin N-demethylation, correlated

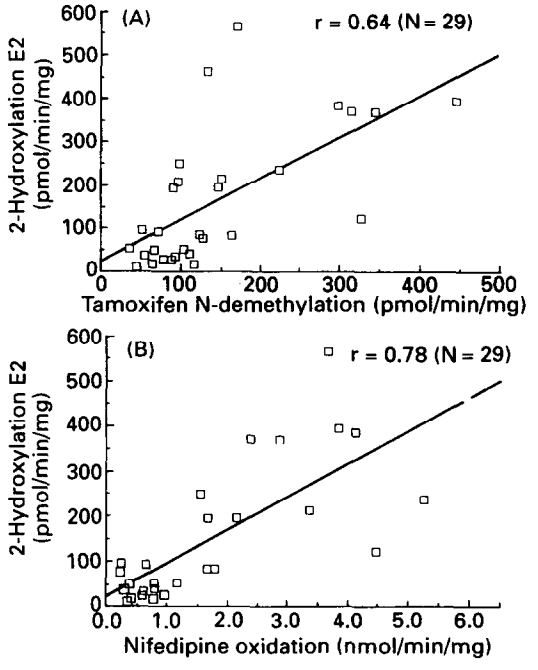

Fig. 5. Correlation of tamoxifen N-demethylation (A) and nifedipine oxidation (B) with 2-hydroxylation of E2 in 29 human liver microsomal samples. Lines were drawn by linear regression and correlation coefficients (r) were calculated.

Table 2. Correlation coefficients of catalytic activities in human liver microsomal preparations

Catalytic activity	Estradiol 2-hydroxylation	Estradiol 4-hydroxylation	N
P450 3A4/3A5 activities			
Tamoxifen N-demethylation*	0.64	0.47	29
Testosterone 6β-hydroxylation*	0.66	0.50	25
Nifedipine oxidation*	0.78	0.73	28
Erythromycin N-demethylation*	0.69	0.53	27
Dextromethorphan N-demethylation	0.52	0.35	28
P450 1A1/1A2 activities			
Caffeine demethylations†	0.14	0.08	28
Phenacetin O-deethylation†	0.13	0.14	30
Acetanilide 4-hydroxylation*	0.24	0.24	30
Ethoxyresorufin O-deethylation†	0.26	0.19	29
Methoxyresorufin O-demethylation†	0.32	0.33	30
P450 2E1 activities			
Nitroso-dimethylamine demethylation	0.11	0.17	25
Butanol oxidation	0.23	0.32	25
Chlorzoxazone 6-hydroxylation	0.00	0.09	28

$r > 0.52$ and $r > 0.40$ corresponds to $P < 0.01$ and $P < 0.05$ significance respectively for 25 samples.

* Results from Jacolot *et al.* [14].

† Results from Berthou *et al.* [13].

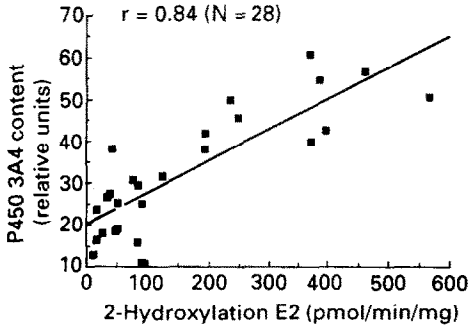

Fig. 6. Correlation between E2 2-hydroxylation activity and immuno-quantitated P450 3A4 immunodetected in human liver microsomes. Intensity units of P450 3A4 are relative arbitrary units determined by densitometry. Correlation coefficient (r) was calculated by the least-squares regression method.

significantly with 2- and 4-hydroxylations of estradiol. In no case was an r value > 0.35 found for substrates markers of P450 1A2 (phenacetin O-deethylation; methoxy-resorufin O-demethylation or caffeine demethylations) or P450 2E1 (nitroso-dimethylamine demethylation, butanol oxidation or chlorzoxazone 6-hydroxylation). The conclusion reached that none of these P450s make a major contribution to E2 2- and 4-hydroxylations.

