Lanosterol 14 α -Demethylase (Cytochrome P-450_{14DM}): Modulation of Its Enzyme Activity by Cholesterol Feeding

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Regulation of the activity of lanosterol 14 α -demethylase (cytochrome P-450_{14DM}) by dietary cholesterol was studied in rats. In male rats fed a 3% cholesterol diet for 1 and 4 weeks, the activity of 24,25-dihydrolanosterol 14 α -demethylase was decreased in the liver. The cytochrome P-450_{14DM} protein content detected by immunoblotting was also decreased by cholesterol feeding. These results demonstrate that dietary cholesterol acts as a repressive factor for lanosterol 14 α -demethylase. Further, the activity was enhanced by preincubation with phosphatase of the enzyme solution from both the control and cholesterol fed rats at the same rate. This result suggests that regulation of the activity involves phosphorylation of this enzyme.

Key words lanosterol 14 α -demethylase; cytochrome P-450_{14DM}; cholesterol; modulation; phosphorylation

Lanosterol 14 α -demethylase (cytochrome P-450_{14DM}) is a biosynthetic cytochrome P-450 (P-450) enzyme that catalyzes the first step in the conversion of lanosterol and 24,25-dihydrolanosterol (DHL) to cholesterol. Cytochrome P-450_{14DM} (P-450_{14DM}) removes the 14 α -methyl groups from lanosterol and DHL, and thus converts them to the corresponding 32-nor-14,15-unsaturated derivatives, 4,4-dimethylcholesta-8,14,24-trien-3 β -ol and 4,4-dimethylcholesta-8,14-dien-3 β -ol, respectively.¹⁾

Raucy *et al.*²⁾ reported the presence of this enzyme in human liver, lymphocytes, and kidney, suggesting that it may play a role in the cellular regulation of cholesterol synthesis. We purified this enzyme from human liver.³⁾ An intermediate in the lanosterol demethylation, 3 β -hydroxylanost-8-en-32-al, was reported to be a suppressor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme of cholesterol biosynthesis.⁴⁾ This suggests that P-450_{14DM} might play an important role in cholesterol biosynthesis. However, the role of this enzyme in cholesterol homeostasis (or biosynthesis regulation) is still unclear. Cholesterol balance is achieved through feedback regulation by cholesterol of several enzymes, acetoacetyl-CoA thiolase,⁵⁾ HMG-CoA synthase,⁶⁾ HMG-CoA reductase,⁷⁾ prenyl transferase,⁸⁾ squalene synthetase,⁹⁾ squalene epoxidase,¹⁰⁾ and of low-density lipoprotein (LDL) receptor.¹¹⁾ Recent genomic studies of the LDL receptor,¹²⁾ HMG-CoA synthase¹³⁾ and HMG-CoA reductase¹⁴⁾ have clarified the mechanisms of regulation by sterols. One mechanism is transcriptional regulation, and these genes contain the sterol regulatory element-1.

Furthermore, HMG-CoA reductase, the rate limiting step for cholesterol biosynthesis, is regulated by phosphorylation and dephosphorylation.¹⁵⁾

In the present study, we determined the contribution of P-450_{14DM} in cholesterol homeostasis. We found that a cholesterol-enriched diet in rats decreased P-450_{14DM} protein and reduced DHL 14 α -demethylase activity in the liver. Furthermore, we obtained evidence that the regulation of the enzyme activity might involve phosphoryla-

tion of this enzyme.

MATERIALS AND METHODS

Materials Cholesterol was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and recrystallized with methanol (purity >99%). Alkaline phosphatase (bacterial, type III) was purchased from Sigma (MO, U.S.A.). Nitrocellulose membrane was purchased from Schleicher and Schuell (Germany). Goat anti-rabbit IgG was obtained from E-Y Laboratories, Inc. (CA, U.S.A.). Rabbit peroxidase anti-peroxidase was obtained from Organon Teknika Corp. (NC, U.S.A.). All solvents and other substances used were of the highest quality commercially available. Anti-pig P-450_{14DM} was prepared as described previously.³⁾

Animals and Treatments Wistar male rats (Sankyo Laboratory, Tokyo, Japan) weighing 150–160 g were fed a cholesterol-enriched chow containing 3% (w/w) cholesterol for 1 and 4 weeks. Control rats were fed the chow without cholesterol.

