Biochemical Pharmacology, Vol. 37, No. 24, pp. 4778–4780, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Substrate specificities of cytochrome P-450, C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$, and contribution to steroid hydroxylase activities in mouse liver microsomes

(*Received* 30 *March* 1988; *accepted* 5 *July* 1988)

Sexually dimorphic steroid hydroxylase activities in liver microsomes are due to sex-dependent expression of forms of cytochrome P-450 (P-450) specific for the corresponding activities. More specifically, testosterone 16α-hydroxylase activity is higher in the liver of male than female 129/J mice. On the other hand, testosterone 15α-hydroxylase activity predominates in liver microsomes prepared from females [1, 2].

To investigate the mechanisms by which the sex-dependent expression of steroid hydroxylases are regulated in mouse liver [2, 3], we previously purified male-specific C-P-450$_{16\alpha}$ and female-specific P-450$_{15\alpha}$. These cytochromes catalyzed only 16α- or 15α-hydroxylations of testosterone respectively.

This report examines 16α- and 15α-hydroxylase activities of C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ through the utilization of various steroid substrates, in addition to testosterone. Furthermore, the contributions of C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ to steroid 16α- or 15α-hydroxylase activities in liver microsomes were estimated as the activities inhibited by C-P-450$_{16\alpha}$ or P-450$_{15\alpha}$ antibodies.

Materials and methods

Animals. Two- to three-month-old 129/J mice were purchased from Jackson Laboratory (Bar Harbor, ME).

Preparation of microsomes, purification of C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ and preparation of antibodies to the P-450s. Mice were killed by cervical dislocation. Liver microsomes were prepared by a method used previously [2]. Male C-P-450$_{16\alpha}$ and female P-450$_{15\alpha}$ were purified from liver microsomes of male and female 129/J mice, respectively, by using specific testosterone 16α- or 15α-hydroxylase activities as the basis of selection of fractions from columns [2, 3]. Antibodies to C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ were raised in New Zealand White rabbits and then enriched by affinity chromatography as described previously [2, 3]. The purification method of Yasukochi and Masters [4] was used to obtain NADPH–cytochrome P-450 reductase from liver microsomes of phenobarbital-treated rats.

Enzyme assay. Steroid 16α- and 15α-hydroxylase activities in microsomes and reconstitution systems were measured by using the assay conditions described for testosterone 16α- and 15α-hydroxylase activities [2]. Instead of methylene chloride, ethyl acetate was used as the extraction solvent only when estradiol or estriol was the substrate. The steroid metabolites in extracts were dried under a stream of nitrogen gas and separated by two-dimensional thin-layer chromatography. To separate metabolites of pregnenolone and dehydroepiandrosterone, the following solvent systems were used: chloroform/ethyl acetate (50/50) in the first dimension and benzene/acetone (72/25) in the second dimension. Other solvent systems used included chloroform/ethyl acetate (35/65) and benzene/acetone (75/25) for progesterone and 4-androstene-3,17-dione (androstenedione); chloroform/methanol (92:8) and benzene/acetone (50/50) for testosterone and estradiol; and chloroform/methanol (80/20) and benzene/acetone (50/50) for estriol and corticosterone. After chromatography, silica plates (20 × 20 cm) were autoradiographed or fluorographed to X-ray films. The positions of 16α- and 15α-hydroxylated metabolites were identified by the comigrations of 16α- and 15α-hydroxylated standards. Color visualization of 16α- and 15α-hydroxysteroids was performed through the reaction of phosphomolybdic acid with the steroid. The hydroxylase activities were calculated from radioactivity recovered from the area of silica gels where the 16α-hydroxylated steroids migrated. Cytochrome P-450 and protein contents were determined by the methods of Omura and Sato [5], and Bradford [6] respectively. Bovine serum albumin was used as the standard for protein assay.

Materials. [4-^{14}C]Pregnenolone (56.0 mCi/mmol), [4-^{14}C]corticosterone (52.0 mCi/mmol), and dehydro[1,2,6,7-^{3}H]epiandrosterone (61.0 mCi/mmol) were purchased from Amersham (Arlington Heights, IL). [4-^{14}C]Progesterone (57.2 mCi/mmol), [4-^{14}C]androstenedione (52 mCi/mmol), [4-^{14}C]testosterone (52 mCi/mmol), [4-^{14}C]estradiol (57 mCi/mmol) and [2,4,6,7-^{3}H(N)]estriol (90 mCi/mmol) were procured from New England Nuclear (Beverly, MA). The standard 16α- and 15α-hydroxylated steroids were obtained from the Reference Collection of the British Medical Council (London, England) and Steraloids, Inc. (Wilton, NH). We purchased pregnenolone, progesterone, androstenedione, dehydroepiandrosterone, testosterone, estradiol, estriol, corticosterone, dilauroylphosphatidylcholine, NADPH and phosphomolybdic acid from Sigma (St. Louis, MO), and silica gel plates from EM Laboratory Inc. (Elmsford, NY).

