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Cholesterol lowering effects of a choleretic phloracetophenone in hypercholesterolemic hamsters

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Abstract

The plasma cholesterol-lowering effect and mechanism thereof of a choleretic phloracetophenone or 2,4,6-trihydroxyacetophenone (THA) were investigated in hypercholesterolemic male hamsters. Intragastric administration of THA (300–600 μmol/kg) twice a day for 7 days to these animals caused a dose- and time-dependent decrease in both plasma cholesterol and triglyceride levels. THA at a dose of 400 μmol/kg reduced the cholesterol and triglyceride levels in plasma to 52% and 25% of the level in corresponding cholesterol-fed controls, respectively, with decreases in both plasma very low density lipoprotein and low density lipoprotein cholesterol but not in high density lipoprotein cholesterol. THA did not significantly alter total hepatic cholesterol content but significantly increased the excretion of both bile acids and cholesterol into the intestinal lumen for elimination. Corresponding to the increase in bile acid excretion, THA caused a seven-fold increase in hepatic cholesterol 7α-hydroxylase activity. These results suggest that THA exerts its cholesterol lowering effect by increasing hepatic cholesterol 7α-hydroxylase activity which increases hepatic conversion of cholesterol to bile acid for disposal via biliary secretion. This compound may have a potential for future development as a therapeutic agent for lowering lipids in hypercholesterolemic patients.

Keywords: Bile acid; Cholesterol; Cholesterol 7α-hydroxylase; (*Curcuma comosa*); Phloracetophenone

1. Introduction

Atherosclerotic vascular disease, particularly coronary heart disease, is known to be one of the leading causes of morbidity and mortality worldwide. A direct relationship between an elevated concentration of low density lipoprotein (LDL) cholesterol in plasma and development and progression of coronary heart disease has been reported (Castelli et al., 1992; Gould et al., 1998). A reduction of plasma LDL-cholesterol levels has been suggested to be an important means for prevention of coronary heart disease development (Gould et al., 1998).

Liver is a major organ which plays a central role in regulating plasma lipoprotein and cholesterol concentrations. It is capable of excreting significant amount of cholesterol which occurs either by direct excretion of cholesterol or by indirect excretion after conversion of cholesterol to bile acids. The hepatic conversion of cholesterol to bile acids and further elimination from the body are the key events for regulation of plasma cholesterol. This conversion is catalysed by hepatic cholesterol 7α-hydroxylase, the first and rate-limiting step in the bile acid biosynthetic pathway (Myant and Mitropoulous, 1977; Russell and Setchell, 1992). Although the details of the control mechanisms of the synthesis of bile acid and the activity of cholesterol 7α-hydroxylase are not clear, they are known to be under the negative feedback control of bile acids returning to the liver via the enterohepatic circulation (Spady et al., 1996; Heuman et al., 1988). An absence of the enterohepatic circulation of bile acids in bile-diverted rats has been demonstrated to activate hepatic cholesterol 7α-hydroxylase activity and leads to an increase in the synthesis of bile acids. Intraduodenal administration of bile acids

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could prevent the effect (Heuman et al., 1988). Likewise, cholestyramine, a bile acid sequestrant, which binds bile acids in the intestinal lumen and limits their enterohepatic circulation increases cholesterol 7α-hydroxylase activity and increases the synthesis of bile acid (Sucking et al., 1991). Therefore, any intervention that activates hepatic cholesterol 7α-hydroxylase activity and enterohepatic excretion of bile acids could be one of the potential pharmacologic strategies to reduce the risk of hypercholesterolemia.

Phloracetophenone (2,4,6-trihydroxyacetophenone, THA), which is the aglycone part of a glucoside from *Curcuma comosa* (family Zingiberaceae), has recently been demonstrated to enhance biliary excretion of bile acids and subsequently decrease plasma cholesterol in rats (Suksamrarn et al., 1997; Piyachaturawat et al., 1998). An earlier investigation of choreletic activities of the compound and its hydroxy analogs showed that THA is the most effective compound in the series to increase biliary excretion of bile acids and lower plasma cholesterol (Piyachaturawat et al., 2000). However, at present the mechanism by which THA increases bile acid excretion and decreases plasma cholesterol is not known. It is possibly related to an increase in hepatic cholesterol 7α-hydroxylase activity as well as activation of a bile acid-dependent choleresis process for disposal of bile acids into feces. The present study was undertaken to examine the effect and, the mechanism thereof of THA for lowering plasma cholesterol, using hypercholesterolemic hamsters. This animal model has been widely used for studying cholesterol metabolism as it has several similarities to humans (Crestani et al., 1993; Horton et al., 1994).