Electrophoresis and Immunoblotting Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using 10% gels by the method of Laemmli.¹⁶⁾ Immunoblotting was performed as described previously.³⁾ Samples were subjected to SDS–PAGE and transferred to nitrocellulose membranes. The nitrocellulose membrane was incubated with a 1:1000 dilution of anti-pig P-450_{14DM}. Immunoreactive proteins were incubated with goat anti-rabbit IgG, followed by rabbit peroxidase anti-peroxidase, and were detected using a chemiluminescence reagent (Renaissance, Du Pont NEN) (MA, U.S.A.). To quantify P-450_{14DM} protein, the intensity of the bands in films was measured with a densitometer.

Enzyme Preparation and Assay of the DHL 14 α -Demethylase Activity Microsomes were prepared from rat livers by the method described previously.³⁾ The microsomes were solubilized with sodium cholate (0.6%) and the supernatant was then applied to an aminohexyl Sepharose 4B column (2 $\times$ 10 cm). P-450_{14DM} containing

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fractions were eluted from the column with potassium phosphate buffer (KPB) (10 mM, pH 7.4) containing 20% glycerol, 0.6% sodium cholate, 0.1 mM EDTA, 1 mM dithiothreitol (DTT) and 0.2% Emulgen 913 and was used as an enzyme preparation after the removal of Emulgen by Bio-bead. The activity of DHL 14 α -demethylase was determined as follows. 200 μ l of P-450_{14DM} containing fraction (210–220 μ g protein), 5 μ l of NADPH-P-450-reductase (1 unit) in 0.1 M KPB (pH 7.4), and 5 μ l of DHL (23 nmol) aqueous solution dispersed with dilauroylphosphatidyl choline (DLPC, 130 nmol) were mixed and sonicated, and 0.19 ml of 0.1 M KPB (pH 7.4) consisting of glucose 6-phosphate (final 40 mM), MgCl₂ (final 0.4 mM), and glucose 6-phosphate dehydrogenase (0.2 units) was added. The reaction was started by adding 0.1 ml of NADPH (final 2 mM) solution in 0.1 M KPB (pH 7.4), and the mixture was incubated at 37 °C under air. The reaction was stopped by adding 2 ml of 20% KOH and 1.5 ml of MeOH. Extraction and quantitation of the reaction product, 4,4-dimethylcholesta-8,14-dien-3 β -ol, was carried out as described previously.³⁾

Treatment of P-450_{14DM} by Phosphatase The P-450_{14DM}-containing fraction eluted from the aminohexyl Sepharose 4B column was applied to a Sephadex G-25 column (PD-10), then eluted with a buffer (50 mM Tris-HCl, 0.4 mM EDTA, 4 mM DTT, 20% glycerol, 0.2% Emulgen 913, pH 7.4) to remove the phosphate, which inhibits phosphatase activity. The eluted enzyme solution (200 μ l, 210–220 μ g protein), after the removal of Emulgen by Bio-bead, was mixed with 10 μ l of 50 mM Tris-HCl buffer (pH 7.4) (A buffer) containing phosphatase, and was preincubated at 37 °C for 30 min. In the control experiment, 10 μ l of A buffer without phosphatase was added. Five μ l of NADPH-P-450-reductase (1 unit) in A buffer and 5 μ l of DHL (23 nmol) aqueous solution dispersed with DLPC (130 nmol) were mixed and sonicated, and 0.18 ml of A buffer consisting of glucose 6-phosphate (final 40 mM), MgCl₂ (final 0.4 mM) and glucose 6-phosphate dehydrogenase (0.2 units) was added. The reaction was started by adding 0.1 ml of NADPH solution (final 2 mM) in A buffer, and the mixture was incubated at 37 °C under air and terminated by adding KOH-MeOH as mentioned above.

