Rapid Restoration of Normal Endothelial Functions in Genetically Hyperlipidemic Mice by a Synthetic Mediator of Reverse Lipid Transport

Kevin Jon Williams, Rosario Scalia, Kirstin D. Mazany, Wendi V. Rodriguez, Allan M. Lefer

Abstract—Endothelial dysfunction is a major pathophysiological consequence of hypercholesterolemia and other conditions. We examined whether a synthetic mediator of lipid transport from peripheral tissues to the liver (ie, the “reverse” pathway) could restore normal endothelial function in vivo. Using assays of macrovascular and microvascular function, we found that genetically hypercholesterolemic apolipoprotein E knockout mice exhibited key endothelial impairments. Treatment of the mice for 1 week with daily intravenous bolus injections of large “empty” phospholipid vesicles, which accelerate the reverse pathway in vivo, restored endothelium-dependent relaxation, leukocyte adherence, and endothelial expression of vascular cell adhesion molecule-1 to normal or nearly normal levels. These changes occurred despite the long-standing hyperlipidemia of the animals and the persistence of high serum concentrations of cholesterol-rich atherogenic lipoproteins during the treatment. Our results indicate that dysfunctional macrovascular and microvascular endothelium in apolipoprotein E knockout mice can recover relatively quickly in vivo and that accelerated reverse lipid transport may be a useful therapy. (Arterioscler Thromb Vasc Biol. 2000;20:1033-1039.)

Key Words: cell adhesion molecules ■ endothelial-derived factors ■ hypercholesterolemia ■ leukocytes ■ vasodilation

In hypercholesterolemic animals and humans, the endothelium of medium-sized muscular arteries, such as the coronary arteries, loses its ability to release biologically available nitric oxide, a vasodilator required for endothelium-dependent relaxation of vessels in response to ADP, acetylcholine, hypoxia, and other stimuli. Furthermore, in hypercholesterolemia, the luminal surface of vascular endothelium displays abnormally high levels of P-selectin and vascular cell adhesion molecule-1 (VCAM-1). On the venous but not the arterial side, these endothelial cell adhesion molecules mediate leukocyte rolling (tethering) and adherence (immobilization), respectively, by binding to counterreceptors on the leukocytes. These changes in the macrovasculature and microvasculature occur early, within 1 to 2 weeks after the onset of arterogenic hypercholesterolemia, well before overt atherosclerosis has developed. In humans, lowering the plasma concentration of LDL and other cholesterol-rich arterogenic lipoproteins that deliver harmful lipids into the vessel wall has been reported to improve endothelium-dependent vasomotor regulation after several months, although full normalization of endothelium-dependent relaxation is uncommon and may require the simultaneous administration of antioxidants or extreme measures to lower LDL levels. Similarly, elevated plasma concentrations of soluble cell adhesion molecules seen in human hyperlipidemias respond only minimally to aggressive drug treatments to lower plasma lipid levels. The therapeutic approach of LDL lowering, with or without the coadministration of antioxidants, presumably attenuates further dysfunction of the endothelium and then relies on endogenous pathways for removal of harmful lipids to gradually improve endothelial function.

We sought a different approach, namely, the determination of whether direct interventions that accelerate lipid transport from the vascular wall to the liver could rapidly restore endothelial function in vivo, even in the continued presence of long-term hypercholesterolemia. Until recently, there was no easy potent method to accelerate reverse lipid transport in vivo without harmful side effects. Drugs that increase plasma concentrations of HDL, the apparent natural mediator of the reverse pathway, are relatively ineffective, and direct injection of HDL or HDL-like artificial particles, though antiatherogenic in animals under some circumstances, is technically arduous. Moreover, injections of small HDL-like particles can provoke the side effect of raising plasma LDL levels, which appears to be a hepatic response to the extra cholesterol load. Adenovirus-mediated gene delivery to rapidly enhance hepatic expression of the scavenger receptor...
BI, a receptor for HDL, leads to the enhanced uptake of HDL lipids by the liver and then to a very large prolonged rise in the plasma concentrations of atherogenic lipoproteins. In the present study, we accelerated reverse lipid transport in vivo through daily intravenous bolus injections of cholesterol-free, large, "empty" phospholipid vesicles (LEVs), where empty indicates the absence of encapsulated drugs. Phospholipid vesicles were shown to act as antiatherogenic cholesterol sinks in plasma before their ultimate removal by the liver (24,27-32) (see also Reference 33). Such particles have little or no effect on erythrocyte composition (30,32) or on the serum parameters of liver function in rodents (D. P. Rosenbaum, unpublished data, 1999). Importantly, the liver can accommodate large amounts of cholesterol from LEVs without causing a rise in plasma concentrations of LDL or suppression of hepatic LDL receptor message. To study the dysfunctional endothelium, we used the apoE knockout mouse, which has been widely investigated because of its spontaneous development of lifelong hypercholesterolemia and accelerated atherosclerosis.44

