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Role of Brain Cytochrome P450 in Regulation of the Level of Anesthetic Steroids in the Brain

MARIA STRÖMSTEDT, MARGARET WARNER, CAROL D. BANNER, PAUL C. MACDONALD, and JAN-ÅKE GUSTAFSSON

Department of Medical Nutrition, Karolinska Institutet, Huddinge University Hospital, Huddinge, Sweden (M.S., M.W., C.D.B., J.-Å.G.), and Cecil H. and Ida Green Center for Reproductive Biology Sciences, University of Texas Southwestern Medical Center, Dallas, Texas 75235-9038 (P.C.M.)

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SUMMARY

The role of brain cytochrome P450 (P450) in regulating the levels of the potent anesthetic steroid 3α-hydroxy-5α-pregnan-20-one (3α-OH-DHP) has been investigated. By analogy with the elimination of androgen from its target tissues, we present evidence that it is 3β-hydroxy-5α-pregnan-20-one (3β-OH-DHP) and not 3α-OH-DHP that represents the major pathway for the formation of more polar metabolites and thus the elimination of the 5α-reduced metabolites of progesterone from target tissues. No polar metabolites were formed when 3α-OH-DHP was incubated with microsomal fractions prepared from rat brain, but 3β-OH-DHP was hydroxylated at the 6α- and 7α-positions. These 3β-diols were not formed to any detectable extent in the liver or kidney but were formed in prostate, pituitary, brain, and breast. The highest catalytic activity, 512 nmol of products formed/g of tissue/hr, was found in the prostate. The corresponding rates in the pituitary, brain, and breast were 71.9, 28.1, and 6.7 nmol/g/hr, respectively. These hydroxylations were confirmed to be P450-catalyzed reactions by solubilization of the P450 from prostate, brain, and breast microsomes and reconstitution of the catalytic activity with NADPH-P450 reductase (EC 1.6.2.4) and lipid. Because 5α-androstane-3β,17β-diol (3β-Adiol) has been shown to be a good substrate for prostate and brain P450, competition experiments were performed to determine whether the same form of P450 is involved in the elimination of 3β-Adiol and 3β-OH-DHP in the brain. These two substrates competed with each other for metabolism in microsomal fractions and in reconstitution experiments with P450 extracted from the brain or prostate. To test the hypothesis that the hydroxylation of 3β-OH-DHP represents a pathway for regulation of the level of 3α-OH-DHP in the brain, the effect of inhibition of the hydroxylation of 3β-OH-DHP on the duration of 3α-OH-DHP-induced anesthesia was examined. The nonanesthetic steroid 3β-Adiol was used as a competitive inhibitor of the metabolism of 3β-OH-DHP. The duration of anesthesia upon intravenous administration of 3α-OH-DHP was increased by 33% when 3β-Adiol was coadministered. We conclude that, in the central nervous system, P450-catalyzed hydroxylation of 3β-OH-DHP is a degradative pathway that plays an important role in regulation of the levels of the neuroactive steroid 3α-OH-DHP.

Progesterone and its metabolites are known to have anxiolytic, sedative, and anticonvulsant properties (for review, see Ref. 1). The most potent anesthetic metabolite has been identified as 3α-OH-DHP (2). Its effects are thought to be mediated through interactions with GABA receptors in the brain, because 3α-OH-DHP and another naturally occurring steroid, 5α-THDOC, are potent stimulators of GABA receptor Cl^- channel complexes (3). An investigation of the structural requirements for GABA receptor-active steroids revealed that maximal activity requires a 5α-reduced, 3α-hydroxylated configuration, with 3α-OH-DHP being >1000-fold more potent than 3β-OH-DHP (4).

Changes in mood and sleep/wakefulness patterns during stress, during pregnancy, and during the menstrual cycle are thought to be due to fluctuations in the levels of metabolites of progesterone and corticosterone (5). The levels of these steroids in the brain are determined both by their rate of synthesis and by their inactivation and/or elimination. The possibility that inactivation occurs by *in situ* metabolism in the brain and the role of brain P450 in this pathway are the subjects of this investigation.

P450 is present in the brain at a concentration of approxi-

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ABBREVIATIONS: 3α-OH-DHP, 3α-hydroxy-5α-pregnan-20-one; 3α-Adiol, 5α-androstane-3α,17β-diol; 3β-Adiol, 5α-androstane-3β,17β-diol; 3β-Adiol OHase, 5α-androstane-3β,17β-diol hydroxylase; 6α-Atriol, 5α-androstane-3β,6α,17β-triol; 7α-Atriol, 5α-androstane-3β,7α,17β-triol; DHP, 5α-dihydroprogesterone; 3β-OH-DHP, 3β-hydroxy-5α-pregnan-20-one; 3β-OH-DHP OHase, 3β-hydroxy-5α-pregnan-20-one hydroxylase; 5α-THDOC, 3α,21-dihydroxy-5α-pregnan-20-one; 6α-diol, 3β,6α-dihydroxy-5α-pregnan-20-one; 7α-diol, 3β,7α-dihydroxy-5α-pregnan-20-one; DHT, 5α-dihydrotestosterone; GABA, γ-aminobutyric acid; GC, gas chromatography; MS, mass spectrometry; HPLC, high performance liquid chromatography; P450, cytochrome P450; P450 reductase, NADPH-cytochrome P450 reductase; LRR, loss of righting response; RRR, return of righting response.

