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INHIBITION OF RAT TESTICULAR HEME SYNTHESIS AND DEPRESSION OF MICROSOMAL CYTOCHROME P-450 BY ESTRADIOL BENZOATE*

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Abstract—Twenty-four hours after a single dose (50 μg, s.c.) of estradiol benzoate (EB), rat testicular microsomal heme and cytochrome P-450 were decreased to 72 and 76% of control levels respectively. Treatment of rats with human chorionic gonadotropin (hCG) resulted in elevated levels of microsomal heme and cytochrome P-450 and increased activity of δ-aminolevulinic acid (ALA) synthase (EC 2.3.1.37). However, the hCG-mediated elevations of testicular microsomal heme and cytochrome P-450 content failed to occur in animals treated with EB. To investigate the possibility that the observed effect of EB was mediated through the pituitary, studies were conducted with hypophysectomized animals. The increased microsomal heme and cytochrome P-450 content mediated by hCG in hypophysectomized animals was again prevented by administration of EB. The elevated activity of testicular mitochondrial ALA synthase produced by hCG in both intact and hypophysectomized animals was not affected by EB. Incorporation of [^{14}C]ALA into microsomal heme was depressed 60% 12 hr following a single dose of EB (50 μg, s.c.). These data suggest that EB depresses testicular microsomal heme and cytochrome P-450 content by inhibiting the synthesis of heme at an enzymatic reaction other than ALA synthase.

It is well recognized that the administration of estrogens to laboratory animals, as well as to men, results in a depression of plasma testosterone levels [1]. The mechanism of estrogen action on the testis was originally thought to be entirely the result of suppression of luteinizing hormone (LH) release from the pituitary [2]. However, after estrogen treatment plasma testosterone levels decline before the depression of plasma LH concentration [1, 3]. Also, it has been demonstrated that estrogens inhibit the human chorionic gonadotropin (hCG)-mediated increase of testosterone synthesis in isolated Leydig cells [1]. Studies with hypophysectomized rats have shown that administration of estradiol depresses testicular 17 α-hydroxylase activity [4]. Furthermore, administration of diethylstilbestrol to hypophysectomized mice results in a decrease in the activities of both 17 α-hydroxylase and 17–20 lyase [5]. These reports suggested that estrogens may act directly on the testis to inhibit testosterone synthesis.

Additional evidence suggesting a direct effect of estrogens on the testis has arisen from studies utilizing gonadotropin-desensitized Leydig cells [1]. The gonadotropin-induced steroidogenic lesion was found to be a decrease in the activity of 17–20 lyase and, to a lesser extent, the 17 α-hydroxylase. Administration of tamoxifen, an anti-estrogen, prior to hCG treatment was shown to prevent the decrease in activities of these enzymes [6–8]. It has also been demonstrated that intratesticular levels of estradiol are increased 30 min after hCG administration [8]. Based on data from these types of studies, investigators have hypothesized that estradiol is responsible for the defect in testosterone synthesis in gonadotropin-desensitized Leydig cells.

Both the 17–20 lyase and 17 α-hydroxylase are known to be cytochrome P-450-dependent enzymes [9]. In 1977, Kremers *et al.* [4], after noting the decrease in testicular 17 α-hydroxylase activity produced by estradiol treatment, suggested that estradiol might act on the biosynthesis of a component of the 17 α-hydroxylase enzyme complex. Indeed, Kalla *et al.* [10] have shown that administration of estradiol to hypophysectomized rats results not only in a decrease in the activities of testicular 17–20 lyase and 17 α-hydroxylase, but also in a decrease in the levels of the hemoprotein, testicular microsomal cytochrome P-450.

In an attempt to elucidate the mechanism by which estradiol benzoate depresses cytochrome P-450 and the subsequent P-450-mediated microsomal enzymatic reactions, we have examined the effects of estradiol benzoate on the synthesis of rat testicular heme.

MATERIALS AND METHODS

Pyridoxal-5′-phosphate, human chorionic gonadotropin (hCG), coenzyme A, porcine heart succinyl-CoA synthetase (EC 6.2.1.4) and β-estradiol-3-benzoate (EB) were obtained from the Sigma Chemical Co., St. Louis, MO. Guanosine triphosphate and δ-aminolevulinic acid (ALA) were obtained from Calbiochem, La Jolla, CA. δ-[4-

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^{14}C]Aminolevulinic acid (42 mCi/mmole) was purchased from the New England Nuclear Corp., Boston, MA, and [2,3-^{14}C]succinic acid (15 mCi/mmole) from ICN Pharmaceuticals, Irvine, CA. Other chemicals were reagent grade.

