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IDENTIFICATION OF LANOSTEROL 14α-METHYL DEMETHYLASE IN HUMAN TISSUES*

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SUMMARY: Lanosterol 14α-methyl demethylase was investigated in human tissues using a radio-HPLC assay to detect the 4,4-dimethyl-5α-cholesta-8,14-dien-3ß-ol (diene) metabolite. The sequence of events leading to the demethylated product in human liver microsomes involves the conversion of the diol to the aldehyde followed by diene formation. Enzyme activity displayed a greater than 10 fold variation among the 9 liver samples studied. Kinetic parameters were determined and shown to differ between two separate liver samples. Addition of inhibitors of yeast lanosterol 14α demethylase, ketoconazole and miconazole, resulted in extensive inhibition of formation of the demethylated metabolite. The enzyme, detected in microsomes isolated from human kidney and lymphocytes, also catalyzed the conversion of dihydrolanosterol to oxylanosterol intermediates and the diene. The presence of this enzyme in microsomes from various human tissues suggests that it may play a role in cellular regulation of cholesterol synthesis.

It has been suggested for some time that oxygenated sterols may be endogenous regulators of HMG-CoA reductase, the rate limiting enzyme in cholesterol synthesis, and consequently the biosynthesis of cholesterol. Several of these oxygenated sterols are precursors or metabolites of cholesterol and there is evidence to suggest that one or more of these sterols may be involved in compensatory changes in HMG-CoA reductase activity and hence, cellular cholesterol balance. The initial reaction in the biosynthesis of cholesterol from lanosterol is the C-32 oxidative demethylation of lanosterol. Three sequential products, a diol, an aldehyde and the conjugated $\Delta^{8,14}$-diene, have been identified during conversion of lanosterol to cholesterol (1,2). A single enzyme is responsible for all three oxidative steps in the removal of C-32 as formic acid (3,4). It has been suggested that the functional importance of the diol and aldehyde, oxylanosterol intermediates, in mammalian systems is to suppress HMG-CoA reductase (5,6). Recent

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Abbreviations used: dihydrolanosterol, 4,4,14α-trimethyl-5α-cholesta-8-en-3ß-ol; diol, lanost-8-ene-3ß, 32-diol; aldehyde, 3ß-hydroxylanost-8-en-32-al; diene, 4,4-dimethyl-5α-cholesta-8,14 -dien-3ß-ol; HPLC, high pressure liquid chromatography; P450, cytochrome P450; PHA, lectin from *Phaseolus vulgaris*; FCS, fetal calf serum; KPO_4, potassium phosphate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

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investigations using Chinese hamster ovary cells, rat hepatocytes, and a 14α-methyl demethylase deficient cell line, AR 45, demonstrate that accumulation of the oxylanosterol products of 14α demethylation has profound effects on the expression of HMG-CoA reductase (5,7). Accumulation of oxylanosterols occurs in response to 14α-demethylase inhibition by miconazole or ketoconazole. Low concentrations of either agent suppress HMG-CoA reductase activity while elevated levels restore reductase activity to control values or higher. This modulatory effect of the demethylase inhibitors upon HMG-CoA reductase was not observed in the lanosterol 14α-methyl demethylase-deficient mutants which fail to accumulate oxylanosterol intermediates. When these inhibitors are administered *in vivo* to rats, similar results to those obtained in culture are observed. Both lanosterol 14α demethylase and HMG-CoA reductase activities are suppressed (8).

Lanosterol 14α demethylase is known to be a P450 enzyme in eukaroytic systems (1,3,4,9), however, the rat is one of the few mammalian species in which the enzyme is characterized (3). In this investigation, we report, for the first time, the 14α demethylation of dihydrolanosterol by microsomes from various human tissues. We provide evidence of variability in activity among individual human liver samples. We also demonstrate the formation of oxysterol intermediates, inhibition of metabolite formation by antifungal agents, and measure kinetic constants. Identification of this enzyme in humans and the formation of oxysterol products opens the possiblity that this enzyme may play a role in regulating cellular cholesterol biosynthesis in humans, as has been suggested for other species.

