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INFLUENCE OF PREGNENOLONE-16α-CARBONITRILE ON THE ANESTHETIC EFFECT AND HEPATIC MICROSOMAL METABOLISM OF PROGESTERONE IN THE RAT

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Abstract—The inhibition of progesterone anesthesia in female rats pretreated for 3 days with pregnenolone-16α-carbonitrile (PCN) was associated with a decreased level of labeled progesterone and its metabolites in brain and serum. When incubated with progesterone, the hepatic microsomes of these animals produced more hydroxylated metabolites and less 5α-pregnane-3,20-dione than those of the controls. Such metabolic alterations may explain why PCN inhibits progesterone anesthesia.

PRETREATMENT with certain steroids [e.g. spironolactone, norbolethone, ethylestrenol, SC-11927 (9α-fluoro-11β,17-dihydroxy-3-oxo-4-androstene-17α-propionic acid-K), PCN (pregnenolone-16α-carbonitrile)] increases the resistance of rats to various anesthetic or toxic compounds.[1,2] This "catatoxic" effect is independent of any presently known hormonal properties, and is associated with a decreased concentration of the toxicant in blood, which is the result of induction of NADPH-dependent mixed-function oxygenases in liver microsomes.[3,4] These findings are in agreement with recent morphologic studies which showed that several catatoxic steroids stimulate smooth-surfaced endoplasmic reticulum proliferation in rat liver cells.[5]

The central depressant effect of progesterone[6] is antagonized by catatoxic steroids, particularly by PCN.[2] Conney *et al.*[7] demonstrated that several drugs and insecticides (e.g. phenobarbital, chlorcyclizine, phenylbutazone, chlordane, DDT), which are potent stimulators of the hydroxylation of progesterone by liver microsomal enzymes, decrease the central depressant action of this steroid hormone. Recent experiments in this laboratory indicate that PCN is a highly active hepatic drug-metabolizing enzyme inducer[8] which causes marked smooth-surfaced endoplasmic reticulum proliferation in the liver.[9] On the basis of these reports, we decided to study the influence of PCN upon progesterone metabolism and its correlation to changes in the anesthetic action of this steroid.

MATERIALS AND METHODS

Female ARS/Sprague–Dawley rats (Madison, Wis., U.S.A.), with a mean initial body weight of 100 g (range 95–105 g) and maintained *ad lib.* on Purina laboratory chow (Ralston Purina of Canada) and tap water, were used throughout these experiments.

First experiment. Eighteen animals were divided into three equal groups, of which one served as untreated controls and the others were given 21·2 mg/kg of PCN [3β-hydroxy-20-oxo-5-pregnene-16α-carbonitrile (Searle)] as a micronized suspension, and 15·2 mg/kg of phenobarbital (B.D.H.), respectively, in 1 ml water, p.o., twice daily for 3 days. On day 4, 10 mg of progesterone (Roussel) was administered, i.p., in 0·5 ml of corn oil, to each group, and the duration of anesthesia (the period between the loss and return of the righting reflex) was measured.

Second experiment. Eighteen rats were divided and pretreated as described in the first experiment. On day 4, the animals were given 10 mg of progesterone-7-^{3}H* (1 μc/mg), i.p., in 0·5 ml of corn oil, and were exsanguinated 1 hr later by aortic puncture under light ether anesthesia. The brains were removed and homogenized in 4 vol. of water. Homogenates (1 ml) and serum (1 ml) were extracted with 8 ml of cyclohexane and, subsequently, with 8 ml of ethyl acetate, and evaporated.[7] The radioactivity of the samples was measured with a Picker multichannel liquid scintillation counter after adding 15 ml of Bray-solution.[10] Recovery of radioactivity in the combined cyclohexane and ethyl acetate extracts of brain and sera was more than 93 and 85 per cent respectively.