Treatment of animals. Male Sprague–Dawley rats (75–85 days of age; 260–290 g) were obtained from Sasco Inc., Omaha, NE. Rats were permitted food (Wayne Lab Blox, Allied Mills, Inc., Chicago, IL) and water *ad lib.* and were maintained on a 12-hr light–dark cycle. In specified experiments, EB was dissolved in acetone–sesame seed oil (1:9, v/v) and injected s.c. (50 μg in 0.2 ml). Control animals received 0.2 ml of vehicle. In other experiments, animals were anesthetized with ether, and a silastic capsule (1 cm in length and 0.10 cm i.d.) was implanted s.c. in the dorsal cervical area. Control animals received empty capsules, and experimental animals received capsules filled with crystalline EB. Capsules of this type containing EB have been found to provide relatively constant blood levels of approximately 140 pg/ml for the duration of implantation [11–13]. hCG was dissolved in 0.9% NaCl and administered s.c. (100 units; 0.5 ml; 12-hr intervals) for the specified length of time. Control animals received 0.5 ml of 0.9% NaCl. Hypophysectomies were performed transauricularly [14], and success was ascertained by visual inspection at autopsy.

Preparation of tissues. Animals were decapitated, and the testes were removed, decapsulated and weighed. The testes from each animal were pooled, and testicular homogenates (10%, w/v) were prepared in 0.25 M sucrose at 5° using a motor-driven glass Potter–Elvehjem homogenizer with a Teflon pestle (0.15 mm clearance). The method of Cammer and Estabrook [15] was used to prepare the mitochondrial and microsomal fractions. Protein was determined by the method of Bradford [16], using bovine serum albumin as the standard.

Measurement of testicular microsomal heme and cytochrome P-450. Microsomal heme was determined from the difference spectrum of the oxidized/reduced pyridine hemochromogen between 541 and 557 nm, using an extinction coefficient of 20.7 $mM^{-1} cm^{-1}$ [17]. The levels of microsomal cytochrome P-450 were determined from the carbon monoxide difference spectrum (450–490 nm) of dithionite-reduced microsomes using an extinction coefficient of 91 $mM^{-1} cm^{-1}$ [18].

Determination of testicular ALA synthase activity. Testicular mitochondrial ALA synthase activity was determined by a radioisotopic method employing [2,3-^{14}C]succinate and a succinyl-CoA generating system previously developed in this laboratory [19]. Recovery of ALA was 91%, and the ^{14}C-labeled material was confirmed to be the [^{14}C]ALA-pyrrole by thin-layer chromatography in two different solvent systems with an authentic ALA-pyrrole standard [19]. Rat testicular ALA synthase activity was found to be localized predominantly in the mitochondrial fraction [19].

Incorporation of [^{14}C]ALA into microsomal heme. The rate of testicular heme synthesis was measured in rats 12 hr after a single, s.c. dose of EB (50 μg) and compared to that in rats which received the vehicle alone. Rats received 6 μCi of [4-^{14}C]ALA [i.p. 0.75 ml total volume of 50 mM Tris–HCl (pH 7.4)–0.9% NaCl] and were killed 90 min later. Incorporation of [^{14}C]ALA into testicular microsomal heme was linear for 120 min. Testes were pooled from two animals, and microsomes were prepared by the method of Cammer and Estabrook [15]. Heme was extracted into ethyl acetate–acetic acid (4:1, v/v) and washed in water and 1.5 N HCl according to the method of Bonkowsky *et al.* [20]. For each sample, an aliquot of the heme extract was added to 6 ml of Amersham ACS scintillation fluid and counted for radioactivity in a Beckman LS8000 liquid scintillation counter (93% efficiency), and another aliquot was assayed for heme.

