Hepatic acyl-coenzyme A:cholesterol acyltransferase activity is decreased in patients with cholesterol gallstones

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Abstract Altered hepatic cholesterol metabolism has been implicated in the etiology of cholesterol gallstones. This hypothesis has been examined by determining acyl-coenzyme A:cholesterol acyltransferase (ACAT) activity in liver biopsies from 31 cholesterol gallstone patients and 12 control subjects. Hepatic ACAT activity in gallstone patients was decreased to one-third that in controls ($P < 0.001$). No differences in hepatic homogenate or microsomal free and total cholesterol concentrations were observed between the two groups. However, marked increases in free (107%) and total (98%) cholesterol concentrations were found in the cytosolic fraction of liver biopsies from gallstone patients. The total phospholipid concentration of the liver homogenate fraction was unchanged in both groups; however, the microsomal total phospholipid concentration was reduced by 17% ($P < 0.01$) in gallstone samples compared with controls. This difference did not result in a significantly increased microsomal cholesterol/phospholipid ratio for the gallstone group (0.180 ± 0.030) compared with the control group (0.169 ± 0.042). These results show that hepatic ACAT activity is significantly decreased in cholesterol gallstone patients. These changes in ACAT activity in livers of patients with cholesterol gallstones are consistent with the known increase in the amount of free cholesterol secreted in the bile of these patients. Thus, the changes in ACAT activity may contribute to the pathogenesis of cholesterol gallstones. —**Smith, J. L., I. R. Hardie, S. P. Pillay, and J. de Jersey.** Hepatic acyl-coenzyme A:cholesterol acyltransferase activity is decreased in patients with cholesterol gallstones. *J. Lipid Res.* 1990. **31:** 1993-2000.

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In developed countries cholesterol gallstones are common and they constitute a major health problem (1). The pathogenesis of cholesterol gallstones is poorly understood; however, it is thought to be multifactorial, with no precise abnormality yet identified. An essential prerequisite for cholesterol gallstone formation is lithogenic bile, bile supersaturated with cholesterol compared with phospholipids and bile salts (2). The presence of nucleating factors in bile (3) has also been suggested. The mechanism by which lithogenic bile is produced in humans is not clear, but changes in hepatic cholesterol metabolism have been implicated (4–14). Factors that influence cholesterol homeostasis in liver include the rates of cholesterol synthesis, cholesterol esterification, conversion of cholesterol to bile acids, the rates of secretion of cholesterol into bile and plasma, the uptake of plasma lipoproteins, and the turnover of membrane cholesterol.

The activities of cholesterol 7α-hydroxylase (EC 1.14.13.7), the rate-limiting enzyme for bile acid synthesis, and 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (EC 1.1.1.34), a rate-limiting enzyme for cholesterol synthesis, in liver biopsies from cholesterol gallstone patients and controls have been determined in a number of studies (7–13). In five studies (7, 9, 10, 12, 13) HMG-CoA reductase activity was significantly increased and in one study (11) no change was observed. The activity of cholesterol 7α-hydroxylase was reported to be significantly decreased in all studies (8–11, 13). The concentrations of cholesterol in the homogenate (7, 9, 12) and microsomal (9, 11, 12) fractions of liver samples have also been determined for several groups of gallstone patients and controls with conflicting results.

Hepatic acyl-coenzyme A:cholesterol acyltransferase (ACAT) is responsible for the intracellular esterification of cholesterol. There have been two reports of its activity in cholesterol gallstone patients (14, 15), but apart from a preliminary study in our laboratory (14), no systematic comparison of hepatic ACAT activity has been made between gallstone patients and controls. In this paper, we report the activities of ACAT and the concentrations of cholesterol and total phospholipids in liver biopsies from 31 cholesterol gallstone patients and 12 control subjects.

