Effects of cytochrome P450 inducers on 17 α -ethinyloestradiol (EE₂) conjugation by primary human hepatocytes

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Aims Our objective was to elucidate further the underlying mechanism responsible for therapeutic failures observed with concomitant administration of the oral contraceptive 17 α -ethinyloestradiol (EE₂) and rifampicin.

Methods We investigated both oxidative and direct conjugative [³H]-EE₂ metabolism by human liver S9 fraction and the effect of known enzyme-inducing drugs using a human hepatocyte induction model *in vitro*.

Results Cofactor dependent [³H]-EE₂ metabolism by human liver S9 fraction produced 2-hydroxy-[³H]-EE₂, 2-methoxy-[³H]-EE₂, and direct [³H]-EE₂ sulphate and glucuronide conjugates. Only two detectable metabolites of [³H]-EE₂ were produced by the S9 fraction in the presence of all cofactors: [³H]-EE₂-3-sulphate (75.7 $\pm$ 7.6% s.d.) and 2-methoxy-³H-EE₂ (2.6% $\pm$ 0.5% s.d.). Human hepatocytes extensively metabolized [³H]-EE₂ to its glucuronide and sulphate conjugates. Small amounts of a 2-methoxy-[³H]-EE₂ 3-conjugate, $\leq$ 10%, was observed but no. 2-hydroxy-[³H]-EE₂ was detected. An unexpected finding in our study was increased [³H]-EE₂-3-sulphate production (1.5–3.3 fold, n =3 donor livers) by hepatocytes pretreated with rifampicin compared to control hepatocytes. No statistically significant increase in [³H]-EE₂-3-sulphation was observed in hepatocytes pretreated with 3-methylcholanthrene, phenobarbitone, dexamethasone, or omeprazole over nontreated hepatocytes. To our knowledge, this is the first observation of sulphotransferase induction by rifampicin in human hepatocytes *in vitro* resulting in increased [³H]-EE₂ sulphation.

Conclusions Our data indicate that the major EE₂ metabolic products formed by human hepatocytes *in vitro* are direct EE₂ conjugates with EE₂ oxidation representing minor pathways. Further studies are required to establish the mechanism of sulphotransferase induction and the clinical relevance of our findings.

Keywords: conjugation, drug–drug interactions, ethinyloestradiol, glucuronidation, hepatocytes, human, induction, metabolism, sulphation

Introduction

Co-administration of certain drugs with oral contraceptives containing 50 μ g or less of 17 α -ethinyloestradiol; 17 α -ethynyl-1,3,5[10]-estratriene-3,17 β -diol; EE₂ [1] have resulted in menstrual bleeding irregularities and pregnancies. Much of the evidence reported for oral contraceptive failure with concurrent drug use is anecdotal and there is considerable controversy concerning which drugs are involved, the mechanism and clinical relevance of this type of drug–drug interaction [2–6].

There are two main mechanisms by which concurrent

drug use are believed to reduce oral contraceptive steroid efficacy; enzyme-induction and/or impaired enterohepatic circulation. The two drug classes most commonly implicated are anticonvulsants and certain antibiotics [2–4, 7, 8]. Following administration of radioactive EE₂ to humans, the drug is primarily recovered in bile or urine as glucuronide or sulphate conjugates (Figure 1). Urinary recovery of EE₂ derived radioactivity reported ranges from 16 to 54% of an EE₂ dose [9–15] and from 28 to 59% is excreted in the faeces [12–15]. Ring 'A' hydroxylation, of which 2-hydroxy-EE₂ is probably the most important primary oxidative metabolite, can be followed by methylation by catechol-O-methyltransferase to form a secondary metabolite, 2-methoxy-EE₂. These two metabolites are excreted in urine and bile as both glucuronide and sulphate conjugates but

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Figure 1 Major human ethinyloestradiol (EE_2) metabolic pathways.

EE_2 -3-glucuronide and EE_2 -3-sulphate are the predominant species found in these two body fluids following an EE_2 dose [9, 16, 17].

There is considerable evidence that the antibiotics rifampicin [18–20] and rifabutin [21] and the anticonvulsant drugs phenytoin [22], phenobarbitone [23], carbamazepine [22] and oxcarbazepine [24] induce metabolism of EE_2 and significantly lower oestrogen plasma concentrations in humans. However, none of these studies reported the metabolic products produced. Individual CYP450 human liver enzymes reported to oxidize EE_2 include: CYP3A, CYP2C, and CYP2E [25, 26]. The work of Guengerich [25] highlighted CYP3A4, known to be induced by numerous drugs, as the major CYP450 enzyme responsible for EE_2 oxidation. Although EE_2 is known to be predominantly excreted in urine and bile as EE_2 -3-glucuronide and EE_2 -3-sulphate, to our knowledge no human liver studies *in vitro* have been reported concerning the relative effects of drugs known to induce liver enzymes on the individual parallel human EE_2 metabolic pathways shown in Figure 1.