Preparation of silastic capsules. Silastic tubing (o.d. 0.22 cm, i.d. 0.10 cm) was obtained from the Dow–Corning Corp. (Midland, MI). Crystalline EB was packed into the tubing, and capsules were cut to 1-cm lengths. The ends were sealed with silicone medical adhesive (Type A, Dow–Corning). After drying for 24 hr, the capsules were placed in absolute ethanol for 1 hr to remove any external EB. The capsules were incubated in 0.9% NaCl for 12 hr at 25° prior to implantation.

Statistical analysis. Data were analyzed by Student's *t*-test to determine the significance of differences between means.

RESULTS

Depression of rat testicular microsomal heme and cytochrome P-450 by estradiol benzoate. The effect of a single dose of EB (50 μg, s.c.) on rat testicular microsomal heme and cytochrome P-450 at 24 and 48 hr is shown in Fig. 1. Microsomal heme and cytochrome P-450 decreased to 72 and 76% of control levels, respectively, 24 hr following EB administration. Microsomal heme and cytochrome P-450 remained significantly lower than controls at 48 hr.

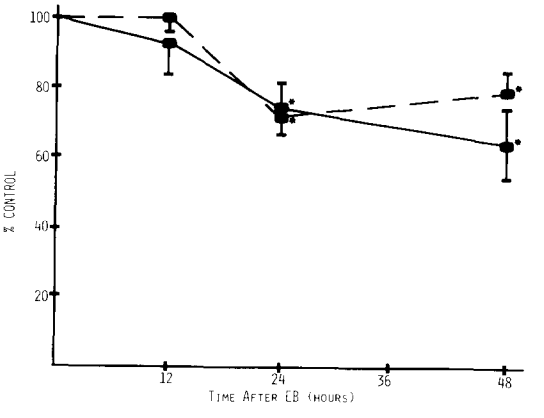

Fig. 1. Effect of EB on rat testicular microsomal heme (■– – –■) and microsomal cytochrome P-450 (■——■). Animals were killed at the specified time intervals after a single dose of EB (50 μg, s.c.). The mean control value for microsomal heme was 0.254 ± 0.010 nmole heme/mg protein. The mean control value for microsomal P-450 was 0.147 ± 0.009 nmole P-450/mg protein. Each point is the mean ± S.E.M. for five to six determinations. An asterisk denotes a significant difference ($P < 0.05$) between values from EB-treated and control rats.

Table 1. Effect of EB on rat testicular mitochondrial ALA synthase activity*

Hours after EB treatment	ALA synthase activity (nmoles ALA/g protein/hr)
Control	270 ± 12
12	259 ± 16
24	261 ± 25
48	286 ± 36

* Rats received a single dose of EB (50 μg, s.c.) and were killed at the specified time intervals. Mitochondrial ALA synthase activity was measured as described in Materials and Methods. Each value is the mean ± S.E.M. of three determinations.

Testicular mitochondrial ALA synthase activity was not altered by EB during this time period (Table 1).

Administration of hCG resulted in an increased activity of testicular mitochondrial ALA synthase and increased levels of microsomal heme and cytochrome P-450 (Table 2). In animals receiving EB from implanted capsules, microsomal heme and cytochrome P-450 were depressed during this time period, but the activity of ALA synthase was unchanged. EB administration antagonized the hCG-mediated increase of testicular microsomal heme and cytochrome P-450 content. However, ALA synthase activity was increased to levels comparable to those of control rats treated with hCG alone.

Effect of estradiol benzoate on rat testicular microsomal heme and cytochrome P-450 in hypophysectomized rats. To eliminate the role of the pituitary in the EB effects on testicular heme, a similar series of experiments was performed using hypophysectomized rats (Table 3). Activity of mitochondrial ALA synthase and levels of microsomal heme and cytochrome P-450 were all decreased ($P < 0.05$) following hypophysectomy (Tables 2 and 3). Treatment of hypophysectomized rats with hCG increased the levels of testicular microsomal heme and cytochrome P-450 and the activity of mitochondrial ALA synthase. Microsomal heme and cytochrome P-450 content and mitochondrial ALA synthase activity were not changed in animals receiving EB from implanted capsules. Administration of hCG to hypophysectomized rats receiving EB from implanted capsules failed to increase microsomal heme and cytochrome P-450, yet it did result in an increased activity of mitochondrial ALA synthase.