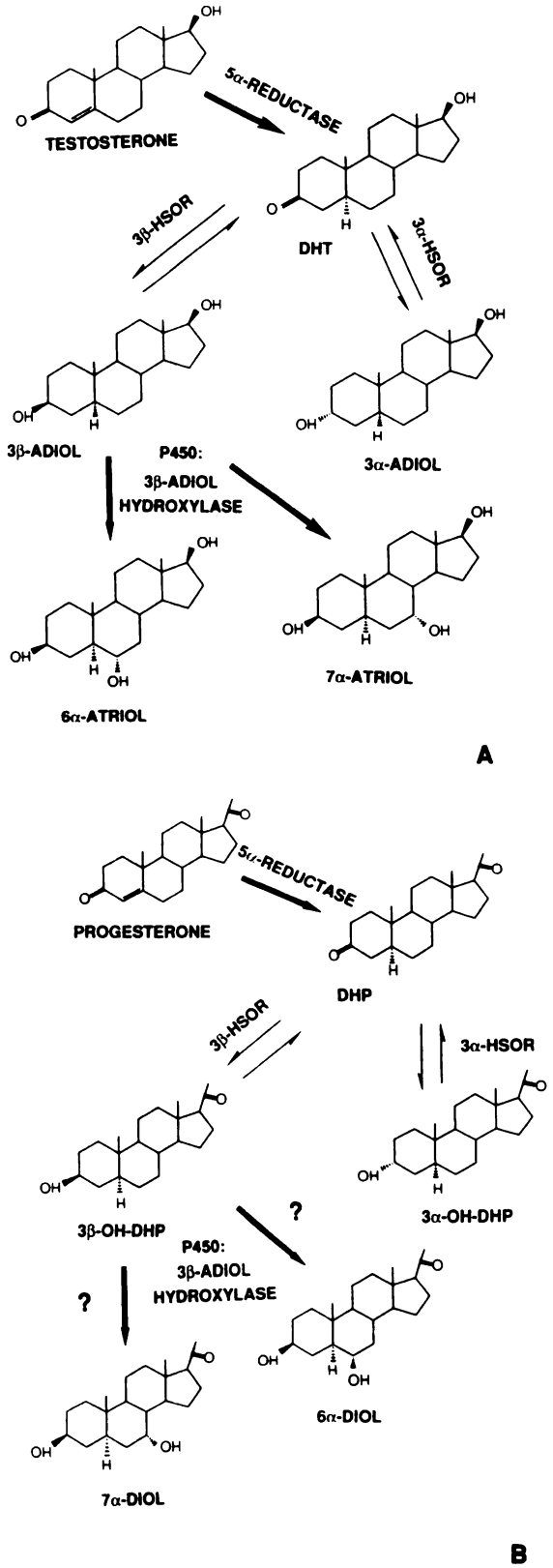

Fig. 1. A, Pathway for the elimination of androgen from target tissues such as prostate, pituitary, and brain. The 3α- and 3β-hydroxysteroid oxidoreductases (*HSOR*) catalyze reversible reactions, but only 3β-Adiol is irreversiby hydroxylated to polar metabolites without androgen activity. This step is catalyzed by the P450 3β-Adiol OHase. B, Pathway for metabolism of progesterone in the brain. Progesterone is rendered inactive as a steroid hormone upon 5α-reduction. DHP can be further metabolized, mainly to 3α-OH-DHP, a potent anesthetic steroid, but also

mately 1% of the liver level (6). One of the major forms of P450 in the brain is 3β-Adiol OHase. This enzyme is not detectable in the liver or kidney but is abundant in the prostate, pituitary, and brain (7–10). The known substrate for this enzyme, 3β-Adiol, is metabolized to 6α-, 7α-, and 7β-triols. 3β-Adiol OHase has a narrow substrate specificity. It does not accept 3α-hydroxysteroids, testosterone, or androstenedione, and DHT is a very poor substrate. The hydroxylation of 3β-Adiol is the final step in the tissue-specific inactivation of the active androgen DHT (11, 12). DHT is converted by hydroxysteroid oxidoreductases to 3α- and 3β-Adiols (Fig. 1A). The equilibrium of the 3α-hydroxysteroid oxidoreductase favors the oxidation of 3α-Adiol to DHT (13). Thus, 3α-Adiol is readily reconverted to the active hormone DHT, but 3β-Adiol is rapidly hydroxylated via the action of P450 and does not accumulate in the target tissues. Progesterone, unlike androgens, is rendered inactive as a steroid hormone upon 5α-reduction. *In vivo* studies have shown that the major metabolites found in rat brain after injection of [^{3}H]progesterone are DHP and 3α-OH-DHP and that 20α-hydroxy-5α-pregnan-3-one is a minor metabolite (14, 15). A similar metabolite profile was reported after *in vitro* incubation of brain tissue with [^{3}H]progesterone or [^{3}H]DHP (16, 17). In rat fetal brain cells from the hypothalamus and olfactory tubercle in culture, the major metabolites of progesterone were again DHP and 3α-OH-DHP. No 20α-hydroxy-5α-pregnan-3-one was detected, but 3β-OH-DHP, as well as two unidentified polar metabolites, were formed (18). One explanation for the reported absence of 3β-OH-DHP formation in adult brain may be the difficulty of separating 3β-OH-DHP from DHP. Another explanation could be that, by analogy with androgen metabolism in prostate (11), hydroxylation of 3β-OH-DHP is an important elimination pathway and the product is rapidly converted to polar metabolites (Fig. 1B). Despite these inconsistencies with the metabolic studies, when the steroids in the brains of adult rats, rabbits, and dogs were extracted and identified, 3α-OH-DHP and 3β-OH-DHP were present in approximately equal amounts (19). In this study, we have examined the pathway for elimination of 3α- and 3β-OH-DHP in the brain and compared it with that of 3α- and 3β-Adiol.

Materials and Methods

Chemicals. 3β-[1α,2α-^{3}H]Adiol (40 Ci/mmol) was obtained from Amersham International Ltd. (Buckinghamshire, Great Britain). 7α-Diol was from the Reference Steroid Collection, MRC (London, UK). Unlabeled 3β-OH-DHP, 3α-OH-DHP, and 3β-hydroxy-5-pregnen-20-one were from Steraloids. All other unlabeled steroids, as well as Cremophor, phenylmethylsulfonyl fluoride, NADPH, and *p*-chloroamphetamine hydrochloride, were purchased from Sigma Chemical Co. (St. Louis, MO). Emulgen 911 was from Kao Atlas (Tokyo, Japan).