2. Materials and methods

2.1. Animals and treatment

Adult male golden hamsters weighing 100–130 g were obtained from the Animals Center, Faculty of Science, Mahidol University (Bangkok, Thailand). They were housed in a room with a 12-h light–dark cycle, room temperature 29–32 °C and were given access to a standard commercial diet (C.P. Pokaphand, Bangkok, Thailand) and water ad libitum. Hypercholesterolemia was induced by maintaining the hamsters on the same commercial diet with daily supplemented feeding of a cholesterol suspension in corn oil corresponding to 2% cholesterol in the diet. The animals were used after 3 weeks when the plasma cholesterol level was higher than 300 mg%. These hypercholesterolemic animals were subjected to different treatments while being maintained on the cholesterol-feeding regimen. THA, dissolved in 10% dimethylsulfoxide in corn oil, was given intragastrically in a final volume of 0.5 ml at doses of 100–600 μmol/kg twice a day for 7 days. The normal diet control and cholesterol control animals received the same volume of solvent in an identical manner. For the time course study, animals were given an effective dose of the compound (400 μmol/kg) and killed at various periods (3–14 days) during the treatment. To follow the change in plasma cholesterol, all animals were fasted overnight and a blood sample was collected from the orbital sinus under light ether anesthesia. At the end of experiment, a blood sample of the overnight fasted animal was collected from the abdominal aorta under ether anesthesia. The plasma was separated and stored at −20 °C for further analysis of lipids and lipoproteins. The liver was immediately excised, weighed and kept for further analysis for triglyceride and cholesterol contents and activity of cholesterol 7α-hydroxylase. Intestinal content of the cecum was collected for determination of excretory cholesterol and bile acid concentrations. The studies were carried out in accordance with the International Guiding Principles for Biomedical Research involving Animals (CIOMS/WHO).

Triglyceride and cholesterol concentrations in liver and plasma were analyzed by using a colorimetric enzymatic method (McGowan et al., 1983; Allain et al., 1974). The lipoproteins in plasma were separated on a density gradient by preparative ultracentrifugation according to the method described by Havel et al. (1955). After centrifugation, the gradient was fractionated and the cholesterol content of each fraction was determined by an enzymatic method (Allain et al., 1974).

2.2. Preparation of liver homogenate and microsomes

The liver was homogenized in 4 volumes of an ice-cold medium containing 250 mM sucrose, 1 mM EDTA and 10 mM Tris–HCl pH 8.0 using a glass-Teflon homogenizer. The liver homogenate was divided into two portions: 3 volumes of the homogenate for preparation of liver microsomes and one volume for lipid extraction. The microsomal fraction was prepared according to the modified method of Hoeg et al. (1984). The homogenate was centrifuged at 12,000 × *g*, 4 °C for 50 min to remove the larger cellular debris. The supernatant was filtered through six layers of gauze and the filtrate was recentrifuged at 100,000 × *g*, 4 °C for 60 min to obtain the microsomal pellet. The pellet was resuspended in buffer containing 150 mM NaCl, 10 mM Tris–HCl pH 8.0 for analysis of cholesterol 7α-hydroxylase activity and endogenous cholesterol level. Total protein content of liver microsomes and homogenate was determined by the method of Lowry et al. (1951). Lipids were extracted by the method of Bligh and Dyer (1959).