Correlation of E2-hydroxylations with immuno-quantified P450 3A4

The 2- (Fig. 6) and 4-hydroxylations (not shown) of E2 correlated significantly with the amount of P450 3A4 immunodetected by a monoclonal antibody anti-human-P450-nifedipine ($r = 0.84$ and $r = 0.70$, respectively; $N = 28$; $P < 0.001$) by means of western blot technique. The regression line failed to intersect the y axis at zero, suggesting the involvement of other P450 isozyme(s) in this hydroxylation reaction. No significant correlation could be measured between 2- and 4-hydroxylations of E2 and the amount of P450 1A2 ($r = 0.02$; $N = 25$) or P450-2E1 ($r = 0.018$; $N = 28$) (results not shown).

Inhibition of E2-hydroxylations by different compounds

To further assess whether or not the 2- and 4-hydroxylations of E2 are catalysed by the P450 enzyme involved in N-demethylation of tamoxifen (i.e. P450 3A4/3A5), the effect of tamoxifen on this reaction was studied. Figure 7 illustrates the competitive inhibition of tamoxifen on the 2-hydroxylation activity with an apparent K_i of 43 μM. Similar curves were obtained for the 4-hydroxylation of E2, giving an apparent K_i of 48 μM.

A weak inhibition of E2 2-hydroxylation was observed when the microsomes were incubated with some selective inhibitors of other human liver cytochromes P450, including caffeine, phenacetin (P450 1A2) all used at concentrations known to block more than 90% of this P450 activity (Fig. 8). Compounds such as cyproterone acetate or omeprazole inhibited the 2-hydroxylation no more than 50% at concentrations up to 500 μM. Compounds known to be metabolized by P450 3A4, namely cyclosporin, triacetyl-oleandomycin (TAO), 17α-ethynylestradiol and diltiazem inhibited this catalytic activity up to 90% at concentrations less than 200 μM. Similar results were observed for the 4-hydroxylation activity. Br028 and Br046 microsomal samples gave similar inhibition results (not shown).

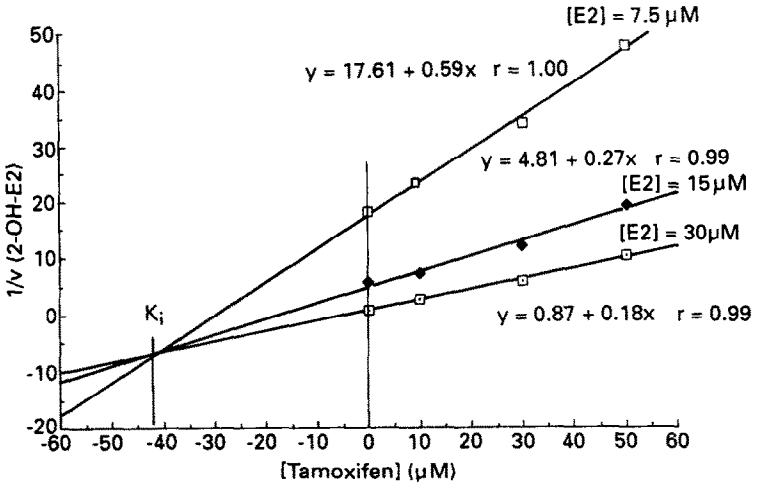

Fig. 7. Competitive inhibition of 2-hydroxylation of E2 by tamoxifen. Liver microsomal preparation from Br035 donor was assayed for estradiol 2-hydroxylation activity in the presence of increasing amounts of tamoxifen. The data were analysed according to the Dixon plot. Uninhibited metabolic rates were 541, 186 and 56 pmol/min/mg protein for estradiol concentrations of 30, 15 and 7.5 μM, respectively.

