Activation of Human Plasma Lipid Transfer Protein by Apolipoproteins†

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ABSTRACT: Activation of human plasma lipid transfer protein (LTP) by apolipoproteins was studied. Pyrene-labeled cholesteryl ester was used as a probe substrate for the transfer reaction between lipid microemulsions, with a diameter of 26 nm, of triglyceride and phosphatidylcholine, and the reaction was monitored as a change in the ratio of the peaks of monomer and excimer in the fluorescence spectrum of pyrene. The transfer of pyrene-cholesteryl ester was hardly catalyzed by highly isolated LTP in the absence of apolipoprotein unless extreme overdose of LTP was given, regardless of the presence of bovine serum albumin. Human apolipoprotein (apo) A-I and apoA-II activated the LTP reaction in a dose-dependent manner. The activation was directly proportional to the titration of the surface of the substrate lipid emulsions by the apolipoproteins when the rate was plotted against the apolipoproteins bound to the surface. Human apoE also activated the LTP reaction in the same manner. The activation by human apoC-III was also proportional to the surface-bound protein, but the rate of the transfer was lower than those with other apolipoproteins. Displacement of apoA-I by apoC-III from the lipid emulsion surface, therefore, resulted in apparent deactivation of the LTP reaction. Thus, LTP requires apolipoproteins for its activation, and the activation seems proportional to the area of the surface of the lipid substrate particles modified by apolipoproteins. ApoA-I, -A-II, and -E are more potent activators than apoC-III for cholesteryl ester transfer.

Plasma lipid transfer protein (LTP)[1] facilitates the transfer of neutral lipids, such as cholesteryl ester and triacylglycerol, among plasma lipoproteins (Morton & Zilversmit, 1982; Tall, 1986). Since the majority of cholesteryl ester in plasma is generated by lecithin:cholesterol acyltransferase (LCAT) on plasma high-density lipoprotein (HDL) (Glomset et al., 1983), the LTP reaction has been thought to play an important role in the regulation of HDL metabolism. Indeed, cholesteryl ester accumulates in HDL, thereby enlarging its size in patients with a genetic defect of LTP (Inazu et al., 1990; Yamashita et al., 1990). It has also been shown that HDL level is lower in transgenic mice of human LTP than in normal mice which have no measurable plasma LTP activity (Agellon et al., 1991). The transfer of lipids by LTP seems based on their equimolar exchange between lipoproteins in vitro (Barter & Jones, 1971, 1980; Morton & Zilversmit, 1983; Kurasawa et al., 1985). Therefore, the net transfer of cholesteryl ester should occur as its heteroexchange with triglyceride between cholesteryl ester-rich and triglyceride-rich lipoproteins, i.e., HDL or low-density lipoprotein (LDL) and very-low-density lipoprotein (VLDL) or its remnant. HDL may become small as a constant influx of triglyceride is provided by LTP from VLDL or its remnant in exchange for newly-generated cholesteryl ester by LCAT since triglyceride is hydrolyzable by lipoprotein lipase or hepatic lipase unless it ends up in the LDL core which is no longer accessible to either lipase. A major portion of VLDL particles and its remnant becomes LDL in plasma that is eventually removed by the liver via the LDL receptor pathway (Bilheimer et al., 1985). Generation of cholesteryl ester on HDL by LCAT and its removal as LDL are both much slower than the rate of lipid transfer by LTP, so that core lipid exchange between plasma LDL and HDL leads to the distribution of these lipids between the two lipoproteins to nearly equilibrium in steady state (Barter & Jones, 1971, 1980; Morton & Zilversmit, 1983; Kurasawa et al., 1985). However, some portion of cholesteryl ester is directly taken up by hepatocytes as remnant particles much more rapidly than LDL, and the net transfer of cholesteryl ester to such particles may be rate limiting for delivery of cholesteryl ester to the liver in this pathway. Assuming that HDL accepts free cholesterol from peripheral cells and esterifies it, LTP plays an important role in the transport of these cholesterol molecules to the liver by transferring them to other lipoproteins. Regulation of this reaction is one of the crucial factors to regulate this hypothetical pathway.

We have reported that apolipoproteins activate the transfer of cholesteryl ester catalyzed by LTP between LDL and triacylglycerol/phospholipid microemulsions with the same size as LDL, using partially isolated human LTP (Nishikawa et al., 1988) and also highly purified LTP (Ohnishi et al., 1990). Unless the surface of the emulsion is covered with apolipoproteins, LTP hardly catalyzes the transfer of the core lipids between LDL and the emulsion. The reaction was activated only by covering the emulsion surface with various apolipoproteins with amphiphilic helices such as apoA-I, -A-II, -C-II, -C-III, and -E. However, the reaction system used in these studies had some technical limits in order to investigate this mechanism further; LDL was used as cholesteryl ester donor because of easy separation from the acceptor particles by selective binding to dextran sulfate–cellulose, but it has apolipoprotein B in itself so that the effect of apolipoprotein could not be completely eliminated, and the reaction was measured between the heterogeneous donor and acceptor lipoprotein particles by terminating the reaction to separate them at certain times of incubation and by determining the

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[1] Abbreviations: LTP, plasma lipid transfer protein; HDL, high-density plasma lipoprotein; VLDL and LDL, very-low- and low-density plasma lipoproteins; apo, apolipoprotein; LCAT, lecithin:cholesterol acyltransferase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; EDTA, ethylenediaminetetraacetic acid; PBS, sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1 mM EDTA.

