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EFFECT OF THE PROGESTOGENS, GESTODENE, 3-KETO DESOGESTREL, LEVONORGESTREL, NORETHISTERONE AND NORGESTIMATE ON THE OXIDATION OF ETHINYLOESTRADIOL AND OTHER SUBSTRATES BY HUMAN LIVER MICROSOMES

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Summary—A number of different progestogens, levonorgestrel (LNG), norethisterone (NET), gestodene (GSD), desogestrel (DG) and norgestimate (NORG) are used in combination with the oestrogen ethinyloestradiol (EE_2) in oral contraceptive steroid preparations. All the progestogens are acetylenic steroids and previous studies have indicated the potential of acetylenic steroids to cause mechanism-based or "suicide" inactivation of cytochrome *P*-450.

We have compared the effects of the different progestogens on EE_2 2-hydroxylation (a reaction catalyzed by enzymes from the *P*-450IIC, *P*-450IIIA and *P*-450IIE gene families) and also the oxidative metabolism of other drug substrates (cyclosporin, diazepam, tolbutamide) by human liver microsomes. On coincubation with EE_2 as substrate, GSD, 3-keto desogestrel (3-KD, the active metabolite of desogestrel) and LNG produced some concentration-dependent inhibition of EE_2 2-hydroxylation (maximum 32% inhibition at 100 μM 3-keto desogestrel). K_i values determined for GSD and 3-KD were 98.5 ± 12.3 and 93.2 ± 10.3 μM (mean $\pm$ SD; $n = 4$), respectively. Preincubation of progestogens in a small volume (50 μl) incubation for 30 min in the presence of an NADPH-generating system enhanced the inhibitory potential of all the steroids (at 100 μM, inhibition was for GSD 39%, 3-KD 46%, LNG 46%, NET 51% and NORG 43%). Inhibitory effects were therefore comparable and also similar to the macrolide antibiotic troleandomycin. The most marked inhibition seen was of diazepam N-demethylation and hydroxylation by GSD (71 and 57%, respectively) and 3-KD (62 and 50%, respectively).

In preincubation studies involving cyclosporin as the substrate, the order of inhibitory potency was GSD > 3-KD > NET > LNG for production of both metabolite M17 and M21.

The results of the study indicate that all the progestogens in common use have the propensity to inhibit a number of oxidative pathways but there is little evidence for one progestogen being more markedly inhibitory than others.

INTRODUCTION

A major route of metabolism of the oral contraceptive steroid ethinyloestradiol (EE_2) is cytochrome *P*-450 dependent 2-hydroxylation to form the catechol oestrogen 2-hydroxy ethinyloestradiol [1–4]. Recent evidence has shown that the reaction is catalyzed by cytochromes from the *P*-450IIC, *P*-450IIE and *P*-450IIIA gene families [5, 6]. The work of Guengerich [5] highlights *P*-450IIIA4 (*P*-450NF) as a major enzyme involved and also shows EE_2 to be a mechanism-based inhibitor of its own metabolism. Mechanism-based or "suicide" inactivation can also occur with other acetylenic steroids including progestogens [7]. Since progestogens are used with EE_2 in combined oral contraceptive preparations, it is important to know whether a progestogen can inhibit/inactivate the isozyme(s) responsible for EE_2 2-hydroxylation. Recently, Jung-Hoffman and Kuhl [8] have suggested, on the basis of higher EE_2 plasma concentrations in women taking an OC containing EE_2 and gestodene compared to those taking EE_2 and desogestrel, that gestodene may have a more pronounced inhibitory effect on EE_2 oxidative metabolism than desogestrel.

The aim of the present work was to compare the effects of various progestogens on EE_2 2-hydroxylation using human liver microsomes *in vitro*. In addition, we have examined the effect of the progestogens on other drug substrates.

