Study on Stereospecificity of Enzyme Reaction Related to Peroxisomal Bile Acid Synthesis in Rat Liver

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We examined stereoselectivities of enzymes related to bile acid formation in hepatic peroxisomes using two stereoisomers of 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and its coenzyme A (CoA) derivatives. The activity of acyl-CoA synthetase for 25*S*-THCA was 1.4-times higher than that for 25*R*-THCA. The difference was also observed after clofibrate-treatment. This activity was located in microsomes, differing from palmitoyl-CoA synthetase located in mitochondria, peroxisomes and microsomes. There was no stereoselectivity in the reaction of peroxisomal fatty acyl-CoA oxidase for THCA isomers, and the activity was one tenth of that for acyl-CoA synthetase. Considering the overall raction of peroxisomal bile acid formation, the stereoselective difference observed in THCA-CoA synthesis should be denied. Thus, the previous finding that the overall formation of bile acid from THCA was not stereoselective was further confirmed. Furthermore, the activity for THCA oxidation was not induced by clofibric acid, suggesting that there would be different isozymes of peroxisomal acyl-CoA oxidase against THCA-CoA and palmitoyl-CoA.

Keywords stereoselectivity; peroxisome; bile acid formation; acyl-CoA synthetase; acyl-CoA oxidase; rat liver

Introduction

The peroxisomal β-oxidation system participates in the metabolism of a wide variety of intrinsic and extrinsic compounds such as fatty acids, bile acids, very long-chain fatty acids, dicarboxylic acids and xenobiotic acyl-compounds.[1-10] Recently, it has been reported that there is a difference in the metabolic pathway and physiological activity between stereoisomers of drugs or biological substances.[11] Peroxisomes also include some enzymes with stereoselectivity for their substrates, such as D-amino acid oxidase and L-α-hydroxyacid oxidase.[12,13] However, there has been no report concerning the stereospecificity of peroxisomal β-oxidation. A precursor of bile acid, 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA), which can be a substrate of peroxisomal β-oxidation, has stereoisomers which are produced during the metabolism of isomeric intermediates.[14] There have already been reports that the formation of cholic acid from THCA was little stereospecific.[15,16] However, the stereospecificity of each enzyme reaction composing the bile acid formation process has not yet been elucidated. Then, using two stereoisomers of THCA and its coenzyme A (CoA) derivatives, we examined whether or not the acyl-CoA synthetase and peroxisomal acyl-CoA oxidase, which relate to the formation of bile acid from THCA, and acyl-CoA hydrolase, which could have an inhibitory affect on the supply of THCA-CoA to bile acid synthesis process, have a stereoselectivity in their reaction.

Materials and Methods

Meterials Clofibric acid (CPIB), acetyl-CoA, palmitoyl-CoA, CoA, nicotinamide adenine dinucleotide (NAD), and bovine serum albumin (BSA, fatty acid free) were purchased from the Sigma Chemical Co., St. Louis, MO, U.S.A. L-Carnitine-HCl was a gift from the Earth Pharmaceutical Co., Ltd., Japan. Racemic THCA and its stereoisomers were prepared according to the method of Tohma and Kurosawa.[17] Each isomer prepared showed a stereochemical purity of over 97% on high performance liquid chromatographic (HPLC) analysis. The details will be reported elsewhere in the near furture. 25*R*-THCA-CoA and 25*S*-THCA-CoA were prepared by the method of Shah and Staple.[18] Briefly, 25*R*- or 25*S*-THCA of 75 μmol dissolved in 6 ml methylenechloride was added 2 ml methylenechloride containing 0.012 ml ethylchloroformate, allowed to stand for 1 h at r.t., evaporated under nitrogen and dissolved in 5 ml of tetrahydrofuran. The mixed anhydride of each THCA obtained was added to 62.5 μmol of CoA-SH in 5 ml of water, stirred for 15 min and acidified with 10% perchloric acid to precipitate CoA thioester. The precipitated CoA thioesters were washed with ether, acetone and water, and then lyophilized. The purities of the CoA thioesters were above 90% by HPLC analysis using a Nova-Pak C18 column. Other chemicals, all of reagent grade, were obtained from Wako Pure Chemicals Co., Ltd. (Japan).