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transfer of cholesteryl ester as a chemical quantity or as radioactivity (Nishikawa et al., 1988; Ohnishi et al., 1990).

In order to overcome these problems, we have established a new system to measure the lipid transfer reaction using the fluorescence of pyrene (Milner et al., 1991). The fluorescence spectrum of the pyrene compound is dependent on its local concentration. The emission fluorescence of pyrene when diluted in a microenvironment appears at about 396 nm from the lowest excited single state, and it is termed monomer fluorescence, and the fluorescence peak at 468 nm appears from excimer formation when concentrated. It has been known that the ratio of the intensity of the excimer fluorescence to that of the monomer fluorescence is a function of the concentration of pyrene in the microenvironment (Förster, 1969; Pownall & Smith, 1973; Charlton et al., 1976). Therefore, pyrene compounds have been used as a hydrophobic probe to estimate the local concentration of lipid in many reports to study lipid transfer among lipid vesicles or among lipoproteins (Roseman & Thompson, 1980; Correa-Freire et al., 1982; Massey et al., 1982; Jones & Thompson, 1989; Pownall & Smith, 1989). The method has an advantage in that the reaction can be monitored continuously without separation of the donor and acceptor particles. In a previous study (Milner et al., 1991), we applied the same strategy to measure pyrene-cholesteryl ester transfer between triolein/phosphatidylcholine microemulsions catalyzed by partially purified human LTP. The emulsion had previously been characterized thoroughly in terms of the method for preparation, the size and structure of the microemulsion, the integrity of cholesteryl ester and free cholesterol, and the binding of various apolipoproteins (Tajima et al., 1983; Yokoyama et al., 1985; Okabe et al., 1988), and in terms of the effect of the lipid transfer reaction on their gross structure (Nishikawa et al., 1988; Milner et al., 1991). Apolipoproteins were completely eliminated from the base-line condition for the transfer reaction using this assay system. We showed the activation of LTP by apoA-I and -A-II and proposed that the activation of LTP may be directly related to titration of the surface of the lipid particle by these apolipoproteins.

In this report, we describe details of the kinetics of LTP activation by various apolipoproteins, apoA-I, -A-II, -C-III, and -E, in this emulsion system with pyrene-cholesteryl ester as a substrate analogue, using highly isolated LTP from human plasma. We demonstrate that the presence of apolipoproteins on the surface of the substrate lipid particles is the essential requirement for the activation of the catalytic amount of LTP and the activation is proportional to the apolipoproteins bound to the lipid surface.

EXPERIMENTAL PROCEDURES

Chemicals. Cholesteryl (pyren-1-yl)-hexanoate and triolein were purchased from Sigma, and egg yolk phosphatidylcholine was from Avanti Polar-Lipids, Inc. Other chemicals used in the present study were of the best quality commercially available.

Preparation of Proteins. Human apoA-I and apoA-II were purified from the HDL fraction of fresh human plasma according to the methods described elsewhere (Yokoyama et al., 1982; Tajima et al., 1983). ApoC-III and apoE were isolated from the human VLDL fraction as described previously (Tajima et al., 1983; Yokoyama et al., 1985). Each apolipoprotein, shown as a single band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), was lyophilized and stored at −75 °C. An aqueous solution of each apolipoprotein was prepared according to the method of Yokoyama et al. (1982), Tajima et al. (1983), and Yokoyama et al. (1985), respectively, and the concentration of each protein was determined on the basis of its absorbance at 280 nm using the respective specific molar extinction coefficient found in Yokoyama et al. (1982), Tajima et al. (1983), and Yokoyama et al. (1985). Lipid transfer protein was purified from human plasma according to the method previously published by Ohnishi et al. (1990). The purified protein consists of 69- and 66-kDa LTP; both have the same specific activity of cholesteryl ester transfer between LDL and HDL (Ohnishi et al., 1990). Judging from the staining density of the protein bands on SDS–PAGE, 90% of the total LTP was 69-kDa protein, and 10% was 66-kDa protein in the particular preparation used, and no other band was observed on the gel. The final preparation, 1.4 mg/mL protein, was stored at −75 °C.