Results and discussion

Table 1 summarizes 16α- and 15α-hydroxylase activities with various steroids in the reconstitution systems. C-P-450$_{16\alpha}$ catalyzed 16α-hydroxylation of dehydroepiandrosterone at an even higher rate than that of testosterone, and also modestly metabolized estradiol and progesterone. Pregnenolone, androstenedione and corticosterone were poorly hydroxylated at their 16α-positions by C-P-450$_{16\alpha}$.

The substrate specificity of P-450$_{15\alpha}$ was higher than that of C-P-450$_{16\alpha}$. Progesterone, followed by testosterone, was the most effective substrate among these steroids. P-450$_{15\alpha}$ also catalyzed 16α-hydroxylation of androstenedione relatively well, but only minor activity was detected when other steroids were introduced. This indicated that Δ^4-steroids with the same A-, B- and C-ring structures as testosterone were effective substrates.

An inhibitory antibody to C-P-450$_{16\alpha}$ or P-450$_{15\alpha}$ was used to determine the contributions of these P-450s in 16α- or 15α-hydroxylation activities of steroids in liver microsomes from the males and females. With the exception of corticosterone, liver microsomes from 129/J male mice catalyzed 16α-hydroxylations of all the other steroids at various rates (Table 2). The C-P-450$_{16\alpha}$ antibody inhibited nearly 100% of testosterone and estradiol 16α-hydroxylase activities. The hydroxylations of the other steroids, however, were decreased to only 20–35% of control levels. These results suggest that, although C-P-450$_{16\alpha}$ is the major isozyme catalyzing the 16α-hydroxylations of these steroids in liver microsomes, there may be other P-450s that are involved in these hydroxylations.

Among the eight steroids tested, only testosterone, androstenedione and progesterone were hydroxylated effectively in liver microsomes from female 129/J mice at the 15α-position (Table 2). The inhibition by P-450$_{15\alpha}$ antibody indicated that P-450$_{15\alpha}$ was the major isozyme responsible for these 15α-hydroxylase activities in liver microsomes from female mice. The 15α-hydroxylations of other substrates such as dehydroepiandrosterone, cortico-

Table 1. Reconstituted steroid 16α- and 15α-hydroxylation activities by C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$

Steroid substrates	16α-Hydroxylation by C-P-450$_{16\alpha}$ (nmol product/min/nmol of P-450)	15α-Hydroxylation by P-450$_{15\alpha}$ (nmol product/min/nmol of P-450)
Pregnenolone	8.70	<0.08
Progesterone	23.9	102
Androstenedione	9.85	45.8
Dehydroepiandrosterone	113	<6.21
Testosterone	73.8	83.7
Estradiol	32.0	0.34
Estriol	—	2.53
Corticosterone	<1.05	<3.33

The reconstitution system for steroid hydroxylation activities consisted of 0.02 nmol of purified P-450$_{16\alpha}$ or P-450$_{15\alpha}$, 3 units NADPH–cytochrome P-450 reductase, 250 nmol NADPH, 25 μg dilauroylphosphatidylcholine, and 100 nmol of radiolabeled steroid substrates. The 16α- and 15α-hydroxylated steroids were separated out by two-dimensional thin-layer chromatography, and their radioactivities were measured by scintillation counting [3]. The results from two separate assays were averaged.

