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EFFECTS OF ETHINYLESTRADIOL AND TESTOSTERONE IMPLANTS ON HEPATIC MICROSOMAL CYTOCHROME *P*450 MONOOXYGENASES OF BIRTH GONADECTOMIZED MALE AND FEMALE DARK AGOUTI RATS

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Summary—Monooxygenases in the cytochrome *P*450 IIIA subfamily are induced by a number of their xenobiotic substrates and by testosterone, an endobiotic substrate of importance in their regulation. 17α-Ethinylestradiol (EE) is also metabolized by these enzymes and in this study Dark Agouti rats were used to examine the effects of subcutaneous implantation of controlled release silastic capsules containing EE to determine if this steroid also induces these enzymes. Data were compared with results obtained from equivalent groups of animals implanted with capsules containing testosterone propionate (TP). Liver microsomes prepared from male and female rats were used to identify intrinsic gender differences in the monooxygenases studied and gender differences in the responses to the implanted steroids were also determined. Effects due to imprinting of growth hormone secretion patterns were controlled by using male and female birth gonadectomized animals. Results obtained from groups with blank implants showed there were no effects due to the silastic implant material itself on the monooxygenases studied. The specific activities of erythromycin *N*-demethylation in liver microsomes of both EE and TP implanted male and female birth gonadectomized animals were enhanced relative to corresponding blank implanted controls consistent with both steroids having an effect to induce activity attributable to cytochrome *P*450 IIIA isoforms. Immunoinhibition studies using microsomes from EE treated female rats with erythromycin as substrate provided further evidence for this steroid having this induction effect. The specific activity of ethylmorphine *N*-demethylation was however not increased in microsomes prepared from the EE implanted female animals and was decreased in the corresponding male preparations. These findings distinguished the response to this steroid from that to TP and suggested induction by this estrogen of an isoform(s) having a more limited range of substrates than has characteristically been found in this subfamily. EE treatment also caused an increase in diazepam C_3 hydroxylase consistent with an effect to induce *P*450 IIIA activity but this was found only in microsomes from birth gonadectomized female animals. This was in contrast to the effect of TP treatment which produced increases in this monooxygenase in both male and female animals. Another gender specific effect of EE was a striking decrease in morphine *N*-demethylase activity seen only in birth gonadectomized male rats. This again contrasted with the effect of TP which caused a marked increase in this activity in liver microsomes of both male and female birth gonadectomized animals consistent with the proposal that testosterone is important in the regulation of this activity. EE also caused decreases in diazepam *N*-demethylase specific activity in both male and female rats and spectral evidence indicated that mechanisms other than heme alkylation may be responsible for the decreased activities of these monooxygenases.

INTRODUCTION

Many synthetic and natural steroids cause induction of hepatic cytochrome *P*450 monooxygenases. Particularly well studied in this respect have been the effects of pregnenolone-16α-carbonitrile [1–5], dexamethasone [3–8] and testosterone [9, 10] on members of the IIIA subfamily of these enzymes. These investigations have been conducted principally using rats, and additional enzyme purification and immunochemical studies using rat liver have shown the response to steroid administration involves the synthesis of multiple but independently regu-

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lated polypeptides in this cytochrome *P*450 subfamily [11, 12]. The two isoforms that are best characterized are identified as IIIA1 and IIIA2 and other studies with rats have shown mephenytoin [13] and macrolide antibiotics [14] to be inducers of these enzymes, in addition to phenobarbital [3]. Rabbits have been used to examine the induction of orthologues of these enzymes by macrolide antibiotics [15] and by rifampicin and dexamethasone [16] and these drugs also elicit equivalent induction responses in the human liver [17, 18]. Evidence for the polymorphic expression in human liver of more than one isoform of this subfamily has been presented [19–21] and a developmentally regulated, fetus-specific, form has been identified [22, 23].

