Phospholipid Liposomes Acquire Apolipoprotein E in Atherogenic Plasma and Block Cholesterol Loading of Cultured Macrophages

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Abstract

A single infusion of phospholipid liposomes promptly and persistently abolished the ability of hypercholesterolemic rabbit plasma to cause cholesteryl ester loading in cultured macrophages. This phospholipid enrichment of plasma caused moderate stimulation of cellular cholesterol efflux and, unexpectedly, almost complete inhibition of cellular uptake of β -very low density lipoprotein (β -VLDL), the major cholesteryl ester-rich particle in hypercholesterolemic rabbit plasma. Cell viability and LDL receptor activity were unaffected. Incubation of liposomes with β -VLDL resulted in transfer of apolipoprotein-E (apoE) to the liposomes; reisolated apoE-phospholipid liposomes then competed efficiently for cellular apoprotein receptors. Thus, a major mechanism by which phospholipid infusions result in diminished accumulation of cholesteryl ester in cultured macrophages is by blocking cellular uptake of β -VLDL. The liposomes deplete β -VLDL of apoE, then compete for receptor-mediated uptake. These results suggest a novel mechanism contributing to the known antiatherogenic effect of phospholipid infusions: infused liposomes acquire apoE, then block uptake of atherogenic lipoproteins by arterial wall macrophages.

Introduction

Cholesterol-fed rabbits develop deposits of cholesterol in their arterial walls and have been used extensively as an experimental model of atherosclerosis. The deposits of cholesterol result from uptake by vessel wall macrophages of β -very low density lipoprotein (β -VLDL), the major cholesteryl ester-rich lipoprotein in the plasma of cholesterol-fed rabbits (1). Macrophage uptake of β -VLDL is mediated by a cell surface receptor that is immunologically identical to the LDL receptor but displays different functional properties (2).

Three decades ago, repeated intravenous administration of dispersed phospholipid was shown to produce rapid, substantial shrinkage of atherosclerotic deposits in cholesterol-fed rabbits (3). This result has been confirmed by several laboratories using a variety of animal models (4–11). Phospholipid infusions have recently been shown to result in the appearance of vesicular lipoproteins in plasma (12). The vesicular lipoproteins remove cholesterol from other lipoproteins and from tissues (12) and they acquire apolipoprotein (apo) E¹ and bind to LDL receptors

(13). These observations support the hypothesis that the antiatherogenic effect of phospholipid infusions results in part from uptake of tissue cholesterol by vesicular lipoproteins, with reverse cholesterol transport to the liver mediated perhaps by hepatic apolipoprotein receptors (11).

The most striking examples of phospholipid-induced regression of experimental atherosclerosis were those in which regression was shown to occur even while the animals were maintained on the high cholesterol diets (9, 10). Thus, the phospholipid infusions converted highly atherogenic plasma into antiatherogenic plasma. Using cultured macrophages as a model of the macrophages in the arterial wall, we investigated the effect of phospholipid infusions on the ability of hypercholesterolemic rabbit plasma to cause cellular cholesterol loading and efflux. We found that phospholipid enrichment of plasma produced a moderate enhancement of cellular cholesterol efflux *in vitro*. However, added phospholipid liposomes unexpectedly produced a dramatic inhibition in cellular uptake of the atherogenic β -VLDL particles.

A preliminary report of this work was presented at the 59th Scientific Sessions of the American Heart Association (14).

Methods

Lipoproteins and liposomes. Rabbit β -VLDL ($d < 1.006$ g/ml) was prepared by ultracentrifugation of hypercholesterolemic rabbit plasma. Human LDL ($d = 1.019$ – 1.063 g/ml) was prepared by ultracentrifugation of fresh human plasma (15). Acetyl-LDL was prepared by reaction with acetic anhydride (16).

