Increased Lipid Transfer Activities in Hyperlipidemic Rabbit Plasma

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Three models of hypercholesterolemia in rabbits were positively associated with plasma cholesteryl ester and triglyceride transfer activities. In cholesterol-fed rabbits, the transfer activities increased two- to three-fold when plasma cholesterol concentration reached about 1500 mg/dl. In hypercholesterolemia induced by feeding a cholesterol-free semisynthetic casein-sucrose diet, plasma cholesterol increased up to three-fold and the increase in cholesteryl ester and triglyceride transfer activities per unit of plasma cholesterol increment was similar to that in cholesterol-fed rabbits. Watanabe heritable hyperlipidemic rabbits, in which hypercholesterolemia is induced by a genetic defect of the LDL receptor, had significantly higher transfer activities than normolipidemic rabbits. However, fasting, which also induced hypercholesterolemia, lowered transfer activities, whereas refasting returned them to prefasting levels. In rats, hypercholesterolemia induced by a high cholesterol diet did not increase cholesteryl ester and triglyceride transfer activities. Although hypercholesterolemia in rabbits may not be the primary cause of increased lipid transfer activities, some mechanism related to hypercholesterolemia appears to be associated with increased cholesteryl ester and triglyceride transfer activities.

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The cholesteryl ester transfer protein has been identified and purified from human\(^1\)–3 and rabbit\(^4\)–5 plasma. Its function in lipoprotein metabolism has been partially characterized in vitro. The transfer protein mediates transfer of cholesteryl ester (CE) and triglyceride (TG) between lipoproteins in plasma, and thus alters the lipid composition of lipoproteins. Some researchers have speculated about the possible interactions between CE transfer activity and reactions such as transesterification\(^6\)–7 and lipolysis\(^8\)–9 of lipoprotein lipids.

The mechanism(s) for the metabolic regulation of CE transfer activity are not known, and equivocal reports have been presented regarding the influence of hyperlipidemia on CE transfer activity. Groener et al.\(^10\) reported elevated CE transfer activity in delipidated plasmas of hyperlipidemic patients compared to delipidated plasmas of normolipidemic subjects. On the other hand, Fielding et al.\(^11\) showed that net transfer of CE mass was lower in hyperlipoproteinemic than in normolipidemic plasmas. In the present study, we have investigated the effects of conditions that cause hypercholesterolemia in rabbits on CE and TG transfer activities. Apparently, both transfer activities increase in hypercholesterolemia, whether it was induced by a cholesterol diet, a cholesterol-free atherogenic diet, or a genetic defect such as in the Watanabe heritable hyperlipidemic rabbits.

Methods

Animals

Male and female New Zealand white rabbits were purchased from Beckens Research Animal Farm, Sanborn, New York. Male Sprague-Dawley albino rats were obtained from Blue Spruce Farm, Altamont, New York. Experimental protocols were approved according to Cornell University guidelines.

Diets

The diets used for rabbits were as follows: for the control diet, Purina Rabbit Laboratory Chow; for a somewhat higher fat diet, the same rabbit chow supplemented with 2.7% Wesson Oil (Hunt Wesson Foods, Incorporated, Fullerton, California); for the high cholesterol diet, rabbit chow supplemented with 0.5% cholesterol (Nutritional Biochemical Company, Cleveland, Ohio) and 2.7% Wesson oil; for a hypercholesterolemic cholesterol-free diet, a casein-sucrose diet described by Ross et al.\(^12\). The casein-sucrose
diet contained 23.2% casein, 63.2% sucrose, and 2.33% corn oil plus vitamins and minerals as described previously. This diet was obtained in pelleted form from U.S. Biochemicals (Cleveland, Ohio) and was stored at 4° C until feeding time.

