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SUPPRESSION OF 5α-ANDROSTANE-3β,17β-DIOL HYDROXYLASE ACTIVITY IN RAT PITUITARY BY COBALT PROTOPORPHYRIN: IMPLICATIONS FOR TESTOSTERONE HOMEOSTASIS

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Summary—Cobalt protoporphyrin (CoPP) administered subcutaneously to adult male rats caused a marked reduction in the conversion of 5α-androstane-3β-17β-diol (3β-adiol) to its main triol derivative (6α-atriol) by homogenates of the pituitary but not of the prostate or brain (ventromedial hypothalamus and cortex). No effect in the brain was observed when this heme analogue was infused intracerebroventricularly. 3β-adiol hydroxylase, the enzyme responsible for the reaction and whose main function is thought to be the elimination of dihydrotestosterone and its metabolites from target tissues, was also inhibited by CoPP and SKF-525A added *in vitro*. The reaction was microsomal and dependent on NADPH. It is proposed that the lack of reciprocal elevation of luteinizing hormone in the face of the low testosterone levels observed following treatment with CoPP may be due, in part, to increased levels of androstanediols. These metabolites accumulate because of increased production from testosterone and decreased conversion to their triol derivatives in the pituitary.

INTRODUCTION

Several cytochrome *P*-450-catalyzed steroid hydroxylations occur in the brain but the level of these activities is low and, apart from their role in the sexual differentiation of the central nervous system [1], very little is known about their other physiological functions. The enzyme 5α-androstane-3β,17β-diol (3β-adiol) hydroxylase, a constitutive form of cytochrome *P*-450, occurs in rat brain with catalytic activity at least 300-fold higher than that reported for any other steroid hydroxylase in this organ [2]. This hydroxylase is also found in the prostate [3–5] and pituitary [6] and is considered to have a major role in the elimination of dihydrotestosterone from its target tissues after its reduction to 3β-adiol.

Subcutaneous (s.c.) administration of high doses of cobalt protoporphyrin (CoPP) leads to a large and sustained induction of hepatic heme oxygenase and a marked reduction in hepatic cytochrome *P*-450 concentration [7]. Additionally, high doses of CoPP lead to a decline in the serum concentration of both testosterone and luteinizing hormone (LH) [8,9] associated with attenuation of the pituitary LH responses to luteinizing hormone releasing hormone (LHRH) [10]. After treatment with this heme analogue, the hepatic hydroxylation of testosterone is decreased, but a compensatory increase in the rates of 3β- and 5α-reduction of testosterone leads to elevated levels of androstanediols [11]. These compounds are known to be potent inhibitors of pituitary LH secretion [12]. This observation, in conjunction with the attenuated LH response to LHRH, may account in part for the lack of reciprocal elevation of LH despite the low serum testosterone levels produced by s.c. treatment with CoPP. However, intracerebroventricular (i.c.v.) administration of small doses of CoPP, although biologically active in the brain and hypothalamus, is without effect on serum testosterone and LH concentrations [13].

The concentration of these biologically active reduced metabolites of testosterone, particularly that of 3β-adiol, may also be raised in the brain and pituitary following administration of CoPP as a result of decreased cytochrome *P*-450-dependent conversion to their hydroxylated triol derivatives. In this paper, we have investigated whether CoPP inhibits triol formation in the pituitary and brain to assess the contribution of

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this putative mechanism to the refractory decreases in LH concentration caused by this metalloporphyrin. We have compared the effects of parenteral versus intracerebroventricular administration of CoPP, studied temporal relationships and looked for changes in the activity of 3β-adiol hydroxylase in the ventral prostate, brain (hypothalamus and cortex) and pituitary after treatment with CoPP.

MATERIALS AND METHODS

Chemicals

5α-[1α,2α-^{3}H]androstane-3β,17β-diol (3β-adiol) (52 Ci/mmol) was purchased from Amersham (Oakville, Ontario) and was shown to be free of radioactive impurities by autoradiography (Hyperfilm-^{3}H, Amersham) after thin-layer chromatography (TLC) (see below). 6α-atriol used as standard was a generous gift from Dr P. Ofner (Lemuel Shattuck Hospital, Boston, Mass.) and SKF-525A (diethylaminoethyl-2,2-diphenylvalerate HCl) was from Smith, Kline and Beckman, Canada (Mississauga, Ont.). Unlabeled steroids were purchased from Steraloids (Wilton, N.H.) and cobalt protoporphyrin (CoPP) from Porphyrin Products (Logan, Utah).