Synthesis of ^{3}H-labeled 3β-OH-DHP. Radiolabeled 3β-OH-DHP was prepared from radiolabeled 5α-pregnane-3,20-dione by limited reduction with potassium borohydride in methanol. All of the substrate was converted to the products of the reaction, i.e., 5α-pregnan-3α-ol-20-one (10%) and 5α-pregan-3β-ol-20-one (90%). The reaction products were separated by column chromatography on celite-ethylene glycol, using isooctane as the mobile solvent. The two components were completely separated by this chromatography. Radiochemical homogeneity was evaluated further by multiple thin layer chromatography

to 3β-OH-DHP, which lacks anesthetic properties. The question of whether 3β-OH-DHP is converted to polar metabolites in the brain, by analogy with 3β-Adiol, and, if so, whether this metabolism is catalyzed by brain P450 is the topic of this investigation.

steps and after acetylation with pyridine and acetic acid anhydride and recrystallization. The acetylated product was recrystallized to a constant specific activity with added nonradiolabeled 5α-pregnan-20-one-3β-acetate, to verify the purity of the radiolabeled substrate. Before use in enzyme assays, the purity of the substrate was checked by reverse phase HPLC using a linear gradient from 55% methanol in water to 100% methanol, with an increase in methanol concentration of 0.75%/min.

Preparation of microsomes. Adult (8–10-week-old) male Sprague-Dawley rats were killed by decapitation and tissues were immediately excised and immersed in ice-cold 0.25 M sucrose containing 50 μM phenylmethylsulfonyl fluoride. Microsomes were prepared from brain, pituitary, liver, and kidney as described previously (6) and from prostate as described by Haaparanta *et al.* (20).

Preparation of total membranes from rat breast tissue. The tissue was homogenized as described for prostate (20) and total membranes were pelleted by centrifugation at 100,000 × *g* for 60 min. The pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.4, and centrifuged at 1000 × *g* for 5 min, and the supernatant was used in the assay.

Assays for hydroxylation of 3β-Adiol and 3β-OH-DHP. Approximately 0.5 × 10^6 dpm of ^{3}H-labeled 3β-Adiol dissolved in ethanol were added to a glass tube together with unlabeled substrate, and the solvent was evaporated under nitrogen. Microsomes were added and the volume was adjusted to 0.5 ml with 50 mM potassium phosphate buffer, pH 7.4. The reaction was started after a 2-min preincubation at 37° by the addition of 2 mM NADPH. Incubations were continued for 30 min at 37° and terminated by the addition of 3 ml of ethyl acetate. The extraction was repeated once with another 3 ml of ethyl acetate. Incubations without NADPH were used as blanks and gave results similar to those of incubations with boiled microsomes. The ethyl acetate fractions were pooled, taken to dryness under nitrogen, dissolved in 50 μl of methanol/water (45:55, v/v), and analyzed using HPLC.

The metabolism of 3β-OH-DHP was assayed in a similar way, but the extract was dissolved in methanol/water (55:45, v/v) before HPLC analysis. The metabolism of both substrates were assayed under saturating concentrations of substrate, which for 3β-Adiol was 10 μM and for 3β-OH-DHP was 50 μM. The catalytic activities for both substrates were linear with time up to 30 min and with protein concentrations up to 0.6 mg of microsomal protein for prostate and 2 mg of microsomal protein for brain.

Protein purification. P450 was solubilized from microsomes or total cellular membranes with Emulgen 911 and sodium cholate and was partially purified on *p*-chloroamphetamine-coupled Sepharose as described previously (7). The recovery of P450 from prostate, brain, and breast was 200, 40, and 30 pmol/g of tissue, respectively. P450 reductase was purified from rat liver microsomes by affinity chromatography on 2′,5′-ADP-Sepharose, according to the method of Yasukochi and Masters (21).

Reconstitution of catalytic activities. For reconstitution of 3β-Adiol and 3β-OH-DHP OHase activities, liver microsomal lipid corresponding to 30 mg of liver tissue, prepared as described (7), was added to a tube containing substrate. P450 from prostate (1–2 pmol), brain (2–5 pmol), or breast (4–8 pmol) was added, followed by 0.08 units of P450 reductase (1 unit = 1 μmol of cytochrome *c* reduced/min), and the volume was adjusted to 1 ml with 50 mM potassium phosphate buffer, pH 7.4. After a 3-min preincubation, the reaction was started by the addition of 2 mM NADPH and was continued for 20 min. Steroids were extracted as described above for incubations with microsomes.

HPLC analysis of substrates and metabolites. 3β-Adiol and 3β-OH-DHP were separated from their metabolites by HPLC using a C_{18} column (46 × 25 cm; Beckman, San Ramon, CA). In the case of 3β-Adiol, products were eluted with methanol/water (45:55, v/v), at a flow rate of 1 ml/min, and the substrate was eluted with methanol after 45 min. The two major polar metabolites formed in prostate and brain were previously identified as 6α-Atriol and 7α-Atriol, both by comigration with authentic standards and by liquid chromatography/MS (10, 11). The products from incubations with 3β-OH-DHP were eluted with methanol/water (55:45, v/v), at the same flow rate, and the substrate was eluted with methanol after 60 min.

Identification by GC/MS of metabolites of 3β-OH-DHP. Peaks 2 and 3 from HPLC separation were converted to trimethylsilyl ether derivatives before analysis by GC/MS. The instrument used was a VG 70–250 double-focusing instrument (VG Analytical Ltd., Manchester, UK) equipped with a Hewlett-Packard 5790A series gas chromatograph (Hewlett-Packard Co., Avondale, PA) and a VG 11–250 data system. The fused silica capillary column (methyl silicone, 25 m × 0.32-mm internal diameter, 0.25-mm film thickness; Quadrex Co., New Haven, CT), in a temperature gradient of 60–230° at 30°/min, was connected directly to the ion source. An all-glass falling-needle injection system was used. Spectra were recorded by repetitive scanning between *m/z* 500 and 200 at a scan rate of 2 sec/decade, with an accelerating voltage of 6 kV, an electron energy of 70 eV, and a trap current of 200 mA. Metabolites were identified by comparison of retention times and mass spectra with those of authentic standards. The following ions characteristic for structural features of trimethylsilyl ether derivatives of dihydroxylated pregnanolones were monitored: *m/z* 478 (M^+), 463 (M − 15), 388 (M − 90), 373 (M − 90 − 15), 298 (M − 2 × 90), and 283 (M − 2 × 90 − 15).