The high cholesterol diet for rats was prepared as described by Mahley and Holcombe.\textsuperscript{13} Cholesterol (Nutritional Biochemical Company), taurocholic acid, 6-N-propyl-2-thiouacil (Sigma Chemical Company, St. Louis, Missouri) were added to lard oil (Sigma Chemical Company), were liquefied at 60° C and stirred. The warm suspension was mixed thoroughly with a commercial pelleted laboratory diet (Agway, Syracuse, New York). The final diet contained 5% lard, 1% cholesterol, 0.1% propylthiouracil, and 0.3% taurocholic acid by weight.

**Feeding Protocols**

**Rabbits**

Six male and six female New Zealand white rabbits, weighing about 3 kg each, were used in a study on the effect of hypercholesterolemia induced by cholesterol feeding on lipid transfer activities. Two rabbits of each sex were given the rabbit chow throughout the study. Four rabbits in each group were fed the fat diet for 28 days for an observation of the possible effects of the 2.7% oil in the cholesterol diet. Subsequently, the rabbits were fed the cholesterol diet for 41 days. For the next 16 weeks, the animals were returned to the unmodified rabbit chow. All rabbits consumed 100 g of diet per day throughout the study.

In a second study, the effect of hypercholesterolemia induced by a cholesterol-free atherogenic diet on the CE and TG transfer activities was observed. Eight rabbits out of 14 female New Zealand white rabbits, weighing 1.6 to 2.1 kg each, were given a casein-sucrose diet ad libitum (consumption was approximately 50 g per day), and the remaining rabbits received 100 g of rabbit chow for 82 days.

**Rats**

To induce hypercholesterolemia, seven male Sprague-Dawley rats weighing 150 to 200 g were each given the high cholesterol diet for 4 weeks. Another four rats were given a control diet (Agway). Food was available at all times. Rats were fasted for 16 hours before being bled.

**Preparation of Lipoprotein-Deficient Plasma**

Blood was obtained from the marginal ear vein of rabbits 16 to 18 hours after the last meal. It was collected into tubes containing 0.01 ml of 0.4 M EDTA and 4% Na\textsubscript{2}C\textsubscript{O} at 4° C, and kept at 4° C. Rats were bled by heart puncture under ether anesthesia. Plasma was prepared by low-speed centrifugation. One ml of plasma was adjusted to density 1.21 by adding d = 1.478 NaBr solution, and was centrifuged in a Beckman 50.3 rotor at 4° C for 3 × 10\textsuperscript{8} g per minute. The infranatant d > 1.21 was recovered from tubes containing 0.01 ml of 0.4 M EDTA and 4% Na\textsubscript{2}C\textsubscript{O} at 4° C (3.99 × 10\textsuperscript{8} g per minute).

A typical assay mixture consisted of labeled LDL and unlabeled HDL, lipoprotein-deficient plasma equivalent to 10 to 30 µl of undiluted plasma, and 1% crystalline bovine serum albumin (BSA) (Miles, Elkhart, Indiana) in stoppered 1.5 ml plastic conical tubes. The volume was adjusted to 0.7 ml with 50 mM Tris/150 mM Na\textsubscript{2}C\textsubscript{O} (pH 7.4), and the tubes were incubated at 37° C for 1.5 hours. The LDL was then precipitated with sodium phosphate and MnCl\textsubscript{2}, and the radioactivity in the supernatant was counted in an aqueous counting scintillant (ACS) (Amersham Corporation). The supernatant radioactivity in the assay blanks, without the plasma sample, was subtracted from that of the other samples; for CE transfer, the blanks were less than 10% of sample values.

The catalyzed transfer of CE and TG from LDL to HDL during the incubation was expressed as transfer activity and calculated as follows: \( \text{HDL}^* = \text{LDL}^* \left( 1 - e^{-\left( k_1 + k_{-1}\right) t} \right) \cdot k_t/(k_1 + k_{-1}) \) (1)
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in which \( k_1 \) = the fractional transfer rate of LDL CE/TG to HDL; and \( k_{-1} \) = the fractional transfer rate of HDL CE/TG to LDL. In this assay system, no net transfer of CE or TG mass was observed. Therefore, if LDL\(_M\) and HDL\(_M\) represent the CE (or TG) mass in LDL and HDL, respectively:

\[ k_1 \text{LDL}_M = k_{-1} \text{HDL}_M \]  