Treatment of animals

Adult male Sprague–Dawley rats (250–300 g), with free access to Purina rat chow and water were injected subcutaneously in the nuchal region with CoPP (50 μmol/kg bw) as described previously [7]. In certain experiments, CoPP (0.4 μmol/kg bw) was injected intracerebroventricularly (i.c.v.) into the brain via chronic indwelling stainless steel cannulae inserted stereotactically under metofane and fentanyl/droperidol anesthesia (Pitman-Moore, Washington Crossing, N.J.) as previously described [12]. Correct cannula placement was confirmed by eliciting drinking behaviour following i.c.v. injection of angiotensin (100 ng in 10 μl saline).

Tissue preparation and incubation

The animals were killed by decapitation and the ventral prostate, pituitary and brain removed. The ventromedial hypothalamus and a section of the cortex were isolated from the brain and used immediately or kept frozen at −68°C together with the prostate and pituitary for later incubation. The tissues were weighed and the appropriate amount homogenized in 0.05 M potassium phosphate (1 ml) pH 7.4 using a Potter–Elvejhem homogenizer with a Teflon pestle. [1,2α-^{3}H]adiol ($4.5–7.0 \times 10^5$ dpm in 0.1 μg) was incubated with the homogenate containing 5–10 mg of tissue (as indicated) with NADPH (1.8 mM) at 37°C with constant shaking for 20 min in a total volume of 1 ml. Radioactive products were extracted three times with equal volumes of diethyl ether, separated by TLC on silica gel using chloroform–ethyl acetate–ethanol (40:10:7 by vol) [13] or benzene–ethanol (9:1 by vol) and visualized by autoradiography. Areas of the TLC plate containing the substrate or its main metabolite (6α-atriol) were scraped into counting vials and radioactivity determined in a liquid scintillation spectrometer. The recovery of [1,2α-^{3}H]adiol added to buffer, extracted and subjected to TLC before reisolation and counting was 60–70% by this method; results have not been corrected for these procedural losses. The conversion of 3β-adiol to its other, less polar metabolites, which accounted for <20% of the ^{3}H-radioactivity in the ether fraction, was not determined. The steroid standards (5–10 μg of 3β-adiol and 6α-atriol) were localized by spraying with Folin–Ciocalteu reagent diluted with water (1:2 by vol) followed by heating on a hot plate. The protein content of the homogenates was determined by the method of Lowry *et al.* [14] using bovine serum albumin as standard. The effectiveness of the systemic treatment with CoPP was checked by preparing liver microsomes and measuring their feminizing effect on 3β- and 5α-reduction of testosterone and 4-androstenedione [11].

Characterization of 6α-atriol

The identity of 6α-atriol shown previously to be the main product formed by brain and prostate [2], was established by its co-elution with the authentic standard (m.p. 230–231°C) in 2 different TLC systems (see above) and also by high-performance liquid chromatography (HPLC). For the latter, products were separated on a reverse phase Beckman C-18 ultrasphere-ODS column using an isocratic mobile phase of acetonitrile–methanol–water (1:3:3 by vol) at a flow rate of 1.0 ml/min and an operating pressure of 2.7×10^3 psi [15]. The purity of the 3β-adiol used as substrate was also confirmed by this technique.

Table 1. Effect of cobalt protoporphyrin on the conversion of 5α-androstane-3β,17β-diol (3β-adiol) to its 6α-atriol derivative by pituitary or prostate homogenates

Tissue and treatment	6α-atriol (%)	Water-soluble products (%)	3β-adiol remaining (%)
Pituitary			
Control (16)	7.1 ± 1.2	24.2 ± 2.1	18.4 ± 2.4
CoPP (17)	2.0 ± 0.2*	17.8 ± 2.4	28.5 ± 3.1***
Prostate			
Control (6)	16.1 ± 1.6	30.9 ± 1.4	6.9 ± 1.6
CoPP (6)	14.6 ± 2.0	26.2 ± 2.9	15.7 ± 3.6**

The ^{3}H-labeled steroid (0.1 μg) was incubated with homogenates of pituitary (5 mg) or prostate (10 mg) + NADPH (1.8 mM) for 20 min at 37°C in a total volume of 1 ml of 0.05 M potassium phosphate, pH 7.4. CoPP (50 μmol/kg bw) was injected 5 days before sacrifice. The number of rats used is shown in parenthesis. Results are expressed per mg wet wt of tissues.
*$P < 0.001$, **$P < 0.05$, ***$P < 0.02$ vs control (Student's t-test).