Human primary hepatocytes represent a relevant experimental system for the evaluation of pharmacokinetic drug–drug interactions. In our laboratory human hepatocytes are routinely used as an experimental model for toxicology, metabolism and pharmacokinetic drug interactions [27–30]. We have previously reported that treatment of primary human hepatocytes with rifampicin

led to duration and dose-dependent induction of the rate of CYP3A-dependent metabolic pathways: lignocaine dealkylation [31] and testosterone 6β -hydroxylation [30].

In this report the disposition of [3H]- EE_2 was investigated in human liver S9 fractions, freshly isolated human hepatocytes, and freshly isolated hepatocytes following exposure to the known P450 enzyme inducer drugs rifampicin (CYP3A4, CYP2C9 and CYP2C19), phenobarbitone (CYP3A4), omeprazole (CYP1A2), methylcholanthrene (CYP1A2), and dexamethasone (CYP3A4) [32] *in vitro*. Unlike most previous human liver studies *in vitro* which used EE_2 at μM concentrations, our study used therapeutically relevant [3H]- EE_2 (1 nM) concentrations [21, 24]. In addition we report the results of [3H]- EE_2 metabolism at clinically relevant concentrations by human liver S9 fractions in the presence or absence of several individual cofactors.

This study was presented in part at the 8th North American ISSX Meeting, Hilton Head, SC, October 26–30, 1997.

Methods

Chemicals

Vitrogen was obtained from Collagen Corp., Palo Alto, CA. Fungizone, L-glutamine, and phenol red were procured from Life Technologies, Inc., Grand Island, NY. The BCA Protein Assay ReagentTM kit (232225), containing Pierce working solution, was purchased from Pierce, Rockford IL. 2α -hydroxytestosterone, 7β -hydroxytestosterone, 16α -hydroxytestosterone, 2β -hydroxytestosterone, 6β -hydroxytestosterone, 16β -hydroxytestosterone, and androstenedione were obtained from Steraloids, Inc., Eilton, NH. Collagenase was purchased from Worthington Biochemicals, Freehold, NJ. 17α -[6,7- 3H (N)]-ethinyloestradiol ([3H]- EE_2), Sp. Ac. = 49.1 Ci mmol⁻¹ was obtained from New England Nuclear, MA. Reference EE_2 metabolites: 2-hydroxy- EE_2 , 2-methoxy- EE_2 , EE_2 -3-sulphate, EE_2 -3,17-disulphate, EE_2 -17-sulphate, and EE_2 -17-glucuronide were kindly provided by Dr W. Slikker, National Center for Toxicology (NCTR), AK. All other chemicals were obtained from Sigma Chemical Co., St Louis MO.

Human liver source

Human donor livers unsuitable for transplantation used in this study were acquired from the International Institute for the Advancement of Medicine (IIAM, coordinated from Exton, PA) or the Washington Transplant Consortium (Washington DC). The human donor demographics and medical histories are listed in Table 1.

Preparation of human liver hepatocytes and S9 fractions

Human hepatocytes were isolated according to the two-step collagenase perfusion procedure of Li *et al.* [33]. Isolated hepatocytes were used either as suspension cultures for metabolism studies or as monolayer cultures for enzyme induction studies. Human liver homogenates were centrifuged at 9000 *g* according to the methods described [34, 35]. The resulting supernatant S9 fraction containing both cytosolic and microsomal enzymes was aliquoted and stored frozen at -70°C until used.

Human liver S9 fraction incubations

Human liver S9 fractions prepared from donor livers 1, 2, 3, and 4 were incubated in the presence of individual cofactors. Each S9 incubation contained 1 mg S9 protein and [³H]-EE₂ (1 nM, 9.2×10^4 d min⁻¹). The following cofactors were added to individual incubations: NADPH generating system [glucose-6-phosphate (10 mM), NADP⁺ (1 mM) and glucose-6-phosphate dehydrogenase (1.2 units ml⁻¹); SAM (1 mM) + NADPH generating system; UDPGA (2.5 mM); or PAPS (100 μM); in a 6 ml 100 mM Tris buffer pH 7.8. Incubations were carried out at 37 $^{\circ}\text{C}$ in air for 40 min in a shaking metabolic water bath. The reaction was terminated and protein precipitated by adding 2 ml acetone to the reaction mixture. Incubation media were pelleted and the supernatant analysed by h.p.l.c. for quantification of individual radioactive compounds. Irreversibly bound [³H]-EE₂ derived radioactivity in the pellet was determined as described below.