(2)

or

\[ k_{-1}/k_1 = \frac{\text{LDL}_M/\text{HDL}_M}{c} \]  

(3)

which, when substituted in equation 1, gives:

\[ \text{HDL}_* = \text{LDL}_* \left( 1 - e^{-k(t+c)} \right)/(1+c) \]  

(4)

or

\[ k_1 t = -\ln((1-\text{HDL}_*/\text{LDL}_*)/(1+c))/(1+c) \]  

(5)

from which \( k_1 \) can be calculated if LDL\(_M\)/HDL\(_M\) is substituted for \( c \).

In the text and tables, \( k_1 \) is expressed as \( k_t \) and the absolute transfer activity is expressed as 100 \( k_t \) times the incubation time (%\( k_t \)), which is 1.5 hours. Responses of the lipid transfer activities to the experimental diets were expressed as relative transfer activities or the ratios of lipid transfer activities in treated rabbit plasmas to those in plasma of control rabbits. Although an inhibitor for lecithin/cholesterol acyltransferase was not included in the assay, there was no significant change in the ratio of CE/free cholesterol in LDL and HDL during the assay.

For the transfer activity assay of rat plasma, conditions were the same as those for rabbit plasmas, except that the lipoprotein-deficient plasma was used up to a volume corresponding to 150 \( \mu l \) of the undiluted plasma. Lipids were extracted from the HDL fraction, were dried down, and were counted in a toluene scintillation mixture.

Lipid Analysis

Lipids from lipoprotein samples were extracted by partitioning into hexane from 42% ethanol in water according to the method of Thompson et al.\(^{18}\) Free cholesteryl and CE were separated on precoated thin-layer chromatography (TLC) plates (Silica Gel 60, E. Merck, Darmstadt, F.R.G.) with hexane/diethyl ether/acetic acid (80:20:1, vol/vol/vol). Lipids were extracted from the silica gel with chloroform/methanol (9:1, vol/vol). Cholesterol was determined by the ferric chloride procedure\(^{19}\) after saponification according to Abell et al.\(^{20}\) Triglyceride was measured by the method of Sardesai and Manning.\(^{21}\)

Results

In a preliminary study, the plasma CE and TG transfer activities of cholesterol-fed and control rabbits were compared. Plasmas from cholesterol-fed rabbits contained twice as much transfer activity as that of control rabbits. Plasmas from rabbits fed cholesterol for 1 month did not differ in transfer activities from those fed cholesterol for 2 months. Similar observations were made in a time-course study summarized in Figure 1. Relative transfer activities for each sex were expressed as the ratio of the average transfer activity of cholesterol-fed rabbit plasmas to the average transfer activity in control rabbit plasmas. Since the measurement of transfer activities was conducted over a long period of time, control transfer activities appeared to be variable. This was especially so when newly prepared substrates were used and a decrease in transfer activities of all samples was observed (Figure 1 B). The transfer activities of the four control animals responded similarly, which indicates that the fluctuations and gradual drifts of transfer activity were due to such factors as differences in composition and age of the substrates. The sudden decrease in transfer activities was reproduced with another preparation of substrates with which the percentage change in transfer activity in 26 different plasma samples was similar to that obtained with the first substrates. Thus, we have used relative transfer activities as a measure of treatment effects compared to controls.