Methods
Preparation of LEVs and Clearance Study
Synthetic 99% pure 1-palmitoyl,2-oleoyl phosphatidylcholine (Avanti Polar Lipids) was chosen because of its fluidity at body temperature yet strong resistance to oxidation. After hydration in saline, the lipid was made into LEVs (~100 to 200 nm) by extrusion, as previously described. For the clearance studies, trace amounts of [3H]cholesterol hexadecyl ether, a nonhydrolyzable lipid that remains associated with liposomes in the absence of cholesteryl ester transfer protein, were included to follow the particles. Wild-type and apoE knockout mice, 3.5 to 4.0 months of age, in the C57BL/6 background (Jackson Laboratory, Bar Harbor, Me) received a single bolus injection of labeled LEVs (300 mg/kg) via the tail vein. Blood samples were taken over a 5-day period; then the animals were killed, and organ radioactivity was determined. All animal studies were conducted in accordance with the Thomas Jefferson University Animal Care and Use Committee.

Endothelium-Dependent Relaxation in Aortic Rings
The basic approach for assessing endothelium-dependent relaxation of isolated aortic rings follows previous descriptions. Thoracic aortas were removed from mice anesthetized with pentobarbital sodium (120 mg/kg); the aortas were then placed into ice-cold Krebs-Henseleit buffer. Surrounding tissue was dissected away, and great care was taken to avoid injury to the endothelium. Aortic rings (~2 mm) were mounted isometrically in aerated baths at 37°C under a resting force of 0.5 g and allowed to equilibrate for 90 minutes before the administration of any agents. Rings were contracted with 10 nM U46619 (9,11-methanoepoxyprostaglandin H2), a thromboxane A2 mimetic, and then relaxed with the agents listed in Figure 1. Isometric contractions were measured with Grass FT-03 force transducers (Grass Instruments). Fresh bath solution was added after each test response to reequilibrate the rings to baseline values. Because A23187, the calcium ionophore, is not completely revers-}

Figure 1. Impairment of endothelium-dependent relaxation in aortic rings from genetically hypercholesterolemic apoE knockout (KO) mice and enhancement to normal levels after treatment with LEVs for 1 week. Values are mean±SEM (n=5 to 7). Statistical comparisons were performed by ANOVA, followed by pairwise comparisons by the 2-tailed t test with the Bonferroni correction. Data from wild-type control mice and LEV-treated apoE KO mice were statistically indistinguishable. *P<0.05, **P<0.02, and ***P<0.001 vs saline-treated apoE KO mice.

Intravital Microscopy
Intravital microscopy to assess leukocyte-endothelium interactions was performed in anesthetized mice by following standard procedures for other species but was adapted in the present study to mouse peri-intestinal venules, as previously described. Each mouse was initially anesthetized with sodium pentobarbital (120 mg/kg IP), and a loop of intestine was exposed through a midline laparotomy and kept moist on a heated microscope stage by superfusion of warm oxygenated Krebs-Henseleit buffer. A second abdominal incision in the right flank was made to administer additional anesthetic, as needed. Mice were allowed to stabilize for 20 minutes after surgery. A Microphot microscope (Nikon Corp) was used to visualize the mesenteric microcirculation, and a 30- to 50-μm-diameter postcapillary venule in peri-intestinal fat on the serosal surface of the intestine was chosen for observation. The image was projected by a high-resolution video color camera (DC-330, DAGE-MTI, Inc) onto a high-resolution color video monitor (Multiscan 200-sf, Sony Corp) and recorded on a videocassette recorder. Red blood cell velocity was determined online with an optical Doppler velocimeter (Microcirculation Research Institute), which allows for calculation of venular shear rates. The numbers of rolling and adhered leukocytes were quantified offline by playback of the videotape. Leukocytes were considered to be rolling if they were moving significantly more slowly than the red blood cells. Rolling was expressed as the number of such cells moving past a designated point per minute (ie, flux). A leukocyte was judged to be adherent if it remained stationary for >30 seconds. Adherence was expressed as the number of such cells per 100 μm of vessel length during 2-minute periods of observation at 0, 15, and 30 minutes after stabilization. The adherence values in each intravital preparation remained stable over this entire time period.