Radioiodinated β -VLDL and LDL were prepared by a modification of the iodine monochloride method (17). By dodecyl sulfate-polyacrylamide gel electrophoresis (18) of the ¹²⁵I- β -VLDL, 71.0% of recovered radioactivity was in apoB, 6.6% was in apoE, 4.5% in apoA-I, and 6.0% in C-apoproteins. [³H]cholesteryl ester-labeled β -VLDL, LDL, and acetyl-LDL were prepared by incubation with labeled HDL (19) and cholesteryl ester transfer protein, followed by ultracentrifugal reisolation of the less dense lipoproteins. By silica-gel thin-layer chromatography (20), > 98% of tritium in the labeled β -VLDL, LDL, and acetyl-LDL was in cholesteryl ester.

10% (wt/vol) dispersions of chromatographically pure egg phosphatidylcholine (Sigma Chemical Co., St. Louis, MO, catalogue No. P-2772) in physiologic saline (0.9%) were prepared by ultrasonic irradiation at 0°C under argon for a total of 40 min, followed by centrifugation to remove fragments of titanium shed by the sonicator probe (21). The sonicated phosphatidylcholine was then sterilized by passage through a 0.45- μ m filter (Millex-GS, Millipore Corp., Bedford, MA). Sonicated phosphatidylcholine liposomes were always used within 24 h of preparation.

Rabbits. New Zealand white rabbits were given a cholesterol-enriched diet (0.5%) to raise plasma cholesterol concentrations to 7–12 mmol/liter. These cholesterol-fed rabbits were each given a single bolus infusion of 300–400 mg of phosphatidylcholine liposomes per kilogram of body weight. Plasma was always obtained just before ($t = 0$ h) and 20 min after each bolus infusion. In a long-term experiment, plasma was also obtained at 1, 2, 4, 8, and 24 h after the bolus infusion. Plasma cholesterol,

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Received for publication 28 October 1986.

1. *Abbreviations used in this paper:* apo, apolipoprotein; DMEM, Dulbecco's modified Eagle's medium; fBSA, fatty acid free bovine serum albumin.

J. Clin. Invest.

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0021-9738/87/05/1466/07 \$1.00

Volume 79, May 1987, 1466–1472

cholesteryl ester, and lipid phosphorus were measured by standard assays (22–24).

Cultured macrophages. Two types of cultured macrophages were used: the J774 macrophage-like tumor line, which accumulates cytoplasmic droplets of cholesteryl ester when incubated with β -VLDL, acetyl-LDL, or unmodified LDL (25), and the resident mouse peritoneal macrophage, which accumulates cholesteryl ester when incubated with β -VLDL or acetyl-LDL (1, 26). Monolayer cultures of the J774 macrophages were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum (FCS). Resident mouse peritoneal macrophages were prepared as needed by cold saline irrigation of the peritoneal cavities of sacrificed mice. Macrophages were separated from other peritoneal cells by their ability to adhere firmly to plastic wells during a 2-h incubation at 37°C (27).

J774 cells were plated into 35-mm dishes, then loaded with [3 H]cholesterol by either an 18-h incubation in DMEM/FCS with [(7- 3 H)cholesteryl ester]-LDL (0.6 mg protein/ml) or by a 2-h incubation in DMEM/FCS with [(7- 3 H)cholesteryl ester]-acetyl-LDL (10 μ g protein/ml). Radioactive media were then removed from each dish, and the cells were washed three times in phosphate-buffered saline (pH 7.4).

To assess the ability of pre- and postinfusion hypercholesterolemic rabbit plasma to cause cellular cholesterol loading and cholesterol efflux, the labeled J774 cells were incubated for 18 h in DMEM supplemented with fatty acid-free bovine serum albumin (fBSA) (control medium) or in control medium supplemented with 10% (vol/vol) pre- or postinfusion hypercholesterolemic rabbit plasma. Additional wells of labeled cells were incubated in control medium supplemented with preinfusion plasma plus 0.6 mg liposomal phospholipid/ml, or with liposomes without plasma.

Cells were then scraped into the media, and media and cells were separated by low-speed centrifugation. Cells were resuspended in buffer and repelleted three times to remove any residual media. Cells and media were separately counted for 3 H-radioactivity; the percentage of total radioactivity (i.e., in cells plus media) remaining in the cells was computed for each well. Cells were assayed for protein by the method of Lowry et al. (28) and for total and unesterified cholesterol by gas-liquid chromatography using β -sitosterol as an internal standard (23). Cholesteryl ester content was calculated as the difference between total and unesterified cholesterol. Cholesterol values were normalized for cellular protein content. Cellular viabilities were determined by trypan blue exclusion (29).