General Assays The P-450 content was determined as described by Omura and Sato.¹⁷⁾ Protein determination was performed according to the method of Lowry *et al.*¹⁸⁾ with serum bovine albumin as a standard.

RESULTS

Effects of Cholesterol Feeding on P-450_{14DM} The immunoblot shown in Fig. 1 demonstrates the effects of cholesterol feeding on hepatic microsomal P-450_{14DM}. Immunoblots resolved two immunoreactive proteins of 51 and 49 kDa. The upper (51-kDa) band is P-450_{14DM}, as described previously.¹⁹⁾ The cross-reacting 49-kDa protein may be the other P-450 isozyme sharing antigenicity with P-450_{14DM}. By cholesterol feeding, the upper band (P-450_{14DM} protein) was greatly decreased, whereas the lower band did not change.

Table 1 shows the effects of cholesterol feeding on DHL

Fig. 1. Immunoblot of Rat Liver Microsomal Proteins

Hepatic microsomes of rats were applied at a level of 5 μ g protein. Electrophoresis was performed and the proteins were transferred to a membrane and stained immunologically with anti-pig P-450_{14DM}. C, control; 1, 1 week cholesterol feeding; 4, 4 week cholesterol feeding.

Table 1. Effects of Cholesterol Feeding on DHL 14 α -Demethylase Activity and the P-450_{14DM} Content of Rat Liver

Treatment	DHL 14 α -demethylase activity (nmol/min/mg protein))	P-450 _{14DM} content
Control	0.176 $\pm$ 0.010	0.694 $\pm$ 0.039
1 week cholesterol	0.109 $\pm$ 0.021* (62% of control)	0.466 $\pm$ 0.022* (67% of control)
Control	0.173 $\pm$ 0.010	0.691 $\pm$ 0.033
4 weeks cholesterol	0.096 $\pm$ 0.019* (55% of control)	0.390 $\pm$ 0.025* (56% of control)

DHL 14 α -demethylase activity was measured as described in Materials and Methods. P-450_{14DM} content is presented as optical density $\times$ mm (peak area) from scanning densitometry after immunoblot analysis. Values represent the mean $\pm$ S.D. of four rats. * Values are significantly different from the corresponding controls ($p < 0.01$).

14 α -demethylase activity using the partially purified P-450_{14DM}. DHL 14 α -demethylase activity was decreased by 1 and 4 weeks cholesterol feeding. The rate of decrease in DHL 14 α -demethylase activity paralleled that in the P-450_{14DM} protein level. DHL is known to be converted to cholesterol when incubated with hepatic microsomes from rat.²⁰⁾ Consequently, to determine the amounts of the 14 α -demethylated metabolite of DHL produced by hepatic microsomes, the addition of two inhibitors of both 14-double bond reductase (*trans*-1,4-bis(2-chlorobenzyl-aminomethyl)cyclohexane dihydrochloride) and 4-methyl sterol oxidase (CN⁻) is necessary in the reaction mixture. As we did not have the former inhibitor, DHL 14 α -demethylase activity was not measured in the microsome fraction. To support the data of Table 1, we determined the conversion rate of DHL to cholesterol using the microsomes.²⁰⁾ The conversion rate of DHL to cholesterol in the microsomes of cholesterol fed rats was suppressed in a similar manner to the result of the experiment using the partially purified P-450_{14DM}.

Table 2 shows the effects of cholesterol feeding on NADPH-P-450 reductase activity and total P-450 content in the microsomes of rats. Cholesterol feeding did not change the NADPH-P-450 reductase activity, nor the total P-450 content.