Preparation of the Lipid Microemulsion. Triolein/phosphatidylcholine microemulsion was prepared by sonication as described previously (Okabe et al., 1988). Triolein (20 mg) and egg yolk phosphatidylcholine (20 mg) were sonicated with 10 mL of 10 mM sodium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.1 mM EDTA (PBS) in a cup cooled with circulated water under a stream of nitrogen. After sonication, the emulsion was isolated by ultracentrifugation at 99 000 rpm for 30 min with a Beckman TL-100 ultracentrifuge and applied to a Sepharose CL-4B gel permeation column. The fractions, whose weight ratio of triolein to phosphatidylcholine was between 1.1 and 1.3, were collected and kept at 4 °C under argon gas. The microemulsion containing pyrene-labeled cholesteryl ester was prepared as described in a previous report (Milner et al., 1991). Cholesteryl (pyren-1-yl)-hexanoate (1 mg) was premixed with 20 mg of triolein and 20 mg of phosphatidylcholine, and the microemulsion was prepared in the same manner as the triolein/phosphatidylcholine microemulsion was prepared except that all procedures were conducted in darkness. The content of pyrene-cholesteryl ester in the core lipids (pyrene-cholesteryl ester and triolein) of the microemulsion was 5.5% (mol/mol).

Measurement of Fluorescence. The fluorescence of the microemulsion solution was measured at emission wavelengths of 396 nm for monomer pyrene and 468 nm for excimer pyrene with an excitation wavelength at 320 nm, using a Hitachi F-2000 fluorescence spectrophotometer. The excitation light was kept shut off until 20 s before the measurement took place, and the fluorescence was integrated for 5 s at each emission wavelength. The interval required for changing the emission wavelength was less than 2 s, and the samples containing microemulsions were kept under a stream of argon (10 mL/min) during measurement.

Measurement of the Lipid Transfer Reaction. The microemulsion without pyrene-labeled cholesteryl ester was preincubated with apolipoproteins in PBS, when they were used, in a final volume of 0.9 mL, and the fluorescence of the solution was measured as background. The microemulsion containing pyrene-labeled cholesteryl ester in 0.1 mL was added to the mixture at a ratio of phospholipid to plain emulsion of 1/9. After preincubation for 5 min at a room temperature, 5 μL of the human LTP solution was added, and the emission fluorescence of the solution was measured for 30 min at appropriate time intervals as described above. The background fluorescence was subtracted from the fluorescence at each wavelength, and the ratio of the emission fluorescence at 468 nm to that at 396 nm was calculated as an indicator of the pyrene concentration of the microemulsion at each time of

the incubation.

Calculation of the Lipid Transfer Rate. The ratio of the emission peak at 468 nm to that at 396 nm (Ex/Mo) was shown as an empirical linear function of the concentration of pyrene in the core of the microemulsion in a previous study (Milner et al., 1991): Ex/Mo (f) of the homogeneous microemulsion can be expressed as $f = a(c/L) + b$, where c is the concentration of pyrene-cholesteryl ester, L is the concentration of total core lipid (triacylglycerol and cholesteryl ester), and a and b are empirical constants. Given that the core lipid concentration of the acceptor particle (plain microemulsion) is sufficiently larger than that of the donor particle (pyrene-containing particle), the initial part of the transfer reaction is expressed as the equation [details of the derivation were described in our previous report (Milner et al., 1991)]:

$$\ln(f - b) = -(2k/L_1)t + \ln(ac/L_1) \quad (1)$$

where k is the average rate for equilibrium transfer of the total core lipid between the donor and acceptor particles and L_1 is the core lipid concentration of the donor particle. The constant b is the ordinate intercept of the linear plot of f against c/L. This value was experimentally obtained as a linear extrapolation to zero pyrene concentration of the ratio of the two peaks at the end point of the transfer reaction (Milner et al., 1991). Thus, the plot of $\ln(f - b)$ against the incubation time t gives the transfer rate k as a slope. This linearized plot was a valid approximation for the initial 50% of the change in the $(f - b)$ value when the donor/acceptor ratio was 1/9 since the error caused by the back-transfer of the pyrene compound from the acceptor particles should only be about 10% at maximum [see the derivation of the equations by Milner et al. (1991)].