Abbreviations: ACAT, acyl-coenzyme A:cholesterol acyltransferase; BMI, body mass index; CSI, cholesterol saturation index; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; LDL, low density lipoproteins; SD, standard deviation; VLDL, very low density lipoproteins.

MATERIALS AND METHODS

Details of subjects

The gallstone group consisted of 31 patients who were having elective cholecystectomy and liver biopsy for confirmed gallstones at the Royal Brisbane Hospital, Brisbane, Australia. The control group (nongallstone) was made up of 12 subjects who were undergoing liver resection for cancer (n = 4) or who were organ donors (n = 8). The four liver resection patients had either metastatic colonic cancer to the liver, hepatocellular carcinoma, hepatic cystadenoma, or cavernous haemangioma. The organ donors consisted of subjects who died as a result of either head injuries, cerebral tumor, meningioma, or hypoxic brain death. Clinical data for each group of subjects are in **Table 1**. The gallstone and control groups were similar except for a greater proportion of males in the control group. The fasting concentrations (mean ± 1 SD) of total cholesterol and triglycerides in plasma from the gallstone group (n = 23) were 5.63 ± 1.24 mM (range 4.10–8.70 mM) and 1.57 ± 0.62 mM (range 0.68–2.63 mM), respectively. Plasma lipid concentrations were not determined for the control subjects because of logistic difficulties and the problem of interpretation in the context of blood volume changes during resuscitation. Plasma lipids are also known to be altered in patients with tumors.

Liver samples

Liver samples were obtained with appropriate consent. For organ donors, consent for use of tissue for scientific purposes was obtained from the next of kin. For patients undergoing liver resection or cholecystectomy and liver biopsy, patient consent was obtained in accordance with a protocol approved by the hospital ethics committee.

Patients undergoing cholecystectomy and liver biopsy were fasted for a minimum of 8 h prior to blood collection and surgery. At operation, a wedge liver biopsy (approximately 0.7 g) was taken (usually from the left lobe) and placed into cold homogenizing buffer (14). Most operations occurred between 1:50 PM and 4:20 PM; three were between 9:00 AM and 10:00 AM (mean 2:04 PM). There was no correlation between ACAT activity and the time the liver biopsy was taken. The biopsy was transported on ice to the laboratory (about 10 min) where the solution was decanted and the biopsy was frozen at −70 °C. Liver samples were homogenized and fractions were prepared as described below.

TABLE 1. Details of subjects

	Control	Gallstone
Age (yr)	43.8 ± 19.0 (n = 12)	51.8 ± 16.6 (n = 31)
Male:female	6:6	7:24
Weight (kg)	63.8 ± 17.9 (n = 4)	67.5 ± 13.0 (n = 31)
BMI	ND	25.1 ± 4.0 (n = 24)

Results are given as the mean ± 1 standard deviation; ND, not determined; BMI, body mass index.

Control liver samples (usually 10–200 g; the majority from the right lobe) were taken between 2:00 AM and 10:37 PM (mean 12:56 PM) as previously described (16). There was no correlation between ACAT activity and the time the liver sample was taken. Liver samples were either treated the same as the gallstone samples as described above, rapidly frozen in liquid nitrogen, and stored at −70 °C, or cut into small pieces and placed in a freezer kept at −70 °C (16). Liver samples were homogenized and fractions were prepared as described below. These different treatments of the liver samples have been shown previously not to affect ACAT activity (14, 16).

All patients (gallstone and liver resection) received similar anesthetic agents, whereas the organ donors (16) received no anesthesia. Subjects from both groups who were found to have metabolic abnormalities or marked histological changes in the liver (e.g., severe fatty changes or cirrhosis) were excluded. Some subjects undergoing liver resection or who were organ donors were found to have gallstones and therefore were also excluded from the control group. Previously, we showed (16) that ACAT activity in liver from organ donors is similar to that in liver from liver resection cases. Thus, liver samples from organ donors are just as suitable as controls compared with samples from liver resection cases when ACAT activity is being compared.