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MATERIALS AND METHODS

Chemicals

17α-[6,7-^{3}H]ethinyloestradiol was purchased from New England Nuclear, Stevenage, Herts, U.K. [2-^{14}C]diazepam and [^{3}H]cyclosporin were obtained from Amersham International, Bucks, U.K. 17α-Ethinyloestradiol, levonorgestrel, diazepam, norediazepam, temazepam, NADPH, glucose 6-phosphate, $NADP^+$, glucose-6-phosphate dehydrogenase were obtained from Sigma Chemical Company Ltd, Poole, Dorset, U.K. 2-Hydroxy ethinyloestradiol was a gift from Professor Knuppen, Lubeck, F.R.G. Gestodene and 3-keto desogestrel were gifts from Schering AG, Berlin and Organon, Oss, respectively. Tolbutamide, 4-hydroxy tolbutamide and chlorpropamide were gifts from Dr M. Schorr, Hoescht AG, Frankfurt, F.R.G. Cyclosporin and metabolites M17 and M21 were gifts from Dr Maurer, Sandoz Basel, Switzerland. Troleandomycin was a gift from Dr M. Lennard, Sheffield, U.K. All other reagents were purchased from BDH Chemical Company Ltd, Poole, Dorset, U.K. and were of analytical grade.

Human livers

Human livers were obtained from renal transplant donors soon after clinical death. Consent for their removal was obtained from the donor's relatives and ethical approval was granted for their use in this study. Livers were stored as 10–20 g portions at −80°C until use. Washed microsomes (105,000 g pellets) were prepared using the classical differential sedimentation method as previously described [4]. Cytochrome *P*-450 was assayed by the method of Omura and Sato [9]. Microsomal protein was determined by the method of Lowry *et al.* [10].

Enzyme Assays

Ethinyloestradiol 2-hydroxylase.

1. Coincubation experiment—Ethinyloestradiol (EE_2) 2-hydroxylation was determined as follows: incubations containing ^{3}H-EE_2 (25 μM; 0.5 μCi), progestogen (0–100 μM), microsomal protein (1.5–2 mg), NADPH (0.6 mM), ascorbate (1 mM) and 0.67 M phosphate buffer (pH 7.4) to a final volume of 2.5 ml were performed at 37°C with gentle agitation for 8 min. EE_2 and metabolites were extracted into ether and quantified by radiometric HPLC [4, 6].

The kinetic parameters (V_{max} and K_m) of ethinyloestradiol 2-hydroxylation (EE_2 concentration, 5–40 μM) were determined in the absence and presence of each of the progestogens (gestodene, 3-keto desogestrel, desogestrel, levonorgestrel and norgestimate; 10–100 μM) by non-weighted regression analysis from Lineweaver–Burk plots ($1/V$ against $1/[S]$). The apparent K_i was determined graphically from a plot of $1/V$ against $[I]$.

2. Preincubation experiments—Human liver microsomes containing 250 pmol of cytochrome *P*-450, were incubated at 37°C for 30 min in the presence of 0.067 M phosphate buffer (pH 7.4), progestogen (10 or 100 μM) and either NADPH (0.6 mM) or an NADPH-generating system (glucose 6-phosphate, 10 mM; glucose 6-phosphate dehydrogenase, 0.5 mM; $NADP^+$, 2 IU) in a total volume of 50 μl. For the subsequent assay of ethinyloestradiol 2-hydroxylase activity, 0.9 ml of a solution composed of 0.067 M phosphate buffer, ascorbate (1 mM), ^{3}H-EE_2 (25 μM; 0.5 μCi) and either NADPH (0.6 mM) or the generating system (as above) was added. The reaction proceeded at 37°C for 8 min with gentle agitation. EE_2 and metabolites were extracted into ether and analyzed by radiometric HPLC.

In one series of preincubation experiments troleandomycin (10 and 100 μM) was used as the alleged inhibitor of enzyme activity.