Effect of 3β-Adiol on 3α-OH-DHP-induced anesthesia. Male, 8-week-old, Sprague-Dawley rats were used. 3α-OH-DHP (7 mg/kg) and 3β-Adiol (7 mg/kg) were dissolved in saline containing 3% ethanol and 21% Cremophor and were administered through a tail vein using a short butterfly needle (0.5-mm diameter; Abbot Ireland Ltd., Sligo, Ireland). The anesthetic state was defined by LRR, that is, when the animal failed to right itself within 10 sec after being placed on its back, and RRR, when the animal righted itself after LRR. The injection was considered successful when a free stream of blood appeared in the tubing when the needle was inserted and no resistance was met during injection.

Results

Identification of metabolites of 3β-OH-DHP formed in the brain. Incubation of prostate and brain microsomes with ^{3}H-labeled 3β-OH-DHP led to the formation of three polar metabolites in the presence of NADPH (Fig. 2A). The major metabolite (peak 3) represented 85% of total polar metabolites and coeluted on HPLC with authentic 6α-diol, as well as with the major metabolite from incubations with pituitary microsomes and a breast total membrane preparation (Fig. 2B).

Peak 2 from incubations with rat prostate and peak 3 from incubations of prostate and brain microsomes were analyzed by GC/MS. GC revealed that HPLC peak 3 consisted of two metabolites, "GC peak 1" and "GC peak 2," in both tissues. The retention time relative to that of 5α-cholestane, the retention index calculated from retention times of a series of C_{26}-C_{32} *n*-alkane standards, and the relative ion intensities for *m/z* 478, 463, 388, 373, 298, and 283 for the three unknown metabolites were compared with those of authentic standards (data for prostate are shown in Table 1). GC peak 1 was identified as 7α-diol and GC peak 2 as 6α-diol. The 7α-diol constituted approximately 60% of HPLC peak 3 from prostate and brain microsomes. The identity of HPLC peak 2 could not be determined, but the fragmentation pattern indicates that this steroid is another monohydroxylated metabolite of the substrate. No attempt was made to identify HPLC peak 1, because too little material was available.

Tissue distribution of 3β-OH-DHP and 3β-Adiol OHase. The 6α- and 7α-hydroxylation of 3β-OH-DHP was

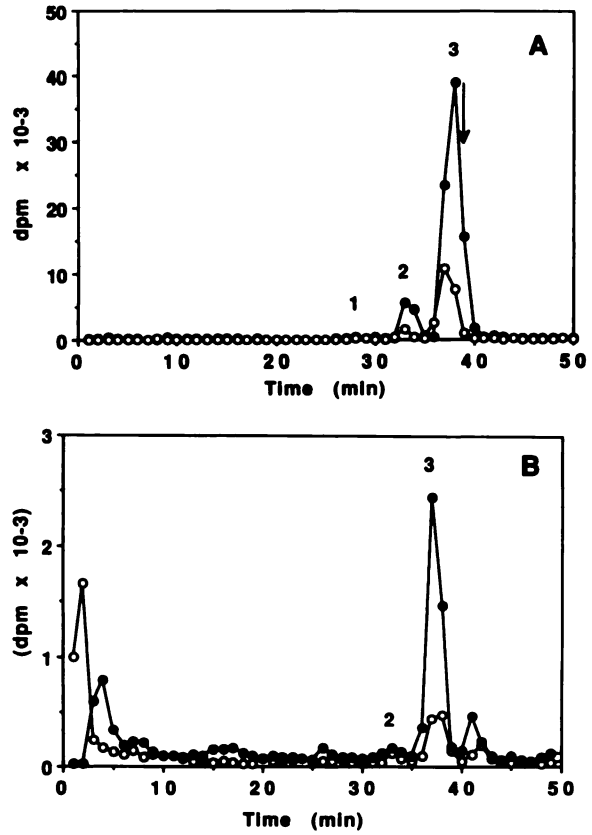

Fig. 2. HPLC profile of metabolites of ^{3}H-labeled 3β-OH-DHP from prostate (●) and brain (○) microsomes (A) and breast (○) and pituitary (●) microsomes (B). Metabolites were separated on a C_{18} column and eluted with methanol/water (55:45, v/v), at a flow rate of 1 ml/min. The substrate was eluted with methanol after 60 min (not shown). *Arrow*, position of elution of authentic 6α-diol.

compared with that of 3β-Adiol in microsomes from different tissues (Fig. 3). Tissues high in 3β-Adiol OHase activity (prostate > pituitary > brain ≫ breast) also had a high capacity for hydroxylation of 3β-OH-DHP, whereas both liver and kidney had low or undetectable activities towards both substrates. A difference between the two substrates is the ratio between the metabolites formed. With 3β-Adiol as substrate the 6α-Atriol is the major metabolite, constituting approximately 65% of total polar metabolites, but with 3β-OH-DHP the major metabolite is the 7α-diol.

Hydroxylation of 3β-OH-DHP in the mouse brain. Mouse brain also formed polar metabolites from 3β-OH-DHP, and the rate of formation of HPLC peak 3 was similar to that in the rat (Fig. 3). GC/MS analysis of HPLC peak 3 from rat and mouse brain microsomes showed that it consisted of both 7α- and 6α-diol in both species but, whereas the rat showed a ratio of 6α-diol to 7α-diol of approximately 1:3, the 6α-diol was only a small fraction of peak 3 in the mouse. This is in agreement with the hydroxylation of 3β-Adiol in the mouse, where 7α is the preferred position, in contrast to the rat, where 6α-Atriol is the major metabolite (22).

Competition of 3β-OH-DHP and 3β-Adiol for P450-catalyzed hydroxylation. Incubations of rat prostate microsomes with ^{3}H-labeled 3β-OH-DHP in the presence of increasing concentrations of unlabeled 3β-Adiol as a competitor, and vice versa, showed that both steroids were metabolized by the same enzyme (Fig. 4). The production of both 6α- and 7α-diol from 3β-OH-DHP was inhibited to the same extent, because similar ratios of 6α:7α were found in incubations performed in the absence and in the presence of 50 μM 3β-Adiol.