Analogous experiments were performed with mouse peritoneal macrophages. Mouse peritoneal macrophages were loaded with [3 H]cholesterol by a 2.5-h incubation in [(7- 3 H)cholesteryl ester]-acetyl-LDL (10 μ g protein/ml). These labeled macrophages were then incubated in DMEM/0.1% fBSA without supplementation (control) or in control medium supplemented with β -VLDL alone or with β -VLDL and added liposomes. Cellular cholesterol masses, viabilities, retained 3 H radioactivity were measured as described above.

Direct measurement of cellular uptake and degradation of β -VLDL. [125 I]- β -VLDL was incubated with J774 or mouse peritoneal macrophages in DMEM with or without plasma, and with or without added liposomes. Cell association of [125 I]- β -VLDL was determined by measuring cellular [125 I]-radioactivity (30). Degradation of [125 I]- β -VLDL was determined by measuring the release of trichloroacetic acid-soluble, chloroform-insoluble [125 I]-radioactivity into the media. Cell-specific degradation was calculated by subtracting spontaneous degradation measured in cell-free wells from the total degradation measured in the presence of cells (30). Spontaneous degradation was at most 3.2 ng [125 I]- β -VLDL protein per 35-mm tissue culture well. These cell-association and degradation data reflect primarily the catabolism of apoB, which contained most of the radioactivity in the [125 I]- β -VLDL. Cell association was determined for [(7- 3 H)cholesteryl ester]- β -VLDL by measuring cellular 3 H radioactivity.

Reisolation of liposomes and β -VLDL after incubation. Unlabeled β -VLDL was dialyzed against 50 mM sodium phosphate buffer, pH 7.4. Phosphatidylcholine liposomes were prepared by sonication in 50 mM sodium phosphate buffer. Aliquots of dialyzed β -VLDL, each containing 18 mg protein, were incubated for 2 h at 37°C with either buffer (control) or with 900 mg of liposomal phospholipid in 50 mM phosphate buffer.

Incubated mixtures were then applied to 2.5 $\times$ 30-cm beds of hydroxyapatite, and unbound material was eluted with 50 mM phosphate buffer. Bound material was then eluted by a linear gradient of phosphate concentrations, from 50 to 650 mM, as previously described (13, 31).

Fractions eluted from the hydroxyapatite columns were analyzed for their content of phospholipid (24), cholesterol, cholesteryl ester (23), protein (32), and protein species (18). Reisolated vesicles were identified by a high content of phospholipid with little cholesteryl ester (13) and no apoB (13, 31). Reisolated β -VLDL was identified by its high content of cholesteryl ester and apoB.

Competitive binding studies. Human foreskin fibroblasts, frozen in the 6th–8th passage, were thawed, plated at 10^5 cells per 35 mm dish, and grown to subconfluency in DMEM/FCS. The cells were then grown to near-confluency in 5% lipoprotein-deficient FCS, to stimulate the activity of LDL receptors (30). Competitive displacement studies were conducted at 4°C between [125 I]-LDL and unlabeled particles isolated from incubated mixtures of β -VLDL with or without liposomes (30).

Statistical analyses. Unless otherwise stated, values for which errors are given are means $\pm$ SEM for three separate determinations ($n = 3$).

Results

Modification by liposomes of cellular accumulation of cholesterol.

