CETP and LCAT Activities are Unrelated to Smoking and Moderate Alcohol Consumption in Healthy Normolipidemic Men

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To distinguish between the effects of smoking and drinking on lipid metabolism, we conducted a cross-sectional study using 52 healthy normolipidemic subjects who either smoked or drank, but not both. The subjects were divided into three groups: Group S, smokers who did not drink ($n=20$); Group D, drinkers who did not smoke ($n=12$); and Group C, controls ($n=20$), who neither drank nor smoked. High density lipoprotein cholesterol (HDL-C) levels and plasma cholesteryl ester transfer protein (CETP) and lecithin:cholesterol acyltransferase (LCAT) activities were measured in all of the subjects, and the values obtained in Group S and Group D were compared to those of controls. Group S had lower ($p<0.01$) HDL₂-C and HDL₃-C levels, and Group D had higher ($p<0.05$) HDL₂-C and HDL₃-C levels than the controls, but there were no significant differences between groups with respect to CETP and LCAT activities. Thus, in healthy normolipidemic men, both smoking and drinking affect HDL-C levels as expected, but do not affect plasma CETP or LCAT activity levels.

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SMOKING and drinking habits have a remarkable effect on lipid metabolism. Several epidemiological studies have made it clear that cigarette smoking is associated with a depression in high density lipoprotein cholesterol (HDL-C) and a reduction in HDL protein components, ie, apolipoprotein A-I (apoA-I) and A-II (apoA-II),^{1–6} while alcohol is associated with an increase in HDL-C, apoA-I, and apoA-II.^{7–14} However, the mechanisms by which cigarette smoking and alcohol intake modify HDL-C metabolism are unknown. Some authors have suggested that these effects are mediated by plasma cholesteryl ester transfer protein (CETP) or lecithin:cholesterol acyltransfer-

ase (LCAT). Dullaart et al¹⁵ reported that cigarette smoking elevated plasma CETP activity while Rönnekaa et al¹⁶ reported that cigarette smoking reduced LCAT activity. Hannuksela et al¹⁷ indicated that alcohol intake sometimes reduces plasma CETP activity and Haffner et al¹⁸ found that alcohol intake elevates LCAT activity. However, the findings in these studies must be interpreted with caution for several reasons: ie, the possible overlap of drinking and smoking habits in many subjects, the fact that several epidemiological measurements were not taken simultaneously, and differences in lipid levels in the subjects. Further, the facts that heavy alcohol consumption often leads to liver dysfunction and thereby reduces the secretion of protein or enzyme mass, and that both heavy smoking and drinking habits affect caloric intake patterns and alter HDL-C levels, cannot be ignored.

Key words:

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Finally, endogenous rather than more precise exogenous measures were used to gauge LCAT activities.

The purpose of the present study is was to reassess the effects of smoking and alcohol consumption on CETP and LCAT levels to determine if these factors are involved in mediating HDL-C levels. In the present investigation, every attempt was made to standardize subjects as much as possible to avoid the problems encountered in earlier studies. In addition, all measurements were taken simultaneously.

METHODS

Subjects

The subjects in this study consisted of 52 normolipidemic adult males chosen from a pool of healthy 30–55-year-old members of the Japanese Ground Self Defense Force. Fasting total cholesterol (TC) and triglyceride (TG) levels in each subject were less than 220 mg/dl and 150 mg/dl, respectively. None had a history of metabolic disease or apparent liver dysfunction, such as elevated serum transaminases, leucine amino peptidase (LAP), or total bilirubin. Finally, none of the subjects had a history of both heavy smoking and drinking.

The subjects were divided into 3 groups, as follows: Group S, 20 non-alcohol-consuming individuals who had a history of smoking at least 20 cigarettes per day; Group D, 12 non-smokers who had a history of consuming a moderate amount (30 g to 100 g) of alcohol per day; and controls, Group C, 20 individuals who neither drank nor smoked. Measures of plasma lipoprotein, CETP and LCAT were obtained from all of the subjects and the mean values in each group were compared to those of the controls.