Cytochrome *P*450 IIIA isoforms have been variously characterized as having high activities relative to other *P*450s in catalyzing the *N*-dealkylation of ethylmorphine [16], diltiazem [24], erythromycin and other macrolide antibiotics [16], and hydroxylation of *S*-mephenytoin [13], and of the benzodiazepines diazepam, midazolam and triazolam [25–27]. Steroid hormones are also substrates of these enzymes and associated catalytic activities are 6β-hydroxylation of androstenedione [9, 10], testosterone [9, 10, 28–30] and cortisol [31], 2-hydroxylation of 17α-ethinylestradiol (EE) [32] and 2- and 4-hydroxylation of 17β-estradiol [19, 33]. Other characteristic oxidations of nifedipine [19], other 1,4-dihydropyridines [34], quinidine [35] and cyclosporin A [17, 36] have also been recorded.

The mechanisms by which steroids influence expression of these enzymes are not fully understood. The effects of pregnenolone-16α-carbonitrile and dexamethasone for example are considered not to be mediated by glucocorticoid receptors [4, 5] and induction by testosterone is recognized to be largely indirect since it requires an intact hypothalamo–pituitary axis [37, 38]. Further characterization of this latter finding showed that male and female growth hormone secretion patterns are different and important in determining the expression of a number of gender-specific cytochrome *P*450 monooxygenases [39, 40]. A male pattern of growth hormone secretion is imprinted by early neonatal exposure to testosterone and growth hormone has been shown to contribute to the differential regulation of *P*450 IIIA isoforms 1 and 2 in rat liver [41]. Gene cloning methods have also shown that form IIIA1 is minimally [42, 43], or not [44] expressed in uninduced animals and that form IIIA2 is male-specific in adult rats but is transiently expressed in immature females [44].

As noted above a number of the substrates of cytochrome *P*450 IIIA isoforms are also inducers of these enzymes but the question of this relationship with respect to estrogens has received little attention.

In the work reported here we examine the effects of subcutaneous implantation of controlled release silastic capsules containing EE on rat liver microsomes to determine if this substrate induces monooxygenase activities attributable to these enzymes. Data were compared with results obtained from equivalent groups of animals implanted with capsules containing testosterone propionate (TP) and effects due to imprinting of growth hormone secretion patterns were controlled by using male and female birth gonadectomized animals.

Dark Agouti rats were used in this study because large gender differences noted in a number of liver monooxygenases in a previous study from this laboratory [25] suggested their utility in characterizing the induction effects of EE and TP.

METHODS

Materials

D-Glucose-6-phosphate (disodium salt) and glucose-6-phosphate dehydrogenase (yeast enzyme, grade 1) were obtained from Boehringer Mannheim Pty Ltd (Sydney, Australia). Ethylmorphine hydrochloride and morphine hydrochloride were products of Macfarlan Smith Ltd (Edinburgh, Scotland). Erythromycin was a gift from Fauldings Pty Ltd (Adelaide, Australia) and oxazepam, temazepam, nordazepam, camazepam and diazepam were all gifts from Roche Products Pty Ltd (Sydney, Australia). Crystalline TP and EE, and rabbit pre-immune IgG, (product code I5006), were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). Rabbit polyclonal antibody prepared against rat liver cytochrome *P*450 IIIA, catalog number 003A0-P-RT, was purchased from Oxygene (Dallas, TX, U.S.A.). Testosterone RIA kit, product code TRK.600, was purchased from Amersham Int. Plc (Amersham U.K.). Silastic tubing (medical grade, 3.18 mm o.d., 1.57 mm i.d.) and medical grade Silastic adhesive type A were purchased from Dow Corning Corp. (MI,

U.S.A.). Penthrane (methoxyflurane U.S.P.) was purchased from Abbott Laboratories Pty Ltd (Sydney, Australia). HPLC grade methanol was purchased from Mallinckrodt Pty Ltd (Clayton, Australia) and HPLC grade ethyl acetate was obtained from Waters Associates Inc. (Milford, MA, U.S.A.). All other chemicals were of reagent grade and water purified using an Elga Spectrum system and having a conductance of 0.07 μS/cm was used throughout.