To determine whether the 6α- and 7α-hydroxylation of 3β-OH-DHP was a P450-catalyzed reaction, partially purified prostate, brain, or breast P450 was mixed with purified liver P450 reductase, liver microsomal lipid, and NADPH, and the catalytic activity was measured. For both substrates, prostate P450, reductase, and NADPH were all required for catalytic activity (Fig. 5A). In addition, the presence of unlabeled 3β-OH-DHP in incubations with 3β-Adiol inhibited the 3β-Adiol OHase activity, and vice versa, further supporting the hypothesis that both substrates are metabolized by the same enzyme. The same requirement for all components of the reconstituted system, as well as competition between the substrates, was seen for both substrates using brain P450 (Fig. 5B) and for 3β-OH-DHP metabolism with breast P450 (data not shown). The presence of both 6α- and 7α-diol was confirmed in reconstitution experiments with prostate P450 by comigration with authentic standards on GC (data not shown). Due to the low turnover number, it was not possible to identify by GC the metabolites formed by brain and breast P450 in the reconstituted system.

Substrate specificity of 3β-OH-DHP OHase. The effect of other, structurally related, steroids on the 3β-OH-DHP OHase activity in the reconstituted system was investigated (Table 2). Incubations were performed in the presence of a 5-fold molar excess of unlabeled competitor. The most potent competitors were 3β-Adiol and 3β,21-dihydroxy-5α-pregnane-20-one. Pregnenolone and 5α-THDOC were less efficient, and the other steroids tested did not compete with the substrate at all.

Role of 3β-OH-DHP hydroxylation in regulation of

TABLE 1

Retention time, retention index, and relative intensities of diagnostically important MS peaks given by the trimethylsilyl ethers of metabolites of 3β-OH-DHP from prostate microsomes and of reference compounds

Sample	Retention time[a]	Retention index[b]	Relative intensity					
			m/z 478	463	388	373	298	283
			% of base peak					
6α-Diol	1.16	2891	93	89	100	52	85	34
7α-Diol	0.95	2782	8	41	100	12	55	32
HPLC peak 2	1.24	2901	4	77	100	17	32	23
HPLC peak 3, GC peak 1	0.95	2785	8	45	100	12	54	29
HPLC peak 3, GC peak 2	1.16	2887	90	84	100	57	73	36

[a] Retention time relative to that of 5α-cholestane.
[b] Retention index calculated from retention times of a series of C_{26}-C_{32} *n*-alkane standards.

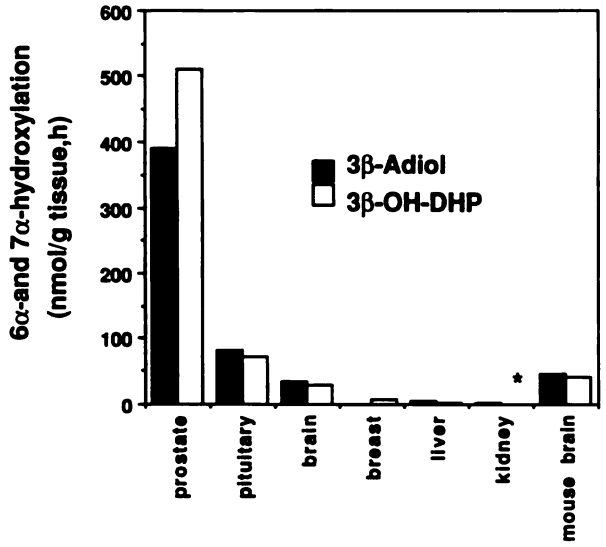

Fig. 3. Comparison of 6α- and 7α-hydroxylation of 3β-Adiol and 3β-OH-DHP in microsomes (or, in the case of breast, a total membrane preparation) from different tissues of the rat and mouse. For prostate and pituitary, tissues from six animals were pooled. For rat and mouse brain, the values represent the means of six and three individual animals, respectively. Kidney and breast values were obtained from one animal. *, Value below the detection limit.

the anesthetic effects of 3α-OH-DHP. To test the hypothesis that the P450-catalyzed hydroxylation of 3β-OH-DHP influences the level of the potent anesthetic steroid 3α-OH-DHP, we studied the effect of a competitive inhibitor of 3β-OH-DHP metabolism on the duration of 3α-OH-DHP-induced sleep (Fig. 6). The competitor chosen was the nonanesthetic steroid 3β-Adiol. Intravenous injection of 3α-OH-DHP at 7 mg/kg, in a volume of 400 μl, induced anesthesia within 90 sec in all rats tested. Injections of 400 μl of 3β-Adiol (7 mg/kg) or 800 μl of vehicle alone had no anesthetic effect. Coadministration of both steroids, in a volume of 800 μl, gave a significant increase in sleeping time. When the duration of anesthesia was measured from the time when injections were started until the time of RRR, a 44% increase ($p < 0.001$) was seen. This way of measuring overestimates the difference between the two groups, because the injection time for the group receiving both steroids was 2–3 min longer than that when only one steroid was administered. When the duration of anesthesia was measured as the time between LRR and RRR, a 33% increase ($p < 0.01$) in sleeping time was seen when 3β-Adiol was coadministered. This way of measuring underestimates the difference between the two groups, because it was difficult to determine whether the criteria for LRR (the animal is unable to right itself within 10 sec after being placed on its back) were fulfilled while the drug was being injected. This error is larger for the group that received both steroids, because of the longer time required for injection of a larger volume. The prolongation of 3α-OH-DHP sleeping time by 3β-Adiol is therefore between 33 and 44%. We conclude that 3α-OH-DHP is converted to DHP and then to 3β-OH-DHP in the brain and that the hydroxylation of 3β-OH-DHP is the major pathway for elimination of 3α-OH-DHP. The most likely explanation for the prolongation by 3β-Adiol of the duration of the anesthetic effect of 3α-OH-DHP is that the competition between 3β-Adiol and 3β-OH-DHP for metabolism by brain P450 that we have demonstrated *in vitro* can occur *in vivo*.