Hepatocyte enzyme induction procedure

Primary human induction studies were performed using the following protocol developed by Li *et al.* [28, 30, 31]: day 1, freshly isolated human liver hepatocytes were transferred to Vitrogen-coated 24-well plates containing

Dulbecco's modified Eagle's media (DMEM) supplemented with insulin, hydrocortisone, L-glutamine, and MEM nonessential amino acids, BSA, fructose, gentamicin, and amikacin. The culture medium was replaced with sandwich medium after 2–4 h of attachment. The cells were sandwiched with collagen and incubated at 37 $^{\circ}\text{C}$ in a 95% air:5% CO₂. Media changes were carried out daily. On day 3 and day 4 a selected enzyme inducer drug for CYP1A (OMP, 3-MC) or CYP3A (DEX, RIF, or PB) was added and on day 5 the medium was replaced with Krebs-Hensleit buffer and individual drug experiments were performed.

Hepatocyte drug induction experiments

The hepatocyte induction model was validated following pretreatment of freshly isolated hepatocytes (0.7×10^6 cells ml⁻¹ density per well) with 3-MC (1 μM), OMP (33.3 μM), DEX (33.3 μM), or RIF (33.3 μM), and non treated hepatocytes. On day 5, media were removed and the hepatocytes were then incubated with KHB containing either 2 μM ethoxyresorufin or 250 μM testosterone for 60 min at 37 $^{\circ}\text{C}$ under a 90% air:5% CO₂ atmosphere. Following incubations, the CYP1A enzyme activities were determined by measuring the ethoxyresorufin-O-deethylase activity according to the method reported by Burke *et al.* [36]. CYP3A enzyme activity determinations were based on the methods reported by Sonderfan *et al.* [37] and Fuane *et al.* [38].

Hepatocytes isolated from donor livers 1, 2, and 3 were used to investigate the induction of [³H]-EE₂ metabolism after pretreatment of hepatocytes (0.7×10^6 cells ml⁻¹ per well) with known enzyme inducer drugs at the following concentrations: for DEX, RIF, or OMP at 0, 0.33, 1.0, 3.3, 10, and 33.3 μM and for 3-MC or PB at 0, 0.01, 0.033, 0.1, 0.33, and 1 mM. The concentrations used were previously shown by Li *et al.* [28, 30, 31] to be near optimal in the induction of the corresponding CYP isoenzymes. The metabolism of

Table 1 Human donor demographics and medical history.

Liver	Age (years)	Sex	Race	Disease	Medication
1	67	F	Caucasian	Hypertension	Captopril, ranitidine
2	44	M	Caucasian	Arthritis	Diltiazem, aspirin
3	61	F	Caucasian	Basal cell carcinoma	None
4	14	F	Caucasian	Asthma	Phenytoin, ranitidine, cefoxitin, desmopressin, methylprednisolone, phenobarbitone, lorazepam, midazolam, levothyroxine
5	53	F	African-American	Hypertension	Lisinopril
6	51	M	Caucasian	Hypertension, diabetes, arthritis, colitis	Phenelzine, triazolam levothyroxine, insulin, quinine, ketoprofen
7	57	M	Caucasian	Hypertension, diabetes	Enalapril

[^3H]-EE₂ was determined in pretreated and non treated hepatocytes following incubation with [^3H]-EE₂ (1 nM, 1.1×10^5 d min⁻¹/well) for 15 min.

[^3H]-EE₂ metabolic recovery experiments were conducted with freshly isolated hepatocytes in suspension from donor liver 5 and with non treated hepatocytes isolated from donor livers 6 and 7 following pretreatment with rifampicin (33.3 μM). Hepatocytes in suspension (donor liver 5) were incubated with [^3H]-EE₂ (1 nM or 100 nM, 1.1×10^5 d min⁻¹/well) in the presence or absence of 5 μM ketoconazole, a known CYP3 A4 competitive inhibitor ($K_i = 0.7 \mu\text{M}$) [39], for 0.1, 1, 4, and 8 h. The rifampicin pretreated and non treated hepatocyte experiments (donor livers 6 and 7) were incubated with [^3H]-EE₂ (1 nM, 1.1×10^5 d min⁻¹/well) for 0.1, 1, 4, and 8 h in the presence or absence of ketoconazole (5 μM).