Animals and experimental procedures

Date mated, pregnant female Dark Agouti (DA) rats were obtained at weekly intervals over a three month period from the University of Queensland central animal breeding house, 2–3 days prior to parturition. Within 6 h of birth individual neonates were identified by gender and were either gonadectomized according to previously described methods [45], or were left untreated. All neonates were reared normally with their dams until 4 weeks of age. At this age they were weaned and individuals were segregated according to gender and assigned to groups of three to six animals which were either left untreated or were furnished, under methoxyflurane anaesthesia, with subcutaneous silastic implants on the dorsum of the neck. Implants were 1 cm long and were either empty (controls) or contained 20 mg EE or 20 mg TP. The procedures for making and fitting the implants and their properties with respect to controlled release of steroids have been described previously [46, 47]. Animals of each gender were assigned at regular intervals over the course of the entire experimental period to each of the five treatment groups (untreated intact, non-implanted birth gonadectomized, blank-implanted birth gonadectomized, EE-implanted birth gonadectomized, TP-implanted birth gonadectomized), and were individually housed and fed a standard laboratory rodent diet to appetite with unlimited access to drinking water. All animals were weighed at weekly intervals and killed by cervical section on the 28th day after weaning; food being withdrawn for 16 h prior to the time of death. In this way all animals furnished with implants containing TP or EE were exposed to steroid for exactly 28 days and all were the same age as blank-implanted and non-implanted animals at the time of death when blood samples were collected and livers removed. Livers were rinsed in ice-cold 1.15% potassium chloride solution, blotted dry and weighed and microsomal suspensions were prepared from individual livers [48]. Microsomal cytochrome *P*450 and protein concentrations were determined [49, 50] and monooxygenase activities measured. Assays of *N*-demethylation of morphine (5 mM) and ethylmorphine (5 mM) were carried out as described [51] using the Hantsch reaction [52] and *N*-demethylation of erythromycin (0.5 mM) was also determined using this procedure [14]. *N*-demethylase and C_3-hydroxylase activities of liver microsomes with 200 μM diazepam as substrate were determined using a reverse phase HPLC method as described previously [25]. The blood samples taken at the time of death were allowed to clot at room temperature and serum was taken and stored at −80°C until analyzed for steroid concentrations. Testosterone assays were conducted using a commercially available kit on serum samples from non-gonadectomized males and non-implanted and TP-implanted gonadectomized male and female animals. Estimates of EE concentrations were made on serum samples from the EE implanted gonadectomized male and female animals after acidic solvent extraction [53] by reverse phase HPLC with fluorescence detection monitored using a Shimadzu model RF-535 detector operating at 280 nm excitation and 308 nm emission. Sample application and mobile phase degassing and delivery systems were as described previously for the assay of benzodiazepines [25]. The mobile phase for the EE assay was methanol–0.02% triethylamine pH 3.0, (55:45 v/v) and separation was effected with a Brownlee Analytical RP-18 Spheri-5 column at a mobile phase flow rate of 1.5 ml/min. EE was quantitated using a standard curve prepared using samples of castrated male sheep serum to which EE had been added over a range from 0 to 50 ng/ml serum.

RESULTS AND DISCUSSION

Serum testosterone concentrations of the normal male and TP-implanted male and female rats were 1.4 ± 0.2 (6), 12.8 ± 1.5 (4) and 14.7 ± 1.7 (6) ng/ml, respectively. The values for non-implanted birth gonadectomized and blank-implanted birth gonadectomized males were at or below the lower limit of detection of the method (0.7 ng per ml). The fluorescence method used for determination of serum EE concentrations in EE-implanted male and female animals allowed only approximate values to be obtained owing to a small fluorescence

response in the region of EE elution in the serum of birth gonadectomized non-implanted male and female animals. EE concentrations were estimated to lie in the range 9 to 27 ng/ml serum for both males and females, demonstrating that all these animals were exposed to high concentrations of this steroid during the implantation period.

The cytochrome *P*450-specific contents and monooxygenase-specific activities of the liver microsomal suspensions of the male and female rats used in this study are shown in Tables 1 and 2, respectively. In neither male nor female rats did birth gonadectomy cause any change in liver cytochrome *P*450-specific content and this parameter was also unchanged in the blank-, TP- and EE-implanted groups. Table 1 shows that birth gonadectomy of males did cause marked reductions in the activities of diazepam C_3 hydroxylase and in erythromycin, ethylmolrphine and morphine *N*-demethylations. The isoform(s) responsible for morphine demethylation remain undetermined at this time but this monooxygenation has been established as male predominant and the proposal has been made that it is regulated by testosterone and the adenohypophyseal axis [54]. Enzymes in the cytochrome *P*450 IIIA subfamily in rats are also male predominant and have been identified as being important in catalyzing the other three reactions [16, 25]. Table 2 shows that birth gonadectomy of female rats did not affect the specific activities of any of these four monooxygenations, in keeping with their established male predominant occurrence, and comparison of the results in Tables 1 and 2 shows that all these activities were as low in the samples from intact females as in the birth gonadectomized males. The enzyme(s) responsible for diazepam *N*-demethylase are unknown at present [25] and in neither gender was this monooxygenation affected by birth gonadectomy.