Animals and Treatment Male Wistar rats weighing around 180 g were divided into control- and CPIB-groups (4 animals/group). The animals of the later group were orally administered CPIB suspended in 0.5% carboxymethylcellulose at 300 mg/kg/d. After 1 week of treatment, the animals were sacrificed by decapitation, and the livers were perfused, removed quickly and 10% (w/v) homogenates were prepared in 0.25 M sucrose. The homogenates were used for the assay of enzyme activities and subcellular fractionation.

Subcellular Fractionation of the Liver The liver homogenate was fractionated by the method of de Duve *et al.*[19] The light mitochondrial fraction (LM fraction) was further centrifuged in a discontinuous sucrose density gradient.[20] Separately, a part of the liver homogenate was centrifuged at $10000 \times g$ for 20 min and subsequently at $105000 \times g$ for 1 h, and the resulting pellet suspended in 0.25 M sucrose was referred to as the microsomal fraction, and the supernatant as the cytosol fraction.

Assay Methods of Enzyme Activities The activity of catalase was determined by the method of Luck.[21] The activities of glutamate dehydrogenase (GDH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome c reductase (cyt c red.) were determined by Beaufay *et al.*[22,23] The acyl-CoA synthetase activity for palmitic acid was determined by Krisans *et al.*[24] The acyl-CoA synthetase activity for THCA was determined by the method of Tanaka *et al.*[25] Briefly, the 1 ml of the reaction mixture composed by 0.5 mM THCA, 1 mM CoA, 100 mM Tris–HCl (pH 8.0), 10 mM adenosine triphosphate (ATP), 15 mM $MgCl_2$, 150 mM KCl, 5 mM dithiothreitol and 0.1 ml of the enzyme preparation was incubated at 37 °C for 30 min. Then, 0.1 ml of 5.5 N HCl was added to the reaction mixture, cooled on ice for 20 min and centrifuged at 3000 rpm for 10 min. The supernatant was filtered and applied on HPLC for determination of the amount of THCA-CoA formed. The conditions of HPLC were as follows: column, Nova-Pak C18 (3.9 mm × 15 cm); solvent, 40—60% acetonitrile in 10 mM tetra-*n*-butylammonium perchorate (TBAP, pH 5.5) (0.5%/min); flow rate, 1 ml/min; detection, absorption at 258 nm. The activity of acyl-CoA oxidase was determined by the method of Reubsaet *et al.*[26] using palmitoyl-CoA or THCA-CoA as a substrate. The acyl-CoA hydrolase was determined by the method of Kawashima.[27] Protein content was determined by the Lowry method using BSA as a standard.[28]

Results and Discussion

THCA, a precursor of cholic acid, is a substrates of peroxisomal β-oxidation in the liver.[9] Because THCA has an asymmetric carbon at the 25 position, there are two stereoisomers, such as 25*R* form and 25*S* form. However, stereospecificity of the process of cholic acid biosynthesis

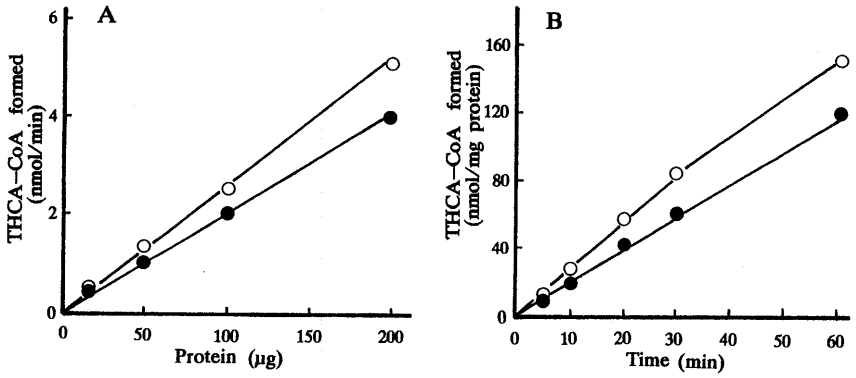

Fig. 1. Protein- and Time-Dependent Formation of THCA-CoA from (25*R*)- and (25*S*)-THCA by Rat Liver Homogenate

A: Protein-dependent formation. B: Time-dependent formation. ●, (25*R*)-THCA; ○, (25*S*)-THCA.