J774 cells were preloaded with [3 H]cholesterol, then incubated for 18 h in DMEM/fBSA (plasma-free control), or in control medium supplemented with 10% (vol/vol) hypercholesterolemic rabbit plasma obtained just before or 20 min after a single intravenous bolus of 300 mg phospholipid/kg body wt (Fig. 1). Incubation with preinfusion, hypercholesterolemic plasma in-

Figure 1. Effect of a phospholipid infusion on the ability of plasma from a hypercholesterolemic rabbit to cause cholesterol loading and cholesterol efflux from J774 macrophages. J774 macrophages were preloaded with cholesterol mass and radioactivity by an 18-h incubation at 37°C with [(7- 3 H)cholesteryl ester]-LDL in DMEM with 10% (vol/vol) FCS. Preloaded cells were washed, then incubated for an additional 18 h in DMEM plus 1.5% fBSA (control), or in control medium supplemented with 10% (vol/vol) hypercholesterolemic rabbit plasma obtained just before (+ 10% PRE) or 20 min after (+ 10% POST) a single bolus infusion of 300 mg liposomal phospholipid/kg body weight. Additional wells of preloaded cells were incubated in 10% preinfusion plasma plus 0.6 mg egg phosphatidylcholine/ml medium (+ Lps + 10% PRE), or egg phosphatidylcholine without plasma (+ Lps). Plasma cholesterol concentrations were 9.8 mmol/liter before the infusion and 9.5 mmol/liter at 20 min after the infusion. Cells and media were separately counted for cholesterol radioactivity following these incubations; the percentage of total radioactivity (i.e., in cells plus media) remaining in the cells was then computed. Displayed are means and standard errors of the means for total cellular cholesterol (□), cholesteryl ester (■), and retained radioactivity (▨) from a representative experiment. These values were determined from three separate tissue culture wells in each experimental group. Cellular viabilities by trypan blue exclusion were 85–94%.

creased the cellular contents of total cholesterol and cholesteryl ester to approximately twice the control values (Fig. 1). In contrast, incubation with postinfusion plasma from the same rabbit resulted in essentially no net cellular accumulation of either total cholesterol or cholesteryl ester compared to control (Fig. 1).

Performing this experiment with a range of cholesterol contents in the preloaded, control cells (101.2, 153.4, and 181.3 nmol total cholesterol/mg protein) and a range of cholesterol concentrations in the preinfusion, hypercholesterolemic plasma (7.3, 9.8, and 11.5 mmol/liter) produced the same results: preinfusion plasmas consistently caused large increases in total cellular cholesterol and cholesteryl ester over control, whereas postinfusion plasmas resulted in total cholesterol and cholesteryl ester contents nearly identical to control (not displayed). Thus, although the preinfusion, hypercholesterolemic rabbit plasmas were extremely potent in cholesterol-loading the cells, plasmas obtained from the same rabbits 20 min after a phospholipid infusion were nearly inert.

Despite the large difference in cellular cholesterol content between J774 cells incubated with pre- and postinfusion hypercholesterolemic plasma, a comparatively small difference was observed in the amount of cholesterol radioactivity retained by these cells (Fig. 1, hatched bars). The cells retained 19% less total cholesterol radioactivity in postinfusion plasma than in preinfusion plasma, but the total cholesterol mass was 44% less. Phospholipid-enrichment of hypercholesterolemic plasma *in vivo* by infusion or *in vitro* by adding liposomes to preinfusion plasma produced nearly identical effects on cellular cholesterol mass and radioactivity (Fig. 1). Phospholipid liposomes without plasma produced parallel losses of cholesterol mass and radioactivity (Fig. 1).

Similar impairment of cholesterol loading was seen with mouse peritoneal macrophages. Mouse peritoneal macrophages were preloaded with [^3H]cholesterol by incubation with acetyl-LDL. Preloaded cells were then incubated for 18 h in DMEM/fBSA (control) or in control medium supplemented with β -VLDL alone (300 μg total cholesterol/ml) or with β -VLDL and liposomes (1.1 mg sonicated phospholipid/ml). Incubation with β -VLDL in the absence of liposomes increased cellular total cholesterol content to $134\pm 1.8\%$ of control, while the cells retained $66.3\pm 0.27\%$ of their preloaded cholesterol radioactivity. Incubation with β -VLDL in the presence of liposomes resulted in a cellular cholesterol content of $98\pm 1.2\%$ of control, and the cells retained $62.3\pm 0.25\%$ of their preloaded radioactivity. Thus, liposomes abolished the ability of β -VLDL to cholesterol-load mouse peritoneal macrophages, while having essentially no effect in the presence of β -VLDL on cellular retention of cholesterol radioactivity.