Repetitive LEV Injections
ApoE knockout mice received daily intravenous bolus injections of LEVs (1000 mg/kg) or the equivalent volume of saline, beginning Friday afternoon and ending the following Friday morning, for a
total of 8 injections over the course of just under 1 week. Notice that this course of therapy is far shorter than one previously used to shrink arterial lesions. 32 Three hours after the last dose, mice were euthanized for the harvesting of aortas or anesthetized for intravitral microscopy. Blood samples for lipid and apoB assays were taken before any injections and just before euthanasia. In control experiments to examine nonspecific effects of LEVs on leukocyte-endothelium interactions, apoE knockout mice were injected with a single dose of LEVs (1250 mg/kg), and wild-type mice were given 8 daily injections of LEVs (1000 mg/kg) and then evaluated with intravitral microscopy.

**LEV-Cytokine Binding**

To determine whether LEVs bind key cytokines, ^125^I-labeled interleukin-1B (0.2 ng/mL) or tumor necrosis factor-α (0.4 ng/mL, Amersham Corp) was incubated for 1 hour at 37°C with serum obtained from LEV-treated apoE knockout mice. The mixture was then subjected to size-exclusion chromatography by passage over a column of BioGel A-15m (1.5×27 cm, Bio-Rad Corp) that had been calibrated to distinguish LEVs, LDL, HDL, and albumin. In parallel experiments using a serum-free system, ^125^I-labeled cytokines were incubated with BSA (0.4%), without or with LEVs (24 mg/mL), and then passed over the BioGel column. Radioactivity associated with LEVs was assessed by gamma counting the column fractions and then correcting for counts that eluted in the LEV size range but in the absence of LEVs (presumably, aggregated labeled material).

**Quantitative Immunohistochemistry**

Quantitative immunohistochemistry of ileal venules was performed as previously described. 9 Tissue sections of ileum were incubated with a primary antibody against either mouse P-selectin (PB 1.3, Cytel Corp) or mouse VCAM-1 (MVCAM.A-429, Endogen) at a concentration of 3,3 μg/mL; saline-treated mice, 3.29 ± 0.30 mg/mL. The short course of LEV treatments did not affect plasma levels of esterified cholesterol (LEV-treated mice, 283 ± 15 mg/dL; saline-treated mice, 272 ± 42 mg/dL; P = NS) or murine apoB (LEV-treated mice, 676 ± 58 mg/L; saline-treated mice, 660 ± 71 mg/L; P = NS), 45 indicating unaltered plasma concentrations of atherogenic lipoproteins (see Reference 24). Nevertheless, LEV treatments of apoE-deficient mice enhanced the responsiveness of their aortic rings to the 2 endothelium-dependent agents up to levels statistically indistinguishable from the responsiveness seen in rings from normolipidemic wild-type control mice (Figure 1).

We next examined microvascular endothelial function. Consistent with prior evidence that hypercholesterolemia in other species upregulates cell adhesion molecules, 6,4,10 we found large increases in leukocyte rolling and adherence in apoE-deficient mice compared with normolipidemic wild-type control mice (Figure 2). Treatment of the apoE-deficient animals with LEVs for 1 week produced a small statistically insignificant rise in rolling but a markedly attenuated leukocyte adherence compared with values seen in normal wild-type mice (Figure 2).

Next, we used 2 methods of assessing endothelial function in mice: (1) endothelium-dependent relaxation of isolated aortic rings and (2) leukocyte rolling and adherence in postcapillary peri-intestinal venules by intravitral microscopy. Vascular rings from wild-type female mice relaxed 61 ± 5% (mean ± SEM, n = 18) to 10 μmol ADP/L, whereas rings from wild-type males relaxed only 9 ± 3% (n = 10), and a sex difference was evident down to 10 nmol ADP/L. The finding is presumably a consequence of higher oxidative stress in male aortas, 40 although this remains a controversial area. 41,42 ADP did not relax deendothelialized mouse aortic rings. All subsequent experiments with aortic rings were conducted with female mice. Leukocyte rolling and adherence showed no sex differences in either wild-type or apoE-deficient mice, which allowed the use of both sexes.