2.3. Assay of cholesterol 7α-hydroxylase activity

Cholesterol 7α-hydroxylase activity was measured by using [^{14}C]cholesterol as the substrate (Princen et al., 1986). Conversion of exogenous [^{14}C]cholesterol into [^{14}C]7α-hydroxycholesterol was determined. The two were separated by thin layer chromatography on silica-gel plates, after

elution with a mixture of benzene/ethylacetate (2:3, V/V). The band corresponding to 7α-hydroxycholesterol was scraped off the plates and extracted three times with diethyl ether. The combined extract was dried and counted for radioactivity. Cholesterol 7α-hydroxylase activity was expressed as percent conversion of radioactivity from exogenous [^{14}C]cholesterol to 7α-hydroxycholesterol by the microsomal enzyme.

2.4. Determination of cholesterol concentration in liver and lipid in cecum

The lipids of liver homogenate, liver microsomes and dried cecal contents were extracted in chloroform/methanol [3:1, V/V] three times. The organic solvent in the extract was pooled and evaporated to dryness under N_2 and was redissolved with isopropanol. Cholesterol concentration was determined using an enzymatic assay method (Allain et al., 1974) and bile acids was determined according to the method of Turnberg and Mote (1969).

2.5. Chemicals

Phloracetophenone or 2,4,6-trihydroxyacetophenone (THA), cholesterol, β-nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, DL-dithiothreitol and 3α-hydroxysteroid dehydrogenase were purchased from the Sigma-Aldrich (St. Louis, MO, USA). [4-^{14}C]Cholesterol was purchased from Amersham (Illinois, USA). Enzymatic reagent kits for determination of total cholesterol and triglyceride were purchased from Bio-Medical Laboratory (BM-Lab, Bangkok, Thailand). All other chemicals and solvents were of analytical grade.

2.6. Statistical analysis

All data are expressed as means ± S.E.M. of individual values from the specified number of animals. The values obtained from different groups were compared using one-way analysis of variance followed by the Student–Neuman–Keul's test. Values of $P < 0.05$ were considered to indicate a statistically significant difference.

3. Results

3.1. Effect on plasma lipids and lipoproteins

Table 1 shows the effects of THA treatment in hamsters supplemented with cholesterol. The cholesterol-fed animals had a body weight comparable to that of normal diet controls. Their liver weights were slightly increased but not significantly different from those of normal diet controls. The plasma lipid levels, both cholesterol and triglycerides increased progressively over the cholesterol-feeding period. After 3 weeks, plasma cholesterol rose to approximately 300 mg% at which level the hypercholesterolemic animals received THA treatment for 7 days. THA treatment decreased both plasma cholesterol and triglyceride in a dose-related manner. THA at a dose of 400 μmol/kg twice a day for 7 days markedly reduced plasma cholesterol and triglyceride to approximately 52% and 25% of those of the corresponding cholesterol-fed control, respectively. THA exerted an effect countering the rise of plasma lipid in cholesterol-fed animals. A further increase in the dose of THA to 600 μmol/kg caused a further reduction of plasma lipids, bringing them close to control values.

The time course of THA treatment for lowering plasma lipids in the hypercholesterolemic animals was studied at a dose of 400 μmol/kg twice a day for 14 days as this dose resulted in approximately 50% reduction at day 7. As shown in Fig. 1, both plasma cholesterol and triglyceride decreased progressively over the period of THA treatment. The maximum effect was attained on day 10 of THA treatment. Prolonging the period of treatment to 14 days did not induce any further decrease of plasma lipids.

Since cholesterol feeding resulted in a marked increase in plasma cholesterol, the profile of plasma lipoprotein cholesterol was analysed in more detail, using gradient ultracentrifugation. The increase in cholesterol was found to occur in the VLDL and LDL fractions, whereas cholesterol

Table 1
Effect of THA on body weight, liver weight, plasma cholesterol and triglyceride levels

Parameters	Normal diet	Cholesterol-fed diet			
		THA (μmol/kg BW twice a day, 7 days)			
		0	300	400	600
Body weight (g)	87.8 ± 2.6	87.0 ± 5.0	86.0 ± 2.5	88.1 ± 4.2	86.0 ± 4.9
Liver weight (g/100 g BW)	3.3 ± 0.1	3.7 ± 0.2	3.7 ± 0.1	3.6 ± 0.2	3.9 ± 0.1
Plasma cholesterol (mg%)	150.9 ± 4.7	372.4 ± 22.6[a]	277.4 ± 16.0[b]	93.4 ± 13.4[b]	173.2 ± 15.5[b]
(% of control)		(100%)	(74.5%)	(52%)	(46.5%)
Plasma triglyceride (mg%)	181.0 ± 6.6	865.9 ± 158.2[a]	395.8 ± 41.5[b]	219.4 ± 32.0[b]	170.3 ± 32.7[b]
(% of control)		(100%)	(45%)	(25%)	(19.7%)

Values are means ± S.E.M. obtained from 10 hamsters; % of control represents the percentage of the value without THA.

