Cholesteryl Ester Enrichment of Plasma Low-Density Lipoproteins in Hamsters Fed Cereal-Based Diets Containing Cholesterol

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Abstract. **Male Syrian hamsters were fed 0.02, 0.03, or 0.05% cholesterol to test the hypothesis that moderate cholesterol intake increases the cholesteryl ester content of the plasma low-density lipoproteins (LDL). Dietary cholesterol levels of 0.02%–0.05% were chosen to reflect typical human intakes of cholesterol. Hamsters were fed *ad libitum* a cereal-based diet (modified NIH-07 open formula) for 15 weeks. Increasing dietary cholesterol from 0.02% to 0.05% resulted in significantly increased plasma LDL and high-density lipoprotein cholesterol concentration, increased liver cholesterol concentration, and increased total aorta cholesterol content. The cholesteryl ester content of plasma LDL was determined as the molar ratio of cholesteryl ester to apolipoprotein B and to surface lipid (i.e., phospholipid + free cholesterol). Increasing dietary cholesterol from 0.02% to 0.05% resulted in significantly increased cholesteryl ester content of LDL particles. Furthermore, cholesteryl ester content of LDL was directly associated with increased total aorta cholesterol, whereas a linear relationship between plasma LDL cholesterol concentration and aorta cholesterol was not observed. Thus, the data suggest that LDL cholesteryl ester content may be an important atherogenic feature of plasma LDL.** [P.S.E.B.M. 2000, Vol 223]

Elevated plasma cholesterol concentration is widely regarded as a primary risk factor in the development of atherosclerosis and coronary heart disease (CHD). However, clinical studies have shown that about 30% of patients with premature CHD exhibit normal plasma total cholesterol and low-density lipoprotein (LDL) cholesterol concentrations (1, 2). Genest *et al.* (3) reported that 46% of CHD patients had desirable plasma total cholesterol levels ≤200 mg/dl (5.2 m*M*) and 42% had desirable LDL cholesterol levels ≤130 mg/dl (3.4 m*M*). Data from the Multiple Risk Factor Intervention Trial involving 361,662 men indicated that 40% of CHD deaths occur in individuals with plasma total cholesterol concentrations <200 mg/dl (4). These studies suggest that other factors contribute to the development of CHD in addition to elevated plasma total and LDL cholesterol concentration.

Several studies suggest that the size of plasma LDL is associated with atherosclerosis development and CHD. Rudel *et al.* (5, 6) first demonstrated in primates that the size of plasma LDL was directly correlated with the extent of coronary artery atherosclerosis. Campos *et al.* (7) have recently reported that CHD patients with plasma total cholesterol levels <200 mg/dl (5.2 m*M*) had significantly larger plasma LDL particles compared to matched healthy controls. The association between CHD and large LDL was found to be independent of lipoprotein cholesterol concentrations in the plasma (7). In a similar study, Sherrard *et al.* (8) reported that although mean LDL particle diameter was the same between CHD patients and control subjects, CHD patients had a broader distribution of LDL sizes and a greater proportion of larger LDL particles. In contrast, it has

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been suggested that small, dense LDL are associated with CHD; however, the association does not persist after adjusting for plasma triacylglycerol and high-density lipoprotein (HDL) cholesterol concentration (7, 9–11). Consequently, disagreement still exists regarding LDL size *per se* and its relationship to atherosclerosis development.

Because LDL are aggregate particles consisting of various lipids and one molecule of apolipoprotein B_{100} (apoB) (12), particle size is directly related to the chemical composition of LDL. In general, the amount of lipid in the core of LDL (i.e., cholesteryl ester and triacylglycerol) relative to apoB and surface lipid (i.e., phospholipid and free cholesterol) determines LDL particle size. Previous studies (13, 14) in primates suggested that LDL size was largely determined by the number of cholesteryl ester molecules within the core of the LDL particle. Rudel *et al.* (15) recently reported that the cholesteryl ester content of LDL was more highly correlated with atherosclerosis development than LDL particle size. These studies suggest that LDL cholesteryl ester content may be a more sensitive indicator of atherogenicity than LDL particle size. Therefore, the present study was conducted to document the changes in plasma LDL particle composition in hamsters fed cholesterol at levels reflecting typical human intakes.