Preparation of liver fractions

Liver homogenates and washed microsomes were prepared from frozen liver as described previously (17). After the sedimentation of microsomes, the remaining clear supernatant (cytosol) was carefully removed using a Pasteur pipette. All fractions were stored at −70 °C until used for protein or lipid analysis or for the measurement of ACAT activity. Some lipid extractions were performed on liver homogenates and microsomes before their storage at −70 °C. ACAT activity determined in the presence of exogenous cholesterol is unaffected by freezing of liver tissue before microsomal preparation, freezing of the microsomal fraction prepared from unfrozen tissue, or freezing both the tissue and the subsequent microsomal fraction (14–16). Although Einarsson et al. (15) reported that either freezing the liver tissue or the microsomal fraction stimulated ACAT activity when determined in the absence of exogenous cholesterol, we found no stimulation of ACAT activity by freezing (14, 16).

Assays

ACAT activity was determined in the absence and presence of exogenous cholesterol (6 μg cholesterol and 150 μg Tween 80 per assay) using the method previously described (14,

16). Endogenous cholesterol in the assay was about 2.2 μg. In previous work (14) we found that 6 μg of exogenous cholesterol resulted in maximal ACAT activity; higher concentrations were inhibitory (14). Free and total cholesterol concentrations in liver fractions were measured by the method of Salè et al. (18). Plasma total cholesterol concentrations were determined colorimetrically using an automated Boehringer Mannheim cholesterol method on a COBAS-BIO centrifugal analyzer (Roche) in the Biochemistry Department of the hospital. Total phospholipids were determined by the method of Hess and Derr (19), modified by substituting Triton X-100 for Sterox, and digesting at 170 °C for 70 min instead of at 190 °C for 25 min. A linear relationship was obtained between absorbance and the amount of a dipalmitoyl phosphatidylcholine standard in the range 1–5 nmol phosphorus. Protein was estimated by the method of Lowry et al. (20) using bovine albumin Cohn fraction V as the standard protein.

Statistical methods

Comparisons between test and control values were assessed by the Student's *t*-test. Correlations between ACAT activity and the various lipid concentrations and patient characteristics were determined by linear regression.

RESULTS

Hepatic ACAT activities

The mean ACAT activity of the gallstone patients was 36% ($P < 0.001$) and 41% ($P < 0.01$) of controls when ACAT activity was determined in the presence or absence of exogenous cholesterol, respectively (**Table 2**). The addition of exogenous cholesterol to the ACAT assay stimulated ACAT activity by 4.3 ± 2.1-fold (mean ± 1 SD; range 1.3–10.6) in gallstone samples. In control samples the corresponding stimulation factor was 6.0 ± 3.9 (n = 11; range 2.0–13.5). The difference between the effects of exogenous cholesterol on ACAT activity in gallstone and control samples was significant at $P < 0.001$.

For some control livers (n = 4) and gallstone livers (n = 8) ACAT activity per gram of liver tissue was determined. ACAT activity (mean ± 1 SD) determined in the presence of exogenous cholesterol for samples from gallstone patients was 437 ± 207 pmol·min^{-1}·g liver^{-1}, while the ACAT activity (mean ± 1 SD) of control samples was 1304 ± 327 pmol·min^{-1}·g liver^{-1}. This difference was statistically significant ($P < 0.001$). Similar results were found for ACAT activity determined in the absence of exogenous cholesterol (gallstone: 107 ± 46 pmol·min^{-1}·g liver^{-1}; control: 223 ± 99 pmol·min^{-1}·g liver^{-1}; $P < 0.05$). The total amount of microsomal protein recovered (mean ± 1 SD) for the two groups was 8.82 ± 2.76 mg·g liver^{-1} for controls and 7.01 ± 2.44 mg·g liver^{-1} for those from gallstone patients. These recoveries were not statistically significantly different. Therefore, ACAT activity in gallstone patients was also approximately one-third that in controls when expressed per gram of liver.