TABLE I. Hepatic Capacity of THCA-CoA Formation in Normal and CPIB-Treated Rats

Treatment	Activity		
	(25*R*)-THCA-CoA	(25*S*)-THCA-CoA	Palmitoyl-CoA
Control	416±24	600±24[b]	10543±855
CPIB	584±20 (1.40)[a]	704±24 (1.17)[a,b]	23224±1348 (2.20)[a]

Activity was assayed using liver homogenate. Results are expressed as means ± S.D. of 3 experiments. Statistical significance: *a*) $p<0.05$ *vs.* control; *b*) $p<0.05$ *vs.* (25*R*)-THCA-CoA. Activity: nmol/min/g liver. Numbers in parentheses represent the relative values to the control group.

from THCA through the peroxisomal β-oxidation system has not yet been established. We examined the stereospecificity of the enzymes related to cholic acid metabolism, such as acyl-CoA synthetase, acyl-CoA oxidase and acyl-CoA hydrolase. Figure 1 shows good correlation between protein amount (A) or reaction time (B) and the amount of THCA-CoA formed. The linearity of the relationship was observed in the range of 0—200 μg on protein amount or of 0—20 min on time. Table I shows the results of the determination of acyl-CoA synthetase activity in these ranges of protein amount and time. The activity of acyl-CoA synthetase toward 25*R*-THCA or 25*S*-THCA in the liver of control rat was 416 or 600 nmol/min/g liver, respectively. These activities were one twentieth and one eighteenth, respectively, compared with that obtained when palmitic acid was used as a substrate. CPIB-treatment induced the activities toward 25*R*- and 25*S*-THCA by 1.4- and 1.17-fold, respectively, with statistical significance. The activity toward palmitic acid was also induced by CPIB. Enzyme activities and protein contents in the 13 fractions collected from the sucrose gradient (1.265—1.105 g/ml) were indicated in Fig. 2. The distribution of the marker enzymes, catalase for peroxisomes, GDH for mitochondria and cyt c red. for microsomes, showed these subcellular particles mainly concentrated in fraction-3, 7 and 10, respectively. The activity of acyl-CoA synthetase for THCA coincided with cyt c red., showing the acyl-CoA synthetase activity for THCA located in microsomes. Furthermore, the activity for 25*S*-isomer was about 1.5-fold higher than that for 25*R*-isomer in the fractions. Furthermore, there was a significant difference in the distribution patterns of acyl-CoA synthetase activities for THCA and palmitic acid. Thus, activation of THCA to its CoA derivatives

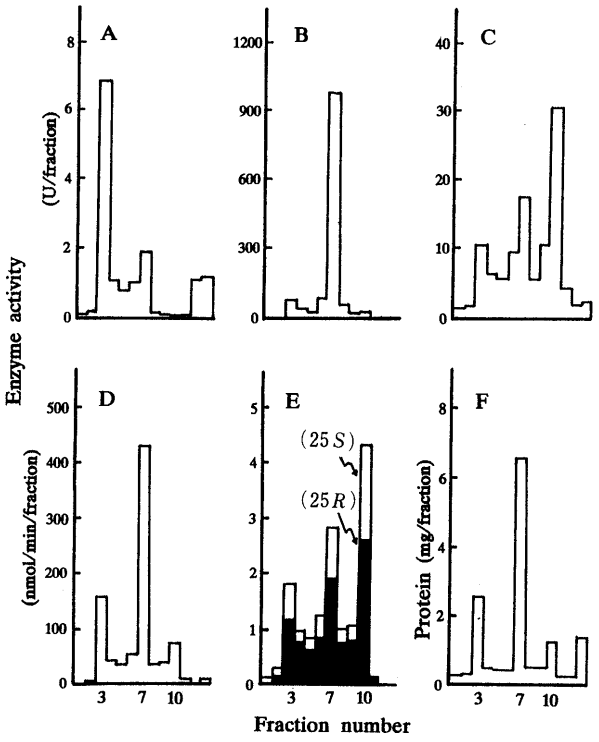

Fig. 2. Sucrose Density Gradient Centrifugation of LM Fraction from Rat Liver

A: Catalase. B: GDH. C: NADPH-cyt c red. D: Acyl-CoA synthetase (palmitate as a substrate). E: Acyl-CoA synthetase (THCA as a substrate). F: Protein.

is performed in microsomes, and then the THCA-CoA formed is transferred into peroxisomes and subjected to β-oxidation.