Tolbutamide 4-hydroxylase. Incubations containing tolbutamide (100 μM), progestogen (0–25 μM), microsomal protein (2 mg), NADPH (0.6 mM), $MgCl_2$ (5 mM), EDTA (1 mM), KCl (1 mM) and 0.067 M phosphate buffer (pH 7.4) to a total volume of 2.5 ml were performed at 37°C with gentle agitation for 8 min. The reaction was terminated by the addition of 6 M HCl (100 μl). Unmetabolised tolbutamide and hydroxytolbutamide were extracted in ether (5 ml) for 10 min. The ether was evaporated to dryness and the residue reconstituted in methanol (75 μl) before analysis by HPLC [11, 12].

Diazepam hydroxylase and N-demethylase. Incubations containing ^{14}C-diazepam (25 μM; 0.5 μCi), progestogen (0–100 μM), microsomal protein (2 mg), NADPH (1 mM) and 0.067 M phosphate buffer (pH 7.4) to a final volume of 2.5 ml were performed at 37°C with gentle agitation for 12 min. The reaction was terminated

by extraction with diethylether (5 ml) of the unchanged diazepam and metabolites. Diazepam, the hydroxylated product temazepam, and the N-demethylated metabolite nordiazepam were quantified using HPLC with spectrophotometric (u.v. at 280 nM) and radiometric detection. Separations were performed using a μ-Bondpak C_{18} radial compression cartridge and a mobile phase of ammonium phosphate buffer (pH 3, 0.05%):methanol:acetonitrile (45:45.10), run isocratically at a flow rate of 1.8 ml/min. Under these conditions the retention times for diazepam, temazepam and nordiazepam were 11, 7.5 and 9 min, respectively.

Cyclosporin hydroxylase and N-demethylase. Human liver microsomes containing 1.5 mg protein, were incubated at 37°C for 30 min in the presence of 0.067 M phosphate buffer (pH 7.4), progestogen (0–50 μM) and NADPH (1 mM) in a total volume of 50 μl. For the subsequent assay of cyclosporin enzyme activity, 0.95 ml of a solution composed of 0.067 M phosphate buffer, ^{3}H-cyclosporin (5 μM; 0.2 μCi) and NADPH (1 mM) was added. The reaction proceeded at 37°C for 20 min and was terminated by extraction into ether and analyzed by radiometric HPLC as previously described [13]. Metabolites were identified according to the retention times of the authentic standards M17 and M21. However, because of the complicated nature of cyclosporin metabolism, this must be regarded as tentative and we await definitive designation of the metabolites.

All results are expressed as enzyme activity (nmol or pmol of metabolite formed per minute per nmol of cytochrome *P*-450 in the incubation). The inhibitory effect of the various progestogens can be seen by comparison with control (no progestogen) incubations.

RESULTS

Results from the coincubation studies of various progestogens with EE_2 are shown in Table 1. On a percentage basis, 3-keto desogestrel produced slightly more inhibition than either gestodene or levonorgestrel.

The K_m value for EE_2 2-hydroxylase determined by regression analysis from Lineweaver–Burk plots with mean (±SD) data from six livers was 20.6 ± 6.4 μM. The K_i values for gestodene, 3-keto desogestrel, desogestrel, levonorgestrel and norgestimate were 98.5 ± 12.3, 93.2 ± 10.3, 53.7 ± 13.6, 39.2 ± 18 and 74.0 ± 39, respectively.

Table 1. Inhibition of microsomal ethinyloestradiol 2-hydroxylase activity by coincubation with various progestogens

		Enzyme activity (nmol of 2-OHEE$_2$ formed/min/nmol *P*-450) Mean ± SD
Control		1.24 ± 0.41
GSD (μM)	25	1.22 ± 0.44 (98)
	50	1.10 ± 0.48 (89)
	75	1.07 ± 0.44 (86)
	100	1.01 ± 0.41 (81)
3-KD (μM)	25	1.07 ± 0.40 (86)
	50	0.93 ± 0.41 (75)
	75	0.89 ± 0.32 (72)
	100	0.84 ± 0.33 (68)
LNG (μM)	25	1.19 ± 0.40 (96)
	50	1.16 ± 0.43 (94)
	75	1.06 ± 0.39 (85)
	100	1.00 ± 0.36 (81)

Numbers in parentheses are the percentage of the control value. Data from 5 livers. GSD = gestodene; 3-KD = 3-keto desogestrel; LNG = levonorgestrel.