TABLE 2. Microsomal ACAT activity in liver biopsies from gallstone and control subjects

Sample	ACAT Activity	
	+ Cholesterol	− Cholesterol
	pmol·min^{-1}·mg protein^{-1} ± SD	
Control (12)	124 ± 35	27 ± 16 (11)
Gallstone (31)	45 ± 26	11 ± 6
P value	< 0.001	< 0.01

When ACAT activities (measured in the absence or presence of exogenous cholesterol) were compared in samples from different lobes of the liver from either controls or gallstones samples, no significant differences were found. For ACAT activity determined in the presence of exogenous cholesterol the results were: control, left lobe: 145 ± 37 pmol·min^{-1}·mg^{-1} (n = 4), right lobe: 121 ± 25 pmol·min^{-1}·mg^{-1} (n = 7); gallstone, left lobe: 58 ± 20 pmol·min^{-1}·mg^{-1} (n = 9), right lobe: 60 ± 17 pmol·min^{-1}·mg^{-1} (n = 6). A similar pattern of results was found for ACAT activity determined in the absence of exogenous cholesterol.

Hepatic cholesterol and total phospholipid concentrations

There were no significant differences in homogenate or microsomal free and total cholesterol concentrations between the control and gallstone groups (**Table 3**). However, compared with the controls, the gallstone samples showed a 107% ($P < 0.001$) and a 98% ($P < 0.001$) increase in cytosolic concentrations of free and total cholesterol respectively. Cytosolic cholesteryl ester concentrations were not significantly different between the two groups, although they tended to be higher in samples from patients with cholesterol gallstones.

There were no significant differences in total phospholipid concentrations in homogenate fractions from control and gallstone samples. However, the microsomal fraction of the gallstone group showed a 17% ($P < 0.01$) decrease compared with the control group. Phospholipids were not detected in the cytosolic fractions from either group. The cholesterol/phospholipid ratio for the microsomal samples was 0.180 ± 0.030 for the gallstone group (n = 24) and 0.169 ± 0.042 for the control group (n = 11). This difference was not statistically significant.

Correlations between ACAT activity and hepatic lipid concentrations

When ACAT activity in control samples was determined in the absence of exogenous cholesterol it was positively correlated with microsomal free cholesterol concentration (**Fig. 1A**; $P < 0.035$), with cytosolic free cholesterol con-

TABLE 3. Concentrations of cholesterol and phospholipids in various fractions of liver from gallstone and control subjects

	Homogenate			Microsomal			Cytosolic		
	TC	FC	PL	TC	FC	PL	TC	FC	CE
	nmol · mg protein^{-1} ± SD								
Control	41.8 ± 12.4 (n = 9)	40.9 ± 12.4 (n = 10)	183 ± 23 (n = 10)	95.2 ± 24.1 (n = 10)	93.9 ± 21.8 (n = 11)	563 ± 82 (n = 11)	5.80 ± 4.22 (n = 11)	3.87 ± 3.02 (n = 11)	2.37 ± 1.13 (n = 9)
Gallstone	45.3 ± 7.1 (n = 26)	40.8 ± 7.0 (n = 24)	188 ± 29 (n = 26)	90.8 ± 22.7 (n = 29)	89.5 ± 21.9 (n = 24)	469 ± 105 (n = 29)	11.46 ± 3.56 (n = 26)	8.02 ± 3.25 (n = 29)	3.59 ± 1.19 (n = 26)
P value	NS	NS	NS	NS	NS	< 0.01	< 0.001	< 0.001	NS

TC, total cholesterol; FC, free cholesterol; PL, total phospholipid; CE, cholesteryl esters; NS, not significant.

centration (**Fig. 1B**; $P < 0.04$), with cytosolic total cholesterol concentration ($P < 0.028$), with cytosolic cholesteryl ester concentration (Fig. 1D; $P < 0.028$), and with homogenate free cholesterol concentration ($P < 0.04$). However, when ACAT activity was determined in the presence of exogenous cholesterol, only a positive correlation with microsomal total phospholipid concentration (Fig. 1C; $P < 0.021$) was found.