Laboratory Determination

Fasting blood samples from each subject were drawn into tubes containing the anticoagulant EDTA (1 g/L), and the plasma was immediately separated by centrifugation at 2500 g for 20 min. Plasma samples were frozen at -30°C and analyzed within 4 weeks. Lipoprotein fractions (VLDL, $d < 1.006$; LDL, $1.006 < d < 1.063$; HDL₂, $1.063 < d < 1.125$; and HDL₃, $1.125 < d < 1.21$) were separated by sequential ultracentrifuga-

tion at 40,000 rpm in a Hitachi 65 Ti rotor using a Hitachi SCH-55, as described by Havel et al.²¹ Cholesterol and triglyceride levels were assayed enzymatically. ApoA-I, A-II, B, C-II, C-III, and E were measured by immunoturbidimetry using commercially available kits.

Assay of Plasma CETP Activity

The activity of CETP was determined by a modification of the method of Albers et al.²² [1, 2-³H]-Cholesterol ester (CE)-labeled HDL was prepared by collecting the $d > 1.21$ g/ml fraction using a sequential ultracentrifugation method after incubating [1, 2-³H]-cholesterol (1.86 TBq/mmol, New England Nuclear, Boston, MA) and the $d > 1.125$ g/ml fraction (0.15 mol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, 0.03% NNA₃ pH 7.4; Buffer A) at 37°C for 24 h. The final cholesterol concentration of this fraction was 100 mg/dl. LDL solution was prepared by taking the 1.006–1.063 g/ml fraction out of the eluate from a dextran cellulose column used for LDL apheresis in a homozygote for familial hypercholesterolemia (FH). The final cholesterol concentration of this fraction was 1000 mg/dl. Plasma (40 μl) from each subject, 200 ml of [1, 2-³H]-cholesterol ester-labeled HDL and 200 ml of LDL solution were incubated at 37°C for 10 h. The radioactivity of CE transferred from HDL to LDL was counted. CETP activity was calculated as $[(b-s)/b \times 100\% / 10 \text{ h} / 40 \text{ ml plasma}]$, where 'b' is the radioactivity of a blank buffer using 0.1% bovine serum albumin/Buffer A, and 's' is that of a sample. All assays were carried out in duplicate.

Assay of Plasma LCAT Activity

Apolipoprotein A-I (apo A-I) was purified according to the method described by Brewer et al.²³ LCAT activity was measured by a modification of the method of Chen and Albers.¹⁹ Egg yolk lecithin (39 mg), 3.7 mg of unlabeled cholesterol and 400 ml of [1, 2-³H]-cholesterol (1.86 TBq/mmol, New England Nuclear, Boston, MA) was mixed and evaporated under a nitrogen stream. Purified apo A-I (1.4 mg) and 600 mg of sodium cholate were dissolved in 3.5 ml of assay buffer (0.15 mol/L NaCl, 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.4) and added to the dried lipids. After incubation at 24°C

TABLE I MEAN LEVELS OF MEASURES OBTAINED FROM THE SMOKING (GROUP S) AND DRINKING (GROUP D) GROUPS, AS COMPARED TO THOSE OF THE CONTROLS (GROUP C)

Measure	Group S Smokers	Group C Controls	Group D Drinkers
N	20	20	12
Age (years)	45.1 ± 4.7	45.0 ± 5.5	44.5 ± 3.8
BMI (kg/dl)	23.8 ± 2.8	24.8 ± 2.7	23.1 ± 2.2
TC (mg/dl)	157.5 ± 35.9	156.1 ± 20.4	157.8 ± 20.8
TG (mg/dl)	96.9 ± 32.8	91.5 ± 19.6	87.0 ± 15.2
HDL-C (mg/dl)	33.4 ± 7.4**	48.6 ± 5.6	56.2 ± 13.9
Plasma apolipoproteins			
Apo A-I (mg/dl)	108.1 ± 25.7**	125.3 ± 18.6	138.4 ± 17.7*
Apo A-II (mg/dl)	40.3 ± 8.6	43.6 ± 7.3	47.4 ± 6.6
Apo B (mg/dl)	99.3 ± 23.0	100.8 ± 15.5	98.1 ± 16.0
Apo C-II (mg/dl)	4.1 ± 2.3	3.4 ± 1.8	3.5 ± 1.3
Apo C-III (mg/dl)	10.3 ± 6.3	8.3 ± 3.6	8.5 ± 3.0
Apo E (mg/dl)	4.8 ± 2.0	4.8 ± 1.7	5.2 ± 2.1
HDL subfractions			
HDL ₂ -C (mg/dl)	17.9 ± 5.4**	23.2 ± 9.2	26.6 ± 5.9*
HDL ₂ -TG (mg/dl)	8.7 ± 3.3	7.2 ± 3.3	9.5 ± 2.2
HDL ₃ -C (mg/dl)	15.5 ± 4.9**	25.4 ± 3.4	29.6 ± 3.2*
HDL ₃ -TG (mg/dl)	4.9 ± 2.2*	7.5 ± 3.5	8.5 ± 3.2
CEPT Activity (%)	16.1 ± 5.0	15.7 ± 4.6	15.4 ± 3.4
LCAT Activity (%)	0.57 ± 0.28	0.54 ± 0.37	0.56 ± 0.29