Effect of estradiol benzoate on the incorporation of δ-[4-^{14}C]aminolevulinic acid into testicular microsomal heme. These data suggest that EB might act directly on the testes to impair the synthesis of testicular heme. This possibility was investigated by measuring the rate of incorporation of [4-^{14}C]ALA into testicular microsomal heme in animals receiving EB (50 μg, s.c.). The effect of EB on the incorporation of [4-^{14}C]ALA into microsomal heme is shown in Fig. 2. The incorporation of [4-^{14}C]ALA into testicular microsomal heme was decreased to 40% of control values 12 hr after administration of EB.

Table 2. Effects of EB on testicular microsomal cytochrome P-450, microsomal heme and mitochondrial ALA synthase activity*

Treatment	Microsomal cytochrome P-450 (nmoles P-450/mg protein)	Microsomal heme (nmoles heme/mg protein)	Mitochondrial ALA synthase activity (nmoles ALA/g protein/hr)
Control	0.163 ± 0.006	0.241 ± 0.015	282 ± 7
hCG	0.231 ± 0.021†	0.307 ± 0.016†	419 ± 25†
EB	0.060 ± 0.008†	0.146 ± 0.033†	278 ± 13
hCG + EB	0.085 ± 0.008†	0.182 ± 0.019†	396 ± 28†

* Rats were implanted (s.c.) with silastic capsules containing EB as described in Materials and Methods. Twenty-four hours after implantation, treatment was initiated with hCG (100 units, 12-hr intervals) and continued for 6 days. Animals were killed 12 hr after the last injection of hCG, and assays were performed as described in Materials and Methods. Each value is the mean ± S.E.M. of six determinations.

† Significantly different from control values ($P < 0.05$).

Table 3. Effect of EB on testicular microsomal cytochrome P-450, microsomal heme and mitochondrial ALA synthase activity in hypophysectomized rats*

Treatment	Microsomal cytochrome P-450 (nmoles P-450/mg protein)	Microsomal heme (nmoles heme/mg protein)	Mitochondrial ALA synthase activity (nmoles ALA/g protein/hr)
Control	0.028 ± 0.005	0.124 ± 0.009	210 ± 6
hCG	0.055 ± 0.005†	0.192 ± 0.015†	272 ± 7†
EB	0.019 ± 0.004	0.098 ± 0.013	201 ± 8
hCG + EB	0.030 ± 0.004	0.130 ± 0.010	283 ± 13†

* Rats were hypophysectomized and implanted (s.c.) with silastic capsules containing EB as described in Materials and Methods. Twenty-four hours after implantation, treatment was initiated with hCG (100 units, 12-hr intervals) and continued for 4 days. Animals were killed 12 hr after the last injection of hCG, and assays were performed as described in Materials and Methods. Each value is the mean ± S.E.M. of five to eight determinations.

† Significantly different from control values ($P < 0.05$).