In contrast, for samples from gallstone patients, ACAT activity determined in the absence of exogenous cholesterol was correlated negatively with cytosolic free cholesterol concentration ($P < 0.02$) and cytosolic total cholesterol concentration ($P < 0.043$). However, similar to control samples, ACAT activity was positively correlated with homogenate free cholesterol ($P < 0.05$). There was no apparent

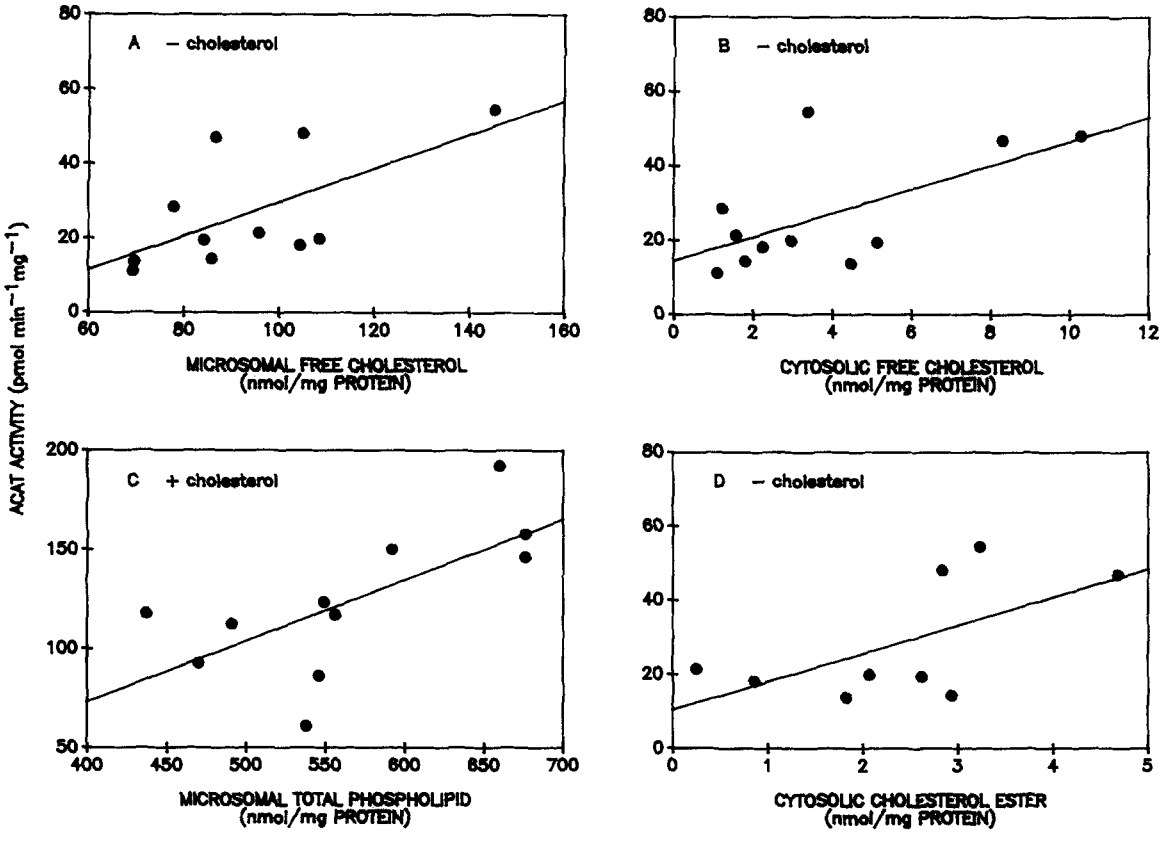

Fig. 1. Correlations between hepatic ACAT activity and hepatic lipid concentrations in control subjects. ACAT activity determined in the absence of exogenous cholesterol (– cholesterol) and presence of exogenous cholesterol (+ cholesterol). (A) Microsomal free cholesterol, n = 11, $r = 0.637$, $P < 0.035$, y = 0.45x – 15.8. (B) Cytosolic free cholesterol, n = 11, $r = 0.627$, $P < 0.04$, y = 3.22x + 14.4. (C) Microsomal total phospholipid, n = 11, $r = 0.682$, $P < 0.021$, y = 0.31x – 50.2. (D) Cytosolic cholesteryl ester, n = 9, $r = 0.613$, $P < 0.028$, y = 7.62x + 10.4.