Calculation of the Level of Surface Saturation of the Microemulsion by Apolipoproteins. The equation for the calculation of the level of the surface saturation by apolipoproteins is

$$\% \text{ saturation} = 100\{[(P_t + K_d + B_0 - \sqrt{(P_t + K_d + B_0)^2 - 4P_tB_0})/2]/B_0\} \quad (2)$$

where $B_0 = B_s \times$ (phospholipid concentration, M), B_s is the maximum level of surface saturation (apolipoprotein/phospholipid, mol/mol), K_d is the dissociation constant of apolipoprotein binding to the microemulsion, and P_t is the total apolipoprotein concentration in the mixture. For calculation of the percent saturation, binding constants were taken from our previous publications: The K_d values, 1.6×10^{-7}, 2.5×10^{-7}, 5.3×10^{-7}, and 5.6×10^{-7} M, were used for apo A-I, apoA-II, apoC-III (Tajima et al., 1983), and apoE (Okabe et al., 1988), respectively, and the B_s values, 2.39×10^{-3}, 9.35×10^{-3}, 1.22×10^{-2}, and 4.01×10^{-3} mol/mol for apoA-I, apoA-II, apoC-III (Tajima et al., 1983), and apoE (Okabe et al., 1988), respectively. The concentration of phospholipid used in the series of this study was 1.32×10^{-5} M (10 μg/mL) or 6.59×10^{-5} M (50 μg/mL). The molar concentration was calculated using the molecular weight of each component (780 for egg phosphatidylcholine, 28 026 for apoA-I, 17 414 for apoA-II, 8764 for apoC-III, and 34 145 for apoE).

Binding of Apolipoproteins to the Surface of the Microemulsion. To study the competitive binding of apoA-I with apoC-III, phosphatidylcholine/triolein microemulsion, 60 μg as phospholipid, was incubated with apoA-I, 16 μg, and with various amounts of apoC-III in a final incubation volume of 200 μL for 2 h at room temperature. The mixture was then centrifuged in a Beckman TL-100 ultracentrifuge at 99 000 rpm at 4 °C for 1 h using a TLA100 rotor, and the top 50 μL was collected by a microsyringe. The proteins in this fraction were analyzed by SDS–PAGE after delipidation with 200 μL of diethyl ether. After proteins were stained with Coomassie Brilliant Blue R-250, the gel was analyzed by a densitometric scanner (CAMAG, TLC Scanner II), and the bound apoA-I was quantitated for each condition relative to the amount bound in the absence of apoC-III. Free apoA-I background was determined by a blank sample incubated without the microemulsion and subtracted.

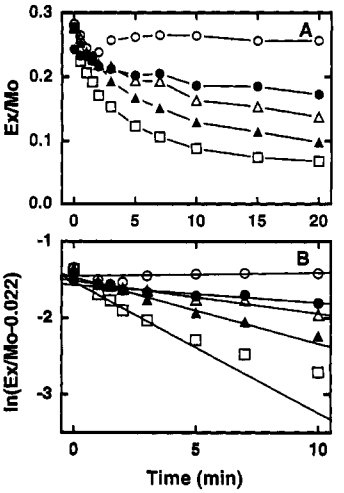

FIGURE 1: Time course of pyrene-cholesteryl ester transfer between lipid microemulsions catalyzed by LTP. The donor microemulsion particle containing pyrene-cholesteryl ester [5 μg of phospholipid and 5.5% (mol/mol) pyrene-cholesteryl ester in the triglyceride core] was incubated with the acceptor particle (45 μg of phospholipid of triolein/phospholipid microemulsion) and 20 μg of apoA-I in PBS, in a final volume of 1 mL, in the presence of human LTP [0 (○), 0.5 (●), 1 (△), 2 (▲), or 5 μg (□)]. (A) The ratio of excimer fluorescence to monomer fluorescence (Ex/Mo) was plotted against the incubation time; (B) the value of ln(Ex/Mo − 0.022) (3–10 min) was plotted against the incubation time. Solid lines represent least-squares linear regression lines for the initial 50% of the reaction. Details of the procedure and data calculation are described under Experimental Procedures and Results.

Other Analytical Methods. Concentrations of lipids were determined using enzymatic assay kits for triacylglycerol, choline-containing phospholipid, and total cholesterol, purchased from Wako Pure Chemical (Richmond, VA). The protein concentration of LTP was determined by a Pierce BCA protein assay kit using bovine serum albumin as a standard. SDS–PAGE was carried out according to the method of Laemmli (1970), and proteins were visualized by staining gel with Coomassie Brilliant Blue R-250.

RESULTS

The donor particle containing pyrene-cholesteryl ester [5 μg of phospholipid and 5.5% (mol/mol) pyrene-cholesteryl ester in the core] was incubated with the acceptor particle (45 μg of phospholipid in the triolein/phospholipid microemulsion) in the presence of 20 μg of apoA-I. The ratio of the excimer fluorescence peak to the monomer fluorescence peak was about 0.28 before the addition of LTP and remained constant in the absence of LTP for at least 30 min (Figure 1A), showing that the nonspecific transfer of pyrene-cholesteryl ester between the microemulsions is negligible without LTP even in the presence of apoA-I. When LTP was added to the mixture, the ratio started decreasing continuously except for fluctuation at the very initial stage due to stirring the solution at zero time. The rate of the reaction increased with increasing

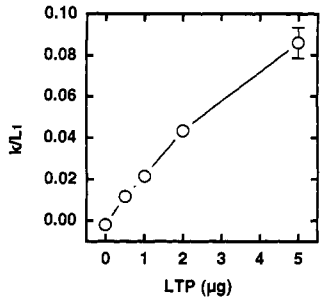

FIGURE 2: Dose dependency of the pyrene-cholesteryl ester transfer reaction on the amount of human LTP with a substrate concentration of 50 μg of phospholipid/mL. The empirical transfer rate constant (k/L_1) calculated as the slope of the least-squares linear regression line in Figure 1B was plotted against the amount of LTP. The error bars represent the standard error of the least-squares regression. The standard error was smaller than the size of the symbols when error bars are not shown.