During the first 28 days, the 2.7% dietary fat had no significant effect on the relative transfer activities (means ± SD of the CE transfer activity for males were 1.31 ± 0.35...
and 1.66 ± 0.62 for 0 and 28 days fat feeding, respectively, and those for females were 1.04 ± 0.22 and 1.08 ± 0.04, respectively). Rabbits fed the cholesterol diet showed a gradual increase of CE and TG relative transfer activities as well as increased plasma cholesterol concentrations. Although the relative TG transfer activity is not shown in Figure 1A, the trend was almost the same as that for CE transfer activity. In Figure 1A, the relative CE transfer activity of males appears to increase more than that for females. In fact, this difference results from the much lower absolute transfer activity in male than in female controls (Figure 1B). After 3 to 11 days of cholesterol feeding, CE and TG relative transfer activities were significantly increased from the initial level for both sexes. The CE and TG transfer activities increased further up to 28 days of cholesterol feeding when the plasma cholesterol concentration reached a plateau. Concomitantly, the average relative CE transfer activities were 2.92 and 3.02 (absolute values, 1349 and 1397 %kt/ml plasma) for male and female rabbits, respectively, when fed the cholesterol diet. When the rabbits were again fed the control diet, the CE and TG transfer activities slowly decreased parallel to the plasma cholesterol concentration. After the rabbits had been fed the control diet for 8 weeks (Day 133, Figure 1), plasma cholesterol concentrations were 291 and 589 mg/dl for males and females, respectively, and the relative CE transfer activity ratios decreased to 2.27 for males and 1.29 for females. After 16 weeks on the control diet (Day 189, not shown in Figure 1), plasma cholesterol concentrations decreased to 51 mg/dl for males and 38 mg/dl for females; relative CE transfer activity ratios also decreased to values that were very close to the ratios at the beginning of cholesterol feeding.

Plasma cholesterol concentrations and relative CE transfer activities of plasmas for individual rabbits during the period of cholesterol feeding are shown in Figure 2. To eliminate the effect of sex differences on the transfer activities, the CE transfer activities of males and females fed cholesterol were divided by the average activities of two chow-fed males and two chow-fed females, respectively. The change in relative CE transfer activities per 100 mg/dl increase in plasma cholesterol was calculated as the mean ± SE of the slopes of individual linear regression lines; for males this was 0.112 ± 0.021 and for females, 0.082 ± 0.022. The slopes for males and females did not differ significantly from one another, but they differed significantly from zero (p < 0.001). The average correlation coefficients between plasma cholesterol and the relative CE transfer activities were 0.94 for males and 0.93 for females.

Hypercholesterolemia was also induced by feeding a cholesterol-free semisynthetic casein-sucrose diet. The daily food intake per animal was 90 g in a previous study. However, in the present study rabbits would consume only about 40 to 50 g per day when food was provided ad libitum. Six control rabbits fed 100 g of rabbit chow gained 965 ± 192 g (mean ± sd), whereas eight rabbits on the casein-sucrose diet gained 753 ± 142 g during 82 days.
(p < 0.025). The plasma cholesterol concentration in rabbits fed the casein-sucrose diet increased from 56 ± 17 mg/dl to 253 ± 74 mg/dl (p < 0.005), whereas that in control rabbits increased from 57.6 ± 16.0 mg/dl to 83.1 ± 36.1 mg/dl over 82 days.

The relationship between the relative CE transfer activity and the plasma cholesterol concentration in the plasmas of individual rabbits fed casein-sucrose diet is shown in Figure 3. The data represent plasmas from eight rabbits sampled on Day 0, 42, 56, and 82. When all data were included for the straight-line regression, the slope did not differ significantly from zero. However, one animal (see Figure 3) showed unusually high initial transfer activities, yielding a slope of 3.3 SD from the mean. When that animal was excluded, the average increase in relative CE transfer activity for a 100 mg/dl increase in plasma cholesterol was 0.123 ± 0.027 (mean ± SE), which is very close to the increments observed for the cholesteryl-fed rabbits. The common slope of the regression lines was significantly greater than zero (p < 0.0025). Plasma TG transfer activities was also elevated in rabbits fed the casein-sucrose diet. The regression of relative TG transfer activity after exclusion of the data from the same animal with very high initial transfer activities gave a common slope of 0.065 ± 0.023 (mean ± se). The slope differed significantly from zero (p < 0.025).