Aortic rings were analyzed from the following groups of female mice: normolipidemic wild-type mice (plasma total cholesterol 56 ± 3 mg/dL) and apoE knockout mice (baseline plasma total cholesterol 528 ± 76 mg/dL) that were treated with daily intravenous bolus injections of either saline or LEVs for 8 consecutive days. Similar to prior results in other systems, 2–4,8,41,44 we found substantially impaired relaxation to ADP, a receptor-dependent endothelium-dependent vasodilator, and to the calcium ionophore A23187, a receptor-independent endothelium-dependent agent, in apoE-deficient mice compared with wild-type control mice (Figure 1, top and middle panels). Relaxation to NaNO_3_ , an endothelium-independent vasodilator, was unaltered in the apoE knockout mice (Figure 1, bottom panel).

At the end of the treatment period, the apoE knockout mice that had been given LEV injections exhibited far higher plasma unesterified cholesterol concentrations (643 ± 29 mg/dL; n = 5) than did the saline-injected apoE knockout mice (150 ± 12 mg/dL; n = 5), reflecting mobilization of tissue stores of cholesterol by the circulating vesicles, as previously described. 24,27–30,32 Plasma phospholipid concentrations were also substantially increased (LEV-treated mice, 24.3 ± 2.5 mg/mL; saline-treated mice, 3.29 ± 0.30 mg/mL). The overall rate of LEV clearance was similar between apoE knockout and wild-type mice (22.6 ± 2.9% and 23.3 ± 2.4% of the injected dose remained in serum in the 2 respective groups of mice; P = NS), with no difference in hepatic accumulation of LEV label. Thus, LEVs mobilize tissue cholesterol and then are cleared by the liver in both apoE knockout and wild-type mice, demonstrating that these particles mediate reverse lipid transport in vivo even in the absence of apoE.

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We next examined microvascular endothelial function. Consistent with prior evidence that hypercholesterolemia in other species upregulates cell adhesion molecules, 6,4,10 we found large increases in leukocyte rolling and adherence in apoE-deficient mice compared with normolipidemic wild-type control mice, indicative of an inflammatory state (Figure 2). Treatment of the apoE-deficient animals with LEVs for 1 week produced a small statistically insignificant rise in rolling but a markedly attenuated leukocyte adherence compared with values seen in normal wild-type mice (Figure 2). In contrast, 1 week of LEV injections into wild-type mice produced no effect on baseline leukocyte-endothelium interactions (Figure 2, legend), indicating the absence of any global inhibitory effect from repeated administration. As a test for nonspecific interactions of LEVs with upregulated cell adhesion molecules or their counterreceptors, the presence of LEVs for 30 minutes in vivo in otherwise-untreated apoE null mice produced the same small statistically insig-
Figure 2. Increased leukocyte rolling and adherence to endothelium of postcapillary peri-intestinal venules in hypercholesterolemic apoE KO mice and effects of treatment with LEVs for 1 week. A, Quantification of leukocyte rolling on endothelium of wild-type mice (open bars, n=7), apoE KO mice treated with saline for 1 week (solid bars, n=4), apoE KO mice treated with LEVs for 1 week (hatched bars, n=4), and apoE KO mice 30 minutes after a single LEV injection (shaded bars, n=6). B, Quantification of leukocyte-endothelial adherence in the same 4 groups of mice. *P<0.01 vs wild-type control mice; †P<0.01 vs apoE KO mice treated with LEVs for 1 week. Values in wild-type mice given daily injections of LEVs for 1 week were 7.38±0.94 rolling cells/min and 1.88±0.66 adhering cells/100 μm (n=4), which are statistically indistinguishable from the data in the leftmost bars of panels A and B (P=0.5).

Discussion

Direct enhancement of reverse lipid transport in vivo represents a novel approach to ameliorate the major sequelae of elevated plasma concentrations of atherogenic lipoproteins.24,25,27–29,32 These sequelae include endothelial dysfunction, platelet hyperreactivity, and atherosclerosis, including the development of lipid-rich rupture-prone plaques. Our data indicate that treatment with LEVs, which accelerate reverse lipid transport in vivo without reducing long-standing elevations in plasma concentrations of cholesterol-rich atherogenic lipoproteins, is sufficient to rapidly restore important indices of macrovascular and microvascular endothelial function to normal. Although it is possible that there are actions of LEVs independent of accelerated reverse lipid transport that may have contributed to the effects we observed, our data eliminated 2 conspicuous mechanistic possibilities, namely, nonspecific interference of LEVs with leukocyte-endothelium interactions and adsorption by LEVs of key cytokines.