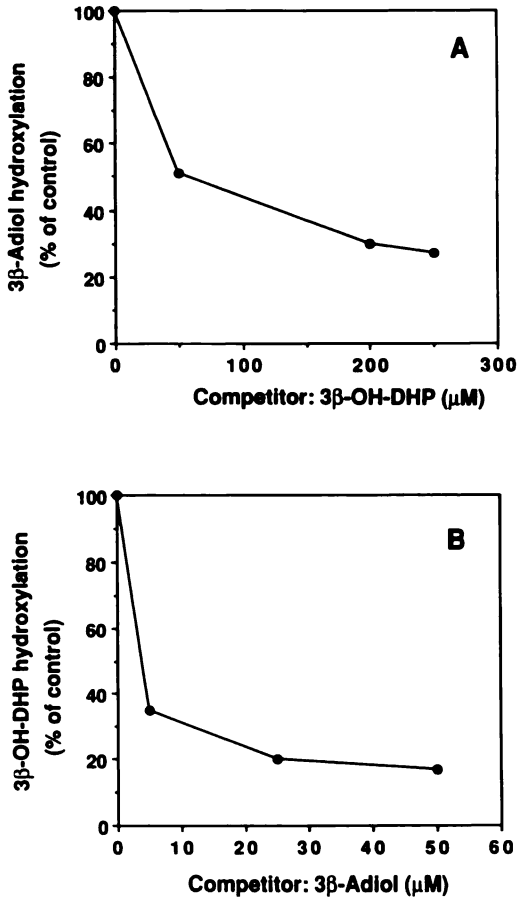

Fig. 4. Competition experiment using prostate microsomes. Assays were performed as described in Materials and Methods. A, Hydroxylation of 3β-Adiol (10 μM substrate concentration) at positions 6α and 7α in the presence of increasing amounts of unlabeled 3β-OH-DHP. B, Hydroxylation of 3β-OH-DHP (50 μM substrate concentration) at positions 6α and 7α in the presence of increasing amounts of unlabeled 3β-Adiol.

Discussion

We have shown that 3β-OH-DHP is hydroxylated in the brain through the action of P450. We have presented evidence that this metabolism is catalyzed by the same form of P450 that in prostate and brain inactivates 3β-Adiol; (i) hydroxylations occur at positions 7α and 6α in both substrates, (ii) the tissue distributions of the catalytic activity are similar for the two enzymes (prostate > pituitary > brain $\gg$ breast), and (iii) competition studies showed reciprocal competition between the two substrates.

Small amounts of polar metabolites that comigrate with the 6α- and 7α-hydroxylated metabolites are formed in liver and kidney, but they have not been identified and the enzymes responsible have not been characterized. It has been suggested that the inactivation of 3α-OH-DHP occurs through conjugation reactions in the liver (23).

The hydroxylation of 3β-Adiol at positions 6 and 7 is not unique to rat prostate, pituitary, and brain but has also been shown in human (24, 25), canine (26), and guinea pig prostate. In the mouse brain and prostate the predominant metabolite of 3β-Adiol is 7α-triol (22). The preference for 7α-hydroxylation was also observed with 3β-OH-DHP as substrate in mouse brain microsomes.

Although it is 3α- and not 3β-OH-DHP that is active at

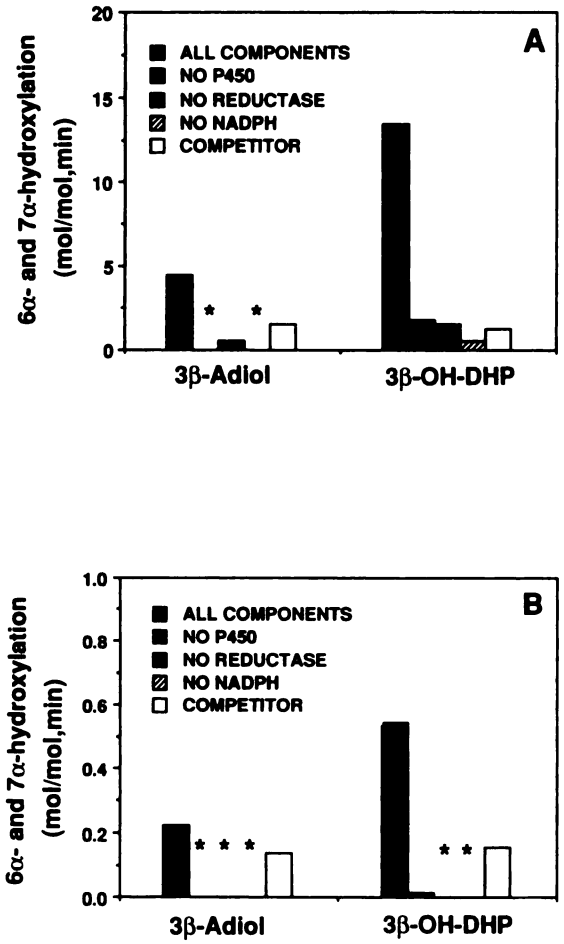

Fig. 5. Reconstitution of 3β-Adiol and 3β-OH-DHP OHase activities using crude P450 fractions from prostate (A) and brain (B). The turnover number is expressed as mol of 6α- and 7α-hydroxylated products formed/mol of P450/min. *, Turnover numbers below the detection limit.

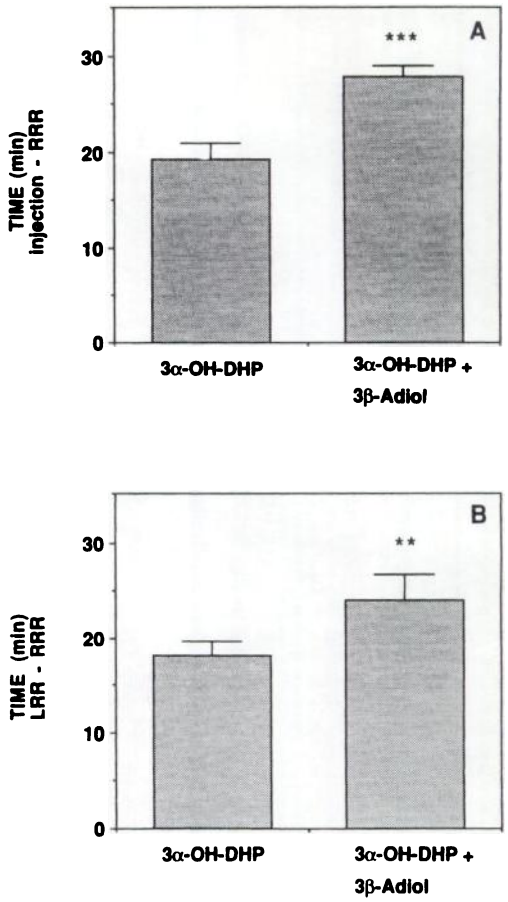

Fig. 6. Effect of 3β-Adiol on duration of 3α-OH-DHP-induced anesthesia. Duration of anesthesia was measured from the time of injection to the time of RRR (A) and from the time of LRR to the time of RRR (B). 3α-OH-DHP alone (7 mg/kg) was given in a volume of 400 μl (n = 5), and 3α-OH-DHP (7 mg/kg) together with 3β-Adiol (7 mg/kg) was given in a volume of 800 μl (n = 3). ***, $p < 0.001$; **, $p < 0.01$.

