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COBALT-PROTOPORPHYRIN CAUSES PROLONGED INHIBITION OF CATECHOL ESTROGEN SYNTHESIS BY RAT LIVER MICROSOMES

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A single injection of cobalt-protoporphyrin (CoPP), which produces a marked and sustained decline in hepatic cytochrome P450 content, reduced the ability of male rat liver microsomes to form catechol estrogens to about 30% of control values within 1 day, as measured by the release of $^{3}H_2O$ from [2-^{3}H]estradiol. Two days after treatment, the apparent K_m of estrogen 2-hydroxylase for estradiol was increased, but other inhibitors of cytochrome P450 function (SKF-525A or piperonyl butoxide) failed to affect the enzyme. Inhibition by CoPP was also demonstrated by measuring the conversion of [4-^{14}C]estradiol to its 2-hydroxylated derivative visualized by autoradiography after chromatographic separation. These findings point to yet another site in the multifaceted action of cobalt protoporphyrin.

Treatment of rats with cobalt-protoporphyrin (CoPP), a synthetic heme analogue, produces an intense and sustained induction of hepatic heme oxygenase (1) and also a pronounced decline in the content of cytochrome P450 (2). These biochemical changes are accompanied by major abberations of endocrine function (1,3) including a significant reduction in serum testosterone, thyroxine and 3,5,3'-triiodothyronine levels but without a reciprocal elevation in either serum luteinizing hormone (LH) or thyroid stimulating hormone (TSH) concentrations (4). It was therefore considered of interest to determine how catechol estrogen formation, the main hepatic cytochrome P450-catalyzed reaction involving estrogens (5), would be affected by such treatment. The enzyme, estrogen 2-hydroxylase, which shows much higher activity in male than in female rats (6) has also been shown to be susceptible to hormonal manipulations including continuous infusion with growth hormone (7). In addition, the products of the reaction, the catechol

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estrogens, influence LH (8) and prolactin (PRL) (9) secretion when administered in pharmacological doses. This report describes the markedly inhibitory effect of *in vivo* treatment with CoPP on the kinetics of estradiol hydroxylation by rat liver microsomes and compares it to the action of SKF-525A and piperonyl butoxide, agents which both inhibit P450-dependent oxidations.

MATERIALS AND METHODS

Chemicals: [2-^{3}H]E_2 was prepared and handled as described previously (10). [4-^{14}C]E_2 (57 mCi/mmol; New England Nuclear Corp., Boston, MA) was shown by chromatography and autoradiography to be free of radioactive impurities. Unlabeled E_2, 2-hydroxyestradiol-17β (2-OHE_2) and testosterone enanthate were purchased from Steraloids (Wilton, NH) and NADPH from Sigma Chemical Co. (St. Louis, MO). CoPP was supplied by Porphyrin Products (Logan, UT), piperonyl butoxide by ICN Pharmaceuticals (Cleveland, OH) while SKF-525A was a generous gift from Smith, Kline and French Laboratories (Philadelphia, PA).

Treatment of animals: Adult male Sprague-Dawley rats were purchased from Charles River (Wilmington, DE) and housed in the Laboratory Animal Research Center at Rockefeller University in temperature (22°C) controlled rooms with a 12/12 hr light/dark cycle. Animals were allowed free access to Purina Lab Chow and water. CoPP was dissolved as described previously (1) and administered s.c. under the loose skin in the nuchal region. SKF-525A (in saline) and piperonyl butoxide (in sesame oil) were also given by this route. Testosterone enanthate (in oil) was injected i.p. 24 hr before the administration of other drugs.

Preparation of liver microsomes: Rats were decapitated and serum from trunk blood stored at -20°C for radioimmunoassay of testosterone (11). Livers were perfused *in situ* with 30 ml of ice-cold saline via the heart, excised, dried and weighed. Homogenization, centrifugation and preparation of the microsomes were as described previously (1). Cytochrome P450 concentrations were measured using fresh microsomes by difference spectroscopy (12) and aryl hydrocarbon hydroxylase (AHH) activity on microsomes stored in liquid N_2 (13). Protein was estimated by the method of Lowry et al. (14).