Although the pathophysiological effects of atherogenic hyperlipidemia on the endothelium are complex and incompletely understood, several potential factors that contribute to this problem have been identified, and there is evidence indicating how these factors can be favorably affected by phospholipid liposomes. The first contributory factor is oxidized substances, chiefly from oxidized LDL. In hypercholesterolemic patients, the best improvements in endothelium-dependent vascular reactivity have been reported after administration of lipid-lowering agents in combination with antioxidants,13,16 and the degree of improvement correlates with the resistance of the patient’s LDL to oxidation.15 Oxidized LDL impairs endothelium-dependent relaxation of isolated coronary arterial and aortic segments,46 and lipid...
components of oxidized LDL, such as lysophosphatidylcholine and oxysterols, reduce the endothelial release of nitric oxide and induce surface displays of endothelial cell adhesion molecules in vitro and in vivo. Incubation of modified LDL with phospholipid complexes impairs its uptake by cells, and toxic oxidized lipids are readily redistributed from oxidized LDL onto LEVs (W.V.R., Tammy R. Dugas, K.J.W., unpublished data, 1998). Furthermore, liposomes made of 1-palmitoyl,2-oleoyl phosphatidylcholine, as we used in the present study, donate large amounts of this oxidation-resistant phospholipid onto native LDL during coincubations in vitro (see References 28 and 52) and presumably remove phospholipids with readily oxidizable polyunsaturated side chains, thereby rendering the LDL resistant to oxidation and oxidation-induced aggregation (W.V.R., Tammy R. Dugas, K.J.W., unpublished data, 1998). Any oxidized or oxidizable lipids acquired by the liposomes in vivo would be transported from lipoproteins and the arterial wall into the liver, which is the site of lipoprotein catabolism.

The second contributory factor in hypercholesterolemia-induced vascular dysfunction is native unoxidized LDL. Recent studies indicate that native LDL induces acute and chronic elevations in cytosolic calcium concentrations, activator protein-1–dependent gene transcription, and VCAM-1 expression in cultured endothelial cells. Increases in cytosolic calcium concentrations by LDL may result in part from structural alterations in intracellular membranes caused by the donation of unesterified cholesterol from LDL to cells. The ability of phospholipid liposomes to extract unesterified cholesterol from cellular membranes has been shown in vitro to reverse changes in membrane structure and calcium flux.

The third contributory factor is low plasma concentrations of HDL. In coronary patients, low levels of HDL are associated with severe impairments of endothelium-dependent vascular reactivity. HDL has been shown to inhibit the oxidation of LDL in vitro, and HDL can block harmful effects of oxidized LDL on endothelial cells because HDL can take up abnormal lipids, such as lysophosphatidylcholine and oxidized sterols. HDL and artificial HDL-like complexes can temporarily alter cultured endothelial cells to be resistant to subsequent activation by cytokines, and the mechanism appears to involve scavenging of cellular pro-oxidant molecules by these particles. In addition, HDL removes unoxidized cholesterol from cells, particularly after the cells have accumulated cholesterol from atherogenic lipoproteins. LEVs substantially enhance the ability of HDL to extract cellular lipids, both through remodeling the HDL into a better lipid acceptor and by acting as a large-capacity sink for lipids shuttled out of cells by HDL.

Our results are consistent with the model that high plasma concentrations of atherogenic lipoproteins induce endothelial dysfunction through several contributory pathways. For example, P-selectin expression and leukocyte rolling in the microvasculature were differently regulated from the other parameters we measured, consistent with prior work. In contrast, endothelium-dependent relaxation, leukocyte adherence, and VCAM-1 expression all returned to normal or nearly normal levels with LEV treatment. Earlier investigation has shown parallel regulation of these parameters, which has been attributed to a role for nitric oxide in both endothelium-dependent relaxation and the suppression of surface expression of endothelial cell adhesion molecules. Moreover, parallel regulation of these specific endothelial functions between the macrovasculature and microvasculature indicates that one can be a marker for the others.

Endothelial dysfunction is a major factor in poor regulation of blood flow, increased platelet aggregation, and recruitment of inflammatory cells into the vessel wall. Overall, our results indicate that direct interventions to enhance reverse lipid transport in vivo, presumably in conjunction with conventional therapies, may be a useful approach to restore normal macrovascular and microvascular endothelial cell physiology in atherogenic hyperlipidemia.

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References


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