Quantification of EE₂ metabolism after treatment with CYP inducers

Both the incubation mixture and cells were stored at -80°C until analysed by h.p.l.c. To analyse the medium, a 500 μl aliquot of medium was added to 2 ml acetone and centrifuged to remove insoluble material. The solvent was then evaporated using first a stream of dry nitrogen and then a vacuum, the residue was reconstituted with 150 μl 15% acetonitrile in water, and 100 μl (10–50 nCi ^3H) was analysed by h.p.l.c. Cells were analysed after releasing them from the flask with 1 ml 0.25% trypsin/0.02% EDTA at 37°C for 10 min. This was added to 2 ml acetone, centrifuged, and dried as above.

Protein bound radioactivity was determined from the radioactivity adhering to the acetone insoluble pellet obtained above. These pellets were washed twice with 5 ml methanol and once with 5 ml water, centrifuging in between. 100 μl acetone washed rat liver homogenate was added to each tube to help maintain the small pellet during the washing procedure. The washed pellets were then disrupted with 500 μl 6 M guanidinium chloride, 10 ml Flo-Scint IV scintillation cocktail was added and the solution was counted for tritium on a Packard Tri-Carb 2500 TR liquid scintillation analyser.

H.p.l.c. conditions consisted of a Hewlett-Packard 1050 h.p.l.c. system with variable wavelength detector. A 250 $\times$ 4.6 mm Alltech Adsorbosphere HS C18 column was used with a mobile phase of 10 mM ammonium phosphate pH 2.7 progressing to 70% acetonitrile/30% 10 mM ammonium phosphate pH 2.7 over 25 min (holding at this composition for 10 min) at a flow rate of 1 ml min⁻¹. Detection was by radioactivity using a Radiomatic Flo-One/Beta detector set for tritium and Ultima-Flo M scintillation cocktail or by u.v. at 265 nm.

Measurement of hepatocyte homogenate protein concentration

Protein content of the freshly isolated human hepatocytes was determined with using a BCA Protein Assay (Pierce Chemical Co., 1993) using BSA as protein standards.

Statistical analysis

Statistical differences between control and induction experiments were determined using the Student's *t*-test. Pairwise comparisons of non treated and treated hepatocyte induction experiments in the presence and absence of ketoconazole were analysed using one way ANOVA and the Tukey test using a confidence interval of 95%. Statistical analyses were performed using SigmaStat for Windows version 2.0 (SPPS Inc., San Rafael CA).

Results

Identification of EE₂ metabolites

EE₂ and its metabolites were identified after h.p.l.c. analysis of the incubation medium by comparing the individual metabolite elution times with synthesized reference compounds (Figure 2a). Following separate incubations of [^3H]-EE₂ with human S9 fractions in the presence of either NADPH, PAPS, or NADPH plus SAM, single h.p.l.c. metabolite peaks were observed with

Figure 2 H.p.l.c. chromatograms of (a) synthesized oestrogen compounds, u.v. detection at 265 nm; (b) extract from 4 h EE₂ (1 nM) incubation with isolated human hepatocytes *in vitro*, radiochemical detection.

elution times corresponding to 2-OH-EE₂ (24.2 min), EE₂-3-sulphate (21.7 min), or 2-methoxy-EE₂ (27.2 min), respectively. Incubation of [³H]-EE₂ with S9 fractions in the presence of UDPGA produced a single peak with an h.p.l.c. elution time of 19.9 min. Although reference EE₂-3-glucuronide was not available and the elution time of the expected [³H]-EE₂-3-glucuronide peak would not be resolved from EE₂-3,17-di-sulphate, the latter compound would not be produced by the S9 fraction in the absence of PAPS cofactor.

Figure 2b is a h.p.l.c. radio-chromatogram of a medium extract following a 4 h incubation of [³H]-EE₂ with human isolated hepatocytes. Peaks observed with elution times of 21.7 min and 26.3 min corresponded to EE₂-3-sulphate and EE₂ reference compounds, respectively. The peak at 19.9 min was identified as EE₂-3-glucuronide similar to that when [³H]-EE₂ was incubated with S9 fraction in the presence of UDPGA. Although this peak could represent EE₂-3,17-sulphate, it is highly unlikely since this compound was not observed following incubation of [³H]-EE₂ with S9 fraction in the presence of PAPS cofactor. Finally, the 2-methoxy-EE₂-conjugate was identified following enzyme hydrolysis by glucuronidase and sulphatase enzymes. The resultant hydrolysed compound had an elution time of 27.2 min corresponding to 2-methoxy-EE₂ (Figure 2a).