Abbreviations: BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein cholesterol; apo, apolipoprotein; CETP, cholesteryl ester transfer protein; LCAT, lecithin cholesterol acyltransferase. *, $p \leq 0.05$; **, $p \leq 0.01$

for 20 min with shaking, the mixture was vigorously dialyzed against the assay buffer. The mixture was then adjusted to 4 ml with assay buffer and this liposome substrate was used within 2 days. Liposome (100 ml), 125 ml of 2% human serum albumin and 235 ml of assay buffer were mixed and incubated at 37°C for 15 min, and then 25 ml of mercaptoethanol and 15 ml of plasma were added to the substrate to initiate the reaction. After incubation at 37°C for 15 h, the mixtures were moved to ice water and 2 ml of pre-chilled methanol was immediately added to stop the reaction. After 2 ml of chloroform and 2 ml of distilled water were added, the mixtures were centrifuged at 2,000 rpm for 20 min at 4°C, and the infranats were collected and dried under a nitrogen stream. Dried lipid samples were resolved in 100 ml of hexane and applied to thin layer chromatographs using petroleum ether: ethyl acetate (85:15, v/v) as a solvent system. The portions that corresponded to free cholesterol (FC) and CE were collected by scraping and the radioactivity was counted in a liquid

scintillation counter (Aloka 3500). LCAT activity was expressed as a fractional activity, ie, $[a/(a+b) \times 100\%/h]$, where 'a' and 'b' are the radioactivities of CE and FC, respectively. LCAT activity was measured in duplicate.

Statistical Analyses

Data were expressed as the mean ± standard deviation. Non-paired Student's t-tests were used to compare mean values in Groups S and D to those in the controls (Group C). Correlations were assayed by Pearson's product-moment correlation coefficient. Differences were considered significant if $p < 0.05$.

RESULTS

Plasma Lipid Levels

There was no significant difference between Groups S and D and the controls with respect to the basic characteristics of the subjects, such as age, body mass index (BMI; body weight/height² (kg/m²)), plasma

total cholesterol level and triglyceride levels. However, as expected, the HDL-C level in Group S was significantly ($P \leq 0.01$) lower than that in Group C (Table I).

Plasma Apolipoprotein (apo) Levels

The mean apo A-I level in the controls (Group C) was significantly ($p \leq 0.01$) higher than that in Group S and significantly lower ($p \leq 0.05$) than that in Group D (Table I). There were no significant differences with respect to apo A-II, B, C-II, C-III, or E.

Correlation Between HDL Cholesterol Levels and Age, BMI, CETP or LCAT

As expected, HDL-C levels and BMI measures were significantly and negatively correlated ($r = -0.59$, $p < 0.05$) in the controls (Group C). On the other hand, there was no significant correlation between HDL-C levels and any of the other measures (age, BMI, CETP and LCAT) within the 3 groups.

HDL Subfractions

Mean HDL₂-C and HDL₃-C levels in the control group were significantly ($p < 0.01$) higher than those in Group S and significantly lower ($p < 0.05$) than those in Group D. The mean HDL₃-TG level in Group S was significantly ($p < 0.05$) lower than that in Group C (Table I).