RESULTS

It is evident (Table 1) that administration of a large dose of CoPP (50 μmol/kg bw) to mature male rats causes a significant decrease ($P < 0.001$) in the conversion of 3β-adiol to its 6α-triol derivative in pituitary homogenates. The concentrations of 7α- and 7β-atriol, which are formed only in small amounts by 3β-adiol hydroxylase [2], were not measured. A concomitant decrease in more polar water-soluble products was observed and the level of metabolism of 3β-adiol was also diminished. No apparent change in the formation of 6α-atriol was observed in the prostate although a significant decrease ($P < 0.05$) in substrate metabolism was produced. The results are generally expressed per mg wet weight of tissue and were not influenced by expressing the values per mg protein, as indicated in Table 3. The identity of the main product (6α-atriol) which had been characterized previously [2], was confirmed by its chromatographic properties (TLC and HPLC) compared to those of the authentic steroid. The characteristics of the hydroxylation reaction in both pituitary and prostate were established from the increased polarity of the products, the absolute requirement for NADPH and the marked inhibition by SKF-525A (0.1 mM) a prototypic inhibitor of cytochrome *P*-450-dependent oxidation [16] (Table 2). In addition, microsomal preparations (resuspended 100,000 *g* pellet) of the pituitary and prostate gave the same products (not shown) and the reaction was abolished by boiling the tissue homogenates. CoPP added *in vitro* caused some inhibition of the reaction even at the lowest concentration (10 μM) tested.

Subcutaneous treatment with CoPP (25 μmol/kg bw), resulted in a marked decrease in the formation of 6α-atriol by the pituitary after 2 or 5 days (Table 3), but was without effect in the hypothalamus or cortex of the brain; within 21 days of exposure to the compound, the effect in the pituitary was lost (data not shown). Intracerebroventricular administration of CoPP was utilized to determine whether the failure of s.c. injections of the compound to influence 6α-atriol formation in the central nervous system (CNS) resulted from exclusion of CoPP by the blood–brain barrier. The dosage of CoPP utilized (0.4 μmol/kg bw) was chosen because it has been shown to produce biological effects following i.c.v. administration [13]. As expected, i.c.v. treatment with

Table 2. Cofactor requirement for the conversion of 3β-adiol to its 6α-triol derivative by pituitary, hypothalamus or prostate homogenates

Additions	6α-atriol (%)	Water-soluble radioactivity (%)	3β-adiol remaining (%)
Pituitary			
Control	12.1	27.2	12.4
NADPH omitted	0.3	3.1	67.6
SKF-525 A	1.7	4.7	27.4
Boiled tissue	0.1	1.7	41.2
CoPP (10 μM)	5.4	11.5	25.6
CoPP (30 μM)	3.3	9.1	37.7
CoPP (100 μM)	1.1	8.3	39.3
Hypothalamus			
Control	6.3	44.2	11.0
CoPP (10 μM)	5.5	41.4	14.6
CoPP (30 μM)	2.0	38.1	12.7
CoPP (100 μM)	0.7	31.8	19.1
Prostate			
Control	13.1	29.0	6.5
NADPH omitted	0.8	3.4	52.4
SKF-525A	4.1	5.9	49.9
Boiled tissue	0.2	2.2	65.2

^{3}H-labeled steroid (0.1 μg) was incubated with homogenates of pituitary or hypothalamus (5 mg) or prostate (10 mg) under the conditions described in Table 1 in the presence or absence of NADPH (1.8 mM), SKF-525A (0.1 mM) or CoPP. Results are the means of determinations from 2 experiments.

Table 3. Effect of mode of administration of CoPP on the hydroxylation of 3β-adiol to 6α-atriol by homogenates of rat pituitary, hypothalmus and cerebral cortex

Tissue	Days after treatment	6α-atriol (%) Subcutaneous CoPP	6α-atriol (%) Intracerebroventricular CoPP
Pituitary			
Control		5.4 ± 0.2 (7)	4.0 ± 0.1 (10)
CoPP	2	2.4 ± 0.2 (9)*	4.1 ± 0.3 (12)
Control		4.6 ± 0.5 (11)	3.5 ± 0.3 (5)
CoPP	5	1.9 ± 0.2 (11)*	4.2 ± 0.2 (5)
Control**		5.4 ± 0.2 (5)	5.2 ± 0.2 (5)
CoPP**	5	2.8 ± 0.3 (5)*	5.8 ± 0.3 (5)
Hypothalamus			
Control		5.8 ± 0.4 (8)	6.0 ± 0.6 (4)
CoPP	5	4.4 ± 0.6 (8)	7.2 ± 0.6 (5)
Control**		4.7 ± 0.3 (5)	6.2 ± 0.6 (5)
CoPP**	5	3.9 ± 0.5 (5)	6.8 ± 0.5 (5)
Cortex			
Control		5.2 ± 0.9 (8)	4.0 ± 0.6 (5)
CoPP	5	4.4 ± 0.4 (8)	4.7 ± 0.4 (5)