EXPERIMENTAL PROCEDURES

Acquisition of human tissues

Human liver samples were obtained within 30 mins of death from the National Disease Research Interchange (NDRI), Phila. PA. None of the subjects had a prior history of drug or alcohol abuse (Table I). Human kidney was obtained from surgical patients by Dr. Martin Black, Temple University School of Medicine, Phila. PA. The livers and kidneys were removed, quick-frozen in liquid nitrogen and stored at -80° C until microsomes were prepared. Human lymphocytes were isolated from 3 units of whole blood obtained from United Blood Services (UBS) Albuquerque, NM. Blood was utilized within 72 hours of being obtained from patients. Lymphocytes were isolated as described (10) using Ficoll-Paque (Pharmacia LKB Biotechnology, Inc.). Cells were cultured at a density of 2 x 10^6 cells/plate in RPMI media containing L-glutamine and 10% FCS. The lymphocytes were then stimulated to proliferate using 20 μg of PHA /10 ml media. After a 48 hour incubation in a 37°C, CO_2 incubator, the lymphocytes were harvested, quick-frozen in liquid nitrogen and stored at -80°C until microsome preparation.

Preparation of microsomes

The liver and kidney samples were slowly thawed and homogenized in 4 volumes of ice-cold 100 mM Tris-HCl buffer, pH 7.4, containing 100 mM KCl, 1 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) with two 40 sec bursts in a Waring Blender. The mixture was further homogenized using a motor-driven teflon/glass tissue grinder. Lymphocyte homogenates were prepared by sonication of the cells using 3 continuous 45 sec bursts with a membrane disruptor. Microsomes were then isolated according to Van der Hoeven and Coon (11). The pyrophosphate-washed microsomes were resuspended in 50 mM KPO_4, pH 7.4, containing 0.1 mM EDTA, 0.1 mM DTT and 20% glycerol and frozen at -80°C until use. Protein concentrations were determined according to Bensadoun and Weinstein (12) and P450 concentrations were measured by the method of Omura and Sato (13). P450 Reductase was assayed by monitoring the rate of cytochrome *c* reduction at 550 nm in 0.3 M KPO_4, pH 7.7 at 30°C, in 1.0 ml incubations containing 20 μg of microsomal protein and 5 mg/ml cytochrome *c*.

Lanosterol 14α -methyl demethylase activity

Lanosterol 14α-methyl demethylase activity was assessed according to a previously published method (3) using 24, 25-[^{3}H] dihydrolanosterol as substrate with the following modifications. Various amounts of microsomal protein were added to the incubation mixture depending on the tissue source; 1 mg of liver, 2 mg of kidney, 2 or 4 mg of lymphocyte. Enzyme assays, initiated by addition of 1 mM NADPH, were performed at 37°C for 15 min and terminated with 0.5 ml 15% KOH in 95% methanol, followed by saponification, extraction and radio-HPLC analysis. Kinetic constants were determined with 1 mg human liver microsomes using substrate concentrations ranging from 10 to 400 μM. Accumulation of diene was linear with time (up to 1 hr) and protein concentration (to 6 mg of microsomal protein) and diene formation increased linearly with substrate concentrations up to 400 μM. Miconazole or ketoconazole (5 μM) was added to the incubation mixture in 10 μl of ethanol.

Materials

Radiolabeled 24, 25-[^{3}H] dihydrolanosterol (DHL) was purified as described (8). NADPH was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Ketoconzole, miconazole, and PHA were obtained from Sigma Chemical Co. (St. Louis, MO), RPMI was from Gibco BRL (Gaithersburg, MD) and fetal calf serum (FCS) was from Hyclone (Logan, UT). All other reagents were of the highest grade commericially available.

RESULTS AND DISCUSSION

Lanosterol 14α-methyl demethylase activity was investigated in microsomes isolated from human liver, kidney and lymphocytes. The results of this investigation are the first demonstration of this P450 in various human tissues. The rates of formation of the demethylated diene varied greater than 10 fold among liver samples from 9 subjects, ranging from 8.17 to 113 pmol/min/mg (Fig. 1), and correlated poorly with aggregate P450 content (r^2=0.48, p>.05) and P450 reductase activity (r^2=0.13, p>0.05)(Table I). Whether the variability in activity of this enzyme effects cellular or extracellular concentrations of cholesterol has not been determined. However, if extracellular concentrations of cholesterol are mediated by this enzyme, serum cholesterol levels may inversely correlate with 14α–methyl demethylase concentrations.