Modification by liposomes of cellular uptake of β -VLDL. The contrasting effects on cholesterol mass and retained cholesterol radioactivity in J774 and mouse peritoneal macrophages strongly suggest that decreased accumulation of cellular cholesterol in the presence of liposomes does not primarily result from enhanced cholesterol efflux, but is rather the result of decreased uptake of β -VLDL cholesterol from the media. To measure directly the cellular uptake of β -VLDL from pre- and postinfusion plasma, J774 macrophages were incubated for 5 h at 37°C in DMEM supplemented with ^{125}I - β -VLDL (0.4 μg protein/ml) and either 10% preinfusion hypercholesterolemic rabbit plasma or 10% plasma obtained from the same rabbit 20 min after a single bolus of 300 mg liposomes/kg body weight. Cell-association and degradation of ^{125}I - β -VLDL in 10% postinfusion hy-

percholesterolemic rabbit plasma were only $13.3\pm 0.2\%$ and $9.4\pm 0.3\%$, respectively, of the values in 10% preinfusion plasma. Similarly, the cell-association of [^3H]cholesteryl ester]- β -VLDL in postinfusion plasma was $26.4\pm 0.5\%$ of the value in preinfusion plasma. These results document that cellular uptake and degradation of β -VLDL is markedly reduced in the presence of liposomes.

The reduction in cell-association and degradation of ^{125}I - β -VLDL also occurred when sonicated phospholipid was added to isolated ^{125}I - β -VLDL in the absence of plasma. In dose-response experiments, there was a progressive decrease in cell-association (not displayed) and degradation (Fig. 2) of isolated ^{125}I - β -VLDL with increasing amounts of liposomal phospholipid. The results were identical for J774 cells (filled circles) and mouse peritoneal macrophages (open circles). The maximum effect was reached at a 43:1.0 mass ratio of liposomal phospholipid to β -VLDL protein, which corresponds to a 2.9:1.0 molar ratio of liposomal phospholipid to β -VLDL cholesterol.

Mechanism of blocked cellular uptake of β -VLDL: possible alterations in the cells. To determine the mechanism of phospholipid-induced blockage of β -VLDL uptake and degradation by model foam cells, we first examined if the effect was the result of alterations in the cells. By trypan blue exclusion, phospholipid liposomes over a wide range of concentrations had no effect on the viability of J774 cells or mouse peritoneal macrophages (Figs. 1 and 2). In addition, J774 cells shown to be nearly incapable of degrading ^{125}I - β -VLDL in the presence of liposomes were capable of fully normal rates of degradation when the liposome-containing media were removed and immediately replaced with ^{125}I - β -VLDL in liposome-free media (Fig. 3). Cell survival and lipoprotein degradation pathways thus had remained normal.

Mechanism of blocked cellular uptake of β -VLDL: alterations in the lipoproteins. We next examined if phospholipid-induced blockage of β -VLDL uptake and degradation was the result of alterations in the lipoproteins in the media. Liposomal phos-

Figure 2. Dose response of added phospholipid on ^{125}I - β -VLDL degradation by cultured macrophages. J774 cells (●) were incubated for 4 h and mouse peritoneal macrophages (○) were incubated for 5 h at 37°C in DMEM supplemented with 0.1% fBSA, 0.5 μg ^{125}I - β -VLDL protein/ml, and the indicated concentrations of sonicated egg phosphatidylcholine. Cell-specific degradation of ^{125}I - β -VLDL protein was then determined. Degradation of ^{125}I - β -VLDL in the absence of added phospholipid (100% on the y-axis) (□) by J774 cells was 0.3 $\mu\text{g}/\text{mg}$ cellular protein and by mouse peritoneal macrophages was 0.8 $\mu\text{g}/\text{mg}$ cellular protein. Displayed are means for values obtained in three separate culture wells in each experimental group. Maximum standard errors of the means were $\pm 3.4\%$ for J774 cells and $\pm 2.1\%$ for mouse peritoneal macrophages. Cell viabilities were 70–90% for the J774 cells and 75–80% for the mouse peritoneal macrophages; viabilities did not vary systematically with the concentration of egg phosphatidylcholine.