Conditions of incubation: [2-^{3}H]E_2 (8 μM; 2.8-3.6 x 10^5 dpm) was incubated for 30 min with constant shaking at 38°C with the resuspended liver microsomes (~2 mg protein) and NADPH (0.3 mM) in 0.1 M tris-HCl buffer (pH 7.4) in a total volume of 4 ml. For the kinetic studies, [2-^{3}H]E_2 at concentrations ranging from 1.0 to 16 μM was incubated with microsomes (0.2 mg protein), except where indicated, for 20 min under the same conditions as described above. After extraction three times with equal volumes of diethyl ether, the amount of ^3H_2O formed was determined as described below. For the experiments with [4-^{14}C]E_2 (8 μM; 1.1-1.5 x 10^5dpm), the incubations were carried out for 30 min in the presence of ascorbic acid (1 mM) to prevent the further oxidation of labile products and the solution extracted with ether (3 x 1 vol). The products in the organic phase were then separated by TLC on silica gel using cyclohexane-ethyl acetate-ethanol (10:9:1) and visualized by autoradiography as described previously (7).

Determination of 2-hydroxylase activity: This was determined by the radiometric assay of Fishman et al. (15). Aliquots (0.5 ml) of the ether-extracted aqueous fraction were allowed to evaporate to dryness in a fume hood at room temperature and ^3H_2O formation determined from the difference between the ^{3}H-radioactivity in the original sample and that in

the dry residue redissolved in 0.5 ml of buffer. This assay gave results identical to those obtained by lyophilization (10). The double reciprocal plots were determined under conditions which gave linear ^3H_2O formation and maintained the amount of available substrate relatively constant.

RESULTS

CoPP administration (50 μmol/kg body wt.) to adult male rats 48 hr or 1 week before isolating their liver microsomes produced a marked inhibition in their ability to hydroxylate estradiol at C-2 to form the corresponding catechol estrogen (Table 1 and Fig. 1). A much smaller effect on this reaction was observed after treatment with SKF-525A (260 μmol/kg body wt.) while even a large dose of piperonyl butoxide (540 μmol/kg body wt.) failed to influence the release of ^3H_2O from [2-^{3}H]E_2. These results were confirmed by the kinetic data (Fig. 2) which showed no change in the V_{max} after treatment with piperonyl butoxide but a small change with SKF-525A without any alteration of the apparent K_m. With CoPP, the K_m was almost doubled (from 3.6 to 6.3 μM) although the V_{max} was not affected significantly. The apparent K_m was also doubled in an incubation with a smaller concentration of microsomes (0.2 mg protein; data not shown). The cytochrome P450 content of the liver was also markedly depleted after treatment with CoPP, but was

TABLE I. Effect of cobalt-protoporphyrin, SKF-525A and piperonyl butoxide on 2-hydroxylation of [2-^{3}H]E_2 by male rat liver microsomes

Treatment	Rats (N)	Release of ^3H_2O (%)
-	4	57.7 ± 8.1
CoPP (48 h)	3	19.2 ± 3.0
CoPP (1 week)	2	13.2 ± 1.0
SKF-525A (48 h)	4	51.7 ± 8.8
Piperonyl butoxide (48 h)	4	65.0 ± 2.2
Non-tissue control	-	1.9

Adult male rats were injected with CoPP (50 μmol/kg), SKF-525A (260 μmol/kg) or piperonyl butoxide (540 μmol/kg) and killed at 48 h or 1 week later. Liver microsomes (2 mg protein) were incubated for 30 min at 38°C with NADPH (0.33 mM) and [2-^{3}H]E_2 (8 μM), and ^3H_2O release measured as described in Materials and Methods. Data are presented as means ± range (N=2) or means ± S.D. (N=3 or 4). Each liver preparation was incubated individually for the hydroxylase assay.