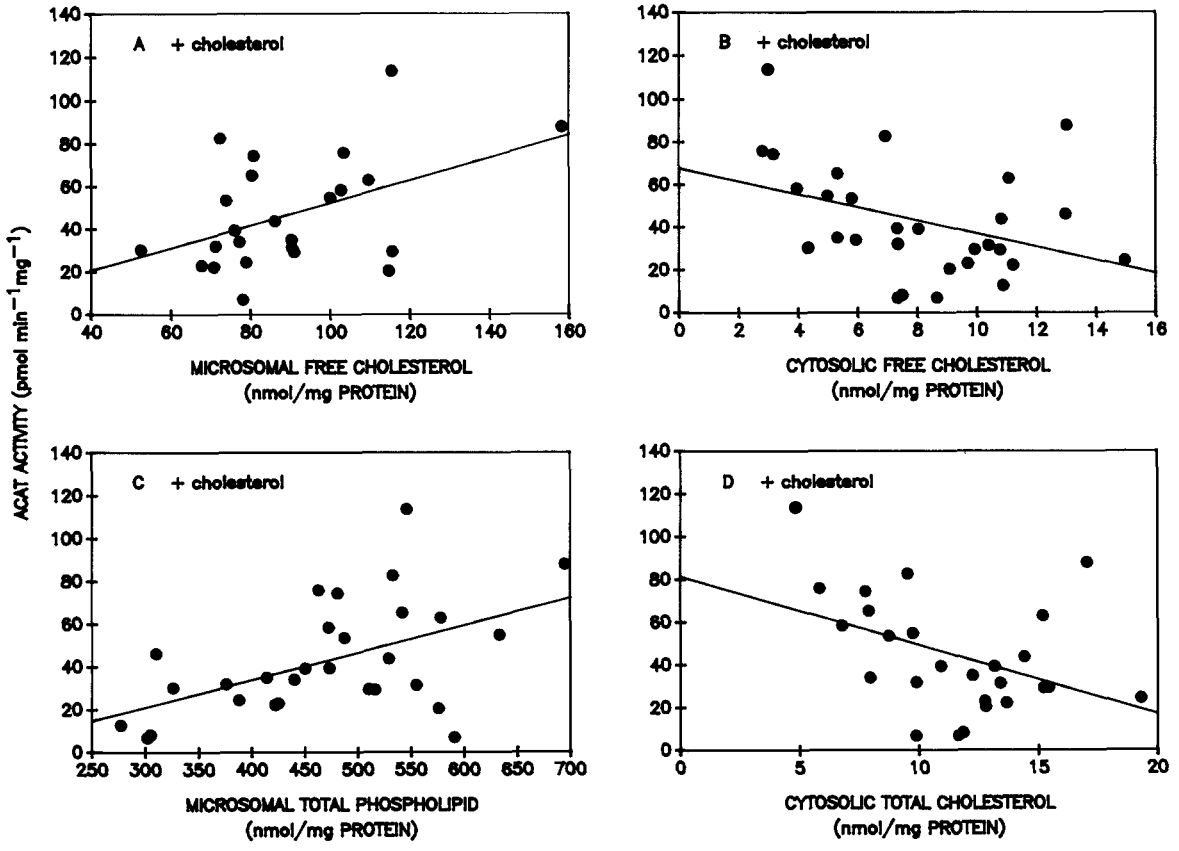

Fig. 2. Correlations between hepatic ACAT activity and hepatic lipid concentrations in gallstone patients. ACAT activity determined in the absence of exogenous cholesterol (− cholesterol) and presence of exogenous cholesterol (+ cholesterol). (A) Microsomal free cholesterol, n = 24, r = 0.454, $P < 0.026$, y = 0.53x − 0.63. (B) Cytosolic free cholesterol, n = 29, r = 0.381, $P < 0.041$, y = 67.6 − 3.09x. (C) Microsomal total phospholipid, n = 29, r = 0.508, $P < 0.005$, y = 0.13x − 17.1. (D) Cytosolic total cholesterol, n = 26, r = 0.424, $P < 0.031$, y = 81.3 − 3.22x.

correlation with microsomal free cholesterol or cytosolic cholesteryl ester concentrations.

When ACAT activity in samples from gallstone patients was determined in the presence of exogenous cholesterol, a positive correlation between ACAT activity and microsomal free cholesterol concentration (**Fig. 2A**; $P < 0.026$) was observed. Negative correlations between ACAT activity and cytosolic free ($P < 0.041$) and total ($P < 0.031$,) cholesterol concentrations (Figs. 2B and 2D) were found. Again, similar to the control samples a positive correlation was found between ACAT activity and microsomal total phospholipid concentration (Fig. 2C; $P < 0.005$).

Correlations between ACAT activity and patient characteristics

No significant correlation of ACAT activity (determined in the absence and presence of exogenous cholesterol) with age was found for the controls. Insufficient data on the subjects' weight and body mass index (BMI) did not allow meaningful correlations to be made on these variables. No statistically significant difference in ACAT activity was found between male and female control subjects.

Regression analyses of the variations of ACAT activity (determined in the absence and presence of exogenous cholesterol) with age, weight, BMI, and plasma total cholesterol concentrations for the gallstone patients showed no correlations. Also, as for controls, no statistically significant difference was found between male and female gallstone patients.