The two other substrates studied on coincubation with progestogens were diazepam and tolbutamide. Results are shown in Table 2 and indicate that at a concentration of 100 μM both gestodene and 3-keto desogestrel produced more than 50% inhibition of diazepam N-demethylation (gestodene 71%; 3-keto desogestrel, 62%) and diazepam hydroxylation (gestodene 57%; 3-keto desogestrel, 50%). Inhibition of tolbutamide hydroxylation by gestodene (25 μM) was 45% and by 3-keto desogestrel was 26%. For both substrates, levonorgestrel was apparently less inhibitory than either gestodene or 3-keto desogestrel.

In preincubation studies involving five different progestogens and EE_2 there were two experimental protocols which involved having either NADPH or an NADPH generating system in the incubation. Results are shown in Table 3 and indicate similar inhibition produced by gestodene, 3-keto desogestrel, levonorgestrel, norethisterone and norgestimate at a concentration of 100 μM. The inhibition produced by the macrolide antibiotic, troleandeomycin is shown in Table 4. At a concentration of 100 μM, EE_2 2-hydroxylase activity was reduced to 62%.

In preincubation studies involving cyclosporin as the substrate, the order of inhibitory potency was gestodene > 3-keto desogestrel > norethisterone > norgestrel for production of both metabolite M17 and M21 (Table 5). The high standard deviation seen in the data with cyclosporin was due to very high metabolite production by one of the livers used in this

Table 2. Inhibition of diazepam (to nordiazepam and temazepam) and tolbutamide (to 4-hydroxytolbutamide) metabolism by coincubation with various progestogens

	Enzyme activity (pmol of metabolite formed/min/nmol *P*-450)		
	Diazepam N-demethylation	Diazepam hydroxylation	Tolbutamide hydroxylation
Control	258 ± 53	550 ± 220	125 ± 70
GSD (μM)			
5	155 ± 33 (60)	328 ± 88 (60)	108 ± 58 (86)
10	140 ± 29 (54)	315 ± 91 (57)	—
15	—	—	81 ± 43 (65)
25	118 ± 17 (46)	308 ± 61 (56)	69 ± 43 (55)
50	85 ± 24 (33)	250 ± 94 (46)	—
100	75 ± 33 (29)	238 ± 104 (43)	—
3-KD (μM)			
5	178 ± 46 (69)	445 ± 183 (81)	110 ± 62 (88)
10	145 ± 44 (56)	390 ± 155 (71)	—
15	—	—	97 ± 45 (78)
25	110 ± 37 (43)	333 ± 159 (61)	92 ± 56 (74)
50	98 ± 33 (38)	303 ± 132 (55)	—
100	98 ± 31 (38)	275 ± 128 (50)	—
LNG (μM)			
5	200 ± 50 (78)	548 ± 217 (100)	111 ± 60 (89)
10	188 ± 45 (73)	535 ± 202 (98)	—
15	—	—	111 ± 66 (89)
25	190 ± 45 (74)	525 ± 198 (96)	102 ± 59 (82)
50	195 ± 49 (75)	518 ± 199 (95)	—
100	183 ± 49 (71)	492 ± 175 (89)	—

Values are mean ± SD. Data from 4 livers.
Numbers in parentheses are the percentage of the control value.