Figure 3. Degradation of ^{125}I - β -VLDL by J774 cells that had been previously incubated with ^{125}I - β -VLDL and liposomes. J774 cells were subjected to two sequential 5 h incubations at 37°C in lipoprotein-free media supplemented with $0.4 \mu\text{g}$ ^{125}I - β -VLDL protein/ml and 0.6 mg liposomal (Lps) phospholipid/ml, as indicated. During the first incubation, degradation of ^{125}I - β -VLDL in the presence of phospholipid liposomes was $3.53 \pm 0.16 \text{ ng protein/mg cell protein}$ ($n = 9$). Degradation of ^{125}I - β -VLDL during the second incubation is shown. Displayed are means and standard errors of the means for three separate determinations.

pholipid (900 mg) was incubated with isolated β -VLDL (18 mg protein). Vesicular particles were then separated from the β -VLDL by hydroxyapatite chromatography.

Two populations of vesicular particles were isolated from the hydroxyapatite column, one that eluted in the initial 50-mM phosphate buffer (vesicles I) and one that eluted at the beginning of the phosphate gradient (vesicles II). Reisolated β -VLDL eluted toward the end of the phosphate gradient. The absence of β -VLDL in vesicles I and II was verified by the lack of apoB, as determined by dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 4), and the low contents of cholesteryl ester ($\leq 0.6 \text{ mass\%}$ in these preparations). Both vesicles I and II acquired unesterified cholesterol from β -VLDL. In addition, both preparations acquired protein from β -VLDL, although the mass ratio of protein to phospholipid in vesicles II was approximately six times the ratio in vesicles I. Because the predominant protein in both preparations was apoE (Fig. 4), the higher mass ratio of protein to phospholipid in vesicles II indicates a higher apoE content.

In competitive displacement studies, vesicles I and II showed potent receptor binding, whereas protein-free phospholipid liposomes had little effect (Fig. 5 A). Of the two preparations of reisolated vesicles, vesicles II showed greater receptor affinity (Fig. 5 A), consistent with its greater apoE content.

Compared to β -VLDL that had been incubated without liposomes then subjected to hydroxyapatite chromatography (control β -VLDL), the β -VLDL reisolated after incubation with liposomes (β -VLDL-PPL) became phospholipid enriched, while losing about half its unesterified cholesterol and a quarter of its protein. The reisolated β -VLDL-PPL still contained apoE detectable by polyacrylamide gel electrophoresis; however, 50%

Figure 4. Protein species acquired by phospholipid vesicles during incubation with β -VLDL. Phospholipid vesicles were incubated with β -VLDL, then reisolated by hydroxyapatite chromatography, as described in the text. Reduced, delipidated samples were subjected to protein electrophoresis on a dodecyl sulfate-polyacrylamide gradient ($6\text{--}16\%$) gel. Lane S, molecular weight standards. Lane VI, vesicles I. Lane VII, vesicles II. The heavily stained bands in lanes VI and VII comigrated with the apoE band of rabbit β -VLDL.

inhibition of ^{125}I -LDL receptor binding required approximately three times as much β -VLDL-PPL as control β -VLDL (Fig. 5 B). Thus, during coincubation, liposomes acquired apoE and receptor reactivity, while β -VLDL lost apoE and receptor reactivity.

Time course of the cholesterol-loading ability of hypercholesteremic rabbit plasma following an infusion of liposomes. For these observations to have relevance to atherosclerosis, infused liposomes must produce sustained alterations in the cholesterol-loading ability of hypercholesteremic rabbit plasma. We therefore examined the cholesterol-loading abilities of plasma samples obtained before and up to 24 h after a single infusion of phospholipid liposomes. After the infusion, plasma phospholipid concentration rose, declined rapidly in the first hour, then declined more gradually from 1 to 24 h (Fig. 6 A). The gradual disappearance of excess phospholipid from the circulation is consistent with prior reports of prolonged half-lives for cholesterol-free liposomes infused in large doses ($> 25\text{--}100 \text{ mg/kg body weight}$) (11, 12, 33, 34).