TABLE 2

Effect of some structurally related steroids on 6α- and 7α-hydroxylation of 3β-OH-DHP in a reconstituted system using prostate P450

Competitor[a]	Turnover no.
	mol/mol/min
None	11.9
3β-Adiol	2.1
3α-OH-DHP	12.9
5α-THDOC	7.6
3β,21-Dihydroxy-5α-pregnan-20-one	1.8
3α-Hydroxy-5β-pregnan-20-one	16.1
3β-Hydroxy-5β-pregnan-20-one	10.7
3β-Hydroxy-5-pregnen-20-one	5.3

[a] All competitors were present at 5-fold molar excess.

GABA receptors, no metabolism of 3α-OH-DHP to polar metabolites could be detected in the brain. We therefore postulate that the 3β pathway is an elimination pathway for progestins, as it is for androgens. The incubations described in these studies were designed to demonstrate the conversion by P450 of 3β-OH-DHP to polar metabolites and to quantitate the extent of the conversion. The incubations were of short duration (30 min) to ensure linearity of the reactions and, therefore, were too short for detection of the conversion of 3α-OH-DHP to DHP and then to 3β-OH-DHP. In experiments of long duration, such as those done *in vivo* or with tissue culture, the conversion of DHP to 3β-DHP has been demonstrated (18). The 3α-hydroxysteroid oxidoreductase is a reversible enzyme that favors the oxidation to DHP. Once DHP is synthesized there is some conversion to 3β-OH-DHP, but this is rapidly eliminated through the action of P450. Our results reveal that an elimination pathway for 3β-OH-DHP exists within the brain. The effect of the nonanesthetic steroid 3β-Adiol on the duration of 3α-OH-DHP-induced anesthesia is supportive evidence for the hypothesis that the 3β pathway is important for regulation of the levels of 3α-OH-DHP.

If the main physiological function of 3β-OH-DHP OHase were the inactivation of progesterone, the enzyme should be highly expressed in the breast, a target tissue for progesterone. The low activity in the breast (5 times lower than that in the brain) supports our hypothesis that the physiological function of the enzyme is in the regulation of tissue levels of 3α-OH-DHP.

Fennessey *et al.* (27) have reported that the main metabolite of progesterone in T47D$_{co}$ human breast cancer cells is 6α-diol. Another human breast cancer cell line, MCF-7, metabolized progesterone to 3α,6α-dihydroxy-5α-pregnan-20-one, indicating that 3α-OH-DHP is also a substrate for 6α-hydroxylation in breast (28). The enzyme responsible for this reaction is clearly different from the one we have investigated in the brain,

because 3α-OH-DHP is neither a substrate nor a competitive inhibitor. We have not identified the position of the hydroxyl group in the metabolite formed in rat breast in this study, because 6α- and 7α-hydroxy metabolites are not separated on HPLC and the amount of product available was not sufficient for GC identification. The low turnover number for hydroxylation of 3β-OH-DHP in the breast (0.12 mol/mol of P450/min), compared with prostate (13.5 mol/mol of P450/min) and brain (0.54 mol/mol of P450/min), indicates that the major part of breast P450 is not 3β-OH-DHP OHase, and this P450 remains to be characterized.

The competition experiments with related steroids gave some information about the structural requirements of substrates for 3β-OH-DHP OHase. In addition to 3β-Adiol, 3β,21-dihydroxy-5α-pregnane-20-one was also an efficient competitor, indicating that the addition of a hydroxyl group at position 21 does not reduce the affinity for the enzyme. This is interesting because 5α-THDOC and 3α-OH-DHP are the two most potent steroids acting at the GABA receptor. Pregnenolone also had some inhibitory effect on 3β-OH-DHP metabolism, which may be of importance for regulation of the level of steroid hormones synthesized in the brain, because it is known that the brain has the capacity to synthesize pregnenolone and progesterone from cholesterol (29, 30). In summary, we have shown that P450 in the brain has the capacity to convert 3β-OH-DHP to the corresponding 6α- and 7α-diols. We suggest that this pathway in the brain may serve to regulate the levels of GABA receptor-active steroids.