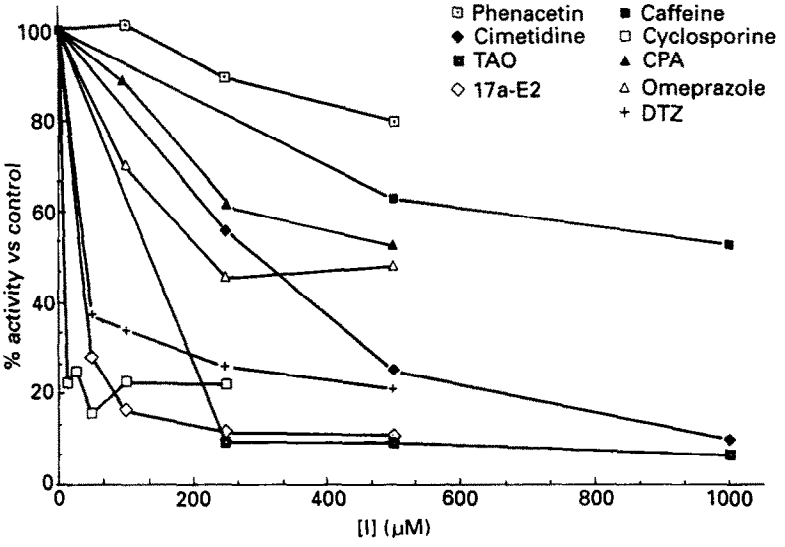

Fig. 8. Inhibition of E2 2-hydroxylation activity in human liver microsomes by different drugs. In all cases, the substrate (45 μM) and inhibitor were both added in 5 μL of methanol to Br035 microsomes and the reaction was initiated by the addition of 1 mM NADPH. Uninhibited metabolic rate was 0.95 ± 0.1 (N = 11) nmol/min/mg microsomal protein. DTZ, diltiazem, CPA, cyproterone acetate; 17a-E2, 17α-ethynylestradiol; TAO, triacetyl-oleandomycin; [I], inhibitor concentration.

Inhibition of E2-hydroxylations by gestodene

Gestodene, a 17α-acetylenic steroid, is known to be a mechanism-based inactivator of P450 3A4. This inactivation process is postulated to result from attack of P450 on the substituted carbon of the acetylene and leads to heme modification. Preincubation of the 30 human liver microsomal samples with 100 μM gestodene dramatically reduced the E2-hydroxylations. In some samples with high hydroxylase activity, the inhibition was up to 95% (Fig. 9) while with other samples the inhibition was rather low. The 2- and 4-hydroxylations were similarly inhibited in almost all the samples (r = 0.71; N = 28). Residual 2- and 4-hydroxylations of E2 after preincubation with gestodene were 21.4% (± 17.3; N = 28) and 25.8% (± 20.6) of control samples, respectively. The lack of complete inhibition

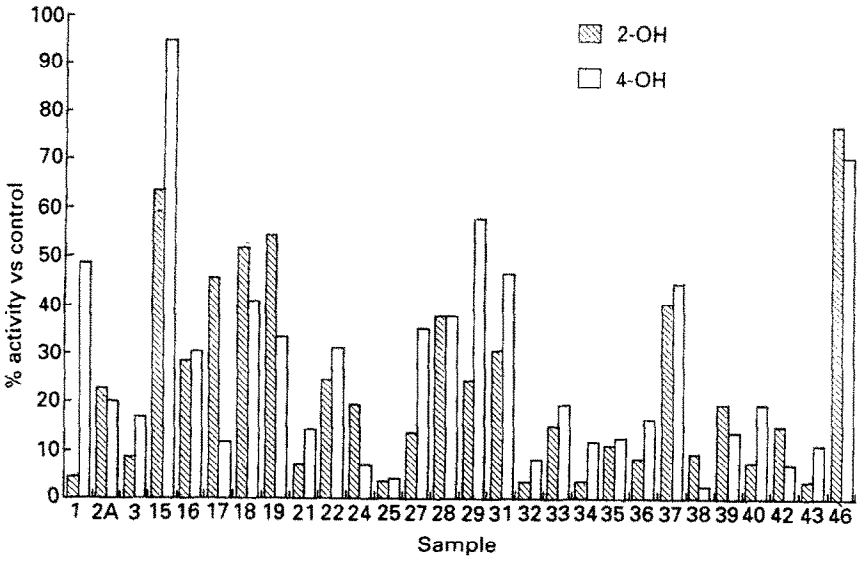

Fig. 9. Per cent of uninhibited 2- (▨) and 4- (□) hydroxylations of estradiol following preincubation with gestodene in microsomal liver samples. The preincubation was carried out with 5 mM NADPH and 100 μM gestodene.