Table II shows the activity of peroxisomal fatty acyl-CoA oxidase for THCA. The activities for both 25*S* and 25*R*-isomers were 69 nmol/min/g liver and one thirteenth of that for palmitoyl-CoA, showing no stereoselectivity of fatty acyl CoA oxidizing system (FAO) reaction to THCA-CoA. Considering the facts that FAO is a rate-limiting enzyme of peroxisomal β-oxidation[29] and the activity of acyl-CoA synthetase for THCA was much higher than that of FAO for THCA-CoA, the difference in acyl-CoA synthetase activity for stereoisomers of THCA should be canceled in the overall reaction process of bile acid formation. A product of FAO reaction is *trans*-$^{24}\Delta$-THCA-CoA, subsequently converted to 24-OH-THCA-CoA with a configuration of 24*R* and 25*R* by enoyl-CoA

Table II. Hepatic Activity of Acyl-CoA Oxidase in Normal and CPIB-Treated Rats

Treatment	Activity		
	(25*R*)-THCA-CoA	(25*S*)-THCA-CoA	Palmitoyl-CoA
Control	69±3	69±3	904±123
CPIB	67±2 (0.97)	68±5 (0.99)	6574±533 (7.27)[a]

Activity was assayed using liver homogenate. Results are expressed as means±S.D. of 4 experiments. Statistical significance: *a*) $p<0.05$ *vs.* control. Activity: nmol/min/g liver. Numbers in parentheses represent the relative values to the control group.

Table III. Hepatic Activity of Acyl-CoA Hydrolase in Normal and CPIB-Treated Rats

Treatment	Activity		
	(25*R*)-THCA-CoA	(25*S*)-THCA-CoA	Palmitoyl-CoA
Control	120±17	88±7[b]	576±28
CPIB	276±2 (2.30)[a]	227±16 (2.58)[a,b]	4203±123 (7.30)[a]

Activity was assayed using liver homogenate. Results are expressed as means±S.D. of 3 experiments. Statistical significance: *a*) $p<0.05$ *vs.* control; *b*) $p<0.05$ *vs.* (25*R*)-THCA-CoA. Activity: nmol/min/g liver. Numbers in parentheses represent the relative values to the control group.

hydratase.[14] However, four stereoisomers of 24-OH-THCA produced by the configuration at 24- and 25-position were equally converted to cholic acid,[30] suggesting that there were no stereospecificities in the reactions of β-hydroxyacyl-CoA dehydrogenase and ketoacyl-CoA thiolase. Thus, 25*R*-THCA and 25*S*-THCA are equally converted to cholic acid *via* peroxisomal β-oxidation.

Surprisingly, although the FAO activity for palmitoyl-CoA was induced by CPIB, no induction in the activity for THCA-CoA was observed. This suggests that at least two distinct enzymes would be involved in the oxidation of palmitoyl-CoA and THCA-CoA, which are also supported by the finding of Miyazawa *et al.*[31] that on analysis of the messenger ribonucleic acid (mRNA) there are at least two isozymes of FAO in peroxisomes. Furthermore, the inducive-response of THCA-CoA synthetase to CPIB was less than that of palmitoyl-CoA synthetase, and the subcellular distribution of these two activities were different from each other. These facts show that enzymes other than those related to fatty acid oxidation in peroxisomes might be involved in bile acid synthesis in peroxisomes.

Table III shows acyl-CoA hydrolase activity in rat liver. The acivity for 25*R*-THCA was higher than that for 25*S*-THCA. Although CPIB induced the acyl-CoA hydrolase for both stereoisomers by 2.3—2.6 fold, this difference in the activities for both stereoisomers was still observed in CPIB-treated rat liver. Although acyl-CoA hydrolase has a role in the degradation of acyl-CoA to its free acid, the activity for THCA-CoA is about one fifth of the activity of acyl-CoA synthetase for THCA, indicating that the hydrolase may not have an affect on the supply of THCA-CoA into peroxisomes.

From these results it was concluded that there is no stereoselectivity in bile acid formation from THCA in hepatic peroxisomes, and that the characteristics of peroxisomes responsible for the metabolism of a wide variety of acyl-compounds was established again in terms of stereoselectivity.

In the process of cholic acid formation from cholesterol, stereoisomers can be produced in the step of hydroxylation of the methyl group at the postion of 27 or 26. However, in the biological system one of the stereoisomers may be produced primarily, and no other isomers may actually be produced. Our present study showed that the process after this hydroxylation reaction does not show stereoselectivity if both stereoisomers are artificially applied.

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