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References

1. Gee, K. W. Steroid modulation of the GABA/benzodiazepine receptor-linked chloride ionophore. *Mol. Neurobiol.* **2**:291–317 (1988).
2. Mok, W. M., S. Herschkowitz, and N. R. Krieger. *In vivo* studies identify 5α-pregnan-3α-ol-20-one as an active anesthetic agent. *J. Neurochem.* **57**:1296–1301 (1991).
3. Majewska, M. D., N. L. Harrison, R. D. Schwartz, J. L. Barker, and S. M. Paul. Steroid hormone metabolites are barbiturate-like modulators of the GABA receptor. *Science (Washington D. C.)* **232**:1004–1007 (1986).
4. Gee, K. W., M. B. Bolger, R. E. Brinton, H. Coirini, and B. S. McEwen. Steroid modulation of the chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of action. *J. Pharmacol. Exp. Ther.* **246**:803–812 (1988).
5. Purdy, R. H., A. L. Morrow, P. H. Moore, and S. M. Paul. Stress-induced elevations of γ-aminobutyric acid type A receptor-active steroids in the rat brain. *Neurobiology* **88**:4553–4557 (1991).
6. Warner, M., C. Köhler, T. Hansson, and J.-Å. Gustafsson. Regional distribution of cytochrome P450 in the rat brain: spectral quantitation and contribution of P-450b,e and P-450c,d. *J. Neurochem.* **50**:1057–1065 (1988).
7. Sundin, M., M. Warner, T. Haaparanta, and J.-Å. Gustafsson. Isolation and catalytic activity of cytochrome P450 from ventral prostate of control rats. *J. Biol. Chem.* **262**:12293–12297 (1987).
8. Gemzik, B., J. Green, and A. Parkinson. Hydroxylation of 5α-androstane-3β,17β-diol by rat prostate microsomes: effects of antibodies and chemical inhibitors of cytochrome P450. *Arch. Biochem. Biophys.* **296**:355–365 (1992).
9. Guiraud, J. M., R. Morfin, B. Ducouret, S. Samperez, and P. Jouan. Pituitary metabolism of 5α-androstane-3β,17β-diol: intense and rapid conversion to 5α-androstane-3β,6α,17β-triol and 5α-androstane-3β,7α,17β-triol. *Steroids* **34**:241–248 (1979).
10. Warner, M., M. Strömstedt, L. Möller, and J.-Å. Gustafsson. Distribution and regulation of 5α-androstane-3β,17β-diol hydroxylase in the rat central nervous system. *Endocrinology* **124**:2699–2706 (1989).
11. Isaacs, J. T., I. R. McDermott, and D. S. Coffey. The identification and characterization of a new $C_{19}O_3$ steroid metabolite in the rat ventral prostate: 5α-androstane-3β,6α,17β-triol. *Steroids* **33**:639–657 (1979).
12. Ofner, P., R. L. Vena, I. Leav, and D. W. Hamilton. Metabolism of C_{19}-radiosteroids by explants of canine prostate and epididymis with disposition as hydroxylated products: a possible mechanism for androgen inactivation. *J. Steroid Biochem.* **11**:1367–1379 (1979).
13. Lundmo, P. I., A. Sunde, and K. J. Tveter. Metabolism of androgens in the seminal vesicles and the different lobes of the prostate in young mature rats. *J. Steroid Biochem.* **22**:513–519 (1985).
14. Karavolas, H. J., and K. M. Nuti. Progesterone metabolism by neuroendocrine tissues, in *Subcellular Mechanisms in Reproductive Neuroendocrinology* (F. Naftolin, K. J. Ryan, and J. Davies, eds.). Elsevier Scientific Publishing Company, Amsterdam, 305–326 (1976).
15. Karavolas, H. J., P. J. Bertics, D. Hodges, and N. Rudie. Progesterone processing by neuroendocrine structures, in *Metabolism of Hormonal Steroids in the Neuroendocrine Structures* (F. Celotti, ed.). Raven Press, New York, 149–170 (1984).
16. Krause, J. E., P. J. Bertics, and H. J. Karavolas. Ovarian regulation of hypothalamic and pituitary progestin-metabolizing enzyme activities. *Endocrinology* **108**:1–7 (1981).
17. Bertics, S. J., P. J. Bertics, J. L. Clarke, and H. J. Karavolas. Distribution and ovarian control of progestin-metabolizing enzymes in various rat hypothalamic regions. *J. Steroid Biochem.* **26**:321–328 (1987).
18. Barnea, A., A. Hajibeigi, J. M. Trant, and J. I. Mason. Expression of steroid metabolizing enzymes by aggregating fetal brain cells in culture: a model for developmental regulation of the progesterone 5α-reductase pathway. *Endocrinology* **127**:500–502 (1990).
19. Mathur, C., V. V. K. Prasad, V. S. Raju, M. Welch, and S. Lieberman. Steroids and their conjugates in the mammalian brain. *Proc. Natl. Acad. Sci. USA* **90**:85–88 (1993).
20. Haaparanta, T., J.-Å. Gustafsson, and H. Glaumann. Isolation of mitochondria, lysosomes and microsomes from the rat ventral prostate with a note on inverted microsomal vesicles. *Arch. Biochem. Biophys.* **223**:458–467 (1983).
21. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH-cytochrome *c* (cytochrome P450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
22. Warner, M., P. Tollet, M. Strömstedt, K. Carlström, and J.-Å. Gustafsson. Endocrine regulation of cytochrome P450 in the rat brain and pituitary gland. *J. Endocrinol.* **122**:341–349 (1988).
23. Sahlberg, B. L., and M. Axelson. Identification and quantitation of free and conjugated steroids in milk from lactating women. *J. Steroid Biochem.* **25**:379–391 (1986).
24. Morfin, R. F., S. Di Stefano, J.-F. Charles, and H. H. Floch. Precursors for 6β- and 7α-hydroxylations of 5α-androstane-3β,17β-diol by human normal and hyperplastic prostates. *Biochimie (Paris)* **59**:637–644 (1977).
25. Gemzik, B., S. Jacob, S. Jennings, J. Veltman, and A. Parkonson. Species differences in 5α-androstane-3β,17β-diol hydroxylation by rat, monkey and human prostate microsomes. *Arch. Biochem. Biophys.* **296**:374–383 (1992).
26. Ofner, P., R. L. Vena, and R. F. Morfin. Acetylation and hydroxylation of 5α-androstane-3β,17β-diol by prostate and epididymis. *Steroids* **24**:261–279 (1974).
27. Fennessey, P. V., A. W. Pike, C. Gonzalez-Aller, and K. B. Horwitz. Progesterone metabolism in $T47D_{co}$ human breast cancer cells. I. 5α-Pregnan-3β,6α-diol-20-one is the secreted product. *J. Steroid Biochem.* **25**:641–648 (1986).
28. Horwitz, K. B., W. P. Pike, C. Gonzalez-Aller, and P. V. Fennessey. Progesterone metabolism in $T47D_{co}$ human breast cancer cells. II. Intracellular metabolic path of progesterone and synthetic progestins. *J. Steroid Biochem.* **25**:911–916 (1986).
29. Hu, Z. Y., E. Bourreau, I. Jung-Testas, P. Robel, and E. E. Baulieu. Neurosteroids: oligodendrocyte mitochondria convert cholesterol to pregnenolone. *Proc. Natl. Acad. Sci USA* **84**:8215–8219 (1987).
30. Jung-Testas, I., Z. Y. Hu, E. E. Baulieu, and P. Robel. Neurosteroids: biosynthesis of pregnenolone and progesterone in primary cultures of rat glial cells. *Endocrinology* **125**:2083–2091 (1989).

Send reprint requests to: Margaret Warner, Department of Medical Nutrition, Karolinska Institutet, Huddinge University Hospital, F60 Novum, S-141 86 Huddinge, Sweden.