DISCUSSION

The present results demonstrate that there is a two- to three-fold decrease in hepatic ACAT activity in cholesterol gallstone patients (Table 2), confirming and extending our previously reported results on a small number of patients (14). This difference was apparent whether ACAT activity was determined in the absence or presence of exogenous cholesterol or whether expressed relative to microsomal protein (Table 2) or per gram liver. This lower ACAT activity and reduced stimulation of ACAT activity by exogenous cholesterol in samples from gallstone patients suggest that these samples may have had less active ACAT protein or less ACAT protein compared with control samples.

Comparison of hepatic ACAT activity and patient characteristics suggest that hepatic ACAT activity is independent of age, sex, weight, BMI, plasma total cholesterol concentration, and the site of the liver biopsy (either the left or the right lobe).

No changes in free or total cholesterol concentrations were observed in the homogenate or the microsomal fraction of the liver samples from gallstone patients and controls (Table 3). These findings are in agreement with one report (12), but not others (7, 9, 11). It should be noted that the number of subjects in the present study is larger than that in the other studies (7, 9, 12); in the study by Carulli et al. (11), only microsomal free cholesterol concentration was determined.

In the present study, liver cytosolic free and total cholesterol concentrations were increased in the gallstone samples compared with the control samples (Table 3). Cytosolic cholesterol concentrations have not previously been reported in liver from gallstone patients or controls. The difference suggests that this component may be of importance in the etiology of cholesterol gallstones and may reflect, in part, the enzymatic changes that occur in hepatic cholesterol metabolism of gallstone patients (7-13).