Tables 1 and 2 also show that the blank-implants had no effect on any of the monooxygenases measured here and statistical analyses of the effects of the steroid hormones on these monooxygenases were made in relation to the values obtained with the blank-implant values.

As indicated above, erythromycin *N*-demethylation is closely identified with cytochrome *P*450 IIIA monooxygenases [16] and the finding of an increase in the specific activity of erythromycin *N*-demethylase in liver microsomes of EE-implanted male and female birth gonadectomized rats compared to blank-implanted controls shown in Tables 1 and 2 strongly suggests that this steroid has actions selectively to cause induction of an isoform(s) in the cytochrome *P*450 IIIA subfamily. The change is much more apparent in females (Table 2) than in males (Table 1) which may be due to the much lower intrinsic level of this activity in females. The advantage of using females to examine induction of "male-specific" cytochrome *P*450 activities has been noted previously [55].

The proposal that EE treatment was able to cause induction of cytochrome *P*450 IIIA isoform(s) was further examined using an immunoinhibition test, the results of which are shown in Fig. 1. These data indicate that the polyclonal antibody raised in the rabbit against purified rat liver cytochrome *P*450 IIIA [2] strongly inhibited approx. 60% of erythromycin *N*-demethylase activity in EE-treated birth gonadectomized female rat liver microsomes. This type of test has been used to explore the involvement of a variety of cytochrome *P*450 species in many microsomal monooxygenations [56–58]. In the present study maximum inhibition was obtained with a relatively low ratio of antibody to microsomal protein, emphasizing the specificity of the interaction under study. The figure shows that inhibition was obtained under preincubation conditions where a rabbit preimmune IgG preparation had no detectable corresponding effect. The antibody preparation is selective for the cytochrome *P*450 IIIA subfamily of closely related isoforms (Oxygene product data) and this test provides strong evidence that EE treatment caused induction of activity attributable to cytochrome *P*450 IIIA subfamily monooxygenase(s). A measure of support for this is to be found in a previous study [59] where EE treatment of rats caused induction of estrogen 2-hydroxylase activity as tested *in vitro* using liver microsomes from the EE-treated rats, with EE itself as substrate. This biotransformation was subsequently [35, 60] found to be a property of rat and human liver cytochrome *P*450 IIIA isoforms, although members of other subfamilies also catalyzed this oxidation [60]. Estradiol is also a substrate for the cytochrome *P*450 IIIA enzymes [19, 36, 60, 61]. However this steroid does not [10] appear to have cytochrome *P*450 IIIA induction properties equivalent to those of EE reported here and it seems at present that no generalizations can be made regarding steroids that are substrates and inducers of these enzymes.

Table 1. Effects of hormone implants on Dark Agouti male rat liver microsomal monooxygenases

Animals and treatments	Microsomal cytochrome P450-specific content (nmol P450/mg protein)	Erythromycin N-demethylase	Ethylmolrphine N-demethylase	Morphine N-demethylase	Diazepam C_3 hydroxylase (nmol temazepam/ min/nmol P450)	Diazepam N-demethylase (nmol nordazepam/ min/nmol P450)
		(nmol HCHO/min/nmol P450)				
Intact mature						
Untreated (6)	1.10 ± 0.07	1.34 ± 0.04[a]	8.40 ± 0.19[a]	0.53 ± 0.11	3.69 ± 0.76[a]	1.18 ± 0.23[a]
Birth gonadectomized						
No-implant (6)	0.94 ± 0.08	0.42 ± 0.08***	3.44 ± 0.37***	0.10 ± 0.04***	0.53 ± 0.25***	1.00 ± 0.04
Blank-implant (3)	0.89 ± 0.08	0.45 ± 0.09	3.50 ± 0.32	0.10 ± 0.05	0.55 ± 0.36	0.99 ± 0.03
EE (5)	1.11 ± 0.05	0.69 ± 0.07*	2.70 ± 0.12**	0.02 ± 0.01*	0.52 ± 0.05	0.78 ± 0.04***
TP (4)	0.97 ± 0.03	1.26 ± 0.19***	6.38 ± 1.04**	1.05 ± 0.21***	2.44 ± 0.74***	0.89 ± 0.12