Materials and Methods

Animals and Diets. Male Syrian hamsters (Charles River, Wilmington, MA) weighing 40–50 g were individually housed in solid-bottom polycarbonate cages with woodchip bedding. All animals were housed in the same room maintained at 25°C with a 12:12-hr light:dark cycle for the duration of the 15-week experiment. Hamsters were fed a modified version of the NIH-07 open formula cereal-based rodent diet (16, 17), prepared by Dyets, Inc. (Bethlehem, PA) according to our specifications. A detailed description of the diets used in this study has been previously published (18). An open-formula, cereal-based diet was chosen over a purified diet for this study to better mimic human diets while maintaining the control needed to manipulate specific constituents. The modified NIH-07 diet contained 15% fat and 0.02%, 0.03%, or 0.05% cholesterol by weight. Treatment groups were designated 0.02%C, 0.03%C, and 0.05%C. The three levels of dietary cholesterol used in this study correspond to 0.11, 0.16, and 0.27 μmol/kcal (0.04, 0.06, and 0.10 mg/kcal). The study protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska.

Experimental Design. Forty-eight hamsters were divided randomly into three groups (n = 16) and given free access to food and water for 15 weeks. Body weight and food intake were recorded weekly. No significant differences in body weight or food intake were detected among treatment groups throughout the study. The animals were sacrificed in random order on three consecutive days during the 16th week. Food was removed 24 hr prior to termination, and the animals were given an overdose of ketamine hydrochloride (≈ 25 mg/100 g body weight). The abdomen and thorax were opened by incision, and blood was collected by cardiac puncture and temporarily placed on ice. The liver was perfused *in situ* with saline, removed and weighed, and immediately frozen at −70°C. The entire aorta was removed by dissection from the heart at the aortic arch to the iliac bifurcation.

Plasma Lipoproteins. Blood was collected by cardiac puncture into 10-ml syringes containing EDTA as an anticoagulant. Red blood cells were removed by centrifugation at 1000*g* for 30 min (4°C). Plasma was collected and adjusted to contain 0.1% NaN_3, 80 μl/ml phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin as preservatives. Approximately 4 ml of plasma were recovered from each hamster and briefly stored at 4°C. Plasma total cholesterol concentration was determined enzymatically (Boehringer Mannheim, Indianapolis, IN) using the microtiter plate method of Carr *et al.* (19).

Plasma lipoproteins were further separated by gel filtration chromatography (20). Whole plasma (1 ml) was applied to 1 × 100 cm columns containing 4% agarose (BioGel A 15 m, BioRad, Inc., Hercules, CA). The sample was eluted at a flow rate of 4.5 ml/hr with 0.9% NaCl, 0.01% EDTA, and 0.01% NaN_3 (pH 7.4). Eluate fractions were collected every 20 min and analyzed for total cholesterol. A typical cholesterol eluation profile of hamster plasma is shown in Figure 1. The fractions corresponding to the column void volume (containing very low density lipoproteins), LDL, and HDL were pooled, and the exact volume was recorded. Total cholesterol recovery from the column was >92% in all cases. The plasma concentration of LDL and HDL cholesterol was calculated after correcting for recovery.

