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SELECTIVE INHIBITION OF CYTOCHROME *P*-450 IN RAT TESTICULAR MICROSOMES: EFFECT OF COBALT-PROTOPORPHYRIN ON PROGESTERONE METABOLISM

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Summary—Cobalt-protoporphyrin (CoPP) administration to adult male rats results in a profound reduction in hepatic cytochrome *P*-450 concentration and activity, and decreased plasma concentrations of testosterone and luteinizing hormone (LH). The metabolism of progesterone by rat testicular microsomes isolated 48 h after treatment *in vivo* with CoPP was compared to that in microsomes from control rats. The conversion of progesterone to 17α-hydroxyprogesterone and 4-androstenedione, which is NADPH-dependent, was reduced by approximately 40% in testicular microsomes following treatment with CoPP (50 μmol/kg body weight) and this inhibition was dose-dependent. The concentration of cytochrome *P*-450 in testicular microsomes and the activity of 7-ethoxycoumarin de-ethylase (a cytochrome *P*-450 dependent function) were also reduced following treatment with CoPP in contrast to two other functional assays of cytochrome *P*-450, aryl hydrocarbon hydroxylase and ethylmorphine demethylase, which were unaffected by treatment with CoPP. Thus, the profound effect of CoPP on androgen homeostasis has been extended to include decreased testicular synthesis of 4-androstenedione in addition to increased hepatic metabolism of testosterone, attenuated pituitary LH release in response to luteinizing hormone-releasing hormone, and failure of testicular response to LH.

INTRODUCTION

Cobalt-protoporphyrin (CoPP) is a synthetic analogue of heme. Parenteral administration of large doses of CoPP (10–50 μmol/kg body weight [body wt]) to adult male rats results in marked induction of heme oxygenase (H.O.) and reciprocal inhibition of the concentration and catalytic activities of cytochrome *P*-450 in the liver [1]. Concomitantly, treatment with CoPP results in suppression of plasma concentrations of thyroid hormones, testosterone and luteinizing hormone (LH) [2], and sustained weight loss [1–4]. However, the effects of CoPP on hormone concentrations are causally independent of both the induction of hepatic H.O. and the weight loss which follows CoPP administration [3].

Inhibition of hepatic cytochrome *P*-450 activities by CoPP also leads to abnormalities in the metabolism of circulating hormones. Thus, inhibition of estradiol 2-hydroxylase results in decreased catechol estrogen synthesis in the liver [5]. The hepatic hydroxylation of testosterone is also decreased, but increased rates of 3β- and 5α-reduction of testosterone lead to increased concentrations of 3α- or 3β-androstanediol (Adiol), 5α-androstan-3ol,17-one and 5α-androstan-3,17-dione [6]. The lack of reciprocal LH elevation observed when testosterone concentrations are lowered by CoPP treatment [2, 3] may result from the increased concentration of Adiol; both 3α- and 3β-Adiol have been shown to be more potent inhibitors of pituitary LH secretion than testosterone [7].

Hypothalamic content of luteinizing hormone-releasing hormone (LHRH) is not affected by CoPP. Pituitary LH secretion in response to exogenous LHRH is attenuated by CoPP both *in vivo*, and *in vitro* in primary pituitary cultures [8]. However, the amounts of LH released from the pituitary in response to *in vivo* LHRH stimulation are insufficient to increase plasma testosterone concentrations; even pharmacological concentrations of LHRH (500 mg/kg body wt) fail to increase testosterone levels, despite peak plasma LH concentrations of 150 mg/ml [8]. Testosterone biosynthesis is known to be dependent on isozymes of cytochrome *P*-450 [9–11] and CoPP is known to inhibit the

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Abbreviations: CoPP (cobaltic-protoporphyrin IX); H.O. (heme oxygenase); LH (luteinizing hormone); Adiol (3α- or 3β-androstanediol); LHRH (luteinizing hormone-releasing hormone); 17αOH-progesterone (17α-hydroxyprogesterone); KP (potassium phosphate); AHH (aryl hydrocarbon hydroxylase); 7-EC (7-ethoxycoumarin de-ethylase); EMD (ethylmorphine demethylase); AD (4-androstenedione); HCG (human chorionic gonadotrophin).

activities of cytochrome P-450 [1, 5, 6]. In the present paper, we have examined this presumed direct effect of CoPP on testicular testosterone synthesis or secretion. Utilizing [^{14}C]progesterone, we have demonstrated CoPP-mediated inhibition of the testicular microsomal cytochrome P-450 activities involved in both exogenous drug metabolism and in the biosynthetic pathway of testosterone.