[a] $P < 0.001$ significant difference from the normal diet.

[b] $P < 0.001$ significant difference from cholesterol-fed control.

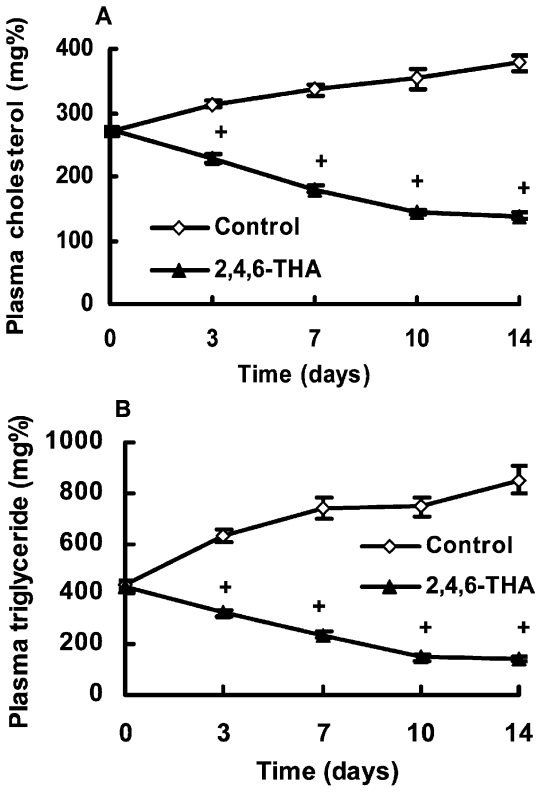

Fig. 1. Effect of THA treatment on concentrations of plasma cholesterol (A) and triglycerides (B) in cholesterol-fed male hamsters. THA was intragastically administered at a dose of 400 μmol/kg, twice a day and the animals were killed at varying periods after treatment (3–14 days). Each value represents the means ± S.E.M. from 10 hamsters. * $P<0.01$, significant difference from normal diet control.

in the HDL fraction was not significantly changed (Fig. 2). Treatment with THA 400 μmol/kg BW twice a day for 7 days significantly reduced the elevated cholesterol in the VLDL and LDL fractions ($P<0.01$) but had no significant effect on the HDL-cholesterol level.

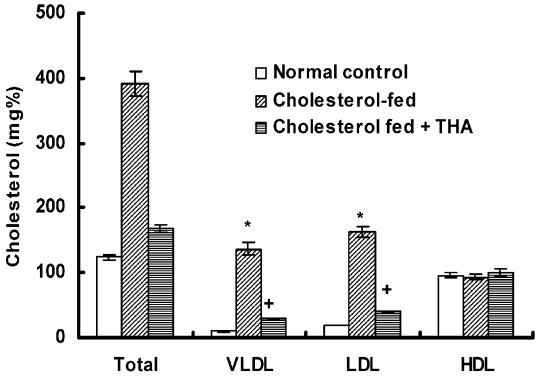

Fig. 2. Effect of THA treatment on cholesterol concentration of plasma lipoproteins in normal diet control, cholesterol-fed with and without THA treatment. Animals received THA treatment at a dose of 400 μmol/kg twice a day for 7 days. Each value represents the means ± S.E.M. from 10 hamsters.