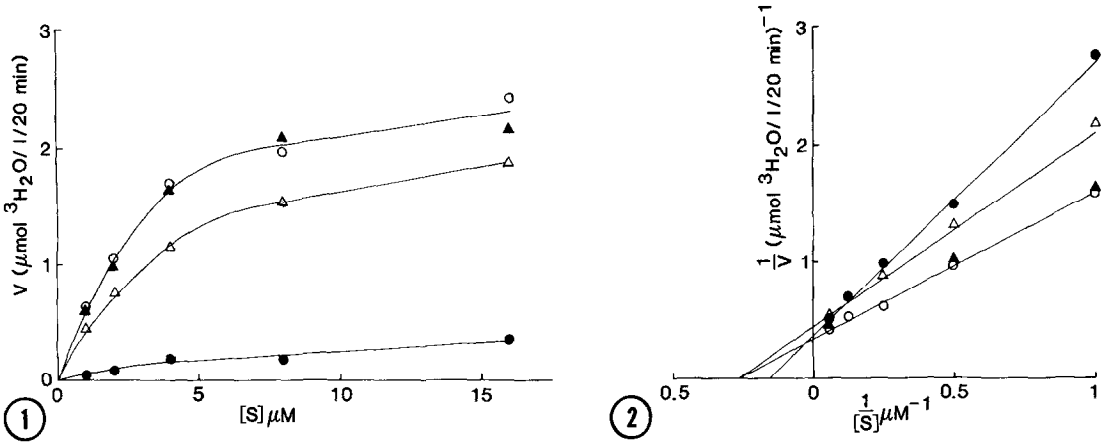

Figure 1. Effect of treatment with CoPP, SKF-525A and piperonyl butoxide on hepatic estrogen 2-hydroxylase activity. Microsomes (0.2 mg protein) from the livers of male rats treated with Saline (○), CoPP (●), SKF-525A (△), or piperonyl butoxide (▲) were incubated with various concentrations of [2-^{3}H]E_2 and NADPH (0.3 mM) and the rate of ^3H_2O formed measured as described in Materials and Methods. Estrogen hydroxylase activity is expressed as μmol ^3H_2O formed/1/20 min.

Figure 2. Lineweaver-Burk plots for the inhibition of hepatic estradiol 2-hydroxylase after treatment with CoPP, SKF-525A and piperonyl butoxide. Experimental conditions and symbols as in Fig. 1. Microsomes from CoPP treated rats were used at a protein concentration of 0.5 mg/ml.

unaffected by SKF-525A and piperonyl butoxide (Fig.3). Nevertheless, the functional activity of cytochrome P450 as indicated by AHH activity was

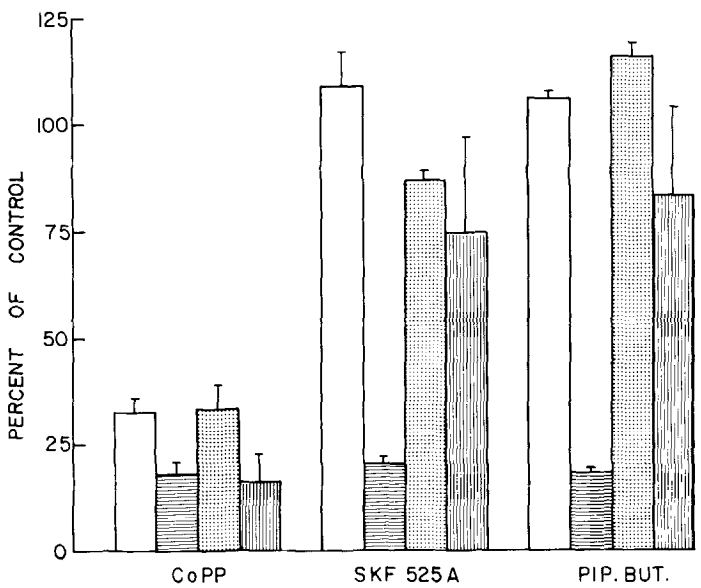

Figure 3. The effect of treatment with CoPP (50 μmol/kg), SKF-525A (260 μmol/kg) or piperonyl butoxide (PIP.BUT.; 540 μmol/kg) on rat hepatic cytochrome P450 concentrations (open bars), AHH activity (horizontal striped bars), ^3H_2O release from [2-^{3}H]E_2 (stippled bars) and serum testosterone concentrations (vertical striped bars). Measurements were performed as described in Methods 48 hr after treatments and compared to results from saline treated controls. All data are expressed as percent ± SEM of control values. N=5, except for estrogen hydroxylase activity where N=2.