Table 2. Inhibition of steroid 16α- and 15α-hydroxylase activities in liver microsomes by C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ antibodies

	Steroid hydroxylation activities (nmol product/min/mg protein)			
	16α-Hydroxylation		15α-Hydroxylation	
Steroids	Control	+ C-P-450$_{16\alpha}$ antibody	Control	+ P-450$_{15\alpha}$ antibody
Pregnenolone	0.27	0.07	ND*	ND
Progesterone	0.80	0.16	1.20	0.26
Androstenedione	0.68	0.18	0.80	0.26
Dehydroepiandrosterone	0.98	0.34	ND	ND
Testosterone	0.61	0.05	0.77	0.09
Estradiol	0.13	0.01	ND	ND
Estriol	—†	—	ND	ND
Corticosterone	ND	ND	ND	ND

Liver microsomes (500 μg) of five male and five female 129/J mice were used to measure steroid 16α- and 15α-hydroxylation activities respectively. To inhibit steroid hydroxylase activities, either C-P-450$_{16\alpha}$ or P-450$_{15\alpha}$ antibody (20 μg) was preincubated with the microsomes prior to the initiation of the activity by the adding of NADPH. For the control, the activities were measured without the presence of the antibodies. The detailed assay conditions have been described previously [3, 7]. Values are the average of two separate assays.

* Not detectable.

† Not measured.

sterone and estriol were not detected, even though the purified P-450$_{15\alpha}$ catalyzed these activities in the reconstitution system.

The fact that both C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ efficiently catalyzed the hydroxylation of multiple steroids suggested that a role of these P-450s is degradation of steroid hormones. In addition, C-P-450$_{16\alpha}$ may be involved in estrogen formation from dehydroepiandrosterone; 16α-hydroxydehydroepiandrosterone in fetal liver is the immediate precursor which then is aromatized to estrogen in placenta. Estradiol 16α-hydroxylation by C-P-450$_{16\alpha}$ may be of interest in view of the proposal by Fishman and coworkers of a close correlation between the formation of 16α-hydroxyestradiol and a high risk of developing mammary tumors in mice [8]. Besides being present in female liver, P-450$_{15\alpha}$ is also present in male kidney at high levels [9]. This suggested that this cytochrome may be involved in the formation of 15-oxyprogesterone which is a more potent antimineralocorticoid [10].

In summary, both C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ catalyzed hydroxylations of multiple steroids, including testosterone, at the 16α- and 15α-position respectively. Δ^4-Steroids with the same A-, B- and C-ring structure as testosterone were specific substrates for 15α-hydroxylation by P-450$_{15\alpha}$. The majority of the 16α- and 15α-hydroxylation of these steroids in mouse liver microsomes was catalyzed by C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$. Thus, steroid 16α-hydroxylase and 15α-hydroxylase would be more appropriate names for C-P-450$_{16\alpha}$ and P-450$_{15\alpha}$ rather than the initially proposed testosterone 16α-hydroxylase and testosterone 15α-hydroxylase.

Pharmacogenetics Section
Laboratory of Reproductive and Developmental Toxicology
National Institute of Environmental Health Sciences
National Institutes of Health
Research Triangle Park
NC 27709, U.S.A.

NOBUHIRO HARADA
MASAHIKO NEGISHI*

* Author to whom correspondence should be addressed.

REFERENCES

1. Ford HC, Lee E and Engel LL, Circannual variation and genetic regulation of hepatic testosterone hydroxylase activities in inbred strains of mice. *Endocrinology* **104**: 857–861, 1979.
2. Harada N and Negishi M, Mouse liver testosterone 15α-hydroxylase (cytochrome P-450$_{15\alpha}$): Purification, regioselectivity, stereospecificity, and sex-dependent expression. *J Biol Chem* **259**: 1265–1271, 1984.
3. Harada N and Negishi M, Mouse liver testosterone 16α-hydroxylase (cytochrome P-450$_{16\alpha}$): Purification, regioselectivity, stereospecificity, and immunochemical characterization. *J Biol Chem* **259**: 12285–12290, 1984.
4. Yasukochi Y and Masters BSS, Some properties of a detergent-solubilized NADPH–cytochrome (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J Biol Chem* **251**: 5337–5344, 1976.
5. Omura T and Sato R, The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
6. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**: 248–254, 1976.
7. Burkhart B, Harada N and Negishi M, Sexual dimorphism of testosterone 15α-hydroxylase mRNA levels in mouse liver: cDNA cloning and regulation. *J Biol Chem* **260**: 15357–15361, 1985.
8. Bradlow HL, Hershcope RJ, Martucci C and Fishman J, Estradiol 16α-hydroxylation in the mouse correlates with mammary tumor incidence and presence of murine mammary tumor virus: A possible model for the hormonal etiology of breast cancer in humans. *Proc Natl Acad Sci USA* **82**: 6295–6299, 1985.
9. Squires EJ and Negishi M, Genetic regulation of testosterone 15α-hydroxylase (cytochrome P-450$_{15\alpha}$) in renal microsomes of female mice. *Biochemistry* **25**: 4913–4918, 1986.
10. Tweit RC and Kagawa CM, 15-Oxygenated progesterone: A new series of synthetic mineralocorticoid antagonists. *J Med Chem* **7**: 524–528, 1964.