To investigate the relationship between CE transfer activity and increased plasma cholesterol concentrations resulting from a mechanism other than dietary manipulation, we compared CE and TG transfer activities in plasmas from controls and Watanabe rabbits. In a preliminary study, we observed that the average CE transfer activity of five male Watanabe rabbits was 1.5 (p < 0.025) times greater than that of six male control rabbits. The average TG transfer activity was 1.2 times greater, but the difference was not statistically significant. In another experiment, 20 plasma samples in 0.04 M EDTA and 0.04% NaN3 were collected from normolipidemic New Zealand white rabbits (five males and five females) and from Watanabe rabbits (five males and five females) 2 to 3 months of age. Blood samples were obtained from control and Watanabe rabbits at the University of Texas Medical Center, Dallas, Texas. These animals were fed a laboratory rabbit diet from Teklad (Winfield, Iowa). The plasma samples were shipped on ice.

The plasma cholesterol concentrations were 12 to 16 times higher in the plasmas from Watanabe rabbits than in normolipidemic rabbits, as shown in Table 1. The average CE transfer activity in plasmas from Watanabe rabbits was 2.6 (p < 0.01) and 1.6 (p < 0.02) times greater than in normolipidemic plasmas in males and females, respectively. Two-way analysis of variance22 with sex as one factor and genetic variant as the other showed significant differences from controls for CE (p < 0.001) and TG (p < 0.025) transfer activities due to the genetic difference, whereas there was no significant difference due to sex or interaction between sex and genetic differences. However, there was no significant correlation between plasma cholesterol and CE transfer activity within Watanabe or within control rabbits.

In the next experiment, we tested the effect of fasting, which had been shown to raise LDL cholesterol concentrations23 on plasma CE transfer activity. Six control and eight casein-sucrose-fed rabbits were divided into two groups each: one group on each diet was fasted during the first week and switched to feeding for the second week; the other group was treated in the reverse sequence. The rabbits lost 430 ± 92 g (mean ± se) during 7 days of fasting. The differences between feeding and fasting were tested by analysis of variance of cross-over designs.24 Plasma cholesterol concentrations increased more than twofold in the controls (p < 0.025) and in some animals that had previously been on a casein-sucrose diet. However, after fasting, both the control and casein-sucrose groups had 12% to 15% (p < 0.05), and 20% to 30% (p < 0.025) less CE transfer activities, respectively (Table 2). In those rabbits that were fed again with their original diets, CE transfer activity returned to the starting level.

It is known that rat plasma does not have substantial CE transfer activity. However, we tested whether hypercholesterolemia can induce transfer activity in this species. Rats fed a high cholesterol diet for 4 weeks had plasma cholesterol concentrations of 225 ± 52 mg/dl (mean ± so), compared to 53 ± 7 mg/dl in controls. There was no difference in CE transfer activity between hypercholesterolemic (5.9 ± 4.6 %kt/ml plasma) and control rats (4.6 ± 4.0).

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Sex</th>
<th>Plasma cholesterol (mg/dl)</th>
<th>Transfer activity (%kt/ml plasma)</th>
<th>Transfer activity (%kt/ml plasma)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>TG</td>
<td>CE</td>
</tr>
<tr>
<td>Control New Zealand</td>
<td>male (n = 5)</td>
<td>48.7 ± 11.5</td>
<td>563 ± 124A</td>
<td>594 ± 99E</td>
</tr>
<tr>
<td>white</td>
<td>female (n = 5)</td>
<td>52.2 ± 8.2</td>
<td>862 ± 206B</td>
<td>901 ± 177F</td>
</tr>
<tr>
<td>Watanabe</td>
<td>male (n = 5)</td>
<td>798 ± 209</td>
<td>1066 ± 341C</td>
<td>1562 ± 579G</td>
</tr>
<tr>
<td></td>
<td>female (n = 5)</td>
<td>640 ± 134</td>
<td>1000 ± 266D</td>
<td>1430 ± 360H</td>
</tr>
</tbody>
</table>

The rabbits were 2 to 3 months old. The transfer activities were measured in lipoprotein-deficient plasma as described in Methods. Values are means ± so. TG = triglyceride; CE = cholesteryl ester.