Table 3. Inhibition of microsomal ethinyloestradiol 2-hydroxylase activity by preincubation with various progestogens. Incubations contained either NADPH or a NADPH regeneration system (see Methods for details). Results are expressed as mean ± SD

	Enzyme activity (nmol of 2-OHEE$_2$ formed/min/nmol *P*-450)	
	NADPH ($n = 5$)	NADPH regenerating system ($n = 6$)
Control	0.83 ± 0.17 (100)	0.87 ± 0.29 (100)
GSD (μM)		
10	0.71 ± 0.13 (86)	0.65 ± 0.29 (75)
100	0.52 ± 0.11 (63)	0.53 ± 0.21 (61)
3-KD (μM)		
10	0.75 ± 0.14 (90)	0.76 ± 0.35 (87)
100	0.55 ± 0.15 (66)	0.47 ± 0.17 (54)
LNG (μM)		
10	0.77 ± 0.16 (93)	0.60 ± 0.11 (69)
100	0.55 ± 0.22 (66)	0.47 ± 0.13 (54)
NET (μM)		
10	0.72 ± 0.17 (87)	0.64 ± 0.17 (74)
100	0.54 ± 0.05 (65)	0.43 ± 0.10 (49)
NORG (μM)		
10	0.63 ± 0.23 (76)	0.64 ± 0.13 (74)
100	0.57 ± 0.04 (69)	0.50 ± 0.11 (57)

Numbers in parentheses are the percentage of the control value.
GSD = gestodene; 3-KD = 3-keto desogestrel; LNG = levonorgestrel; NET = norethisterone; NORG = norgestimate.

Table 4. Inhibition of microsomal ethinyloestradiol 2-hydroxylase activity by preincubation with troleandomycin

		Enzyme activity (nmol of 2OHEE$_2$ formed/min/nmol *P*-450) in liver			
		L8	L9	B1	Mean ± SD
Control		2.17	0.75	1.19	1.37 ± 0.73
Troleandomycin (μM)	10	1.47	0.46	1.02	0.98 ± 0.51 (72)
	100	1.17	0.42	0.95	0.85 ± 0.39 (62)

Numbers in parentheses are the percentage of the control value.

Table 5. Inhibition of cyclosporin metabolism (to M17 and M21) by preincubation with various progestogens

		Enzyme activity (pmol of metabolite formed/min/nmol *P*-450)	
		Cyclosporin hydroxylation (to M17)	Cyclosporin N-demethylation (to M21)
Control		60 ± 47	21 ± 14
GSD (μM)	5	54 ± 48 (90)	18 ± 13 (86)
	10	44 ± 46 (73)	16 ± 13 (76)
	20	37 ± 39 (62)	14 ± 11 (67)
	50	25 ± 18 (42)	9 ± 5 (43)
3-KD (μM)	5	55 ± 47 (92)	20 ± 14 (95)
	10	54 ± 48 (90)	19 ± 16 (90)
	20	47 ± 51 (78)	18 ± 15 (86)
	50	42 ± 45 (70)	15 ± 12 (71)
LNG (μM)	5	57 ± 51 (95)	22 ± 16 (100)
	10	56 ± 47 (93)	20 ± 12 (95)
	20	53 ± 47 (88)	18 ± 13 (86)
	50	48 ± 42 (80)	17 ± 11 (81)
NET (μM)	5	55 ± 49 (92)	21 ± 15 (100)
	10	54 ± 48 (90)	20 ± 12 (95)
	20	53 ± 48 (88)	18 ± 13 (86)
	50	44 ± 43 (73)	17 ± 13 (81)

Values are mean ± SD. Data from 4 livers.
Numbers in parentheses are the percentage of the control value.

study. The liver had been obtained from a patient who had received phenobarbitone and phenytoin for 20 yr.