might be due to catalysis of these hydroxylations by other cytochromes P450 in some samples. The percentage inhibition of 2-hydroxylation by gestodene did not correlate with the hepatic levels of P450 3A ($r = 0.42$; N = 25) or P450 1A2 ($r = 0.22$, N = 25).

Activation of E2-hydroxylations by testosterone and α-NF

On the opposite, the 2- and 4-hydroxylations of E2 were activated by α-NF addition in the 0–20 μM range (Fig. 10). The effect of testosterone on E2-hydroxylations was dependent upon substrate concentration (Fig. 10). When 25 or 12.5 μM testosterone were added to incubation medium containing 30 μM E2, 94% (± 16) and 95% (± 16) of E2-hydroxylations vs control were observed for four samples studied, i.e. FH-2A, Br032, Br035 and Br043. On the opposite, at 15 μM E2 concentration, when 25 and 12.5 μM testosterone were added to the medium, E2-hydroxylations were activated up to 185% (± 27) and 115% (± 34) vs control for the same four samples.

Immuno-inhibition of 2- and 4-hydroxylations of E2 in human liver microsomes

In order to confirm the role of P450 3A4 as the major enzyme involved in the hydroxylations of E2, immuno-inhibition experiments were carried out on two microsomal preparations, FH-2A and Br035 samples. Results are reported in Fig. 11. The two hydroxylations of E2 were inhibited by more than 80% of control activity with 10 mg immune IgG per nmol P450.

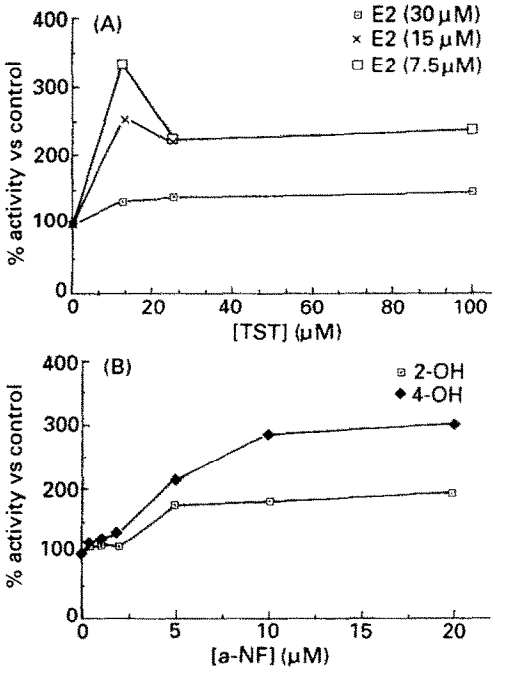

Fig. 10. Effect of testosterone (A; TST) and α-NF (B; a-NF) on the hydroxylations of E2. Liver microsomal preparation from Br035 donor was assayed for E2 hydroxylations in the presence of increasing amounts of testosterone (A) or α-NF (B), both added in 5 μL methanol. E2 concentrations were 7.5, 15 and 30 μM (experiment A) and 25 μM (experiment B).