Human liver S9 fraction incubations in vitro

[³H]-EE₂ drug metabolism by human liver S9 fractions prepared from donor livers 1, 2, and 3 were investigated in the presence or absence of individual cofactors. Formation of [³H]-EE₂ metabolites was quantified by h.p.l.c. [³H]-EE₂ incubation with three individual donor liver S9 fractions (Table 2) in the presence of NADPH produced 3.5–5.1% 2-hydroxy-EE₂ and 4.6–9.5% of [³H]-EE₂ derived radioactivity was covalently bound to S9 protein. When [³H]-EE₂ was incubated with human liver S9 fractions in the presence of UDPGA, 5.7–8.7% of the added [³H]-EE₂ was converted directly to [³H]-EE₂-3-glucuronide. In the presence of PAPS [³H]-EE₂ was rapidly and extensively converted, 67.2–78.7%, to [³H]-EE₂-sulphate. Minimal covalent binding was observed in the presence of either UDPGA (0.9–1.4%) or PAPS (1.1–1.8%).

Further cofactor incubation experiments were carried out using human liver S9 fraction prepared from donor liver 4 to investigate cofactor dependence on the disposition of [³H]-EE₂. The results listed in Table 3 for incubations of [³H]-EE₂ (1 nM) in the presence of three individual cofactors NADPH, UDPGA, or PAPS produced similar results to those listed in Table 2 for donor livers 1, 2, and 3. When SAM and NADPH were added to the media, 2-methoxy-[³H]-EE₂ was the predominate

Table 2 EE₂ (1 nM) incubation (40 min) with human liver S9 fraction. Individual metabolites formed as percent total added radioactivity: cofactor dependence.

Donor	Cofactor	Metabolite	Pellet*
<i>2-hydroxy-EE₂</i>			
1	NADPH	3.5 (0.7)	8.8 (0.6)
2	NADPH	5.3 (2.8)	9.5 (0.7)
3	NADPH	5.1 (3.1)	4.6 (0.3)
<i>EE₂-3-glucuronide</i>			
1	UDPGA	5.7 (0.8)	1.0 (0.1)
2	UDPGA	8.7 (1.2)	0.9 (0.1)
3	UDPGA	8.7 (2.2)	1.4 (0.1)
<i>EE₂-3-sulphate</i>			
1	PAPS	n.a.	n.a.
2	PAPS	78.7 (3.3)	1.8 (0.1)
3	PAPS	67.2 (2.3)	1.1 (0.1)

Numbers in parentheses are $\pm$ s.d., $n=3$ determinations. n.a. = not available.

*Pellet represents [³H] covalently bound to cellular protein.

molecular species observed (mean = $15.6 \pm 3.1\%$ s.d.) and covalent binding was reduced to a mean of $7.3 \pm 0.1\%$ s.d. compared with $15.2 \pm 7.3\%$ s.d. when only NADPH was present. When SAM and NADPH were present, 2-hydroxy-[³H]-EE₂ represented a mean $1.9\% \pm 0.7\%$ s.d. of metabolized [³H]-EE₂. When all four cofactors were incubated with [³H]-EE₂, small amounts of covalently bound radioactivity ($1.7 \pm 0.1\%$ s.d.) and 2-methoxy-[³H]-EE₂ ($2.6 \pm 0.5\%$ s.d.) were observed, however, similar to the data shown in Table 2 the major metabolite formed was [³H]-EE₂-3-sulphate (mean = $75.7 \pm 7.6\%$ s.d.). No 2-methoxy-[³H]-EE₂-3-conjugate was detected.

Validation of human hepatocyte induction model

The isolated liver hepatocyte induction model was tested using freshly prepared hepatocytes from donor liver 1. Production rates of 6 β -hydroxytestosterone from testosterone by human liver hepatocytes pretreated with rifampicin or dexamethasone were significantly greater ($P < 0.001$) than that observed for control hepatocytes as shown in Figure 3a. Likewise Figure 3b shows that pretreatment of hepatocytes with 3-methylcholanthrene or omeprazole significantly increased the conversion rate of ethoxyresorufin to desethyl ethoxyresorufin ($P < 0.001$) compared with control hepatocytes.

Rates of [³H]-EE₂ metabolism by inducer pretreated and non treated isolated human hepatocytes

Freshly prepared hepatocytes from the same individual donor livers 1, 2, and 3 used in the S9 experiments were

Metabolite	NADPH	SAM+ NADPH	UDPGA	PAPS	All
2-Hydroxy-EE ₂	5.6 (3.2)	1.9 (0.7)	n.d.	n.d.	n.d.
2-Methoxy-EE ₂	n.d.	15.6 (3.1)	n.d.	n.d.	2.6 (0.5)†
EE ₂ -3-glucuronide	n.d.	n.d.	13.3 (0.7)	n.d.	n.d.
EE ₂ -3-sulphate	n.d.	n.d.	n.d.	69.5 (6.2)	75.7 (7.6)
Pellet‡	15.2 (0.4)	7.3 (0.1)	1.5 (0.1)	1.3 (0.1)	1.7 (0.1)

Numbers in parentheses are $\pm$ s.d., $n=3$ determinations. n.d.=not detected.