The significance of difference by Student's t test: A vs B (p < 0.025); A vs C (p < 0.025); E vs F (p < 0.01); E vs G (p < 0.01); F vs H (p < 0.02).
Discussion

The present studies show that in rabbits plasma CE transfer activity is related to VLDL and LDL cholesterol. Since the CE transfer protein is known to mediate the net transfer of CE from HDL to VLDL, which is in turn catabolized to LDL, it is not clear whether increased VLDL and LDL cholesterol is the result of CE transfer activity in plasma or whether mechanisms that increase plasma cholesterol affect production or turnover rates of CETP.

In the present study, we have tested four different conditions that cause hypercholesterolemia in rabbits and found that three conditions were associated with elevated plasma CE and TG transfer activities. In cholesterol-fed rabbits, there was a strong correlation between plasma cholesterol concentration and both transfer activities. In most of the casein-sucrose-fed rabbits, in which the plasma cholesterol concentration increased very slowly up to 250 mg/dl, the relationship between plasma cholesterol and transfer activities was similar to that in cholesterol-fed rabbits. Watanabe rabbits had significantly higher transfer activities than normolipidemic rabbits. Fasting, however, which raised plasma cholesterol up to 200 mg/dl, resulted in decreased CE and TG transfer activities in rabbits. Transfer activities in control rabbits differed by a factor of two, but these differences did not show a significant correlation with plasma cholesterol concentrations.

In general, our results agree with the observation by Groener et al. They reported a strong correlation between CE transfer activity and VLDL and LDL cholesterol concentrations in human plasma; transfer activity was consistently higher in plasmas from hyperlipidemic patients than in those from normolipidemic subjects. However, Fielding et al. reported a lower net transfer of CE mass from HDL to VLDL and LDL in hyperbetalipoproteinemia than in normolipidemia. It is difficult to interpret these observations in terms of transfer activities because: 1) net transfer was measured instead of unidirectional transfer; 2) net transfer probably depends not only on the underlying plasma transfer activities, but also on differences in substrate concentrations and compositions in the plasmas of individual patients; and 3) if transfer activities are high in vivo, the substrates would be closer to the equilibrium state and, therefore, show lower net transfer rates when subsequently tested in vitro.

In studies on the effects of cholesterol or casein-sucrose feeding on the transfer activities, problems were encountered in standardizing the measurement of transfer activities over a long period of time during which substrates aged or had to be replaced by new ones. Therefore, we compared transfer activities of treated animals to the average transfer activities of control animals at each time point. Although transfer activities in individual animals fluctuated, their activities relative to each other seemed to be fairly constant (Figure 1B). In the fasting experiment we used a standard plasma sample of pooled rabbit plasma, which was kept at −20°C in small aliquots for each assay. We expressed the relative CE transfer activity as the CE transfer activity of plasma from each animal divided by the CE transfer activity in the standard plasma sample. The transfer activities during storage at −20°C were found to be stable for at least 2 to 3 months when the same control animals were tested repeatedly.

Transfer activities can be increased due to increased amounts of transfer protein, to activation of the protein, or to a decreased amount of inhibitor in plasma. Previously, we reported the presence of an inhibitor protein for lipid transfer activity in human plasma. A similar protein is also present in rabbit plasma. However, even after the inhibitor protein was removed by CM cellulose column chromatography, the specific activity of CE and TG transfer after cholesterol feeding increased similarly, as in unfractionated plasmas (unpublished observation). These results indicate that the increased CE transfer activity in hypercholesterolemia was not due to changes in the level of inhibitor protein in plasma.

Humans and rabbits have CE transfer protein and are susceptible to atherosclerosis compared to other species. Rats and dogs, which do not have measurable CE transfer
activity, are fairly resistant to the disease. In the present study, high CE and TG transfer activities were observed in three models of hypercholesterolemia that are known to promote atherosclerosis. High CE and TG transfer activities in conjunction with hypercholesterolemia may be related to the development of atherosclerosis.

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References


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