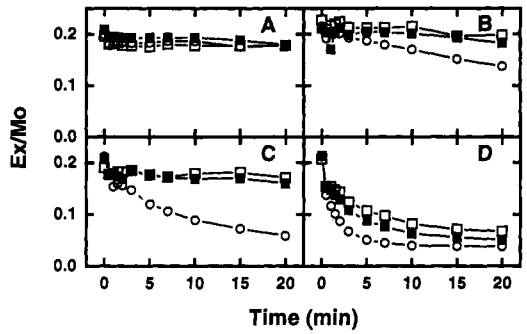

FIGURE 3: Pyrene-cholesteryl ester transfer between microemulsions catalyzed by human LTP with or without apoA-I or bovine serum albumin. One microgram of phospholipid of the donor particle was incubated with 9 μg of phospholipid of the acceptor particle in the absence (□) or presence of bovine serum albumin (■) or apoA-I (○). The reaction was initiated by adding various amounts of human LTP [(A) 0 μg; (B) 0.05 μg; (C) 0.2 μg; (D) 1 μg].

amounts of LTP from 0.5 to 5 μg (Figure 1A). The data were analyzed according to the linearized eq 1 described under Experimental Procedures. In order to obtain the b value for this particular experimental condition, 10 μg of LTP was added to the same mixture. The ratio of the peaks decreased to 0.048 within 10 min and remained constant for 30 min (data not shown) so that 0.022 was obtained as the constant b for the equation by linear extrapolation of the ratio to zero pyrene concentration (eq 1). The values of ln(Ex/Mo − 0.022) for each set of the experimental data were plotted against time for the initial part of the reaction because of the approximation assumed for derivation of eq 1 (Figure 1B) (Milner et al., 1991). Indeed, these plots gave reasonably good straight lines for the initial 50% of the reaction, and the empirical rate constant k/L_1 was calculated as the slope of the plots in Figure 1B using a least-squares linear regression program. Figure 2 is the plot of the rate constant against the concentration of LTP, demonstrating that the rate constant was directly proportional to the concentration of LTP.

The transfer reaction was also measured with a low concentration of microemulsion. The donor particles (1 μg of phospholipid) were incubated with the acceptor particle (9 μg of phospholipid) and LTP in the presence or absence of apoA-I or bovine serum albumin (Figure 3). In the absence of LTP, the ratio of the excimer fluorescence peak to the monomer fluorescence peak remained constant regardless of the presence of apoA-I and bovine serum albumin (Figure 3A), showing that the nonspecific transfer of pyrene-cholesteryl ester is negligible. When 0.05 or 0.2 μg of LTP was added to the reaction mixture, the transfer reaction was measurable

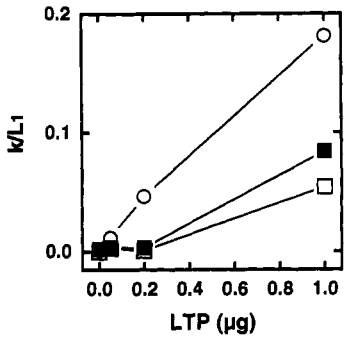

FIGURE 4: Dose dependency of the pyrene-cholesteryl ester transfer reaction on the amount of human LTP with a substrate concentration of 10 μg of phospholipid/mL. The empirical transfer rate constant (k/L_1) calculated from the slope of the linearized plot of the data shown in Figure 3 was plotted against the amount of LTP in the absence (□) or presence of bovine serum albumin (■) or apoA-I (○). The standard error was smaller than the size of the symbols.