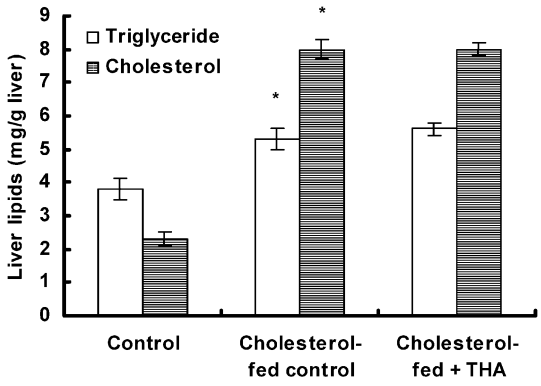

Fig. 3. Effect of THA treatment on hepatic cholesterol and triglycerides concentrations in normal diet control, cholesterol-fed with and without THA treatment. Animals received THA treatment at a dose of 400 μmol/kg twice a day for 7 days. Each value represents the means ± S.E.M. from seven hamsters. * $P<0.05$, significant difference from normal diet control.

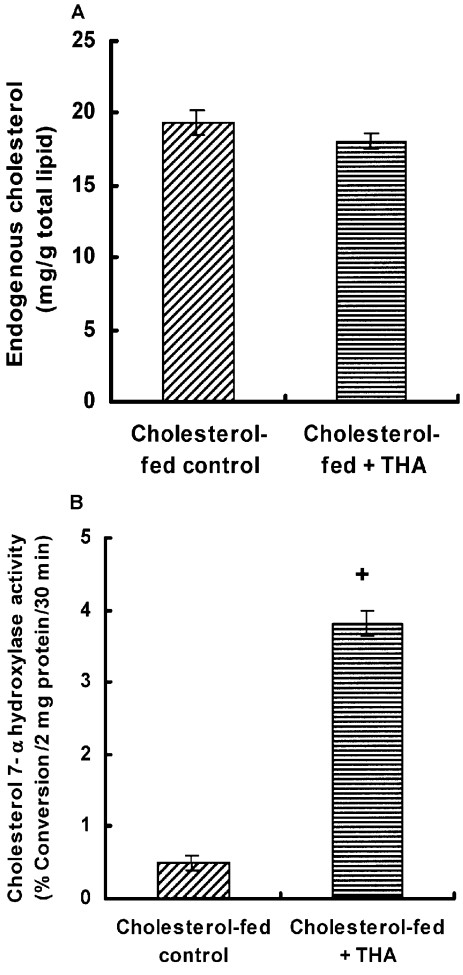

Fig. 4. Effect of THA treatment on (A) endogenous cholesterol content and (B) activity of cholesterol 7-α hydroxylase enzyme in liver microsomes. Animals received THA treatment at a dose of 400 μmol/kg twice a day for 7 days. Each value represents the means ± S.E.M. from seven hamsters. + $P<0.01$, significant difference from cholesterol-fed control.

3.2. Effect on liver lipids

Cholesterol feeding significantly increased liver contents of both triglyceride and cholesterol. The hepatic triglyceride and cholesterol contents were increased from 3.8 ± 0.3 and 2.3 ± 0.2 mg/g liver in normal diet to 5.3 ± 0.3 and 8.0 ± 0.3 mg/g liver, respectively, in the cholesterol-fed animals. THA at a dose of 400 μmol/kg twice a day for 7 days did not significantly affect triglyceride and cholesterol contents in liver. Total cholesterol content was maintained at 8.0 ± 0.2 mg/g liver (Fig. 3).

3.3. Effect on hepatic cholesterol 7α-hydroxylase activity and the excretion of lipids

Since THA has been reported to stimulate bile acid excretion (Piyachaturawat et al., 1998, 2000), we examined if the THA-induced decrease in plasma cholesterol was due to activation of hepatic cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis. Fig. 4 makes it evident that THA treatment had no significant effect on the amount of endogenous cholesterol in liver microsomes when compared to that in the cholesterol-fed control group. Despite the availability of a similar content of endogenous cholesterol substrate, the activity of cholesterol 7α-hydroxylase in the THA-treated group was seven-fold higher than that in the cholesterol-fed control.

To determine if there was any effect of THA on sterol elimination, the excretion of cholesterol and bile acids was estimated by determining their concentrations in cecal contents. As shown in Fig. 5, the cecal bile acid and cholesterol excretion was significantly higher in cholesterol-fed animals than in animals on a normal diet. THA treatment caused a further significant increase in bile acid excretion, as compared to that of the corresponding cholesterol-fed controls. A similar pattern of increased cecal cholesterol excretion was also observed with THA treatment.