Reduced ACAT activity, as reported in this study, coupled to an increase in activity of HMG-CoA reductase and cholesterol synthesis and a decrease in the activity of cholesterol 7α-hydroxylase may lead to an increase in intracellular free cholesterol. The increased cytosolic cholesterol level found in the liver of gallstone samples may reflect this and be evidence of an altered precursor pool of free cholesterol destined for secretion into bile and/or plasma lipoproteins in these patients. Patients with cholesterol gallstones are well known to have an increased biliary output of cholesterol (21) and a positive correlation was reported between cholesterol saturation index (CSI) of gallbladder bile and plasma VLDL-cholesterol in cholesterol gallstone patients (22). A similar correlation between levels of plasma LDL (the major product of VLDL degradation) and bile CSI has been shown in normal subjects (23). The mechanism of direction of cholesterol into the biliary and lipoprotein pathways is not known; however, it may involve a cytosolic sterol carrier protein such as SCP_2 (24, 25).

Although a slight decrease in microsomal total phospholipid concentration was observed in liver samples from gallstone patients, no differences in the microsomal cholesterol/phospholipid ratio or in the microsomal cholesterol concentration were detected between gallstone and control samples. This may indicate that the lower ACAT activity in gallstone patients is not due to a net change in the microsomal lipid concentration or composition. However, since alterations in membrane lipid composition are reported to correlate with changes in ACAT and HMG-CoA reductase activities (26-31), further analyses of the microsomal lipids are required to exclude this possibility. However, ACAT activities can vary with microsomal lipid concentration within both the control and gallstone patient groups (Figs. 1 and 2).

Based on our data, a more likely explanation for the reduced hepatic ACAT activity observed in gallstone patients is that a lower amount of enzyme is present compared with controls. This notion is supported by our finding that hepatic ACAT activity is reduced in gallstone patients compared with controls regardless of whether the enzyme is assayed with exogenous cholesterol under apparent V_{max} conditions or whether the activity is assayed using the endogenous cholesterol as substrate.

Animal studies have shown that changes in hepatic cholesterol metabolism can result in a change in biliary cholesterol concentration or a change in the origin of biliary cholesterol. Chronic administration of progesterone (an inhibitor of ACAT) increased biliary cholesterol secretion in the rat (5). Turley and Dietschy (32) found that the origin of biliary cholesterol could be altered by stimulating or decreasing cholesterol synthesis in rats. Stone et al. (4) found that acute changes in rat hepatic enzyme activities produced acute changes in biliary cholesterol concentration and/or in the origin of biliary cholesterol. The relevance of these animal models to humans is unclear; however, they do show that the net effect of alterations of hepatic enzymes of cholesterol metabolism may be to alter secretion of biliary cholesterol.

The difficulty of in vivo manipulations in humans leaves uncertainty about the relationship between hepatic cholesterol metabolism and regulation of biliary cholesterol secretion in man. Maton, Reuben, and Dowling (33) found no correlation between HMG-CoA reductase activity and cholesterol secretion, whereas Key et al. (13) found a direct correlation between HMG-CoA reductase activity and cholesterol secretion. Recently, Duane et al. (6) demonstrated in humans that treatment with simvastatin, an inhibitor of HMG-CoA reductase, resulted in 24% reduction in the biliary cholesterol saturation index of gallbladder bile. Additional studies in humans are required to elucidate the roles of enzymes of hepatic cholesterol metabolism in regulating biliary cholesterol secretion.

This study demonstrates that hepatic ACAT activity is significantly decreased in cholesterol gallstone patients. This decrease in ACAT activity, together with earlier reports of increases in hepatic HMG-CoA reductase and decreases in cholesterol 7α-hydroxylase activities in gallstone patients, suggests that the net effect of these changes is to increase the availability of free cholesterol within the hepatocyte for efflux into the bile. These results further support the hypothesis that an altered hepatic cholesterol metabolism is involved in the etiology of cholesterol gallstones.

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