Direct Visualization of Lipid Deposition and Reverse Lipid Transport in a Perfused Artery
Roles of VLDL and HDL

J.C. Rutledge, A.E. Mullick, G. Gardner, I.J. Goldberg

Abstract—The major goal of this study was to determine the interactions of VLDL surface and core lipids with the artery wall. We first demonstrated in vitro that surface lipid in VLDL could be traced using the phospholipid-like fluorescent probe 1,1-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine (DiI). The core of VLDL particles was traced by fluorescently labeling apolipoprotein B with TRITC. The labeled VLDLs were perfused through rat carotid arteries, and accumulation of the fluorescently labeled VLDL components in the arterial walls was determined by quantitative fluorescence microscopy. Addition of lipoprotein lipase increased the accumulation of both DiI and TRITC by 2.3-fold. Histological examination showed that DiI and TRITC were primarily localized to the endothelial layer; however, DiI also accumulated as small “lakes” deeper in the artery, in a subendothelial position. Addition of HDL to the perfusion decreased the accumulation of surface lipid and apolipoprotein B–containing particles and eliminated the DiI lakes. Moreover, the increase in endothelial layer permeability associated with lipolysis was attenuated 21% by HDL. If VLDL surface lipid first was allowed to accumulate in the arterial wall, its subsequent rate of loss was more than twice as fast if HDL was included in the perfusate. These studies directly demonstrate atherogenic effects of VLDL lipolysis and their inhibition by HDL. (Circ Res. 2000;86:768-773.)

Key Words: triglycerides ■ atherosclerosis ■ lipoproteins ■ apolipoprotein B ■ cholesterol

The key initial event in atherogenesis is the accumulation of lipid within the vessel wall. This is thought to result from retention of apolipoprotein B–containing lipoprotein particles,1 because they bind to negatively charged matrix proteins such as proteoglycans that are found in the artery wall.2 Non–lipoprotein-associated lipids are also found in lesions. More than a decade ago, Lupu et al3 identified lipid that was not associated with lipoprotein-like particles in early lesions. These “lipid whorls” contain phospholipid and some free cholesterol. The origin of this lipid is unknown.

The mechanisms by which triglyceride-containing lipoproteins, VLDL and chylomicrons, are atherogenic are unclear. It is unlikely that these large particles directly enter the arterial wall. However, several other processes that occur during the metabolism of these lipoproteins along the arterial wall are potentially atherogenic. These include the following. (1) Unlike nascent triglyceride-rich lipoproteins, the smaller remnants may infiltrate into the vessel.4 (2) Lipolysis of triglycerides might compromise the barrier function of the endothelial layer.5,6 (3) Lipids that are dissociated from the surface of triglyceride-rich lipoproteins may accumulate within the artery.5,7 (4) VLDL and chylomicron remnants could be selectively retained in the artery wall.7 HDLs are inversely correlated with the development of complications of atherosclerosis, such as myocardial infarction.8,9 However, thus far, there has been no direct demonstration that HDL will remove lipids from the artery wall. Therefore, the reverse cholesterol transport pathway as a protective effect of HDL must be considered hypothetical. Additionally, HDL has a number of other protective actions in vitro: HDLs contain antioxidant enzymes such as paraoxonase,10 and HDLs may reduce LDL association with matrix proteins.11 Further, in vivo data show that HDLs are a marker for other lipoprotein abnormalities, and, in some studies, HDL is positively correlated with the removal rate of post-prandial lipoproteins.12,13

Despite a large body of in vitro data on the interactions of lipoproteins with cultured cells, there are limited data directly assessing the effects of lipoproteins and lipolysis products on whole arteries. Lipoprotein lipase (LpL) is located primarily on capillary endothelial cells, and immunohistological studies also have shown this protein on macrophages and smooth muscle cells within atherosclerotic plaques.14,15 Our data show that surface lipid accumulates in the intimal layer partially in the form of small regions that have been termed “lakes.” HDL abrogated lipolysis-induced changes in endo-
the intimal layer permeability and removed VLDL-derived surface lipid from the artery wall. Therefore, the first step in reverse lipid transport could be observed within an intact artery.