Following the infusion, the plasma concentration of unesterified cholesterol initially fell. This fall was seen in all phospholipid-infused, hypercholesteremic rabbits, and coincided with the initial, rapid clearance of phospholipid. From 20 min to 24 h after the phospholipid infusion, the plasma concentration of unesterified cholesterol steadily rose (Fig. 6 A), consistent with the findings of previous studies (3, 5, 11, 12, 35–38). Plasma cholesteryl ester concentration rose slightly following the infusion and returned to the preinfusion value by 24 h (Fig. 6 A).

Impairment of the ability of hypercholesteremic plasma to cause cholesterol accumulation in cultured macrophages lasted for hours following a phospholipid infusion (Fig. 6 B). Plasma obtained 4 h after a phospholipid bolus behaved identically to plasma obtained 20 min after the bolus. By $8\text{--}24 \text{ h}$, some of the original loading ability had returned, but cellular accumulation

Figure 5. Competitive displacement of ^{125}I -LDL by sonicated phospholipid and by particles reisolated from incubated mixtures of β -VLDL with buffer or with sonicated phospholipid. Incubations and reisolations are described in the text. Human neonatal foreskin fibroblasts grown to near confluency in DMEM with lipoprotein-deficient serum were incubated for 2 h at 4°C with media containing $1.0 \mu\text{g}$ ^{125}I -LDL protein/ml and the indicated concentrations of competing particles. The cells were then washed, and dextran-releasable radioactivity was measured, from which the mass of dextran-releasable ^{125}I -LDL protein was calculated. Binding of ^{125}I -LDL in the absence of competing articles (100% on the y-axes) ($\square$) was $89.8 \text{ ng } ^{125}\text{I}$ -LDL protein/mg cellular protein. (A) $\times$, sonicated phospholipid; $\circ$, reisolated vesicles I; $\bullet$, reisolated vesicles II. (B) $\times$, control β -VLDL; $\circ$, β -VLDL-PPL. Each point represents the mean of two determinations.

of cholesterol was still far less than in preinfusion plasma (Fig. 6 B). Efflux of $[^3\text{H}]$ cholesterol from the cells was essentially identical in all plasma samples from preinfusion to 24 h post-infusion, despite considerable variation in the cholesterol/phospholipid molar ratio in these plasmas (Fig. 6 B).

Discussion

We conclude that a single intravenous bolus of phospholipid promptly abolishes the ability of plasma from cholesterol-fed rabbits to cause cholesterol accumulation in model foam cells. The effect persists for at least 24 h after the infusion, and primarily results from blockage of cellular uptake of β -VLDL.

Blockage of β -VLDL uptake is the result of two modifications of the plasma lipoproteins. First, a vesicular lipoprotein is formed (11, 12) that contains apoE but has no cholesteryl ester core. Vesicular lipoproteins are internalized and degraded by cultured cells (13), but they have a far lower ratio of total cholesterol to apoE than is found in β -VLDL. Thus, these particles competitively block receptor-mediated uptake of cholesteryl ester-rich

Figure 6. Time course of the cholesterol-loading ability of hypercholesterolemic rabbit plasma after an infusion of liposomes. J774 macrophages were preloaded with cholesterol mass and radioactivity by a 2-h incubation at 37°C with $[^3\text{H}]$ -cholesteryl ester]-acetyl-LDL in DMEM with 10% FCS. Cells were washed, then incubated for an additional 18 h in DMEM, 0.1% fBSA, and 10% hypercholesterolemic rabbit plasma obtained just before ($t = 0$ h) or at the indicated times after a bolus infusion of 400 mg liposomal phospholipid/kg body weight. (A) Concentrations of phospholipid (Δ), cholesteryl ester ($\square$), and unesterified cholesterol ($\circ$) in the plasma samples. The maximum phospholipid concentration (shown in parentheses) was calculated based on the measured dose of phospholipid, a preinfusion phospholipid concentration of 3.36 mmol/liter , and an estimated 50 ml plasma/kg body weight. (B) Mean cellular contents of total cholesterol (Δ), cholesteryl ester ($\bullet$), and retained radioactivity ($\square$) following the 18-h incubation with plasma obtained at the indicated times. Three separate culture wells were used in each experimental group. Maximum standard errors of the mean were $\pm 8.0 \text{ nmol/mg}$ for total cellular cholesterol, $\pm 7.7 \text{ nmol/mg}$ for cellular cholesteryl ester, and $\pm 0.6\%$ for retained radioactivity.