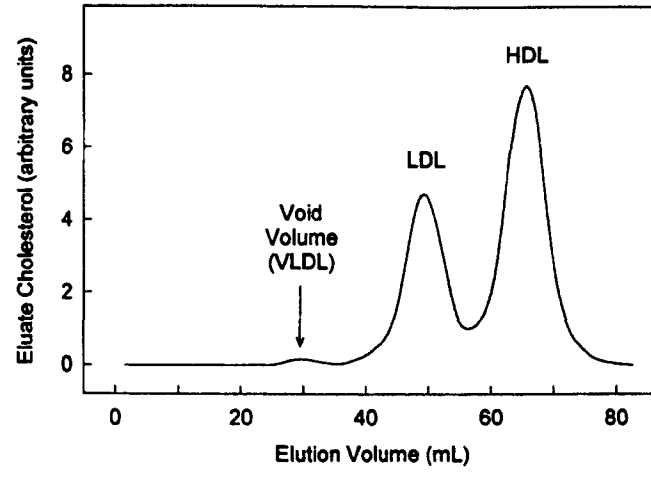

Figure 1. Cholesterol elution profile of hamster plasma using gel filtration chromagraphy. One ml of whole plasma was applied to a 1 × 100 cm column containing 4% agarose (BioGel A 15m, BioRad, Inc., Hercules, CA). The sample was eluted at a flow rate of 4.5 ml/hr with 0.9% NaCl, 0.01% EDTA, and 0.01% NaN_3 (pH 7.4). Eluate fractions were collected every 20 min and analyzed enzymatically for total cholesterol (Boehringer Mannheim, Indianapolis, IN). Fractions corresponding to VLDL, LDL, and HDL were pooled for further analysis.

Plasma LDL composition was determined using enzymatic reagents for free cholesterol (Wako Chemicals USA, Inc., Richmond, VA), total cholesterol, and triacylglycerol (Boehringer Mannheim, Indianapolis, IN) (19). Esterified cholesterol in LDL was calculated as the difference between total and free cholesterol values. LDL phospholipids were determined by measuring inorganic phosphorus using the method of Morrison (21). Apolipoprotein B_{100} associated with LDL was measured immunoturbidimetrically (Sigma Chemical Co., St. Louis, MO). LDL particle diameter was calculated according to the equation of Van Heek and Zilversmit (22). LDL cholesteryl esters were isolated by thin-layer chromatography (TLC) after extraction into chloroform/methanol (2:1, v/v) (23). The lipids were developed on the TLC plate using a solvent system of hexane/diethyl ether/acetic acid (70:30:2). The LDL lipids were visualized with Primulin (Sigma Chemical Co., St. Louis, MO) and the cholesteryl esters scraped into screw-cap tubes. Fatty acid methyl esters were prepared for quantitation by gas chromatography (24).

Tissue Lipids. Approximately 1.5 g of frozen liver was minced and transferred to a glass tube on an analytical balance. The entire aorta was also minced and transferred to a glass tube. Tissue lipids were extracted into chloroform/methanol (2:1, v/v) according to the method of Folch *et al.* (23). Liver total cholesterol, free cholesterol, triacylglycerol, and phospholipids were quantitated using the same procedures described above for plasma LDL lipids. Liver cholesteryl ester fatty acids were measured using a combination of TLC and gas chromatography as described for LDL cholesteryl esters.

Statistical Analyses. After confirming that the data were normally distributed, one-way analysis of variance was used to compare the experimental end points of the three treatment groups. Statistical differences among the means were considered significant at $P < 0.05$ as determined by the Tukey multiple comparison procedure. All statistical analyses were performed on a personal computer using SigmaStat (SPSS Inc., Chicago, IL).

Results

Plasma LDL and HDL cholesterol concentrations were significantly increased in hamsters fed 0.05% cholesterol (Table I). Increasing dietary cholesterol from 0.02% to 0.05% caused an increase in LDL cholesterol concentration of 64%, whereas HDL cholesterol increased only 19%; the LDL/HDL ratio tended to increase although not significantly ($P = 0.069$). Increasing dietary cholesterol from 0.02% to 0.03% did not significantly increase LDL or HDL cholesterol concentration. The majority of plasma cholesterol was transported in HDL under these dietary conditions. As an indicator of atherosclerotic lesion development, the amount of total aorta cholesterol was significantly higher in the 0.05%C group compared to hamsters fed 0.02% cholesterol.

Plasma LDL were isolated by gel filtration chromatography, and the concentration of individual chemical components was determined. Table II shows the physical characteristics of LDL expressed as the molar ratio of individual components. The molar ratio of cholesteryl ester to apoB significantly increased in accord with dietary cholesterol, as did the ratio of cholesteryl ester to surface lipid. Because plasma LDL contain one molecule of apoB (12), the molar ratio of cholesteryl ester to apoB represents the number of cholesteryl ester molecules per LDL particle. Conversely, the ratio of LDL triacylglycerol to apoB was highest in the 0.02%C group. In addition, overall LDL particle diameter, as calculated from the mass percentage of each component (22), tended to increase with dietary cholesterol, although the trend was not significantly different among treatment groups ($P = 0.134$).