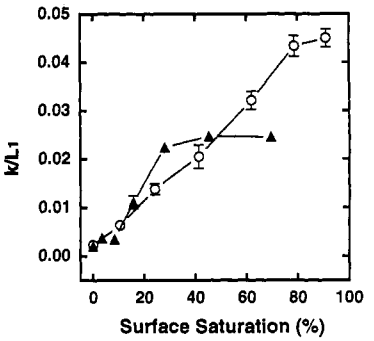

FIGURE 5: Correlation of the rate of pyrene-cholesteryl ester transfer by LTP to the extent of surface saturation of the substrate microemulsions (50 μg of phospholipid/mL) with apolipoprotein. The donor particle (5 μg of phospholipid) and the acceptor particle (45 μg of phospholipid) were incubated with 2 μg of LTP in the presence of various amounts (0–50 μg) of apoA-I (○) or apoE (▲). The empirical transfer rate constant (k/L_1) was calculated as the slope of the linearized plot (eq 1) and was plotted against the extent of surface saturation of the substrate emulsion with apolipoproteins, calculated using eq 2 as described under Experimental Procedures. The error bars represent the standard error of the least-squares regression. The standard error was smaller than the size of the symbols when error bars are not shown.

only in the presence of apoA-I (Figure 3B,C). The transfer was catalyzed only by the excess amount of LTP (1 μg) for the microemulsion (10 μg of phospholipid) in the presence or absence of bovine serum albumin (Figure 3D). The apparent ratio of the excimer peak to the monomer peak reached 0.038 when the excess amount of LTP was incubated with the microemulsion in the presence of apoA-I, so that the zero-extrapolated value, 0.019, was used as the constant b for eq 1 in this experimental condition in order to calculate the empirical rate constant. The rate constant was plotted against the concentrations of LTP and demonstrated to be linearly proportional to the concentration of LTP in the presence of apoA-I (Figure 4).

The transfer rate was measured with various concentrations of apoA-I between 0 and 50 μg in the same conditions as Figure 1: donor particle (5 μg of phospholipid), acceptor particle (45 μg of phospholipid), and 2 μg of LTP. The rate constant was calculated by eq 1 using b = 0.022 for each concentration of apoA-I. The percent saturation of the microemulsion surface was calculated according to eq 2 using the binding parameters listed under Experimental Procedures for each condition as well. The rate constant was plotted against the percent saturation of the surface (Figure 5). The plots appeared to be linear when up to 90% of the surface was

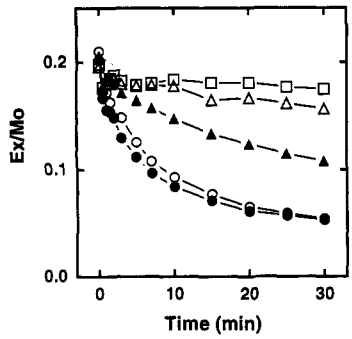

FIGURE 6: Effect of various apolipoproteins on pyrene-cholesteryl ester transfer between microemulsions by LTP. The donor particle (1 μg of phospholipid) and the acceptor particle (9 μg of phospholipid) were incubated with 0.2 μg of LTP in the presence of 5 μg of apoA-I (○), apoA-II (●), apoC-III (△), or apoE (▲) and in the absence of apolipoprotein (□). The ratio of excimer fluorescence to monomer fluorescence, Ex/Mo, was plotted against the incubation time.

covered with apoA-I. The maximum activation was more than 20-fold of the rate measured in the absence of apolipoproteins in this condition.

Human apoE also activated the LTP in a similar manner to apoA-I in the same condition. The same method of analysis was applied for the LTP reaction activated by apoE, and the calculated coverage of the emulsion surface was less than 65% even with 50 μg of apoE due to its relatively high apparent K_d (Okabe et al., 1988). In this condition, it was shown in Figure 5 that the rate constant was almost linear against the surface coverage of the emulsion with apoE with the same slope as that of apoA-I activation.

The activation of LTP by various apolipoproteins, 5 μg, in terms of pyrene-cholesteryl ester transfer between microemulsions (1 μg of donor and 9 μg of acceptor phospholipid) was investigated (Figure 6). ApoA-I and apoA-II gave almost the same extent of activation. ApoC-III, on the other hand, showed much less effect of the activation. Activation by apoE in this condition was again to the same extent as apoA-I and -A-II, consistent with the results of the experiments with higher concentration of substrates.

The rate of the reaction was measured in the presence of various concentrations of apoA-I, -A-II, -C-III, and -E. The rate constants were calculated using the constant $b = 0.019$ and eq 1 for each reaction, and the percent surface saturation of the emulsion with apolipoproteins was also calculated using the binding parameters listed under Experimental Procedures and eq 2 (Figure 7). The plots of the rate constants against the surface saturation with apolipoproteins were linear to the surface coverage with apolipoproteins up to more than 50% saturation except for apoE.

To confirm that enhancement of the transfer reaction by apolipoproteins was due to the surface coverage with apolipoprotein, the apoA-I-activated LTP reaction was modified by displacing apoA-I from the surface by apoC-III (Figure 8). One microgram of phospholipid of donor particles and 9 μg of acceptor particle phospholipid were preincubated with 3 μg of apoA-I, and then an additional 0–30 μg of apoA-I and apoC-III was incubated with 0.2 μg of LTP. When apoA-I was added further to the reaction mixture, the rate constant increased slightly as apoA-I saturated the rest of the lipid emulsion surface, being consistent with the original entire activation profile by apoA-I. ApoC-III deactivated the reaction to the level of activation by apoC-III itself as its concentration increased.