MATERIALS AND METHODS

Chemicals

[4-^{14}C]Progesterone (57.2 mCi/mmol)], [4-^{14}C]androstenedione (52 mCi/mmol) and [4-^{14}C]testosterone (50 mCi/mmol) were purchased from New England Nuclear Corporation (Boston, Mass) and shown to be free of radioactive impurities by chromatography and autoradiography. 17α-Hydroxyprogesterone (17αOH-progesterone) standard and NADPH were purchased from Sigma Chemical Company (St Louis, Mo.) and cobalt-protoporphyrin from Porphyrin Products (Logan, Ut.).

Treatment of animals

Adult mycoplasma-free Sprague–Dawley male rats (250–300 g) were purchased from Charles River (Wilmington, Del.) and acclimatized for at least 1 week in the Laboratory Animal Research Center at The Rockefeller University in a temperature controlled (22 ± 1°C) room with a 12/12 h light/dark cycle with free access to Purina rat chow and water. CoPP was dissolved in 0.1 N NaOH, diluted with saline and the pH adjusted to 7.4–7.8 prior to subcutaneous injection in the nuchal area as described previously [1]; control animals received an equal volume of vehicle. Animals were sacrificed 48 h after injection; this time point was used because previous studies have demonstrated that the nadir in testosterone and LH concentrations occurs 48 h after CoPP treatment [3]. Animals were perfused *in situ* with 30 ml of ice-cold 0.9% saline via the heart, then the testes were dissected, washed and weighed after removal of the capsule. Eight rats were used in each experimental group and the testes of two rats were pooled giving rise to four samples per group. Pooled testes were minced coarsely with scissors, then homogenized in 3 vol 0.1 M potassium phosphate (KP) buffer pH 7.4 containing 0.25 M sucrose by 8 passes in motor-driven Potter–Elveheim glass homogenizers with Teflon pestles. Homogenates were centrifuged at 4°C for 20 min at 10,000 g; the resulting supernatants were decanted and centrifuged at 4°C for 60 min at 100,000 g. Resulting microsomal pellets were rehomogenized in one half the original homogenate volume of 0.1 M KP, pH 7.4. Cytochrome P-450 concentrations [12] and H.O. activities [13] were determined immediately; aryl hydrocarbon hydroxylase (AHH: [14]), 7-ethoxycoumarin de-ethylase (7-EC: [15]), ethylmorphine demethylase (EMD: [16, 17]) and [^{14}C]progesterone metabolism were determined on samples frozen in liquid nitrogen. A Perkin–Elmer Lamda 7 scanning spectrophotometer with the PECSS computer program was utilized for the determination of cytochrome P-450 concentrations, and H.O. and EMD activities; a Hitachi MPFIV fluorescence spectrophotometer with an R928 photomultiplier tube was used for the determination of AHH and 7-EC activities. Protein content was determined by the method of Lowry *et al.*[18] using bovine serum albumin as a standard.

[^{14}C]Progesterone metabolism

[^{14}C]Progesterone (2 μg) was incubated with microsomal suspensions (0.2–0.8 mg protein) with or without NADPH (0.13 mM) at 37°C with constant shaking in a total volume of 4 ml. Radioactive products were extracted three times with equal volumes of diethyl ether, examined by thin-layer chromatography (TLC) on silica gel using benzene–heptane–ethyl acetate (5:2:3 by vol) and visualized by radioautography. Areas of the TLC plate containing the substrate or its metabolites were scraped into counting vials and radioactivity determined in a liquid scintillation spectrometer. The recovery of added material was 75–85% by this method; results have not been corrected for those procedural losses. The identities of products on TLC were established utilizing ^{14}C-labelled standards, or unlabelled standard in the case of 17αOH-progesterone. With the latter, identity was established by reverse isotope dilution; 20 mg of 17αOH-progesterone was mixed with approximately 100,000 dpm of the ethanol eluate of the corresponding area on the TLC plate, and recrystallized from ethanol:H_2O (1:1). Radioactivity (in 0.5 mg) was 1998, 2213 and 2137 dpm respectively in the 1st, 2nd and 3rd recrystallizations.

Statistical analysis

The significance of differences in means was tested utilizing Student's t-test, ANOVA or Duncan's multiple comparison test, which was performed on the Rockefeller University Hospital Clinfo System.