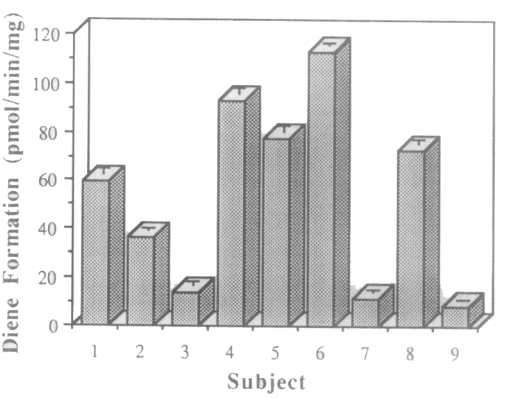

Figure 1. Human liver microsomal 14α demethylation of dihydrolanosterol.
Dihydrolanosterol 14α demethylase activity was performed using nine different human liver microsomal samples as described in "Experimental Procedures". One mg of microsomal protein and a substrate concentration of 50 μM were added to the incubation mixture. Values represent the mean ± S.E., p<.05.

TABLE I

HUMAN SUBJECTS

Subject	Gender	Age	Drug/Alcohol History	Microsomal	
				P450 (nmol/mg)	P450 Reductase (nmol/min/mg)
1	F	--	none[a]	0.301	68.2
2	F	54	none	0.374	81.4
3	M	48	butylol, ancef	0.335	104
4	Unknown			0.315	58.4
5	F	37	ancef	0.384	48.7
6	F	47	none	0.744	89.1
7	M	59	theophylline, terbutaline, smoker	0.309	39.8
8	F	--	none, obese with fatty liver	0.560	117
9	M	65	none	0.246	86.1
10	M	--	none	0.066	23.1
11	F	37	none	0.093	21.9
12	--	--	lymphocyte microsomes	ND[b]	11.2

[a] none indicates that the individual was not recently exposed to any drugs

[b] ND indicates not detectable

P450 concentrations and P450 reductase activity were determined as described in "Experimental Procedures". Each value represents the mean of two determinations.

The route of metabolism of DHL in human microsomes is similar to that shown for rat (3). Formation of the diol and aldehyde metabolites is followed by conversion to diene, as suggested by conversion to both aldehyde (41.2 pmol/min/mg) and diene (62.7 pmol/min/mg) from the diol substrate. The similarities in the metabolic profile of DHL between rat and human suggests that the enzyme is involved in cholesterol production in a similar manner in both species. Although the rates of DHL demethylation for rat liver microsomes are about two fold higher than the mean rate of demethylation determined for the 9 human liver samples investigated in this study, this may be due to an inductive effect of cholestyramine upon the 14α-methyl demethylase in the rat studies (3). Whether this enzyme is inducible by cholestyramine in human liver remains to be determined.

K_m and V_{max} values were determined from double reciprocal plots of the data obtained on microsomes from subjects 6 and 9. These microsomes exhibited the highest and lowest rate of

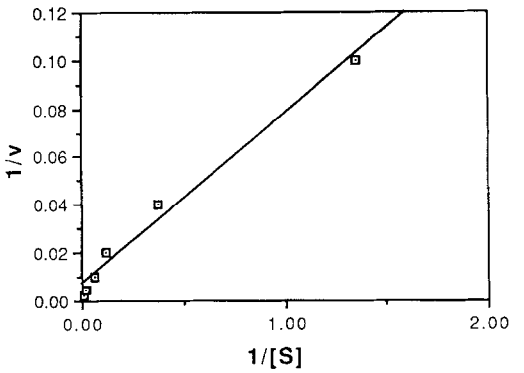

Figure 2. Kinetic Analysis of lanosterol 14α demethylase activity by human liver microsomes A representative Lineweaver-Burk plot of lanosterol 14α demethylation by microsomes from subject #9. Demethylase activity was performed as described in Experimental Procedures with varying concentrations of lanosterol (10-400 μM). Each point represents the mean of a duplicate assay at the substrate concentration indicated; the difference between the duplicates was <5% in all cases. The double reciprocol plot revealed linearity with an r^2= 0.98, p<0.01, Vmax=149 pmol/min/mg microsomal protein, and a k_m = 435 μM.