Third experiment. Twelve rats were divided into two equal groups, one of which was treated with PCN as in the first experiment. On the fourth day, all animals were decapitated, and the livers were removed, sliced and washed at 0–3°. Liver samples (1 g) were then homogenized in a 3-vol. 0·25 M ice-cold sucrose solution. The homogenates were centrifuged at 9000 *g* for 20 min, and the supernatants were further centrifuged at 105,000 *g* for 60 min. The microsomal pellets were rinsed once in 3 ml of 0·05 M tris–HCl buffer, pH 7·5, containing 1·15% KCl. After discarding the supernatant, the pellets were stored overnight in deep freeze, and were resuspended the next morning by adding 4 ml of KCl–tris buffer. Each 1-ml suspension contained the microsomes of 250 mg of wet liver.

The enzymatic biotransformation of progesterone was studied *in vitro* for 5 min, at 37°, with a NADPH-generating system,[11] using 0·7 μmole progesterone-21-^{14}C* (0·5 μc) and a 1-ml liver microsomal suspension containing approximately 5–6 mg microsomal protein. The incubation mixture consisted of a final volume of 5·7 ml, from which 2·5 ml was extracted with 20 ml of cyclohexane and re-extracted with 20 ml of ethyl acetate. The extracts (15 ml) were evaporated and measured for radioactivity as in the second experiment.

Another 2·5 ml of incubation mixture was extracted with 30 ml of dichloromethane, and 20-ml samples were evaporated and redissolved in 0·1 ml of methanol. Thin-layer chromatography of 10 μl of the methanol extracts and of a solution of reference steroids was performed on 6060 Silica gel plastic plates (Eastman Kodak Co., Rochester, N.Y., U.S.A.), using cyclohexane–chloroform–glacial acetic acid (7:2:1) or benzene–ethyl acetate–acetone (10:1:1) solvent systems.[12] Both of these systems produced similar results; hence, only the values obtained with the cyclohexane–chloroform–glacial acetic acid system have been described.

The plates were radioautographed by storing them in contact with Kodak X-ray film for 4 days. Radioactive fractions were eluted with methanol and counted as

* Purchased from New England Nuclear Corp. (Boston, Mass., U.S.A.) with a stated purity of 98 per cent. A single spot was obtained on the radioautogram, when tested by thin-layer chromatography.

described earlier; it was found that 93 ± 5% of progesterone-21-^{14}C was recovered on the chromatograms. Nonradioactive reference standards were visualized with a 2,4-dinitrophenylhydrazine reagent.[13]

The amount of microsomal protein was determined according to the method of Lowry *et al.*[14] Cytochrome P-450 content was measured with a UNICAM SP 800 recording spectrophotometer after adding 2 ml of pH 7·5, 0·5 M phosphate buffer to 1 ml microsomal suspension.[15]

The differences were statistically evaluated by Student's *t*-test.

RESULTS

Progesterone produced deep anesthesia for 228 ± 14 min in the controls. The righting reflex was maintained in animals pretreated with PCN or phenobarbital, and only slight ataxia was observed.

TABLE 1. EFFECT OF PCN AND PHENOBARBITAL ON THE BRAIN AND SERUM LEVEL OF PROGESTERONE AND ITS METABOLITES*

		Brain		Serum	
Group	Pretreatment	Cyclohexane-extractable compounds (μg/g)	Ethyl acetate-extractable compounds (μg/g)	Cyclohexane-extractable compounds (μg/ml)	Ethyl acetate-extractable compounds (μg/ml)
1	Control	9·21 ± 1·11	5·10 ± 0·38	3·69 ± 0·55	7·47 ± 0·79
2	Phenobarbital	4·30 ± 0·30†	3·89 ± 0·30†	1·28 ± 0·20†	4·46 ± 0·57†
3	PCN	3·61 ± 0·36†	2·75 ± 0·25†,‡	0·93 ± 0·09†	1·60 ± 0·13†,‡

* Values are expressed as Means ± S.E. Group 2 was treated with 15·2 mg/kg of phenobarbital and group 3 with 21·2 mg/kg of PCN (equimolecular doses) twice daily for 3 days. On the fourth day, all animals were given 100 mg/kg of progesterone-7-^{3}H, i.p., and were exsanguinated 1 hr later. Homogenates of brain and sera were extracted with cyclohexane and ethyl acetate, and the radioactivity of the samples was measured as described in Materials and Methods.