The analytical procedures are described in the Methods section. Results for each treatment group are means ± SD of analyses of microsomal samples prepared from individually processed livers of each of the rats in each treatment group. The numbers of rats in each treatment group are given in parentheses. Student's *t* test was used for statistical analyses. The significance of difference levels shown for the results obtained for the hormone-implanted groups were calculated relative to the values obtained for the blank-implant group, whereas those for the no-implant birth gonadectomized group values were calculated relative to the intact mature untreated group values.

[a]Data taken from Reilly *et al.* [25].

***$P < 0.001$; **$P < 0.002$; *$P < 0.01$.

Table 2. Effects of hormone implants on Dark Agouti female rat liver microsomal monooxygenases

Animals and treatments	Microsomal cytochrome P450-specific content (nmol P450/mg protein)	Erythromycin N-demethylase	Ethylmorphine N-demethylase	Morphine N-demethylase	Diazepam C_3 hydroxylase (nmol temazepam/ min/nmol P450)	Diazepam N-demethylase (nmol nordazepam/ min/nmol P450)
		(nmol HCHO/min/nmol P450)				
Intact mature						
Untreated (6)	0.79 ± 0.02	0.24 ± 0.06[a]	2.51 ± 0.08[a]	0.10 ± 0.06	0.24 ± 0.01	0.98 ± 0.03
Birth gonadectomized						
No implant (5)	0.85 ± 0.05	0.28 ± 0.05	3.01 ± 0.32	0.13 ± 0.03	0.26 ± 0.08	0.95 ± 0.11
Blank implant (4)	1.04 ± 0.06	0.26 ± 0.01	3.20 ± 0.13	0.19 ± 0.01	0.23 ± 0.02	0.92 ± 0.02
EE (5)	1.01 ± 0.02	1.13 ± 0.13***	2.92 ± 0.18	0.11 ± 0.01	0.74 ± 0.06***	0.79 ± 0.04***
TP (6)	1.04 ± 0.09	1.63 ± 0.07***	7.63 ± 0.23***	1.01 ± 0.04***	2.96 ± 0.15***	0.95 ± 0.05

The analytical procedures are described in the Methods section. Results for each treatment group are means ± SD of analyses of microsomal samples prepared from individually processed livers of each of the rats in each treatment group. The numbers of rats in each treatment group are given in parentheses. Student's *t* test was used for statistical analyses. The significance of difference levels shown for the results obtained for the hormone-implanted groups were calculated relative to the values obtained for the blank-implant group, whereas those for the no-implant birth gonadectomized group values were calculated relative to the intact mature untreated group values.

[a]Data taken from Reilly *et al.* [25].

***$P < 0.001$.

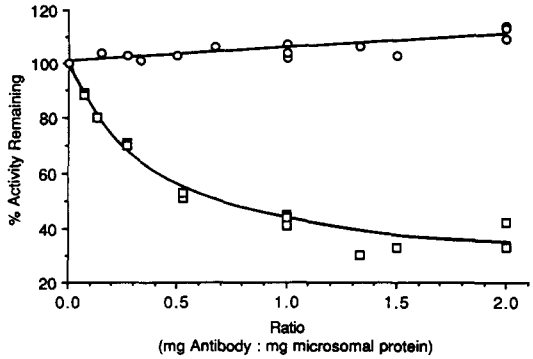

Fig. 1. Microsomes prepared from a birth gonadectomized EE-implanted female rat were incubated at 27°C for 15 min with either Sigma rabbit preimmune IgG (○) or Oxygene rabbit anti-rat *P*450 IIIA antibody (□) at the indicated ratios of antibody: microsomal protein. These mixtures were then used in assays of erythromycin *N*-demethylase as described [14]. The Sigma and Oxygene antibody products were each reconstituted to a protein concentration of 2 mg/ml with sterile normal saline immediately before use and the cytochrome *P*450 concentration of the preincubation mix was 2 μM. Values for individual assays are shown except for the results of analyses of the uninhibited activity (100% activity remaining) which were carried out in triplicate and had a value of 1.11 ± 0.09 nmol formaldehyde/nmol *P*450/min.