^{3}H-labeled steroid (0.1 μg) was incubated with homogenates of pituitary (5 mg), hypothalamus or cortex (10 mg) under the conditions described in Table 1. The animals were treated with CoPP (25 μmol/kg bw s.c., or 0.4 μmol/kg bw i.c.v.) and killed at the times indicated. The number of rats used is shown in parenthesis.

*$P < 0.001$ vs control (Student's t-test).

**Values expressed per mg of protein.

CoPP was without effect on 6α-atriol formation in the pituitary, which is outside the blood–brain barrier. However, this method of administration of the metalloporphyrin was also without effect on 6α-atriol formation in the hypothalamus and cortex of the brain (Table 3).

DISCUSSION

Previous studies have clearly demonstrated that a large dose of CoPP, a synthetic heme analogue which produces a profound decrease in cytochrome *P*-450 concentration and activities in the liver [7], also affects the hypothalamic–pituitary–testicular axis, resulting in decreased serum concentrations of both testosterone and LH [8–11]. The decrease in LH levels did not occur in castrated animals [10]. To account for the lack of expected reciprocal elevation of LH, despite low serum testosterone levels, we had proposed [11] that, after treatment with CoPP, this regulatory function of testosterone might be subsumed by the increased amounts of its reduced metabolites (androstanediols) produced in the liver. These steroids are known to be potent inhibitors of LH secretion [12] and would act in concert with the attenuated response of the pituitary to LHRH in treated rats [10]. However, CoPP could also increase the concentration of bioactive reduced dihydrotestosterone (DHT) metabolites directly in the brain and pituitary by inhibiting their cytochrome *P*-450-catalyzed hydroxylation to triols and other polar metabolites. In this manuscript, we have demonstrated that s.c. treatment with large doses of CoPP results in marked reduction in 6α-atriol formation in the pituitary and commensurate increases in the concentration of residual unmetabolized 3β-adiol. In contrast, i.c.v. administration of CoPP is without effect on pituitary 6α-atriol formation, a finding which is consistent with the failure of this route of administration of the metalloporphyrin to inhibit serum testosterone and LH concentrations [10]. Taken together, those results support our hypothesis that the lack of compensatory increase in LH concentrations observed in testosterone-depleted CoPP-treated animals may result from increased levels of DHT metabolites secondary to inhibition of 3β-adiol hydroxylase in the pituitary. Elevated levels of androstanediols derived from the liver [11] would also inhibit LH release and our current results indicate that these metabolites of testosterone would persist longer because of inhibition of their conversion to atriols after treatment with CoPP. This metalloporphyrin also affects other pituitary functions including LHRH-stimulated release of LH which is attenuated [10]. However, which of these multiple mechanisms of action of CoPP predominates is not known.

No effect on 6α-atriol formation was seen in prostate or CNS tissues from animals treated s.c. with CoPP. Furthermore, i.c.v. administration of CoPP also failed to inhibit 3β-adiol hydroxylation in the CNS. It is already known that the most active steroid hydroxylase in the brain is that which converts 3β-adiol to its

6α-,7α- and 7β-hydroxy derivatives and that this enzyme is also found in the prostate [3–5]. It has high substrate-specificity, is distributed evenly throughout the central nervous system and is not altered by hormonal treatment [2] unlike some of the other cytochrome *P*-450 enzymes occurring in the brain [17, 18]. The constitutive nature of 3β-adiol hydroxylase in the brain [2] may account for the failure of CoPP to affect 6α-atriol formation, perhaps due to differential regulation of the enzyme activity in different tissues. Alternatively, the lack of effect of i.c.v. CoPP may have been due to the low dose of CoPP infused (0.4 μmol/kg bw). The prolonged and presumably non-specific inhibition of cytochrome *P*-450 in the pituitary is likely to contribute to the mechanism by which CoPP affects various endocrine events, including LH secretion. Our current studies provide a pharmacological approach to the elucidation of such physiological processes.

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