demethylase activity, respectively (Fig. 2). The extrapolated V_{max} (333 pmol/min/mg microsomal protein) and K_m (167 μM) of microsomes from subject 6 were considerably different from those observed for subject 9 (V_{max}=149 and K_m= 435). Thus, microsomes which exhibited a low rate of demethylase activity not only had a lower V_{max} but also a 3-fold higher K_m than those determined for microsomes which exhibited a high rate of activity.

Antimycotic drugs such as miconazole and ketoconazole are known to inhibit mammalian lanosterol 14α-methyl demethylase activity (14,15). Both antimycotics effectively inhibited human enzyme activity in the present investigation. Five μM of each inhibitor decreased the formation of the diene to 10-20% of that formed by microsomes in the absence of inhibitors. Because these inhibitors produce similar inhibition in yeast, rat and human microsomes, the active site of this enzyme may be conserved between yeast and mammals.

Lanosterol 14α-methyl demethylase activity was also assessed in extrahepatic tissues. Microsomes obtained from human lymphocytes were investigated for their ability to form the diene end-product. Incubation mixtures containing 2 mg of lymphocyte microsomal protein formed only the diol and aldehyde intermediates, however, at 4 mg microsomal protein both the aldehyde, which was the more prominent metabolite, and diene were detected (Table II). Additionally, microsomes from human kidney samples were examined. Diene formation was about three-fold greater than the diol or aldehyde, which were formed in approximately equal amounts (1.43 ± 0.39 and 2.25 ± 0.48 pmol/min/mg, repectively) when microsomes prepared from whole kidney (subject 10) were utilized (Table II). However, in kidney microsomes prepared from cortex (sample 11), the diol was formed at a greater rate than was the aldehyde (7.69± 0.55 vs 3.17 ± 1.12 pmol/min/mg, repectively). Moreover, diene formation was only 1.4 fold greater than formation of the diol (Table II).

TABLE II

LANOSTEROL 14α-METHYL DEMETHYLASE ACTIVITY IN EXTRAHEPATIC TISSUES

Microsome Source	Microsomal Protein	Diol	Aldehyde	Diene
		(pmol/min/mg microsomal protein)		
Lymphocytes	2 mg	3.08 ± 0.43	1.38 ± 0.63	0
	4 mg	0	1.73 ± 0.28	1.62 ± 0.33
Whole kidney (subject 10)		1.43 ± 0.39	2.25 ± 0.48	6.08 ± 0.45
Kidney cortex (subject 11)		7.69 ± 0.55	3.17 ± 1.12	10.2 ± 0.87

Dihydrolanosterol 14α-methyl demethylase activity was assessed in microsomes obtained from lymphocytes, whole kidney or kidney cortex. The assay was performed as described in "Experimental Procedures" using 50 μM DHL and 2 mg of kidney microsomal protein or 2 and 4 mg of microsomal protein obtained from lymphocytes. Values represent the mean ± S.E., $p<.05$.

Although the information concerning 14α-methyl demethylase activity in extrahepatic tissues is limited, its detection as shown here suggests that cholesterol biosynthesis via this pathway probably occurs in all human cell types. Tabacik et al. (16) show that cultured human lymphocytes accumulate the oxylanosterol aldehyde intermediate, which inhibits HMG-CoA reductase expression. However, they were unable to demonstrate further metabolism to the diene. By isolating microsomes from cultured lymphocytes, we have demonstrated that the lymphocyte enzyme is capable of converting DHL to both the aldehyde and the demethylated product and therefore, is probably similar to the liver enzyme. Moreover, we have shown that human liver lanosterol 14α-methyl demethylase appears to resemble that of rat in its production of oxylanosterol intermediates. This suggests that since rat lanosterol 14α-methyl demethylase regulates the expression of HMG-CoA reductase by producing oxygenated sterols (5), the human enzyme may also play a regulatory role in cholesterol biosynthesis.

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