DISCUSSION

Kinetics of 2- and 4-hydroxylations of E2 in liver microsomes

Hydroxylations of E2 occurs primarily at the 2-

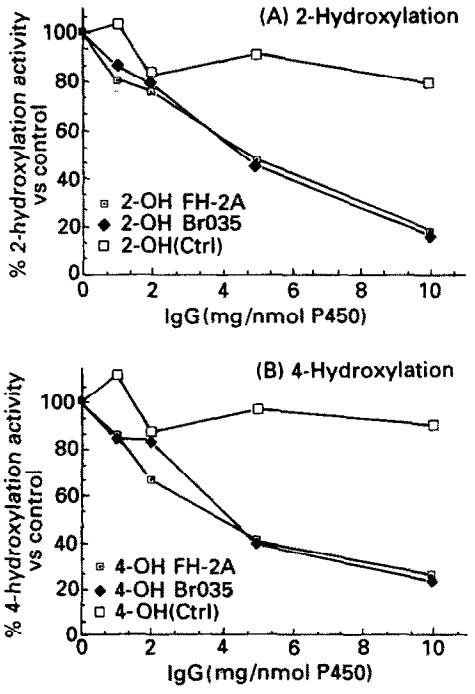

Fig. 11. Inhibition of 2- (A) and 4- (B) hydroxylations of estradiol in microsomal preparations from FH-2A (⊡) and Br035 (╥) samples by polyclonal anti-P450-NF (3A4) antibody. Liver microsomes (120 and 50 pmol P450, respectively) were incubated for 20 min at room temperature in the presence of increasing amounts of immune IgG anti-P450-NF or non-immune IgG (Ctrl □). Inhibited activities for the 2-hydroxylation were 1380 and 975 pmol/min/mg and for the 4-hydroxylation 253 and 194 pmol/min/mg for FH-2A and Br035 samples respectively; E2 concentration was 45 μM.

and 4-positions in the liver microsomes. The 2-hydroxy metabolite represented 33.7% (± 7.5) of total compounds formed. The 2-hydroxy/4-hydroxy metabolite ratio was 3.2 (± 1.5). Earlier reports [6] indicated that human liver microsomes produced 2- and 4-OH-E2 at a ratio of about 1:1. This discrepancy may be due to the assay methods used. Indeed, the tritium release method previously used [6, 9] to quantify 2- and 4-hydroxylation activities may induce significant errors because of isotopic exchange of tritium bound to C-2 or C-4 atoms. The HPLC method used in this study for separation of ^{14}C-labelled hydroxylated metabolites did overcome such a problem. Furthermore, ascorbic acid added into the incubation media prevented oxidation of the catechols formed into *o*-quinones. Other metabolites could be detected, such as estriol (22 ± 7%) and estrone (16.4 ± 5%). Formation of these two metabolites did not involve cytochrome P450 because it was not inhibited by CO bubbling or gestodene addition. On the opposite, 2- and 4-hydroxy derivative formation was inhibited up to 70% when the incubation mixture was bubbled with CO. Furthermore, as these reactions needed NADPH, they were clearly mediated by cytochromes P450 of liver microsomes.

Some values of metabolic rates of E2-hydroxylations have been reported previously. Either they concerned only two human samples [6, 8] or they were measured with a very high concentration of substrate, 25 mM [5]. This present study reports results of metabolic rates from 30 human samples which allowed the greater inter-individual variation of 2- and 4-hydroxylations to be determined: they varied 56- and 17-fold, respectively. No significant difference of hydroxylation activity could be determined according to gender: 188 ± 168 (N = 25) and 137 ± 172 (N = 4) pmol/min/mg for male nd female, respectively.

The quite similar K_m for the 2- and 4-OH-E2 formation (15 and 11 μM, respectively) and the significant correlation between their metabolic rates were consistent with the involvement of the same enzyme(s) in these two hydroxylation reactions.