† Significantly different ($P < 0·05$) from group 1.

‡ Significantly different ($P < 0·05$) from group 2.

TABLE 2. EFFECT OF PCN ON THE LIVER MICROSOMAL PROTEIN AND CYTOCHROME P-450 CONTENT AS WELL AS ON THE PRODUCTION OF POLAR PROGESTERONE METABOLITES*

Treatment	Microsomal protein (mg/g)	Cytochrome P-450 (ΔO.D. 450–490 mμ/mg microsomal protein)	Production of ethyl acetate-extractable metabolites of progesterone (mμmoles/mg microsomal protein)
Control	20·44 ± 0·55	0·041 ± 0·0016	4·76 ± 0·50
PCN	22·20 ± 0·55†	0·070 ± 0·0057†	9·51 ± 0·82

* Values are expressed as Means ± S.E. Group 2 was treated with 21·2 mg/kg of PCN twice daily for 3 days. On the fourth day, all rats were killed, and liver microsomes were incubated with 0·7 μmole of progesterone-21-^{14}C as described in Materials and Methods.

† Significantly different ($P < 0·05$) from the control group.

Table 1 indicates that, in comparison with the controls, phenobarbital pretreatment markedly diminished the quantity of labeled progesterone metabolites extracted with cyclohexane and ethyl acetate from brain and serum. PCN was more active than phenobarbital in decreasing the brain and serum levels of these metabolites of progesterone.

Table 2 demonstrates that PCN increased the liver microsomal protein and cytochrome P-450 content. Incubation of progesterone-21-^{14}C with these microsomes showed that the production of ethyl acetate-extracted (polar) metabolites of progesterone was significantly enhanced.

Thin-layer chromatography of the dichloromethane extract from the incubation mixture revealed the presence of progesterone and its several polar metabolites (Fig. 1). Polar fractions I and II were found to have the same R_f values as 6β- and 16α-hydroxy-progesterone respectively. The nonpolar fraction had the same chromatography behavior pattern as 5α- and 5β-pregnane-3,20-dione, which are Δ^4-reduced metabolites of progesterone.

Table 3 demonstrates that, as compared to the controls, PCN diminished the production of nonpolar reduced metabolites and markedly increased the quantity of hydroxylated compounds (polar fractions I and II). The strongest effect of PCN was on the 6β-hydroxylation of progesterone (polar fraction I).

TABLE 3. EFFECT OF PCN ON PROGESTERONE BIOTRANSFORMATION BY LIVER MICROSOMES*

Products of progesterone metabolites (mμmoles/mg microsomal protein)	Control	PCN
Nonpolar fraction (5α-pregnane-3,20-dione)	4·92 ± 0·15	3·05 ± 0·30†
Polar fraction I (6β-hydroxy-progesterone)	1·09 ± 0·05	3·16 ± 0·50†
Polar fraction II (16α-hydroxy-progesterone)	3·60 ± 0·29	4·97 ± 0·57†

* Values are expressed as Means ± S.E. The liver microsomes of the controls and of rats given PCN (21·2 mg/kg twice daily for 3 days) were incubated with progesterone-21-^{14}C as described in the footnote of Table 2. The individual incubation mixtures were extracted with dichloromethane and chromatographed on TLC plates in a cyclohexane–chloroform–glacial acetic acid (7:2:1) solvent system. Radioactive fractions were eluted and counted as described in Materials and Methods.

† Significantly different ($P < 0·05$) from the control group.