β -VLDL, without causing significant cellular accumulation of cholesterol. Second, β -VLDL loses apoE to this vesicular particle, and thereby directly loses receptor affinity. Further loss of receptor affinity might occur through the compositional changes in β -VLDL surface lipids (cf. 39). As an additional effect on cellular cholesterol balance, liposomes introduced into hypercholesterolemic rabbit plasma produced a variable and small enhancement of cholesterol efflux from cultured macrophages. Liposomes in vivo produce a more consistent enhancement of cellular cholesterol efflux (ref. 12; see also Fig. 6 A).

Our finding of blocked β -VLDL uptake by cultured macrophages may be relevant to cholesterol metabolism in arterial wall macrophages. The arterial wall of cholesterol-fed rabbits expresses large numbers of receptors for β -VLDL (1). Receptor-

mediated uptake of β -VLDL by the arterial wall in vivo may be susceptible to blockage by infused liposomes by the mechanisms we demonstrated in vitro. In contrast, the liver of cholesterol-fed rabbits contains few lipoprotein receptors, and these are saturated (40). Despite impaired hepatic receptors (40), substantial hepatic uptake of β -VLDL has been demonstrated in cholesterol-fed rabbits (41), and the total catabolic rate of β -VLDL in cholesterol-fed rabbits is at least ten times greater than in normal rabbits (42). These observations suggest that a significant portion of hepatic uptake of β -VLDL in cholesterol-fed rabbits is receptor independent, and would not be susceptible to blockage by apoE-containing liposomes. Thus, in the cholesterol-fed animal, infused liposomes might direct β -VLDL away from arterial wall macrophages and toward the liver.

Atherogenic β -VLDL appears in the plasma of cholesterol-fed animals during the period of cholesterol feeding and can persist for months following restoration to a normal diet (4, 42). During the persistent hypercholesterolemia that follows cessation of cholesterol-rich diets, there is continued synthesis of β -VLDL and continued arterial wall uptake of β -VLDL (42). Regression and prevention of experimental atherosclerosis by phospholipid infusions have been documented during continued cholesterol feeding (7–10) and during this phase of persistent hypercholesterolemia (4). Our demonstration in vitro that liposomes block uptake of β -VLDL by macrophages suggests that the antiatherogenic effects of phospholipid infusions during atherogenic hyperlipidemia may result in part from blockage of β -VLDL uptake by arterial wall foam cells, while hepatic clearance is preserved.

Phospholipid liposomes also inhibit cholesterol-loading of cultured macrophages by plasma rich in LDL, the major atherogenic lipoprotein in many types of human plasma. Incubation of J774 macrophages with LDL-rich plasma from a Watanabe Heritable Hyperlipidemic rabbit (26) increased cellular total and esterified cholesterol contents to 346% and 718% of control, respectively. Incubation with plasma plus 1.2 mg sonicated egg phosphatidylcholine/ml medium resulted in total and esterified cholesterol contents that were only 131 and 154% of control (Williams, K. J., T. Donnelly, T. Parker, and A. R. Tall, unpublished data). As a general concept, phospholipid-rich, cholesterol-poor particles, such as vesicular lipoproteins or phospholipid-enriched HDL, may acquire apoE in atherogenic plasma, then compete with atherogenic particles for receptor-mediated uptake by foam cells.

Acknowledgments

This work was supported by National Institutes of Health grant HL22682 and by a Clinician-Scientist Award to K. J. Williams from the American Heart Association and E. R. Squibb & Sons Company, with funds contributed in part by the AHA-New York City Affiliate.

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