Materials and Methods

Transfer of Fluorescent Probes During Lipolysis

To assess the movement of VLDL surface and core lipid during lipolysis, VLDL was labeled with 2 different fluorescent probes: 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine (DiI) and cholesteryl BODIPY-FL C12 (Molecular Probes, Inc). BODIPY cholesterol is a more hydrophobic molecule and will trace esterified cholesterol that is found in the core of the lipoprotein. Ten micrograms of protein of DiI- or BODIPY-FL cholesteryl ester–labeled VLDL was incubated in 150 μL of PBS or PBS-1% BSA for 2 hours at 37°C. The incubations were carried out with and without addition of 1 μg of purified bovine LpL and 10 μg of human HDL protein. The solutions were separated at 4°C by ultracentrifugation in 6-mL tubes using a Beckman 40.3 rotor; VLDL was isolated at d<1.063 g/mL, and LDL plus IDL was subsequently isolated at a density of 1.063 g/mL. Recovered fluorescence then was determined in each fraction.

Measurement of Macromolecule Accumulation

The perfused artery preparation and experimental apparatus are as we have described previously. Several measurements were made during and after perfusion of each carotid artery with fluorocently labeled molecules (Figure 1).

Effects of HDL on Endothelial Layer Permeability

The actions of HDL on lipolysis-mediated increases in endothelial layer permeability were determined by measuring the accumulation of a water-soluble, nonlipid reference molecule (M, 4400 dextran, labeled with TRITC). Initially, the rate of dextran accumulation was determined during perfusions of dextran in 1 perfused carotid artery from a rat. Then, VLDL was added to the perfusate and the rate of dextran accumulation again determined. Next, LpL was added to the perfusate and the rate of dextran accumulation again determined. In the other artery from the same animal, a parallel experiment was performed, but with HDL added to the VLDL perfusate. Vessels were compared before and after LpL administration, in the absence and presence of HDL.

An expanded Materials and Methods section is available online at http://www.circressaha.org.

Results

Lipolysis Leads to Transfer of DiI From VLDL to HDL

To assess the behavior of the fluorescent labels during lipolysis of VLDL, in vitro lipolysis experiments were performed using DiI- and BODIPY-labeled VLDL. Recovery of fluorescent DiI and BODIPY from VLDL incubated in PBS with LpL and/or HDL is shown in Figure 2. Control incubations and incubations with only LpL led to recovery of most of the label, >80%, in the d<1.063 g/mL supranate.
The recovered label in the VLDL and LDL fractions are shown added together to best illustrate the label remaining in VLDL and remnant lipoproteins, ie, the label in the d<1.063 g/mL lipoproteins. The presence of BSA or HDL alone had no effect (data not shown). However, when the Dil-VLDL was incubated with LpL and HDL, >60% of the label was reisolated in the d>1.063 g/mL infranate, ie, with HDL and the nonlipoproteins contained in the 1.21 g/mL infranate. A similar shift of label did not occur with the BODIPY. Therefore, Dil remained with the VLDL during the control incubations and during incubations with LpL. If, however, an acceptor for lipolysed surface lipid such as HDL was included in the incubation, the majority of the Dil disassociated from VLDL and associated with the d>1.063 g/mL infranant. In contrast, the BODIPY remained with the VLDL. It is well known that VLDL surface lipid transfers to HDL during lipolysis. Therefore, it appears that the Dil is a marker for surface lipid transfer.