Liver free and esterified cholesterol concentrations were significantly increased in hamsters fed 0.05% cholesterol (Table III). Compared to the 0.02%C group, the magnitude of increase for cholesteryl esters (212%) was much greater than for free cholesterol (11%). Liver triacylglycerol concentration was significantly lower in the 0.05%C group, whereas liver phospholipid concentration was not altered by dietary cholesterol within the range used in this study.

The concentration of individual cholesteryl ester species within the liver and plasma LDL are presented in Figure 2. The four cholesteryl ester species shown in Figure 2 accounted for 95% of all cholesteryl ester species in the liver and >97% in plasma LDL. In all cases, animals fed 0.05% cholesterol had significantly higher values than animals fed 0.02% or 0.03% cholesterol. Cholesteryl palmitate was the most abundant species in the liver of hamsters fed 0.02% and 0.03% cholesterol. However, cholesteryl oleate

Table I. Plasma and Aorta Cholesterol Concentration in Hamsters Fed Cereal-Based Diets Containing 0.02%, 0.03%, and 0.05% Cholesterol for 15 weeks

	LDL cholesterol	HDL cholesterol	LDL/HDL ratio	Aorta cholesterol
	mM		mol/mol	nmol/aorta
0.02%C	0.99 ± 0.05^{a}	2.09 ± 0.08^{a}	0.48 ± 0.02	39.1 ± 2.7^{a}
0.03%C	1.07 ± 0.07^{a}	2.05 ± 0.06^{a}	0.53 ± 0.04	47.2 ± 2.6^{ab}
0.05%C	1.62 ± 0.11^{b}	2.48 ± 0.13^{b}	0.72 ± 0.12	50.9 ± 2.6^{b}

Note. Values represent means ± SEM ($n = 16$ for each treatment). Means within the same column having different superscripts are significantly different ($P < 0.05$) using one-way analysis of variance and the Tukey multiple comparison test. 0.02%C, 0.03%C, and 0.05%C represent the percentage of dietary cholesterol in each treatment.

Table II. Physical Characteristics of Plasma LDL in Hamsters Fed Cereal-Based Diets Containing 0.02%, 0.03%, and 0.05% Cholesterol for 15 Weeks

	CE:apoB ratio	TG:apoB ratio	CE:surface lipid ratio	TG:surface lipid ratio	LDL particle diameter
			mol/mol		Å
0.02%C	1043 ± 124^{a}	518 ± 49^{a}	0.762 ± 0.108^{a}	0.354 ± 0.029	172 ± 9
0.03%C	1205 ± 164^{ab}	372 ± 28^{b}	1.064 ± 0.105^{ab}	0.346 ± 0.028	186 ± 9
0.05%C	1549 ± 142^{b}	389 ± 36^{ab}	1.185 ± 0.076^{b}	0.300 ± 0.021	197 ± 9

Note. Values represent means ± SEM (n = 16 for each treatment). Means within the same column having different superscripts are significantly different ($P < 0.05$) using one-way analysis of variance and the Tukey multiple comparison test. 0.02%C, 0.03%C, and 0.05%C represent the percentage of dietary cholesterol in each treatment.

Table III. Liver Lipid Concentration in Hamsters Fed Cereal-Based Diets Containing 0.02%, 0.03%, and 0.05% Cholesterol for 15 Weeks

	Phospholipid	Free cholesterol	Cholesteryl ester	Triglyceride
		μmol/g		
0.02%C	51.3 ± 1.2	6.86 ± 0.12^{a}	1.03 ± 0.10^{a}	11.2 ± 1.2^{a}
0.03%C	51.2 ± 0.8	7.35 ± 0.12^{b}	1.22 ± 0.14^{a}	12.1 ± 1.5^{a}
0.05%C	50.5 ± 0.9	7.60 ± 0.18^{b}	3.21 ± 0.80^{b}	7.3 ± 0.5^{b}

Note. Values represent means ± SEM (n = 16 for each treatment).
Means within the same column having different superscripts are significantly different ($P < 0.05$) using one-way analysis of variance and the Tukey multiple comparison test. 0.02%C, 0.03%C, and 0.05%C represent the percentage of dietary cholesterol in each treatment.