The curvature seen in the double reciprocal plot of metabolic rate vs E2 concentration suggests that the substrate itself activates the human cytochromes P450 involved in the 2- and 4-hydroxylations. Such curvilinear curves were already described for the 6β-hydroxylation of progesterone by human and rabbit liver microsomes [24, 25] and also for the E2 2-hydroxylation by P450 3A5 isoform [26]. The effect of α-NF as an activator of E2-hydroxylations, allowing the double reciprocal plot to linearize, was observed previously when incubating progesterone with rabbit liver microsomes [25]. This activation was also seen with other steroids, such as testosterone and progesterone (results not shown), even though these compounds were expected to be 6β-hydroxylated by P450 3A4 [14, 27, 28] and thus inhibit E2 hydroxylations. The α-NF effect on cytochromes P450 is complex. It is known to specifically inhibit P450 1A1/1A2 [29] and specifically stimulate 3A4 [25, 30]. Here, α-NF decreased the apparent K_m for E2 2-hydroxylation from 22 to 15 μM. Furthermore, the stimulation of hydroxylation activities was shown to be concentration-dependent up to 10 μM; the 2- and 4-hydroxylations activities increased about 2- and 3-fold, respectively. As evidence exists that in human liver microsomes P450 3A4 appears to be more responsive to α-NF than most other enzymes [31], all our results suggest that P450 3A4 was the major form involved in the hepatic formation of catecholestrogens. Moreover, as P450 1A2 activity was inhibited by α-NF and methoxy- and ethoxyresorufin dealkylase activities were not inhibited by E2 (results not shown), it may be suggested that P450 1A2 was not, or only very weakly, involved in the E2-hydroxylations in the microsomal preparations studied.

The kinetics of α-NF-mediated enhancement of rabbit liver P450 3A activities has been evaluated in a mechanistic sense, and it appears that this agent exerts is effect by an allosteric phenomenon [25]. As the same effect was observed in human liver microsomes with α-NF but also with testosterone or progesterone, it seems that all these compounds are allosteric effectors. The fact that testosterone or progesterone stimulated the hepatic hydroxylations of E2 might be relevant in clinical practice and

so, merits further attention concerning their physiological concentrations.

P450 3A4/3A5 as the major enzymes involved in E2-hydroxylations

All the results reported in this study demonstrated clearly that cytochromes P450 from the 3A subfamily are the major enzymes involved in the 2- and 4-hydroxylations of E2. This conclusion was derived from the following observations summarized as follows:

1. Correlation of 2- and 4-hydroxylation activities with tamoxifen N-demethylation, testosterone 6β-hydroxylation, nifedipine oxidation and erythromycin N-demethylation, all these activities known to be supported by P450 3A4/3A5 [6, 14, 27, 28, 32]. Dextromethorphan N-demethylation, though a minor metabolic pathway, was also correlated with all these activities, confirming previous results [33]. In all the cases, the 4-hydroxylation of E2 was less significantly correlated with these monooxygenase activities. This lack of correlation was probably due to the low metabolic rate relatively to the 2-hydroxy metabolite.

2. Correlation of 2- and 4-hydroxylation activities with liver P450 3A4/3A5 content determined by western blot ($r = 0.84$ and 0.70, respectively) but no significant correlation with P450 1A2 and 2E1 determined in the same samples.

3. Inhibition of 2- and 4-hydroxylations of estradiol by anti-P450 3A4 antibody.

4. Inhibition by selective inhibitors of P450 3A4, including troleandomycin and gestodene known to be suicide substrates of this enzyme [18, 34, 35]. Cimetidine was also shown to inhibit 2- and 4-hydroxylations of E2 although high levels were required. This *in-vitro* result is in agreement with the *in-vivo* interaction between cimetidine and estradiol [36]. Recently, cimetidine has been shown to inhibit P450 3A4 selectively [30].

5. Inhibition by different substrates specifically metabolized by P450 3A4: nifedipine [6], tamoxifen [14], 17α-ethynylestradiol [37, 38], diltiazem [39] and cyclosporin [40, 41].

6. Activation of these hydroxylations by α-NF known to enhance P450 3A4 activities [30, 31]. The human P450 3A gene family contains four members 3A3, 3A4, 3A5 and 3A6, presenting more than 85% homology in their primary amino acid sequences [7]. The P450 3A4 isoform is the most important member of the family in human adult liver while P450 3A5 is a minor form expressed in only about 15% of the general population [28]. The P450 3A6 is expressed in the fetus [42] and the P450 3A3 constitutes a very minor form in human liver. Accordingly, our results suggest that the E2-hydroxylations are mainly supported by P450 3A4 although the contribution of the 3A5 isoform cannot be excluded.