To clarify the participation of phosphorylation and dephosphorylation in DHL 14 α -demethylase activity, the enzyme solution containing P-450_{14DM} was treated with phosphatase. Table 3 shows the effects of phosphatase treatment on DHL 14 α -demethylase activity. In the control rats, DHL 14 α -demethylase activity was enhanced by phosphatase treatment. In the cholesterol-fed rats,

Table 2. Effects of Cholesterol Feeding on NADPH-P-450 Reductase Activity and Total Content of P-450 in Rat Liver Microsomes

Treatment	NADPH-P-450 reductase (nmol/min/mg protein)	Total P-450 (nmol/mg protein)
Control	107 ± 12	0.56 ± 0.03
1 week cholesterol	126 ± 13 (118% of control)	0.46 ± 0.10 (82% of control)
Control	111 ± 12	0.58 ± 0.05
4 weeks cholesterol	120 ± 17 (108% of control)	0.54 ± 0.11 (93% of control)

Table 3. Effects of Phosphatase Treatment on P-450_{14DM}

	DHL 14 α -demethylase activity (nmol/min/mg)	
	– phosphatase	+ phosphatase ^{a)}
Control	0.150 ± 0.016 (100)	0.248 ± 0.030 (165)
1 week cholesterol	0.098 ± 0.012 ^{b)} (100)	0.164 ± 0.021 ^{c)} (167)
Control	0.155 ± 0.012 (100)	0.257 ± 0.021 (166)
4 weeks cholesterol	0.081 ± 0.011 ^{b)} (100)	0.130 ± 0.015 ^{c)} (160)

Rats were fed ordinary chow (control) or chow containing 3% cholesterol. The values for P-450_{14DM} activity are the mean ± S.D. of four rats. Values in parentheses are relative to that without phosphatase. a) 10 units of phosphatase were added. b) Significantly different from the control (without phosphatase) $p < 0.01$. c) Significantly different from the control (with phosphatase) $p < 0.01$.

the DHL 14 α -demethylase activity was also enhanced by phosphatase treatment. However, the activity of the cholesterol-fed rats was not enhanced to the control value (phosphatase treatment) by preincubation of the enzyme with phosphatase. Further, the DHL 14 α -demethylase activity of the purified rat P-450_{14DM} was stimulated by treatment with phosphatase in a reconstituted system containing NADPH-P-450-reductase (data not shown). The mobility of the purified rat P-450_{14DM} detected by immunoblotting was unchanged by treatment with phosphatase (data not shown). The lower band in Fig. 1 is not thought to be the phosphorylated P-450_{14DM}.

DISCUSSION

The present results showed that the cholesterol feeding to rats decreased the amount of immunodetected P-450_{14DM} protein. Cholesterol feeding decreased the DHL 14 α -demethylase activity, and the decrease is not dependent on NADPH-P-450 reductase activity. As shown in Table 2, reductase activity was not reduced in rats after 1 and 4 weeks cholesterol feedings. Also, the total P-450 content was not changed by 1 and 4 weeks cholesterol feeding. In the previous paper,²¹⁾ we reported that the conversion of lanosterol to cholesterol in rat hepatic subcellular preparations (S-10) was not inhibited markedly by the addition of cholesterol *in vitro*. These results suggest a repressive role of cholesterol in the regulation of P-450_{14DM} protein.

Further, the findings of the present study suggest that rat liver DHL 14 α -demethylase activity is regulated by phosphorylation and dephosphorylation. Dephosphoryla-

tion of the enzyme significantly increased the activity. The effect of dephosphorylation is similar to that of HMG-CoA reductase,¹⁵⁾ the rate-limiting enzyme for cholesterol biosynthesis. Cholesterol 7 α -hydroxylase,²²⁾ the rate-limiting enzyme for cholesterol metabolism, is also regulated by phosphorylation and dephosphorylation in the opposite direction to that of HMG-CoA reductase. We propose that these rate-limiting enzymes of cholesterol biosynthesis and metabolism and P-450_{14DM} are regulated in a coordinated manner. Cholesterol feeding had no effect on the phosphorylation of P-450_{14DM} under these conditions.

The present study suggests that cholesterol feeding influences P-450_{14DM} by regulating its expression. To clarify the mechanism of the effect of cholesterol feeding on P-450_{14DM}, *i.e.* whether it is due to transcriptional or post-transcriptional regulation, further studies on mRNA levels and protein synthesis and degradation must be done. Recently, a partial amino acid sequence of rat P-450_{14DM} was reported by Aoyama *et al.*²³⁾

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