### Accumulation and Localization of VLDL Surface Label During Lipolysis in Perfused Arteries

To examine the action of lipolysis on the VLDL surface components, we studied Dil-VLDL perfused into individual carotid arteries from male rats. Seven carotid arteries were taken from 4 rats (technical problems occurred with 1 carotid artery). During 3 alternating 10-minute perfusions of Dil-VLDL (0.056 mg/mL cholesterol) and the nonfluorescent buffer solution, little or no accumulation of Dil-VLDL was detected (0.15±0.014 [SEM] mV/min; 0.1 ng cholesterol/cm² min⁻¹). When LpL (10 μg/mL) was added to the perfusate, the rate of Dil accumulation increased after the 10-minute perfusion with Dil-VLDL. Previous experiments have shown that LpL in this concentration will extensively hydrolyze VLDL. This effect was maintained during the 2 subsequent 10-minute perfusions of Dil-VLDL. Comparison of all control perfusions of Dil-VLDL with all Dil-VLDL+LpL perfusions showed significantly greater Dil accumulation after LpL was added to the perfusate (0.33±0.048 mV/min; 23 times greater than control, P<0.05 by paired t test; Figure 1). No change in lumen diameter was observed during monitoring using our fluorescence optical system for the duration of the experiment. Therefore, LpL markedly increased Dil accumulation in vessels. Previous experiments from our laboratories have documented a dose-response relationship of LpL on LDL accumulation in the artery. To localize Dil-surface accumulation in the artery wall, we examined sections of the artery at the end of the experiment. As shown in Figure 3, Dil fluorescence accumulated in the intima, with the endothelium and the subendothelial layers most intensely labeled. Further, much less intense sites of homogeneous localized accumulations of Dil were observed below the endothelial layer. These lakes were not seen when VLDL protein was fluorescently labeled and treated with LpL (below). These observations indicate that remnant particles do not contribute to the lakes and suggest that the lakes are composed of surface lipid that is associated with Dil. Thus, lipolysis markedly increased Dil-surface lipid accumulation on the endothelium and in the subendothelial space and led to collections of label deeper in the wall.

### Accumulation and Localization of VLDL Remnants in the Artery Wall

We next assessed whether TRITC-VLDL would accumulate in the artery wall before and after lipolysis. Arteries perfused with TRITC-VLDL (0.058 mg cholesterol/mL) alone had little accumulation at control (0.197±0.046 mV/min; 1.25 ng/cm² min⁻¹). Addition of LpL (10 μg/mL) to the perfused artery significantly increased TRITC accumulation in the artery wall (0.546±0.076 mV/min; 3.46 ng/cm² hour⁻¹, a 2.7-fold increase; n=4 animals). Localization of TRITC showed an intense accumulation in the endothelial layer (Figure 4). Because there was little accumulation of TRITC-VLDL and a 2.8-fold increase in TRITC accumulation after treatment with LpL, we assume that VLDL remnant particles account for the large increase in TRITC accumulation. In contrast, our previously published study showed accumulation of LDL primarily in the subendothelial space after treatment with LpL. Therefore, lipolysis increased remnant accumulation in the artery wall in the same general area that VLDL surface components accumulated after lipolysis. However, no TRITC accumulations (lakes) were detected in the subendothelial space.

### Actions of HDL on Endothelial Layer Permeability

We next examined the actions of HDL on lipolysis-mediated increases in endothelial layer permeability by determining the accumulation of a water-soluble, nonlipid reference molecule (M, 4400 dextran, labeled with TRITC). Initially, the rate of dextran accumulation was determined during perfusions of dextran (0.07 mg/mL) in 1 perfused carotid artery from a rat (n=4 animals). Then, VLDL (0.1 mg cholesterol/mL) was added to the perfusate and the rate of dextran accumulation again determined. Next, LpL (10 μg/mL) was added to the perfusate and the rate of dextran accumulation again determined. In the other artery from the same animal, a parallel experiment was performed, but with HDL (0.1 mg cholesterol/mL) added to the VLDL perfusate. A comparison in the rates of dextran accumulation before and after LpL administration revealed that vessels containing HDL had reduced dextran accumulation after LpL was added to the perfusate (Figure 5). Comparison of the change in rate of dextran accumulation (24% versus 3%) was significantly different (P<0.05, n=8 arteries). Therefore, addition of HDL to the perfusion prevented the increase in dextran accumulation seen with lipolysis.

### Removal of Lipolysis Products From the Artery Wall by HDL

Because our in vitro experiments showed that HDL accepted Dil-surface lipid from VLDL after lipolysis, we reasoned that HDL would remove Dil-labeled surface lipid from the artery wall as well. Parallel experiments were performed in the right and left carotid arteries from a single rat. One artery served as the control (no HDL added to perfusate), and the other artery was perfused with HDL. First, the artery was perfused for 10 minutes with Dil-VLDL (0.13 mg/mL cholesterol), and the rate of Dil accumulation was measured. Then, lipolysis was induced by adding LpL (10 μg/mL) to the perfusate containing...
After 1 hour of perfusion with the DiI-VLDL and LpL, the DiI-containing vessel was perfused with either buffer or buffer+HDL (0.15 mg/mL cholesterol). The decay in fluorescence intensity was monitored during this washout period (15 minutes). The rate of DiI efflux was determined by nonlinear regression techniques, assuming a 2-compartment model of fluorescent decay. This efflux rate constant ($d$) of DiI removal from the artery wall was compared in vessels with and without HDL. In each animal, the efflux rate of DiI was greater with the vessel perfused with HDL (0.9±0.027 versus 0.24±0.016 hours$^{-1}$; $P<0.05$, n=8 vessels; Figure 6). Therefore, HDL effectively removed the DiI that accumulated in the artery.