The binding of apoA-I in the absence and the presence of apoC-III is also shown in Figure 8. ApoC-III displaced apoA-I

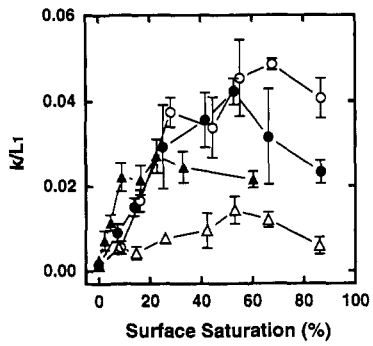

FIGURE 7: Correlation of the rate of pyrene-cholesteryl ester transfer by LTP to the extent of surface saturation of the microemulsions (10 μg of phospholipid/mL) with various apolipoproteins. The donor particle (1 μg of phospholipid) and the acceptor particle (9 μg of phospholipid) were incubated with 0.2 μg of LTP in the presence of various amounts (0–30 μg) of apoA-I (○), apoA-II (●), apoC-III (△), and apoE (▲). The rate of pyrene-cholesteryl ester transfer (k/L_1) was calculated as the slope of the linearized plot (eq 1) and was plotted against the extent of surface saturation of the substrate emulsion calculated according to eq 2. The error bars represent the standard error of the least-squares regression. The standard error was smaller than the size of the symbols when error bars are not shown.

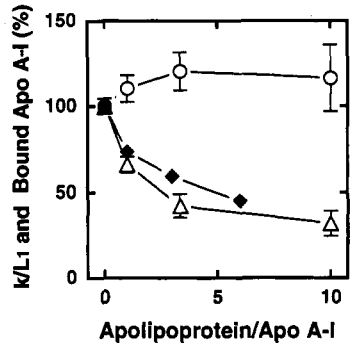

FIGURE 8: Effect of apoC-III on the apoA-I-activated LTP reaction with microemulsion substrate and on apoA-I binding to substrate particles. For the transfer reaction, the donor particle (1 μg of phospholipid) and acceptor particle (9 μg of phospholipid) were incubated with apoA-I (3 μg) and LTP (0.2 μg) in the presence of various amounts of additional apoA-I (○) and apoC-III (△) in a final volume of 1 mL. The empirical rate constant of pyrene-cholesteryl ester transfer (k/L_1) relative to that without additional apolipoproteins (0.0143 = 100%) was plotted against the additional amount of apolipoproteins. For displacement of apoA-I by apoC-III from the surface of the lipid microemulsion, the lipid microemulsion (60 μg of phospholipid) was incubated with apoA-I (16 μg) and the indicated amounts of apoC-III in a final incubation volume of 200 mL at room temperature for 2 h. The error bars represent the standard error of the least-squares regression. The lipid-bound apolipoproteins were floated to the top fraction, 50 μL, by ultracentrifugation, delipidated, and quantitated by densitometric scanning of the stained protein bands in SDS–PAGE. The bound apoA-I is shown as a relative amount to that measured without apoC-III (♦).

in a dose-dependent manner within the range of their concentrations studied (up to 6 times apoA-I by weight), providing further direct supporting evidence for the hypothesis that the surface-bound apolipoproteins are responsible for the activation. The decrease of the bound apoA-I by apoC-III was almost superimposable on the decrease of the transfer activity by apoC-III (Figure 8) as a function of the apoC-III/apoA-I ratio.

DISCUSSION

In a previous study (Milner et al., 1991), we established the method to monitor the lipid transfer reaction using pyrene-cholesteryl ester as a probe substrate analogue. The pyrene

compound was incorporated into the core of the triolein/phosphatidylcholine microemulsion with an average diameter of 26 nm, and the ratio of excimer fluorescence to monomer fluorescence of the compound was empirically shown to be directly proportional to its concentration in the core triglyceride (Milner et al., 1991). When the transfer of the core lipids was catalyzed by human LTP, this ratio decreased as the reaction proceeded, and the rate constant calculated from the time course kinetics was proportional to the LTP concentration. This method has many advantages over the conventional methods of the transfer assay using radiolabeled lipid. The method enables us to reconstitute the reaction system with relatively homogeneous donor and acceptor lipid microemulsion particles since both microemulsions have the same surface with the same curvature and have virtually similar binding properties to apolipoproteins (Okabe et al., 1988). The method does not require separation of the donor and acceptor particles to measure lipid transfer, and thereby the reaction can be continuously monitored in the same incubation mixture on real time. The method provided us for the first time with a tool to study the effect of various factors on the LTP reaction since the structure of the substrate particles mimics plasma lipoproteins and the base-line condition does not include any apolipoproteins.