However, other P450 isoforms may make some contribution to E2-hydroxylations, especially in individuals with very low amounts of P450 3A4. Indeed, study of E2 metabolism by cytochromes P450 synthesized by human hepatoma Hep G2 cells transfected with different cDNAs established that at least six human cytochromes P450 are capable of hydroxylating this estrogen: 1A2, 2C9, 3A3/3A4/3A5 and 4B1 [8, 26]. Recent studies with cells transformed with cDNAs encoding human P450 1A1 [43] and 1A2 [44] clearly demonstrated that these isoforms are involved in the 2-hydroxylation of E2 at a similar turnover of about 0.7–1.5 min^{-1}. Similarly P450 3A4 catalysed the same reaction with a turnover of about 1.3–3.3 min^{-1} [8, 26, 30]. This observation does not mean that E2 was hydroxylated by all these cytochromes P450 in human liver microsomes because the levels of P450 3A4 vs P450 1A2 are of one order of magnitude in the liver [45]. From these values, it can be estimated that the 2-hydroxylation is mediated at about 80% by P450 3A and 10% by P450 1A enzymes while the 4-hydroxylation is mediated up to about 95% by P450 3A enzyme. Such theoretical values are in full agreement with our results. So, assuming that the residual E2 hydroxylations after gestodene preincubation reflect the contribution of cytochromes P450 other than P450 3A forms, this contribution was estimated to about 25% ($\pm$ 20). Moreover, phenacetin or caffeine which are specific substrates of P450 1A2 [22, 46, 47] inhibited the E2-hydroxylations no more than 20–30% at concentrations as high as 500 μM. This observation may explain that cigarette smoking, inducing P450 1A2, alters the hepatic estrogen metabolism in female heavy smokers [48, 49]. But, as the extent of inter-individual variation of the level of P450 1A2 was by two orders of magnitude in the human liver [45], that could explain the lack of correlation between estradiol hydroxylations and P450 1A activities or content in the samples studied.

Drug interactions

In humans, the aromatic hydroxylations of E2 are the most prominent catabolic pathways quantitatively [1]: 30% of administered [^{3}H]E2 was 2-hydroxylated. The identification of P450 3A4 as the major catalyst in E2 2- and 4-hydroxylations allows some predictions regarding potential drug interactions to be made. Therefore, it can be assumed that these hydroxylations should be modified by all the drugs, steroids and carcinogens metabolized by this cytochrome P450, namely steroids such as testosterone, progesterone, cortisol, E2 and ethynylestradiol or drugs such as nifedipine, quinidine, erythromycin, cyclosporin, midazolam, tamoxifen, diltiazem or carcinogens such as aflatoxin B1 [35] senecionine [50]. In theory, estradiol hydroxylations should be decreased during administration of one of these xenobiotics. However, it should be kept in mind that the extent of such an effect is likely to be modulated by various factors such as dose liver biodisponibility and relative K_m of estradiol and xenobiotics.

Levels of P450 3A4 vary widely among individuals. The known inducibility of the enzyme by barbiturates, rifampicin, some macrolide antibiotics [34] and synthetic corticoids [51] can explain the high levels of the enzyme in some individuals. Mechanism-based inactivation of P450 3A4 can be seen with TAO [34], 17α-ethylnylestradiol [37, 38] and gestodene [18, 35] and therefore can explain the low level of the enzyme in some samples. Accordingly, E2-hydroxylations should be altered by such drugs in the same manner as 17α-ethylnylestradiol and

cyclosporin metabolisms are altered by suicide substrates of progestogens family [52].

From the results reported herein, it should be pointed out that E2 metabolism can be seriously altered by various drugs. So, this observation may be relevant in clinical practice, especially during menopause treatment by E2 reproducing the physiological scheme. Further studies are needed to determine whether humans exposed to inducers or inactivators of P450 3A4 will be more or less susceptible to alteration of E2 metabolism.

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