DISCUSSION

Previous studies[16,17] have demonstrated that the hepatic enzyme systems involved in the hydroxylation of steroid hormones are closely related to those responsible for the biotransformation of several drugs and toxic compounds. It has also been shown that phenobarbital enhances both of these actions of the hepatic microsomes.[16] The accelerated production *in vitro* of 6β- and 16α-hydroxy-progesterone in our experi-

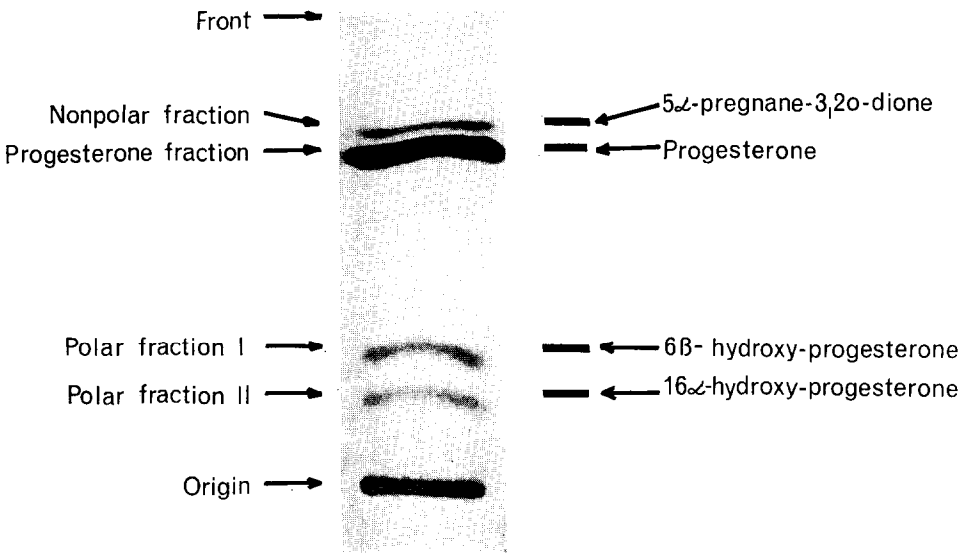

FIG. 1. Separation of progesterone-21-^{14}C and its metabolites by thin-layer chromatography. Progesterone-21-^{14}C was incubated with the liver microsomes of a PCN-treated rat. Extracts from the incubation mixture were chromatographed in a cyclohexane–chloroform–glacial acetic acid (7:2:1) solvent system. The spots on the right side of the autoradiogram indicate the place of reference steroids in the same solvent system.

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ments, as well as the increase in microsomal protein and cytochrome P-450 content, clearly indicate that PCN also stimulates the hydroxylation of this natural steroid hormone by liver microsomes.

In addition, we found that PCN markedly decreased the level of progesterone and its metabolites in brain and serum, and inhibited the anesthetic effect of this hormone. In agreement with previous findings, phenobarbital[7,12] was also effective in these respects, but PCN proved to be more active. It is reasonable to assume that these alterations are related to the stimulation of steroid hydroxylation by liver microsomal enzymes. The influence of these enzymes is underlined by the fact that SKF 525-A, a drug-metabolizing enzyme inhibitor, increases the central depressant effect of progesterone.[12] It is also well known that the highly polar hydroxylated metabolites of progesterone are excreted more easily, and that this change is particularly advantageous for their elimination through the bile in many species, including rats.[18,19]

There is yet another metabolic pathway of progesterone which is also dependent on the enzymes of the liver, that is the reduction of the 4–5 double bond of this steroid. It has been shown that the enzymes of hepatic microsomes catalyze 5α-reduction whereas soluble enzymes are responsible for the 5β-reduction of various Δ^4-3-ketosteroids.[20] While phenobarbital failed to influence the 5α-reduction of progesterone by liver microsomes,[12] it would be interesting to point out that, in our experiments, PCN decreased the production of 5α-pregnane-3,20-dione. Since this metabolite has an anesthetic effect,[12] its decreased production may have additional importance in the abolition of progesterone anesthesia.

Recent experiments have shown that PCN significantly increases bile flow,[21] which may be another factor responsible for the elimination of progesterone metabolites. Further studies are, however, necessary to elucidate the mechanism of this action of PCN in relation to its protective effect against various toxic compounds and anesthetic agents.

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