*Donor liver 4.

†2-Methoxy-EE₂-3-conjugate not detected.

‡Pellet represents [³H] covalently bound to cellular protein.

Table 3 EE₂ (1 nM) incubation (40 min) with human liver* S9 fraction.

Individual metabolites as percent total radioactivity added: cofactor dependence.

Figure 3 Rates of metabolite formation following pretreatment of hepatocytes prepared from Donor 1 with 3-methylcholanthrene (1 μ M), omeprazole (33 μ M), dexamethasone (33 μ M), rifampicin (33.3 μ M) or non treated. (a) conversion rates of testosterone (250 μ M) to 6 β -hydroxy-testosterone, 60 min incubation; (b) conversion rates of ethoxyresorufin (2 μ M) to resorufin, 60 min incubation. Each data point represents three individual determinations. Error bars are $\pm$ s.d.

either non treated or pretreated with varying concentrations of the P450 enzyme inducer, rifampicin. Following the treatment period, [³H]-EE₂ (1 nM) conversion rates to [³H]-EE₂-3-glucuronide and [³H]-EE₂-3-sulphate were determined. As shown in Figure 4a, pretreatment of hepatocytes with rifampicin at 3.3, 10, and 33.3 μ M concentrations resulted in significant increases in the conversion rates of [³H]-EE₂-[³H]-EE₂-3-sulphate compared with non treated hepatocytes ($P<0.01$). With the exception of 0.3 μ M rifampicin treated sample, $P=0.03$, no significant differences

Figure 4 Rates of (a) [³H]-EE₂-3-sulphate or (b) [³H]-EE₂-3-glucuronide formation by non treated or pretreated human hepatocytes with varying rifampicin concentrations and following a 15 min [³H]₂-EE₂ (1 nM) incubation with: (■) Donor liver 1; (□) Donor liver 2; (▒) Donor liver 3. $n=3$ determinations $\pm$ s.d. for each experiment.

($P>0.2$) were observed between treated and non treated hepatocytes in the production of [³H]-EE₂-3- glucuronide (Figure 4b).

In addition to the rifampicin experiments, human liver hepatocytes prepared from donor livers 3 and 4 were pretreated with varying concentrations of 3-methylcholanthrene, omeprazole, phenobarbitone or dexamethasone. Although some individual experiments showed statistical differences between rates of [³H]-EE₂ conversion to [³H]-EE₂-3-glucuronide and [³H]-EE₂-3-sulphate for pretreated compared with non treated hepatocytes, no consistent significant pattern was established.

[³H]-EE₂ metabolic time profiles for rifampicin treated and non treated human hepatocyte experiments

Mass balance experiments were conducted with non treated freshly prepared hepatocyte suspensions from liver donor 5 and with rifampicin pretreated or non-treated hepatocytes from donor livers 6 and 7 to determine the fate of [³H]-EE₂ derived radioactivity added to the medium. The recovery of [³H]-EE₂ derived radioactivity was examined following incubation of [³H]-EE₂ (1 nM) with hepatocytes for 0.1, 1.0, 4.0, or 8.0 h in the presence or absence of ketoconazole (5 µM). H.p.l.c. analysis of media extracts using radiochemical detection provided metabolic time profiles for the recovery experiments. Results of this analysis are shown for donor liver 5 and donor liver 6 in Figure 5 and Figure 6, respectively. Results obtained for donor liver 7 were similar to those observed for donor liver 6 (data for donor liver 7 not shown). [³H]-EE₂ metabolic peaks identified in the h.p.l.c. radiochromatograms were [³H]-EE₂-3-sulphate, [³H]-EE₂-3-glucuronide, and a 2-methoxy-[³H]-EE₂-3-conjugate. no 2-hydroxy-[³H]-EE₂ or its conjugates were observed in any of the [³H]-EE₂ hepatocyte incubations.

One way ANOVA and pairwise comparison using the Tukey Test were performed on the 1 h and 8 h time points to establish the effect of rifampicin pretreatment and addition of 5 µM ketoconazole to each set of donor liver experiments. Parent drug remaining in the media following a 8 h incubation was ≤10% for all incubations shown in Figure 5 and Figure 6. No statistical differences

Figure 5 Media metabolites recovered after incubations of [³H]-EE₂ (1 nM) on day 0 with human hepatocytes in suspension prepared from Donor liver 5. a) Hepatocytes and [³H]-EE₂ (1 nM) b) Hepatocytes, [³H]-EE₂ (1 nM), and ketoconazole (5 µM). Symbols represent: (○) EE₂; (▽) 2-methoxy-EE₂-3-conjugate; (△) EE₂-3-glucuronide; (□) EE₂-3-sulphate. Each data point represents three individual determinations and error bars are s.d.