In a previous work (Milner et al., 1991), we demonstrated that apoA-I and -A-II activated the LTP reaction using partially purified LTP in the same reaction system. In order to calculate the rate of the reaction, a simplified empirical method for analysis of the data was developed employing some approximations in the derivation of the equation to calculate the rate. This simplified analysis we have employed gave a reasonably good approximation for the rate of the lipid transfer reaction, based on fitting the data to the linearized plot for the initial 50% of the reaction and the linear relation of the rate constant of the reaction to the amount of LTP.

Thompson and his colleagues derived equations to solve this problem by a more theoretical approach (Roseman & Thompson, 1980; Correa-Freire et al., 1982; Jones & Thompson, 1989). However, there were some technical difficulties for direct application of their method to analyze the data obtained from the emulsion system; it was difficult to incorporate pyrene-cholesteryl ester more than several percent in the core of the emulsion by the procedure used in the present technique, and thereby the projection of the calibration to "maximum excimer intensity" that is necessary for the data analysis did not seem to be justified; inevitable heterogeneity of the lipid composition of the emulsion particle may also be a factor to make it somewhat irrelevant to apply their equation directly to our assay system.

In this report, we have used highly isolated LTP from human plasma and studied intensively the essential effects of apolipoproteins on the lipid transfer reaction catalyzed by pure LTP using this system. Lipid transfer by LTP between the microemulsion was negligible in the absence of apolipoproteins unless an excess amount of LTP was provided. ApoA-I, -A-II, and -E activated LTP in this reaction system, showing that these apolipoproteins are essentially required for the LTP reaction. The activation by apoC-III was to a less extent than that by apoA-I, -A-II, and -E. Thus, the enhancement of the LTP reaction is somewhat specific for certain apolipoproteins.

Activation of transfer was demonstrated to be directly proportional to the extent of surface coverage of the microemulsion by apolipoproteins that was calculated according to the binding parameters of these apolipoproteins for the same lipid microemulsions. This was clearly shown for apoA-I, apoA-II, and apoC-III. In the case of apoE, however, the activation seems to have already reached the maximum when the surface is titrated only 30–40% according to the calculation using the available binding constants (Okabe et al., 1988). Although apoA-I and apoA-II self-associate in an aqueous phase, their dissociation is rapid, and the proteins become mostly monomeric when they are diluted to the concentration used in the binding study (Yokoyama et al., 1982; Tajima et al., 1983). Self-association of apoC-III is negligible (Tajima et al., 1983; Yokoyama et al., 1985). Thus, binding parameters measured for these proteins are valid to describe the equilibrium between the bound protein and the monomer in the aqueous phase even for such low concentrations of the proteins as used in this study (Tajima et al., 1983). However, apoE strongly forms self-associated tetramer, and it seems to dissociate only in very diluted solution (Yokoyama et al., 1985). The dissociation constant measured for apoE was, therefore, an apparent and empirical value to describe the equilibrium between the bound protein and the mostly tetrameric nonbound protein. This perhaps gives us significant overestimation of the dissociation constant for the equilibrium between bound and monomeric nonbound protein (Yokoyama, 1990). In the experiment with the lower substrate concentration in this study, the apoE concentration is so low that a substantial part of nonbound apoE may be monomeric and therefore the bound apoE concentration may be underestimated by calculation using the binding constants available. This has probably caused apparent deviation of the data with apoE from direct titration of the surface to a greater extent in the analysis of the data with the low substrate concentration than the high substrate concentration.

ApoC-III was capable of displacing apoA-I from the surface of the substrate lipid microemulsion. Displacement of apoA-I by apoC-III from the lipid particle deactivated the apoA-I-activated LTP reaction to the level of activation by apoC-III, providing further supporting evidence that the surface concentration of the activator on the emulsion is a direct regulatory factor for this activation.

Thus, we conclude that LTP is activated by certain apolipoproteins, as they titrate the surface of phospholipid/triolein microemulsions. This mechanism is analogous to activation of LCAT by apolipoproteins or their model peptides (Yokoyama et al., 1980). Details of the activation of LCAT are still unknown, and the LTP activation also remains to be answered. Apolipoproteins cover the surface of lipid particles, filling gaps between phospholipid head groups by hydrophobic interaction (Yokoyama et al., 1980; Shen et al., 1972). Without apolipoproteins, hydrophobic acyl chains are directly exposed to the aqueous phase at these gaps, generating high interfacial energy, so that hydrophobic core lipids may come to this interface with great difficulty. When apolipoproteins fill these gaps, this energy barrier is reduced, and the core lipids would have a much greater chance to come close to this interface. If LTP and apolipoproteins are readily exchangeable at this site, LTP has more chance to meet substrate nonpolar lipids. This hypothesis must be tested in further investigations.

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