Biochemical Pharmacology, Vol. 37, No. 24, pp. 4780–4783, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

Inhibition of bovine serum amine oxidase activity by aminoalkylaminoanthraquinones

(*Received* 11 *April* 1988; *accepted* 22 *July* 1988)

Anthraquinones, mainly their alkyl derivatives are known to interact with nucleic acids [1]. Amidine derivatives of anthraquinone have been synthesized and their activity against *Entamoeba histolytica* has been demonstrated [2]. More recently bis (substituted amino-alkylamino) anthraquinones have been synthesized [3, 4], at least one of which possessed antineoplastic activity against leukemia and melanoma tumors. The antibacterial activity of 1-amino-4-hydroxy-anthraquinone has also been demonstrated [5]. Previous studies in our laboratory demonstrated that diaminoalkyl-aminoanthraquinones inhibited the growth of leishmanial promostigotes grown *in vitro* [6]. It has been shown that a three carbon diamine derivative is more active than the two or four carbon diamine analogues [6]. It has also been observed that the antiviral activity of aminoalkylaminoanthraquinones can be reduced by adding either putrescine or spermidine to a suspension of T_2 coliphages (unpublished data). These findings suggested that the antiviral activity of the aminoalkylanthraquinone is related to polyamines. Polyamines and diamines are ubiquitous organic cations which are present in all prokaryotic or eukaryotic cells analyzed [7, 8]. Polyamines interact with nucleic acids (including phage DNA) and affect their structure and function [7]. Replacing polyamines by aminoalkylaminoanthraquinones may distort the structure of the nucleic acids and thus interfere with their biological activity.

In this study we tested the effect of various aminoalkylaminoanthraquinones on the activity of polyamine oxidizing enzymes. It will be shown that the oxidation of the polyamines spermidine and spermine is dramatically inhibited by these compounds.

Experimental

Materials. Table 1 shows the structures of the substituted anthraquinones investigated in this study. The preparation of these compounds has been previously described [9, 10].

N-Methyl-2-benzothiazolone hydrazone was obtained from EGA Chemie (Steinheim, F.R.G.) and putrescine, spermidine and spermine hydrochlorides were from Sigma (St. Louis, MO). Pea seedling diamine oxidase was purified by the method of Hill [11] (specific activity 10 units/ml; 100 units/mg; 1 unit catalyses the oxidation of 1 μmol putrescine/hr). Pig kidney diamine oxidase (0.1 unit/mg 10 units/ml) was obtained from Sigma. Bovine serum amine oxidase (SAO) was purified as previously described [12] and its activity assayed [13] using benzylamine as substrate (110 units/ml; 50 units/mg).

Methods. The substituted anthraquinones were tested for their ability to inhibit the oxidation of putrescine by both diamine oxidases and the oxidation of spermidine and spermine by SAO. The oxidation of the amines was quantitated colorimetrically by coupling of the resulting aldehydes with *N*-methyl-2-benzothiazolone hydrazone [14]. The experimental details were as follows. Enzyme solution (100 μl) was added to a mixture of buffer (100 μl of 0.2 M Tris–HCl, pH 7.3), substrate (100 μl of 0.1 M aqueous solution of putrescine, spermidine or spermine) and inhibitor (100 μl of 2–200 μM aqueous solution). The final volume (400 μl) was incubated in a water bath at 37° for 1 hr, after which *N*-methyl-2-benzothiazolone hydrazone (0.5 ml of 0.4% aqueous solution) was added. The reaction was linear over 1 hr of incubation. The solution was kept at room temperature (25°) for 30 min before addition of ferric chloride (2.5 ml of 0.2% aqueous solution). After standing a further 15 min at room temperature, the intensity of the resulting blue colour was measured at 660 nm (Bausch and Lomb Spectronic 1001 spectrophotometer). For the oxidation product of spermidine, $\varepsilon = 6.25 \times 10^3 \mathrm{l \cdot mol^{-1} cm^{-1}}$ and for spermine, $\varepsilon = 12.5 \times 10^3 \mathrm{l \cdot mol^{-1} cm^{-1}}$ [14] was used to quantitate the amount of product formed.