Figure 6 Media metabolites recovered after incubation of [³H]-EE₂ (1 nM) with human liver hepatocytes prepared from Donor liver 6: a) Non treated hepatocytes b) Rifampicin treated (33 µM). c) Non treated hepatocytes and ketoconazole (5 µM). d) Rifampicin treated (33 µM) and ketoconazole (5 µM). Symbols represent: (○) EE₂; (▽) 2-methoxy-EE₂-3-conjugate; (△) EE₂-3-glucuronide; (□) EE₂-3-sulphate. Each data point represents three individual determinations and error bars are s.d.

were observed in metabolic profiles obtained for donor liver 5 hepatocytes following incubations of [³H]-EE₂ on day 0 in the absence (Figure 5a) or presence (Figure 5b) of 5 µM ketoconazole. Pretreatment of hepatocytes prepared from donor liver 6 (Figure 6b) with rifampicin resulted in a statistically significant increase in production of [³H]-EE₂-3-sulphate from [³H]-EE₂ compared with untreated hepatocytes (Figure 6a). Similarly, in the presence of 5 µM ketoconazole, increased amounts of [³H]-EE₂-3-sulphate were observed for rifampicin treated hepatocyte (Figure 6d) compared with non rifampicin treated hepatocytes (Figure 6c). Addition of ketoconazole (5 µM) inhibitor appeared to have no effect on the induction of [³H]-EE₂ sulphation by rifampicin. There was a statistically significant decrease in the production of [³H]-EE₂-3-glucuronide in the rifampicin pretreated hepatocyte experiments compared with controls. Ketoconazole appeared to have no effect on either sulphation or glucuronidation.

Production of the 2-methoxy-[³H]-EE₂-3-conjugate

was minimal (less than 5%) in hepatocytes prepared from all three donor livers under all incubation conditions. A statistical analysis of the [^3H]-2-methoxy-EE₂-3-conjugate was not attempted due to the low number of radioactive counts observed in the h.p.l.c. radiochromatograms. Unlike the S9 studies, there was no detectable [^3H]-EE₂ derived radioactivity covalently bound to cellular protein.

Discussion

Numerous reports in the literature have implicated drug–drug interactions for decreased effectiveness of EE₂ and therapeutic failures of oral contraceptives (for recent reviews see Helms *et al.* [2]; Shenfield & Griffin [3], and Orme *et al.* [4]). The two main mechanisms by which simultaneous administration of oral contraceptive pills and other drugs are believed to reduce oral contraceptive steroid efficacy are enzyme induction and impaired enterohepatic circulation. EE₂ is excreted in urine and bile after oxidation and direct conjugation reactions, however, most investigations have focused on induction of the major CYP450 oxidative pathway that converts EE₂ to the catechol, 2-hydroxy-EE₂. Individual CYP450 enzymes reported to catalyse this reaction include: CYP2E, CYP2C, and CYP3A [25, 26]. The work of Guengerich [23] highlighted CYP3A4, known to be induced by numerous drugs, as the major CYP450 enzyme responsible for EE₂ oxidation. In this regard, evidence exists in the literature implicating that coadministration of rifampicin and oral contraceptives can result in therapeutic failures [18–20].

Urinary and biliary excretion of radioactivity following human ingestion of [^3H]-EE₂ has been studied by a few investigators and their results suggest that EE₂ oxidation may represent a minor elimination pathway compared to direct EE₂ conjugation [13–16]. The available data suggest that factors other than induction of EE₂ oxidative enzymes could contribute to the observed therapeutic failures when rifampicin is coadministered with oral contraceptive pills.

Our investigation examined the fate of [^3H]-EE₂ when incubated with human liver S9 fractions and freshly isolated human hepatocytes and points out the importance of using intact liver cells when evaluating relative contributions of parallel metabolic pathways that include direct conjugation of the parent drug. Similar to other authors, we demonstrated that human liver fractions in the presence of NADPH are capable of producing both 2-methoxy-EE₂ and 2-hydroxy-EE₂ and that during EE₂ oxidation, a highly reactive species is formed which covalently binds to cellular protein [40–46]. Formation of EE₂-3-glucuronide or EE₂-3-sulphate, was also observed when the appropriate cofactor was added to the

medium as reported by others [47, 48] (Tables 2 and 3). However, when all cofactors including NADPH were added to the medium EE₂-3-sulphate predominated representing 76% of the metabolized EE₂ (Table 3). Although our results with S9 human liver fractions *in vitro* suggest the importance of EE₂ conjugation, the artificial nature of the system, i.e. addition of cofactors, limits reliable extrapolation of our results to humans *in vivo*.