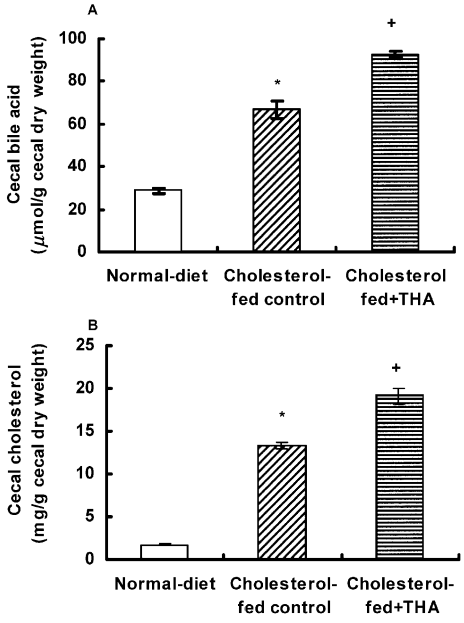

Fig. 5. Cecal excretion of bile acids (A) and cholesterol (B) in normal diet control, and cholesterol-fed with and without THA treatment. Animals received THA treatment at a dose of 400 μmol/kg twice a day for 7 days. Each value represents the means ± S.E.M. from seven hamsters. $^{*}P < 0.01$, significant difference from normal-diet control. $^{+}P < 0.01$, significant difference from cholesterol-fed control.

4. Discussion

The present study examined the effect and mechanism of THA to lower plasma cholesterol and triglycerides in hypercholesterolemic hamsters. THA treatment led to a significant dose-dependent decrease in both plasma cholesterol and triglycerides. The compound selectively reduced the elevated level of cholesterol in VLDL and LDL fractions whereas cholesterol in HDL remained unchanged. Total hepatic cholesterol content was also not affected. Although, the mechanism whereby THA lowered plasma lipid is still unknown, a number of possibilities should be considered. Lowering of plasma cholesterol might be associated with interference with the synthesis as well as secretion of lipoprotein into plasma and/or with acceleration of removal of the circulating cholesterol for excretion.

THA has previously been demonstrated, using a bile fistula, to effectively stimulate a bile acid-dependent choleresis and subsequently lead to lower plasma cholesterol in rats (Piyachaturawat et al., 1998, 2000). It is likely that an accelerating hepatic uptake of cholesterol for clearance might be a potential mechanism of THA to lower plasma cholesterol. THA might have a stimulatory effect on the conversion of cholesterol to bile acids. To investigate this possibility, the activity of cholesterol 7α-hydroxylase which catalyses the rate-limiting step in the bile acid synthesis was determined. Interestingly, in the present study, THA treatment caused a dramatic increase in the activity of hepatic cholesterol 7α-hydroxylase. Corresponding to the increase in cholesterol 7α-hydroxylase activity, excretion of cecal bile acids and cholesterol concentration were increased by THA treatment. The decrease in plasma cholesterol is determined by the output of cholesterol and bile acids in feces, which depends on the size and turnover rate of the hepatic bile acid pool. In the present study, the cecal concentration of cholesterol and bile acids was determined as evidence to support the increase in cholesterol 7α-hydroxylase activity by THA. It reflected a change in cecal content, not output. The reason for a marked decrease in plasma lipid with a modest increase in bile acid excretion observed in this study was not clear. It is possible that the size of the bile acid pool was altered with change in cholesterol feeding. The size of the bile acid pool in the male hamsters was reported to increase when they were fed a high amount of cholesterol and to contract when the hamsters were fed a high dose of cholestyramine (Turley

et al., 1997). Determination of the size and turnover rate of the hepatic bile acid pool after THA treatment might help to clarify this point.