RESULTS

Administration of CoPP to rats is known to cause weight loss [1–4] and seminal vesicle weights are decreased 7 days after CoPP treatment [2]. Consequently, it was important to determine if any effects of CoPP on testicular progesterone metabolism were attributable to testicular atrophy or loss of microsomal protein. No discernible effect of CoPP on testicular weight or microsomal protein content was observed even at the highest dose (50 μmol/kg) when examined 48 h after treatment (data not shown). Table 1 shows the effects of varying doses of CoPP on heme pathway and drug-metabolizing enzymes in testicular microsomes. The concentration of cytochrome P-450 was significantly decreased ($P < 0.05$) by about 35% with all doses of CoPP examined. In

Table 1. The effects of CoPP treatment on heme pathway and drug-metabolizing enzymes in testicular microsomes

	Cytochrome *P*-450 (nmol/mg protein)	Heme oxygenase (nmol bilirubin/mg protein/h)	Aryl hydrocarbon hydroxylase (nmol 8-OH benzo(α)pyrene/mg protein/h)	7-Ethoxycoumarin de-ethylase (μmol/mg protein/h)	Ethylmorphine demethylase (μmol formaldehyde/mg protein/h)
Control	0.18 ± 0.007	7.85 ± 0.41	0.21 ± 0.01	0.095 ± 0.003	0.070 ± 0.005
CoPP 10 (μmol/kg)	0.12 ± 0.003*	8.43 ± 0.26	0.21 ± 0.01	0.064 ± 0.002*	0.060 ± 0.004
CoPP 25 (μmol/kg)	0.12 ± 0.004*	7.89 ± 0.41	0.24 ± 0.02	0.059 ± 0.002*	0.057 ± 0.008
CoPP 50 (μmol/kg)	0.11 ± 0.007*	7.55 ± 0.36	0.25 ± 0.05	0.052 ± 0.005*	0.066 ± 0.003

Values are means ± SEM for 4 rats per group. *$P < 0.05$ utilizing Dunnett's multiple comparison test.

contrast to the marked induction observed in liver [1], testicular H.O. was unaffected by any dose of CoPP administered. 7-EC activity was decreased by 30–45% ($P < 0.05$) in a dose-dependent fashion by CoPP, but the activities of AHH and EMD were unaltered (Table 2).

The products formed from the incubation of [^{14}C]progesterone with untreated testicular microsomes are depicted in Fig. 1. Increasing the concentration of microsomal protein resulted in incremental decreases in the concentration of progesterone and inverse increases in the concentrations of 17αOH-progesterone and 4-androstenedione (AD). At the highest concentration of microsomal protein, a linear increase in the concentration of 17αOH-progesterone was not observed, presumably due to more complete metabolism to AD and testosterone. The effect of CoPP (50 μmol/kg body wt) on the metabolism of [^{14}C]progesterone by testicular microsomes is illustrated in Fig. 2. In the absence of NADPH, there was no discernible metabolism of progesterone, consistent with the known dependence of cytochrome *P*-450 on a supply of NADPH. In the presence of NADPH, metabolism of progesterone was inhibited by approximately 40% in microsomes from CoPP-treated rats compared to control rats (Fig. 2).

The effect of increasing dosages of CoPP on testicular microsomal metabolism of progesterone is shown in Table 2. The accumulation of both 17αOH-progesterone and AD was inhibited progressively in a dose dependent fashion with increasing doses of CoPP; conversely, unmetabolized progesterone increased reciprocally. The time-course of progesterone metabolism by testicular microsomes from control or CoPP-treated (50 μmol/kg body wt) rats is shown in Fig. 3. Accumulation of AD was linear over 45 min in both control and CoPP-treated microsomes. 17αOH-Progesterone accumulation appeared to peak after 30 min of incubation with untreated microsomes, presumably due to further metabolism to AD and testosterone; this effect was not observed in CoPP-treated microsomes (Fig. 3).

DISCUSSION

The results of this study indicate that *in vivo* treatment with CoPP decreased the concentration of testicular microsomal cytochrome *P*-450. However, in marked contrast to hepatic microsomes [1], no induction of testicular microsomal H.O. activity was observed following treatment with CoPP. A possible explanation for the refractory nature of testicular H.O. activity is afforded by the recent demonstration that the rat testis contains two H.O. genes, one of which is expressed at high constitutive levels and is unaffected by prototypic agents which induce H.O. activity in the liver [19–21]; the other gene is expressed at low levels [21], but is inducible by HCG [22], arsenate [23] and hypophysectomy [24]. Interestingly, HCG also causes decreased levels of cytochrome *P*-450 in the testis [22]. The fact that CoPP inhibits testicular cytochrome *P*-450 concentration and activities without affecting H.O. activity argues strongly against the hypothesis that CoPP-mediated alterations in cytochrome *P*-450 are caused by increased H.O. activity leading to relative heme deficiency and increased concentrations of apo-cytochrome *P*-450.