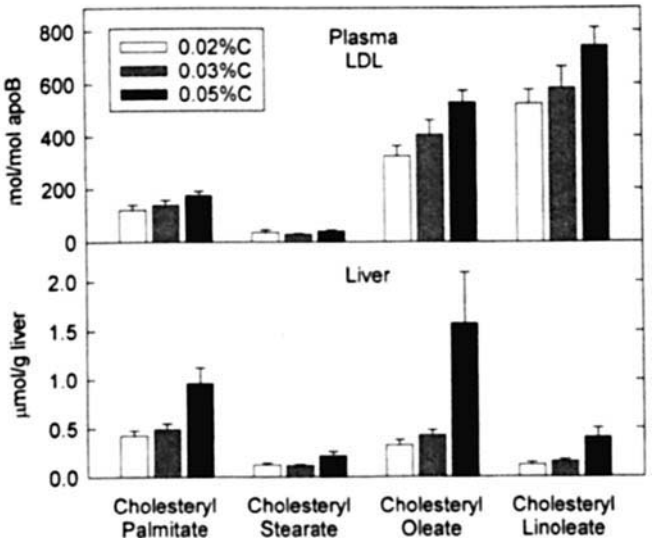

Figure 2. Individual cholesteryl ester species present in the liver and plasma LDL of hamsters fed cholesterol-containing cereal-based diets. Data are presented as mean ± SEM. 0.02%C, 0.03%C, and 0.05%C represent the amount of cholesterol present in the hamster diets. For each cholesteryl ester species in the liver, animals fed the 0.05%C diet had significantly higher values ($P < 0.05$) than 0.02%C and 0.03%C groups. In plasma LDL, only cholesteryl oleate was significant in the 0.05%C group compared with the 0.02%C group.

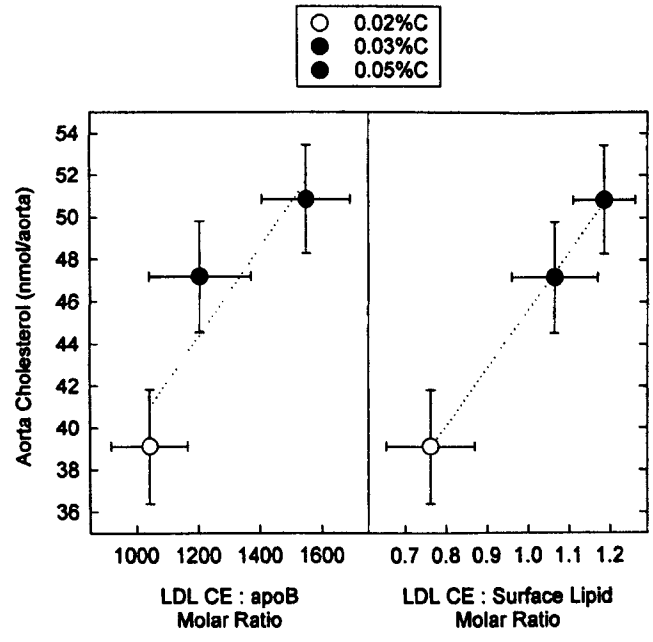

Figure 3. Relationship between total aorta cholesterol and LDL cholesteryl ester content. The LDL cholesteryl ester content is expressed as the molar ratio of cholesteryl ester to apoB (left panel), and the molar ratio of cholesteryl ester to LDL surface lipid (right panel). CE = cholesteryl ester.

increased disproportionately in the 0.05%C group and became the most abundant cholesteryl ester species in the liver. Cholesteryl linoleate was the most abundant species in plasma LDL in each treatment group.

Figure 3 illustrates the relationship between LDL cholesteryl ester content and aorta cholesterol. LDL cholesteryl ester content is expressed two ways; as the molar ratio of cholesteryl ester relative to apoB (left panel) and surface lipid (right panel).