The increase in cholesterol 7α-hydroxylase activity in the present study could have been caused by a direct effect of THA on cholesterol 7α-hydroxylase or an indirect effect of interfering with the feedback regulation by bile acids. Two possible mechanisms might account for the direct action of THA on the increase in cholesterol 7α-hydroxylase activity. In the short term, THA might directly increase phosphorylation or decrease dephosphorylation of the enzyme protein in which the enzyme is active in the phosphorylated state. Phosphorylation has been reported to occur within 30 min (Nguyen et al., 1996). THA action also peaked at a similar period (Piyachaturawat et al., 1998, 2000). However, it is also possible that prolonged treatment with THA could stimulate the transcription level and lead to increase mRNA coding for cholesterol 7α-hydroxylase. In addition, the increased hepatic cholesterol 7α-hydroxylase activity, promoting the elimination of bile acids might be an indirect effect of THA caused by interference with returning of bile acids to the liver via the enterohepatic circulation. In normal animals, most bile acids excreted into the small intestine are reabsorbed along the length of the small intestine, with the ileum being the major site of active transport (Lewis and Root, 1990). Less than 10% of bile acids are excreted in feces and this is generally replenished by hepatic synthesis from cholesterol. The return of bile acid to the liver acts as a feedback inhibitory signal to cholesterol 7α-hydroxylase enzyme by repression of gene transcription (Horton et al., 1994; Stange et al., 1998). In the present study, the increased loss of bile acids might have triggered an increase in bile acid synthesis. This could also occur by inhibition of uptake of bile acids by either hepatocytes or enterocytes or possibly via a non-systemic inhibition in the intestinal lumen, a mechanism exerted by bile acid sequestrants such as cholestyramine. Cholestyramine, which is an anion exchange resin binding to bile acid in the intestinal lumen, prevents the absorption of bile acid and increases in cholesterol 7α-hydroxylase enzyme activity (Sucking et al., 1991). However, THA is unlikely to have such a non-systemic action, since it is a small compound and is rapidly and readily absorbed after intraduodenal administration. Moreover, its choleretic action showed dose-dependence and reached a peak within 15–30 min (Piyachaturawat et al., 1998). Further study, with a comprehensive analysis of liver cholesterol 7α-hydroxylase content, expression, activity and phosphorylation state of the enzyme would help to clarify the mechanism of action of THA.

Although THA was rapidly absorbed into the circulation, it remains to be determined if THA affects the interaction between bile acids and their transporter. Currently, research on cellular membrane transporters for bile acids and lipid is being done to increase our understanding of the mechanism responsible for their uptake into the liver and intestinal cells. A variety of compounds have recently been developed and demonstrated to selectively inhibit the intestinal bile acid transport system (Ichihashi et al., 1998; Izzat et al., 2000). In the present study, it was not clear whether THA interfered with uptake of the returning bile acids, as a means of providing control of enzyme activity. Further detailed investigation of these interactions of THA remains to be done.

Regarding the effect of THA to lower plasma triglycerides, there are two possibilities. Firstly, the reduction of plasma VLDL might relate to a reduced synthesis as well as assembly of VLDL in liver. The increasing hepatic cholesterol 7α-hydroxylase activity in THA-treated hamsters which diverted the hepatic cholesterol pool toward the bile acid synthesis supports this contention. Secondly, THA might increase catabolism of VLDL by stimulating hepatic lipase and/or lipoprotein lipase activity. In addition, the increased hepatic cholesterol 7α-hydroxylase activity and the bile acid excretion by THA were also possibly related to an increase in hepatic LDL-receptors, which led to an increased clearance of plasma VLDL and LDL. Were these the case, cholesterol taken up via LDL receptors must be directed into a pool for biliary excretion rather than into a hepatic pool since THA did not alter the hepatic cholesterol content. Further study, to determine the activity of hepatic lipase, lipoprotein lipase and LDL receptor number in THA-treated animals would tell us by which pathway the compound might exert its lipid-lowering influence.

In conclusion, THA is an effective hypocholesterolemic agent for hamsters with diet-induced hypercholesterolemia. These effects are especially interesting as the effect is primarily associated with lowering of VLDL and LDL levels whereas HDL levels remained unchanged. THA exerts its action by increasing hepatic cholesterol 7α-hydroxylase activity and enhancing the excretion of bile acids. The modulation of cholesterol 7α-hydroxylase activity with an increase in bile acid-dependent choleresis and elimination from the body by THA might offer a new therapeutic means for lowering lipid in patients with hypercholesterolemia.

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