All the rat liver cytochrome *P*450 IIIA isoforms that have been isolated have very similar catalytic profiles and ethylmorphine *N*-demethylation is numbered among their characteristic activities. Some members of this subfamily however are recognized primarily from immunochemical evidence [11, 12] and little is known about their substrate specificities. On the basis of the lack of induction of ethylmorphine *N*-demethylase in females and the reduction in this activity in males in the present study it appears that the isoform(s) induced by EE may be among these and may have a more restricted range of catalytic activities than is associated with the better characterized forms.

Comparison of the results of EE treatment on diazepam C_3 hydroxylase shown in Table 2 with those given in Table 1 shows a gender dependent effect; only in female animals was diazepam C_3 hydroxylase increased. Evidence has been presented [25] to indicate that this monooxygenase is also characteristic of cytochrome *P*450 IIIA enzymes. The reason for the gender dependence of this response to EE was not further explored here, but two recent reports have identified gender dependence of the induction effects of a number of commonly used inducers in rats [62, 63] indicating this may be a characteristic feature of the induction response to xenobiotics requiring further study.

Animals in all groups except those administered EE grew normally and had ratios of liver to body weight in the range 2.1 to 2.4%. EE-implanted animals did not gain weight in the last three weeks after implantation but their livers were in the same size range as animals in the other groups. The liver to body weight ratios of the EE-treated animals were, in consequence, very high (in the range 4.4 to 4.6%). This effect of EE administration in rats has been reported previously [64–66] and is considered to be due to cholestasis [67] and to increased uptake of low density lipoprotein by liver [68] as a consequence of increased expression of low density lipoprotein receptors [69]. The livers from the EE-treated animals however appeared normal in size, shape and colour and microsomes prepared from them also appeared normal. In addition absolute absorbance spectra of liver microsomes of blank- and EE-implanted animals were identical (results not shown) and indistinguishable from published spectra of normal rat liver microsomes [49]. These results show that green pigment formation, indicative of heme *N*-alkylation and cytochrome *P*450 loss due to self-catalyzed inactivation with EE as substrate [70, 71], was not detectable in microsomes prepared from the EE-treated groups of animals. Such loss of heme is characteristic of exposure to a variety of compounds with terminal acetylenic or olefinic functional groups [72, 73]. Although the rats in the present study were exposed to sustained high doses of EE via the implants the finding of no decrease in cytochrome *P*450-specific content of liver microsomes from EE-treated animals (Tables 1 and 2) and no spectral changes in the microsomes that would be indicative of green pigment formation may be due to the relatively low concentrations of steroid in the present study compared to those employed to characterize green pigment production [70, 71] and demonstrate cytochrome *P*450 loss [35].

These observations are of relevance in considering the reductions caused by EE in the specific activities of some of the monooxygenases studied here. In Tables 1 and 2 EE treatment is shown to cause decreased diazepam *N*-demethylase in male and female animals and decreases also in the specific activities of morphine and ethylmorphine *N*-demethylase are shown for male rats in Table 1. The basis for these gender effects was not explored further in this study but the spectral evidence indicating no reduction in cytochrome *P*450-specific con-

tent and lack of green pigment formation in microsomes from the EE-treated animals indicates that the reduced monooxygenase-specific activities found in these preparations may not be attributable to isoform-specific cytochrome P450 heme loss. An alternative mechanism may be enzyme protein alkylation by a metabolite of EE resulting from a process analogous to that proposed for acetylenic fatty acids and secobarbital [74, 75]. EE could also however be causing suppression of production of the relevant enzymes; this being a well established, but relatively unexplored, action of a number of cytochrome P450 monooxygenase inducing agents [76].

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