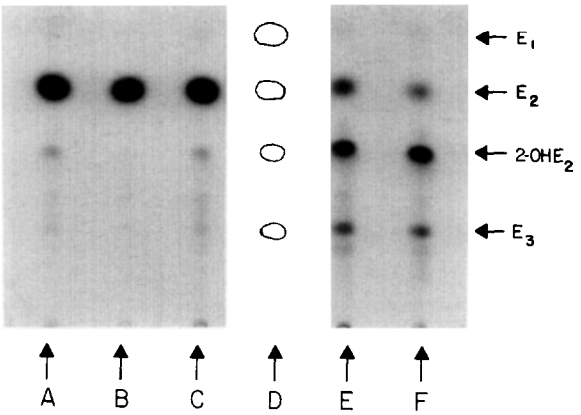

Figure 4. Autoradiogram of products formed from [4-^{14}C]E_2 by liver microsomes of male rats treated with CoPP for 1 day (A), 3 days (B) and 5 days (C), or with saline (E and F). Estrogen standards (D) were visualized as described in Methods.

inhibited to the same extent by all three compounds (Fig. 3). In contrast, the precipitous fall in serum testosterone concentrations following CoPP treatment (4,16) was not seen following administration of the other test compounds (Fig.3). These findings were essentially unchanged even 7 days after chemical treatments (data not shown). To determine whether the decrease in serum testosterone concentrations produced by CoPP had a direct effect on catechol estrogen formation, rats were pretreated with testosterone enanthate (40 mg/kg), which increased the concentration of serum testosterone from 0.61 $\pm$ 0.15 to 10.72 $\pm$ 2.25 ng/ml) in CoPP-treated animals without producing any changes in the metabolism of either ^{3}H- or ^{14}C-labeled estradiol. The inhibitory action of CoPP on the 2-hydroxylation of E_2 was confirmed by separating the products formed from the ^{14}C-labeled estrogen after incubation with liver microsomes and visualizing them by autoradiography (Fig. 4). The yield of catechol estrogen was greatly decreased and much more of the substrate remained unchanged after a 30 min incubation.

DISCUSSION

It might have been predicted that an agent such as CoPP which markedly lowers liver cytochrome P450 levels would also influence the hydroxylation of

compounds which are metabolized by the microsomal monooxygenase system. However, it is of interest that, in the case of estradiol 2-hydroxylase, the affinity of the enzyme for its substrate was also affected by such treatment. This might indicate a differential effect of CoPP on the isozymes involved in steroid hydroxylation. It has been proposed (17) that estrogen 2-hydroxylase consists of more than one form of the enzyme acting on the same substrate with different affinities and sex-specific forms of cytochrome P450 can also be induced by steroid hormones, appearing at sexual maturation (18,19). In addition, prolonged treatment with growth hormone has been shown to decrease the liver microsomal content of male-specific cytochrome P450 and increase the female-specific form (20). Under these conditions, the apparent K_m of estrogen 2-hydroxylase from male liver microsomes was increased significantly (unpublished results). A similar mechanism may underlie the observed changes in apparent K_m following CoPP administration.

Cytochrome P450 has also been identified in the brain (21,22) although its specific localization and function are not fully defined. However, if treatment with CoPP also results in a significant depletion of cytochrome P450 in this organ or in the hypothalamus, it could result in changes in catechol estrogen formation with concomitant effects on LH and PRL secretion (8,9). The effect of CoPP in reducing serum thyroxine and 3,5,3'-triiodothyronine levels (4) might be correlated with the observed effect of decreased catechol estrogen formation in hypothyroidism and the reverse situation in hyperthyroidism (23). However, the dramatic decrease in serum testosterone concentration without a compensatory increase in LH secretion produced by treatment with CoPP (4) will require investigating the metabolism of testosterone under these conditions and such studies are in progress. Treatment of rats with testosterone to maintain circulating testosterone at or above normal levels did not reverse the decrease in estrogen 2-hydroxylase activity of CoPP-treated male rats even though androgen is known to increase the level of this enzyme in ovariectomized female rat liver (6). This again points to an independent effect of the CoPP

on cytochrome P450 synthesis with a secondary decrease in steroid hormone hydroxylation leading to altered circulating hormone levels.

In view of the long-standing controversy about the physiological importance of the catechol estrogens, it would be of considerable interest to study the endocrine consequences of inhibiting the formation of these metabolites of estradiol in the female rat. The long-term depletion of estrogen 2-hydroxylase produced by treatment with CoPP could provide answers to this question. In addition, this heme analogue could be used to test the hypothesis that catechol estrogens are involved directly in estrogen carcinogenesis in the hamster kidney (24). Finally, as suggested previously (3), the effects of CoPP should make it possible to study which isozymes of cytochrome P450 are most readily degraded or are most rapidly resynthesized and how this affects the metabolism of specific steroid hormones.

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