Cytochrome *P*-450-dependent drug-metabolizing activities in testicular microsomes were also reduced by treatment with CoPP as evidenced by the reduced activity of 7-EC, but AHH and EMD activities were unaffected. This is in contrast to the effects of CoPP in hepatic microsomes where both EMD [1] and AHH [5] activities were inhibited. A possible explanation for this discrepancy is that the accumulation of

Table 2. Percentage conversion of [^{14}C]progesterone to 17α-hydroxyprogesterone and 4-androstenedione by rat testicular microsomes

	17αOH-Hydroxyprogesterone	4-Androstenedione	Progesterone
Control	35.8 ± 1.2 $n = 8$	5.4 ± 2.5 $n = 6$	13.5 ± 1.5 $n = 5$
CoPP 10 (μmol/kg)	33.9 ± 2.2 $n = 4$	4.5 ± 0.6 $n = 4$	17.4 ± 2.0 $n = 4$
CoPP 25 (μmol/kg)	27.8 ± 3.6* $n = 4$	3.4 ± 0.5 $n = 4$	25.0 ± 4.8* $n = 4$
CoPP 50 (μmol/kg)	21.8 ± 1.3* $n = 7$	2.8 ± 0.7 $n = 5$	26.7 ± 2.9* $n = 4$

Values are mean ± SEM; n = the number of replicate determinations; *$P < 0.05$ utilizing Dunnett's multiple comparison test.

CoPP in the testis is less than that in the liver, perhaps due to the tight blood-testicular barrier. Comensurate with this hypothesis is the greater reduction in cytochrome P-450 concentrations observed in the liver (60% [5]) vs the testis (40%) when determined 48 h after s.c. administration of CoPP 50 μmol/kg body wt. This factor might also be relevant to the lack of H.O. induction by CoPP in the testis. Inhibition of cytochrome P-450-dependent activities by CoPP in testicular microsomes was also demonstrated by the inhibition of NADPH-dependent progesterone metabolism. The maximal reduction of progesterone metabolism was approximately 40%, in good agreement with the decreases observed in other cytochrome P-450 indices.

Taken together, these results provide clear evidence that *in vivo* treatment with CoPP leads to rapid reductions in the concentration, and differential reductions in the activities, of testicular microsomal cytochrome P-450. Testicular weight and microsomal protein content were not reduced by CoPP after 48 h, rendering an indirect effect of CoPP unlikely. The diminished metabolism of progesterone therefore suggests that CoPP treatment causes a direct reduction in AD and testosterone biosynthesis. This observation is consistent with the previously described failure of testicular response to LH released in response to *in vivo* LHRH administration in CoPP-treated rats [8].

The results presented above suggest that both 17α-hydroxylase and 17,20-desmolase in testicular microsomes are inhibited by CoPP. This coordinate reduction in enzyme activities was also observed

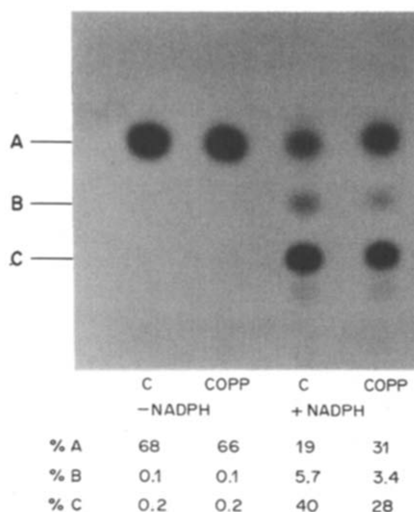

Fig. 2. Autoradiogram of the effect of CoPP on the metabolism of [^{14}C]progesterone by rat testicular microsomes. [^{14}C]Progesterone (2 μg) was incubated for 30 min at 37°C with or without NADPH (0.13 mM) and testicular microsomes ($\approx$0.4 mg protein) prepared from rats 48 h after treatment with saline or CoPP (50 μmol/kg body wt). TLC, radioautography, identification of the products and numerical presentation of the percentages of radioactivity in the products are as described in Fig. 1.