Plasma CETP Activity and LCAT Activity

Neither Group S nor Group D differed significantly from Group C with respect to plasma CETP or LCAT levels (Table I).

DISCUSSION

Hypercholesterolemia and hypertriglyceridemia affect atherogenesis, as do low levels of HDL-C. Therefore, it is important to clarify the mechanisms by which HDL-C metabolism is regulated. Plasma HDL-C metabolism is generally controlled by the synthesis rates of the protein components of HDL (apo A-I, A-II, and other apoproteins), lipoprotein lipase (LPL), hepatic lipase (HL), CETP, and LCAT. Cigarette smoking and alcohol ingestion habits are known to affect HDL-C levels without changing any other serum lipid levels. Therefore, we decided to reexamine the

relationship between CETP and LCAT levels and smoking and alcohol consumption habits.

Our data indicate that smoking and drinking affect HDL-C levels, as expected, but have no detectable effect on CETP or LCAT activity in normocholesterolemic and normotriglyceridemic subjects. These findings conflict with those of Dullaart et al¹⁵ who found that CETP activity was increased in male cigarette-smoking IDDM patients with microvascular complications. In their study, since subjects received insulin therapy, lipoprotein metabolism might have been altered by the exogenous insulin. Haffner et al¹⁸ suggested that smoking was strongly and inversely correlated with LCAT mass and reduced HDL₃ cholesterol levels. However, they did not provide any information regarding LCAT mass or activity. Gylling et al²⁴ investigated the in vivo plasma compartment kinetics of Apo A-I in smokers versus non-smokers, and showed that the residence time for Apo A-I in smokers was less than that in non-smokers without any significant difference in plasma transport rates. Although the cause of this result has not been identified, it is possible that the reduction in HDL concentration observed in smokers may have been the result of an increase in the catabolism of Apo A-I or a change in lipase activities beyond the change in CETP or LCAT activity.

Alcohol intake elevates HDL-C⁷⁻¹⁴. Our results are consistent with these reports in that HDL-C levels were higher in Group D than in the controls. However, there were no significant differences between Group D individuals and normal controls with respect to plasma CETP activity or LCAT activity. Hannuksela et al¹⁷ found reduced CETP activity in alcohol abusers and reported that the activity and concentration of CETP show a strong positive correlation. However, the daily alcohol intake in their subjects was high enough (154 g/day) as to suggest liver dysfunction or at least an altered diet (especially a lower intake of polyunsaturated fatty acid). Hojnacki et al²⁵ reported findings that may explain the difference between moderate and heavy drinkers: a moderate (12% calories) regular daily consumption of ethanol caused a reduction of body weight and an elevation of HDL₂/HDL₃ ratio,

apoA-I/B ratio, LCAT activity and reduction of LDL cholesterol. The same weekly dose consumed consistently during single sittings (very heavy drinking) caused an elevation of LDL cholesterol and apoB and a reduction of LCAT activity, along with weight loss and depletion of body fat. However, LCAT activity was not exogenously assayed in their study. Taskinen et al¹⁴ reported that the alcohol-induced elevation of HDL-C occurred predominantly in the HDL₂ subfraction, although HDL₃ was also elevated; the elevation of HDL-C depended mainly upon the elevation of LPL and HL activities. We previously observed an increase in HDL-C after the loading of a moderate amount of alcohol (30 g/day) for 4 weeks in healthy normolipidemic men without a significant change in CETP or LCAT activity, but with an increase in LPL activity²⁶. This alcohol-loading study supports our speculation that the increase in HDL concentration associated with moderate alcohol consumption is mainly due to a change in lipase activity. To substantiate this point, further investigation of this change and the change in the protein component of HDL that is induced by smoking and alcohol consumption in normolipidemic subjects is needed.

As expected, a significant correlation was observed between HDL-C levels and BMI in the controls (Group C). There were no correlations between HDL-C levels and any other parameters (age, BMI, CETP and LCAT). However, due to the small sample size, we hesitate to draw any firm conclusions from these findings.

In conclusion, moderate smoking and drinking habits had no detectable effects on CETP or LCAT activity in normocholesterolemic and normotriglyceridemic subjects. Therefore, it is unlikely that these factors are responsible for changes in the HDL-C level.

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