**Discussion**

Lipolysis of VLDL leads to the production of free fatty acids, diglycerides, and monoglycerides. LpL also is an A1 phospholipase and leads to production of lysolecithin. Our
studies indicate that in this setting of lipolysis of DiI-labeled VLDL, DiI is a marker for surface lipid transfer. This is not surprising, as DiI is more structurally similar to phospholipids than to the hydrophobic core lipids that would remain within lipoproteins during lipolysis. Thus, the accumulation of DiI-labeled VLDL in the artery wall may not reflect lipoprotein-receptor interactions if lipolysis is concurrently proceeding.

Our fluorescence histological studies showed that both DiI and TRITC significantly accumulated in the artery wall after treatment with LpL. We saw little or no accumulation of DiI-VLDL or TRITC-VLDL on or in the artery wall before addition of LpL. This relatively low level of accumulation before LpL is added indicates that primary VLDL-endothelial cell interactions play a relatively minor role in the large increase in DiI and TRITC accumulation. Our previous studies have shown that the great majority of VLDLs undergo lipolysis at the concentration of LpL and VLDL used in these experiments. Only a small percentage of the LpL binds to the cells; however, marked lipolysis occurred under these conditions. Thus, it appears that the VLDL surface component and the VLDL remnant localized to the intima.

Remnant particle localization to the intima is related, at least in part, to the large size of these particles that prevents further penetration into the artery. We expected the DiI-surface component to be either randomly distributed to the entire artery or present in a gradient with the intensity of signal correlating inversely with the distance from the lumen. Because the DiI and TRITC fluorescence were discretely localized in the intima, this suggested that the postlipolysis VLDL surface lipid and apolipoprotein B bind to specific intimal components. These high-affinity interactions may have prevented further penetration of the lipids into the artery.
it is widely believed that lipoproteins are retained within arteries by association with negatively charged glycoconjugates, i.e., vessel wall proteoglycans. If correct, then negatively charged lipids such as phospholipids, fatty acids, and lysolecithin should be repelled from these proteins. Perhaps basic regions of other matrix molecules interact with these lipids. This might prevent their further diffusion into the artery wall.

Atherosclerotic plaque contains large amounts of non–lipoprotein-associated (free) lipid;\textsuperscript{1,2,22} however, the source of the free lipid is unclear. Free lipid could be derived from lipid-filled macrophages that have undergone cell death or apoptosis.\textsuperscript{23} Our study demonstrates that lipolysis products from triglyceride-rich lipoproteins can also provide free lipid that collects within the artery wall. These regions containing free lipid, we hypothesize, are the progenitors of the lipid whorls found in atherosclerotic plaques. In addition, arterial wall free lipid may initiate processes that promote atherosclerosis, such as inflammation and mitogenesis.\textsuperscript{24} For these reasons, when lipolysis occurs along the artery wall, rather than in the capillaries, it may be atherogenic.

In summary, there are a number of processes that occur during lipolysis of VLDL and chylomicrons that could be deleterious to the artery.\textsuperscript{25} Many of these effects may, in vivo, be tempered by the presence of HDL. Previous studies have shown that free fatty acids can increase transendothelial movement of albumin.\textsuperscript{26} Further, lipolysis also affects the barrier function of the endothelium,\textsuperscript{6} and our current studies demonstrate that HDL modulates endothelial permeability. This may occur because HDL scavenges lipolysis products,\textsuperscript{8} substances that may injure the endothelium and increase its permeability. The net effect may be to prevent permeability-related increases in lipoprotein transfer into the artery wall. A second major action of HDL, shown in these studies, is amelioration of the accumulation of non–lipoprotein-associated surface lipid within the artery, the so-called lakes shown in our micrographs. In our studies, HDL prevents the development of these lipid accumulations and can increase the removal rate of lipid once it is present in the vessel. This later process is a direct demonstration in an artery of “reverse lipid transport,” a process previously shown to occur only in cultured cells.

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