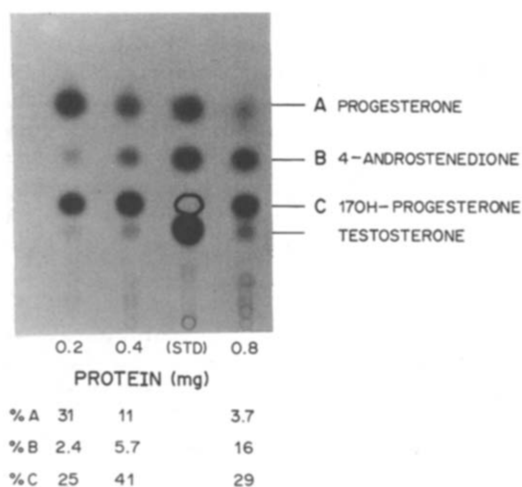

Fig. 1. Radioautogram of the products formed from [^{14}C]progesterone by rat testicular microsomes. [^{14}C]Progesterone (2 μg) was incubated with NADPH (0.13 mM) and the indicated amounts of testicular microsomal protein for 30 min at 37°C. The radioactive products were separated by TLC, visualized by radioautography and compared to authentic standards as described in Methods. The percentages of the added radioactivity in each product were determined as described in Methods and are presented numerically underneath the radioautogram.

following treatment of animals with HCG [25] and with testosterone [26]. However, administration of ketoconazole, an antifungal agent which inhibits cytochrome P-450 via its imidazole moiety, leads to inhibition of cholesterol side-chain cleavage enzyme and C-17,20-lyase, but 17α-hydroxylase is unaffected [27] Thus, imidazole-containing compounds which inhibit cytochrome P-450 [27–30] display differential inhibitory properties which are distinct from CoPP. Additionally, CoPP administration may result in inhibition of the testicular LH-regulated mitochondrial cholesterol 20,22-desmolase side-chain cleavage enzyme, which is also a cytochrome P-450 isozyme [10, 11]. However, in contrast to the liver [1], testicular mitochondrial δ-aminolevulinic acid synthase was not decreased following CoPP (data not shown) which renders mitochondrial accumulational of CoPP in the testis questionable. Another possible contribution to decreased testosterone synthesis following CoPP treatment is the observation that plasma estradiol concentrations may be elevated secondary to inhibition of hepatic estradiol 2-hydroxylase [5]; estradiol inhibits both microsomal 17,20-desmolase and 17α-hydroxylase [31, 32].

Administration of CoPP to adult male rats is known to decrease plasma testosterone and LH concentrations [2, 3], decrease pituitary LH secretion in response to LHRH [8], decrease testicular response to LH, [8], decrease hepatic estradiol metabolism [5], and increase hepatic testosterone metabolism [6]. This paper demonstrates that another action of CoPP is inhibition of testicular androgen synthesis. Most

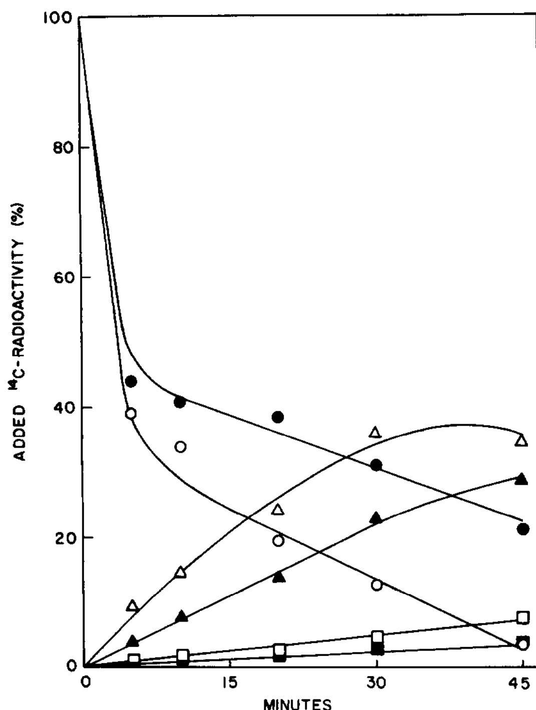

Fig. 3. Time-course of [^{14}C]progesterone metabolism by testicular microsomes from control and CoPP-treated rats. Testicular microsomes ($\approx$0.4 mg) were isolated 48 h after treatment of rats with saline (open symbols) or CoPP (50 μmol/kg body wt. closed symbols). Microsomes were incubated for the times indicated and products identified and radioactivity determined as described in Methods, ○, ● = progesterone; △, ▲ = 17αOH-progesterone; and □, ■ = 4-androstenedione. Averages of 2 determinations per point are presented.

of these actions are explicable on the basis of inhibition of cytochrome P-450 function and underscore the critical contribution of the mixed function monooxygenase system to hormonal homeostasis.

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