Discussion

The results of this study support the hypothesis that moderate cholesterol intake increases the cholesteryl ester content of the plasma low-density lipoproteins (LDL) in hamsters. Cholesteryl ester enrichment of LDL particles was determined by measuring the complete chemical composition of plasma LDL, then calculating the molar ratio of cholesteryl ester relative to apoB and surface lipid. More-

over, there was a direct relationship between LDL cholesteryl ester enrichment and aorta cholesterol concentration, whereas a linear relationship between plasma LDL cholesterol concentration and aorta cholesterol concentration was not observed. Thus, the data suggest that LDL cholesteryl ester content may be an important atherogenic feature of LDL.

An LDL particle is composed of a monolayer of free cholesterol and phospholipid on the surface, with nonpolar cholesteryl ester and triacylglycerol molecules located in the particle core (25). Consequently, the volume of cholesteryl ester and triacylglycerol molecules within the core can determine the size of LDL particles (25). We also estimated the diameter of LDL particles based on LDL composition data and the equation of Van Heek and Zilversmit (22). Whereas the mean diameter of LDL tended to increase with increasing cholesterol intake, the trend was not statistically significant. The increase in LDL cholesteryl ester content that occurred with increased cholesterol intake was accompanied by decreases in LDL triacylglycerol. Perhaps an "exchange" of cholesteryl ester for triacylglycerol with increased cholesterol intake limited significant changes in LDL particle size. Campos *et al.* (7) recently reported that patients with CHD had significantly larger LDL compared with control subjects after adjusting the data for triacylglycerol and HDL cholesterol concentration. Although Campos *et al.* (9) and others (10, 11) originally proposed an association with small dense LDL and CHD, the association disappeared after adjusting for triacylglycerol and HDL cholesterol. The apparent discrepancy may be due to the increased ability of human LDL to exchange cholesteryl ester for triacylglycerol (26). After clearance of the transferred triacylglycerol, LDL particle size may be reduced (26). This finding suggests that changes in LDL size *per se* may not be as sensitive to dietary manipulation as changes in overall particle composition, namely LDL cholesteryl ester content.

The individual cholesteryl ester species present in the liver and in LDL particles were separated and quantitated. Because cholesteryl oleate is the primary product of the cellular cholesteryl ester synthesis (27), the presence of cholesteryl oleate in plasma LDL reflects hepatic origin as we have previously reported (28). In contrast, LDL cholesteryl linoleate arises primarily within the plasma *via* intravascular esterification (29). In the latter case, cholesterol esterification occurs in HDL, and the preferred substrate for the reaction is linoleic acid. The resulting cholesteryl esters in HDL are then transferred to LDL by cholesteryl ester transfer protein. The liver cholesteryl ester distribution in Figure 2 clearly shows cholesteryl oleate as the primary product of esterification in the liver when hamsters were fed 0.05% cholesterol. Although cholesteryl linoleate also increased in the liver in the 0.05%C group, the absolute amount was much lower than cholesteryl oleate or cholesteryl palmitate. In contrast, cholesteryl linoleate was the most abundant species in plasma LDL regardless of cholesterol intake. The second most abundant species in LDL was cholesteryl oleate, suggesting hepatic origin. This finding provides indirect evidence that the cholesteryl esters of LDL in cholesterol-fed hamsters arise from both liver secretion and intravascular synthesis.

The distribution of cholesterol among plasma lipoproteins in hamsters is dependent on several factors, including alterations in the diet (30–35), the strain of hamster (35, 36), and whether the animals have been fasted (37). In the present study, hamsters were fed cereal-based diets with relatively modest levels of cholesterol and fasted prior to blood sampling. Increasing the amount of dietary cholesterol from 0.02% to 0.05% caused LDL cholesterol concentration to increase to a greater extent then HDL cholesterol (Table I). Although one study (31) reported LDL cholesterol concentration to be unaffected by changing dietary cholesterol from 0 to 0.05%, the majority of hamster studies (30, 33–35, 37) concur with our observation that the primary plasma response to increasing dietary cholesterol is a disproportionate increase in LDL cholesterol concentration relative to HDL cholesterol. This observation is important with regard to cholesterol feeding studies in humans in which LDL cholesterol concentration is selectively increased in hyperresponding individuals (38–40). The main purpose of our study design was to feed cholesterol at levels representing typical human consumption to document the compositional changes in plasma LDL in relation to atherosclerosis development. In this regard, the Syrian hamster appears to be an appropriate and useful animal model for feeding studies of this type.

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