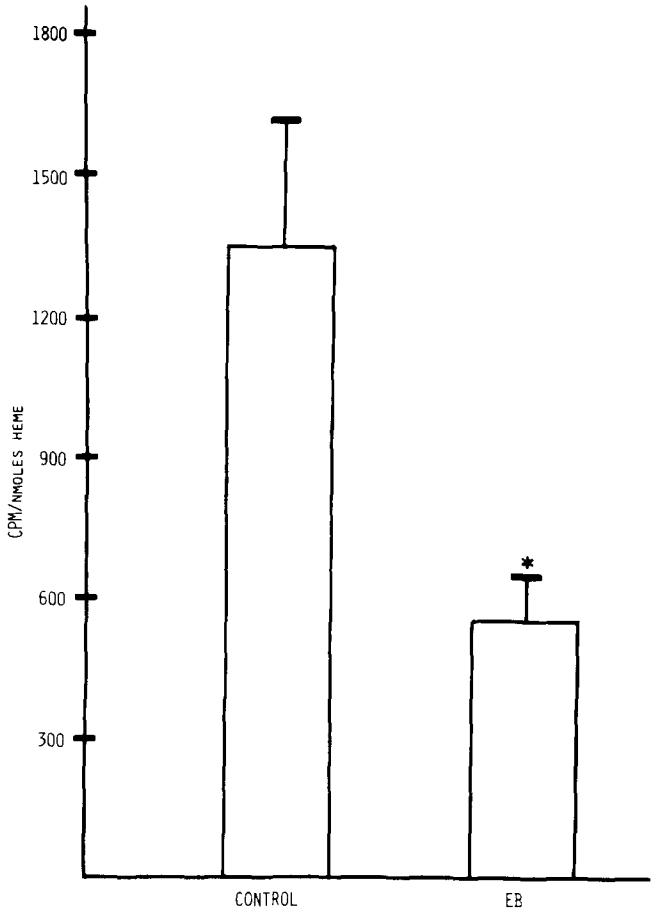

Fig. 2. Effect of EB on the incorporation of [^{14}C]ALA into rat testicular microsomal heme. Rats received 6 μCi of [^{14}C]ALA intraperitoneally 12 hr after administration of EB (50 μg, s.c.). Rats were killed 90 min later, the microsomal heme was extracted and assayed, and the radioactivity was measured as described in Materials and Methods. Each value is the mean ± S.E.M. of six determinations. An asterisk denotes a significant difference ($P < 0.05$) between values from EB-treated and control rats.

These results suggest that EB depressed testicular microsomal heme and cytochrome P-450 by inhibiting the synthesis of heme.

DISCUSSION

It has been established that estradiol can act on the testis, independently of the pituitary, to impair testicular testosterone synthesis [1]. This defect has been localized at the cytochrome P-450-dependent enzymes, 17–20 lyase and 17 α-hydroxylase [1]. In this paper, we have presented data which indicate that EB inhibits the testicular synthesis of heme, the prosthetic group of cytochrome P-450. The resultant decrease in testicular heme levels and the subsequent decrease in the levels of cytochrome P-450 could, therefore, account for diminished activities of the testicular steroidogenic reactions that are mediated by this cytochrome. The time course of depressed testicular microsomal cytochrome P-450 levels described herein coincides with the reported decreases of rat testicular microsomal 17 α-hydroxylase activity and testosterone levels in both plasma and the testis [3, 4, 10]. Thus, inhibition of testicular heme synthesis is one plausible mechanism by which estrogens might impair the synthesis of testosterone. The increased ALA synthase activity produced by hCG administration was not antagonized by EB in either intact or hypophysectomized animals. Thus, EB would impair the testicular heme biosynthetic pathway at an enzymatic step other than ALA synthase.

Heme is also the prosthetic group for microsomal cytochrome b_5. However, the content of this cytochrome would not be expected to change appreciably during the experiments described herein because of the long half-life (12 days) reported for rat testicular microsomal cytochrome b_5 [9]. In contrast, the half-life for rat testicular microsomal cytochrome P-450 is only 3.3 days [9].

It is apparent that the levels of microsomal heme and cytochrome P-450 and the activity of ALA synthase in hypophysectomized rats are all appreciably lower than in intact animals, which suggests that the synthesis of heme in the rat testis might be dependent on the presence of pituitary gonadotropins. Indeed, the activity of rat testicular ALA synthase has been reported to increase after administration of hCG [19]. Thus, it is possible that the decrease in testicular microsomal heme and cytochrome P-450 content

following hypophysectomy is associated with a decreased rate of heme synthesis in the testis.

It has been proposed previously that a compensatory increase of hepatic ALA synthase activity follows depression of heme levels through feedback repression and/or inhibition [21, 22]. Recently, this phenomenon has also been demonstrated in the rat testis utilizing an inhibitor of testicular heme synthesis, 1,2-dibromo-3-chloropropane [23]. In the present report, administration of EB resulted in a decrease of testicular microsomal heme levels without any alteration of the activity of ALA synthase. However, testicular ALA synthase activity is increased following the administration of hCG [19], which indicates that this enzyme may be regulated by pituitary gonadotropins. Therefore, the presence of LH may be necessary for an increase in testicular ALA synthase activity. EB has been reported to depress plasma LH levels [2], which may explain why the decrease in testicular microsomal heme and cytochrome P-450 in intact animals after EB treatment is not followed by a compensatory increase in ALA synthase activity.

The evidence presented herein suggests that the impairment of testicular steroidogenesis by EB might be associated with an inhibition of heme synthesis, and that testicular heme synthesis may play a significant role in the rate of testicular testosterone production. Therefore, an understanding of the regulation of heme synthesis in the testes would appear valuable in learning how various hormones, drugs and toxicants affect testicular function.

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