Freshly isolated human hepatocytes represent a more relevant model for predicting the relative contributions of parallel metabolic pathways than sub cellular liver fractions since hepatocytes contain the full complement of metabolizing enzymes and cofactors present in the intact liver. In addition, they offer the capability to investigate induction of liver enzymes and the resulting effect on a drug's metabolism. The results of our EE₂ human liver hepatocyte metabolism experiments *in vitro* are consistent with a number of observations reported in humans and demonstrate the advantage of hepatocytes for these types of drug metabolism studies. Firstly, we observed that the EE₂ oxidation leading to 2-hydroxy-EE₂ and a secondary metabolite, 2-methoxy-EE₂, followed by conjugation may represent a minor metabolic pathway similar to that observed in human subjects. Under our experimental conditions, no 2-hydroxy-EE₂ was detected. Although it is possible that some 2-hydroxy-[^3H]-EE₂ was formed and then reacted to form covalently bound species, the mass balance indicates that it could be only a very small fraction of total metabolism. Also, less than 10% of metabolized [^3H]-EE₂ was observed as a 2-methoxy-[^3H]-EE₂ conjugate (Figures 5 and 6). Secondly, we have demonstrated that human liver hepatocytes are capable of forming EE₂-3-glucuronide and EE₂-3-sulphate. A comparison of the data in Figure 5a and Figure 6a demonstrates the individual variation in the relative amounts of EE₂-3-glucuronide and EE₂-sulphate produced and is similar to observations reported for EE₂ conjugates excreted in human urine and bile following EE₂ administration [13]. The rapid metabolism of EE₂ to EE₂-3-sulphate by human liver hepatocytes shown in Figures 5 and 6 points out the importance of obtaining early time points during hepatocyte incubation experiments.

Direct conjugation and excretion of EE₂ as opposed to EE₂ oxidation represents the predominant elimination pathway for this drug in both human hepatocytes *in vitro* and humans *in vivo*. These observations suggest that mechanisms other than induction of P450 enzymes by known inducer drugs may be partly responsible for therapeutic failures observed with administration of oral contraceptives.

We observed no induction of EE₂-3-glucuronide formation in rifampicin treated hepatocytes over non

treated. Although Li *et al.* [49] observed a slight increase (0.3 fold) in zidovudine glucuronidation by rifampicin treated hepatocytes, this was only observed in one of two livers examined. A novel finding of our investigation supports this hypothesis in that metabolism of EE₂ to EE₂-3-sulphate by human liver hepatocytes prepared from three individual donor livers was induced following pretreatment with the known enzyme inducer drug, rifampicin. Kern *et al.* [50] observed increased sulphation of *p*-nitrophenol following rifampicin (50 µM) pretreatment of rat or human hepatocytes for 7 and 12 days. Increases in the sulphation of *p*-nitrophenol by pretreated hepatocytes were 2.0–2.5 fold (rats) and 2.0 fold (human) compared with controls. Our study did not directly address the individual enzyme responsible for EE₂ sulphation and there has been some controversy over the identity of human ST(s) responsible for the sulphation of oestrogens and EE₂. Falany *et al.* [51] reported that EE₂ at 1.5 µM was sulphated to form EE₂-3-sulphate by human cytosolic phenol sulphotransferase (P-PST) expressed in bacteria. A distinct human oestrogen sulphotransferase (hEST-1) was isolated by Falany *et al.* [52] from human liver and expressed in two bacterial expression systems. This enzyme which maximally sulphates beta estradiol and estrone at concentrations of 20 nM is also capable of sulphating EE₂. Frobes-Bamforth & Coughtrie [53] presented evidence for the existence of a sulphotransferase in adult human liver which sulphates both estrone and EE₂ but not planar phenols. Based on this information, it appears likely that at 1 nM EE₂ concentrations the enzyme described by Frobes-Bamforth is responsible for the conversion of EE₂ to its sulphated conjugate observed in our study. If this assumption is correct, this is the first demonstration of induction of oestrogen sulphotransferase by rifampicin in human hepatocytes, however, further studies will be required to confirm this hypothesis.

In summary, our results show that primary human hepatocyte cultures possess extensive sulphation and glucuronidation activities and therefore can be applied as an experimental model for the evaluation of drug–drug interactions involving phase II conjugating pathways. Our study represents the first to suggest induction of EE₂-3-sulphation by pretreatment of human hepatocytes with rifampicin. The major EE₂ metabolic products observed in human hepatocytes *in vitro* are direct EE₂ conjugates with EE₂ oxidation representing minor pathways. Further studies are required to establish the clinical relevance of our findings.

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