DISCUSSION

In order to establish the relative roles of different *P*-450 isozymes in the 2-hydroxylation of EE_2 in human liver, we previously used a series of *P*-450 antibodies to inhibit EE_2 metabolism *in vitro* and also examined the correlation of enzyme activity in different livers with the relative concentration of *P*-450 isozymes as determined by Western blotting [6]. Our experiments provided evidence that EE_2 2-hydroxylation is catalyzed by cytochromes from at least three gene families (*P*-450IIC, *P*-450IIE and *P*-450IIIA). Guengerich [5] has also provided data from purified enzyme, immunoinhibition and correlation studies that a member of the *P*-450IIIA family $P\text{-}450_{NF}$ (*P*-450IIIA4) is a major enzyme involved in EE_2 2-hydroxylation. Knowing which isozymes are involved in the metabolism of a particular drug is very important for a rational understanding of drug interactions. For example, if a second drug is added to an already established therapy and it is also metabolized by the same isozyme or inactivates that isozyme, then an elevation of plasma drug concentrations could be seen. Such a scenario is relevant to the OC field, since if the progestogen in the combined OC inhibits or inactivates the major isozyme metabolizing EE_2, then raised EE_2 plasma concentrations could be evident in subjects taking that preparation.

The data from the present study indicate that all the progestogens have, at least some propensity to inhibit EE_2-hydroxylation. At a concentration of 100 μM (which is several orders of magnitude higher than therapeutic plasma concentrations of 5–50 nM), 3-keto desogestrel produced comparable inhibition to gestodene on either coincubation with EE_2 or 30 min preincubation with microsomes before EE_2 was added. The preincubation protocol allows the possibility of metabolism of the progestogen to a more inhibitory metabolite. In our study we followed exactly the procedure described by Guengerich [5]. Both gestodene and levonorgestrel produced more inhibition after preincubation. However, the data displayed in Table 3 do not support the contention of large differences in inhibitory potency existing within the various progestogens used in modern combined OCS, at least when EE_2 is the substrate being studied. The results presented here are therefore at variance with those recently published by Guengerich [14]. The latter has contended that gestodene is a particularly potent mechanism-based inactivator of *P*-450IIIA4 [14].

We also examined cyclosporin metabolism, since there is good evidence [15] that a major isozyme involved in formation of M17, M21 and M1 is *P*-450IIIA4 ($P\text{-}450_{NF}$). Thus, it would appear that both EE_2 and cyclosporin are metabolized by the same isozyme. In these

studies we found gestodene (after preincubation) to be more inhibitory than 3-keto desogestrel, levonorgestrel or norethisterone.

Recent evidence has indicated that tolbutamide is metabolized by two related enzymes *P*-450IIC8 and IIC9 [16, 17]. Since we had previously found that EE_2 is also metabolized by members of the IIC gene family [6] we examined tolbutamide as an alternative substrate. The order of inhibitory potency was gestodene > 3-keto desogestrel > levonorgestrel. We also studied the two pathways of diazepam metabolism. Recent evidence has pointed to enzymes from the *P*-450IIIA family and also *P*-450IIC_{10} being involved in diazepam metabolism [18, 19]. Gestodene and 3-keto desogestrel produced fairly comparable inhibition (50% or greater for both pathways at 100 μM) and this was greater than that seen with levonorgestrel.

The overall picture which emerges from these *in vitro* studies is that all the progestogens have the propensity to inhibit EE_2 2-hydroxylation and also the metabolism of other drugs, but there is no clear evidence of one progestogen being more markedly inhibitory than the others. It should also be noted that the extent of inhibition will be dependent on the substrate concentration used (i.e. more inhibition at lower substrate concentration). Therefore, for example, although it may appear that the metabolism of diazepam is inhibited to a greater extent than that of EE_2, we used a diazepam concentration (100 μM) which is less than the K_m (200–240 μM) for either the N-demethylation or hydroxylation [20] and an EE_2 concentration (25 μM) greater than the K_m (this study).

An important question is, how far does inhibition seen in the test tube relate to what happens *in vivo?* This is particularly relevant when the concentrations used *in vitro* are very much higher than those seen in the patients. It should also be considered, that for EE_2 even if all the 2-hydroxylation pathway was inhibited, there is the possibility for compensatory conjugation (sulphation and glucuronidation) to occur.

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