Lipid Droplet Biogenesis and Function in the Endothelium

Andrew Kuo, Monica Y. Lee, William C. Sessa

Rationale: Fatty acids (FA) are transported across the capillary endothelium to parenchymal tissues. However, it is not known how endothelial cells (EC) from large vessels process a postprandial surge of FA.

Objective: This study was designed to characterize lipid droplet (LD) formation in EC by manipulating pathways leading to the formation and degradation of LD. In addition, several functions of LD-derived FA were assessed.

Methods and Results: LD were present in EC lining the aorta after the peak in plasma triglycerides initiated by a gavage of olive oil in mice, in vivo. Similarly, in isolated aorta, oleic acid treatment generates LD in EC ex vivo.

Cultured EC readily form LD largely via the enzyme DGAT (diacylglycerol O-acyltransferase 1) and degrade LD via ATGL (adipocyte triglyceride lipase) after FA loading. Functionally, LD-derived FA are dynamically regulated and function to protect EC from lipotoxic stress and provide FA for metabolic needs.

Conclusions: Our results delineate endothelial LD dynamics for the first time in vivo and in vitro. Moreover, LD formation protects EC from lipotoxic stress, regulates EC glycolysis, and provides a source of FA for adjacent cells in the vessel wall or tissues. (Circ Res. 2017;120:1289-1297. DOI: 10.1161/CIRCRESAHA.116.310498.)

Key Words: endothelial function ■ endothelium ■ fatty acids ■ lipid droplet ■ lipids ■ metabolism ■ triglycerides

Endothelial cells (ECs) line the entire circulatory system and play a critical role in controlling the transport of oxygen and nutrients. Fatty acids (FA) transport across blood vessels lining metabolically active tissues release FA from triglyceride (TG)-rich lipoproteins via metabolism by lipoprotein lipase, which docks on the luminal surface of capillary endothelium together with GPIHB1 (glycosylphosphatidylinositol-anchored high-density lipoprotein–binding protein 1).6 After TG metabolism to FA, CD36 can serve as a transporter for FA into ECs, providing an important energy source for metabolically active tissues, such as the heart and skeletal muscle.7 Several factors have been reported to regulate FA uptake and transport in EC, including vascular endothelial growth factor B,14 peroxisome proliferator activated receptor-γ,15 and 3-hydroxyisobutyric acid,16 implying dynamic regulation of FA by locally produced substances. However, there is little information investigating how EC store, metabolize, and use FA.

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Lipid droplets (LDs) are intracellular compartments that serve as fat reservoirs.8 On exposure to excessive amounts of FA, cells can esterify FA into TG-rich LDs via the Kennedy pathway to prevent lipotoxicity.7 Because all of the enzymes involved in TG synthesis, including GTPases (glycerol-3-phosphate acyltransferases), AGPAT (acyl-CoA: 1-acyl-glycerol-3-phosphate acyltransferase), lipins, and DGATs (diacylglycerol O-acyltransferases), reside on the endoplasmic reticulum (ER), the de novo generation of LDs is believed to occur in the ER membrane.9 Continuous accumulation of TG between the ER leaflets leads to their detachment from the ER, forming cytosolic LDs, and the dynamics of LDs is tightly regulated by their associated proteins.9 Conversely, lipids in LDs can be subsequently hydrolyzed by a subset of lipases, namely adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL), in a process called lipolysis. FA released during lipolysis can further be used to provide energy or substrate for cellular lipid synthesis.10

Although adipocytes are the most active cells in storing and metabolizing LDs, LD formation can occur in all eukaryotic cells tested, especially under pathological conditions of FA excess. For instance, obese individuals with chronically elevated levels of circulating nonesterified FA develop LDs within skeletal muscle and liver, and the presence of LDs is posited to promote insulin resistance.11 Although the presence of neutral lipids (TG and cholesterol esters) in lipid globules has been documented in EC lining mammalian atheromas,12-15 the biogenesis and metabolism of LDs in EC have not been thoroughly investigated.

Thus, the purpose of this study is to characterize the ability of ECs to generate and metabolize LD. Here, we show that ECs readily form and degrade LDs in response to changing levels of TG, in vivo. Using both cultured EC and en face imaging of ECs in large vessels, we demonstrate that ECs rely on DGAT1

Original received December 16, 2016; revision received January 17, 2017; accepted January 24, 2017. In December 2016, the average time from submission to first decision for all original research papers submitted to Circulation Research was 13.4 days.

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The online-only Data Supplement is available with this article at http://circes.ahajournals.org/lookup/suppl/doi:10.1161/CIRCRESAHA.116.310498/-/DC1.

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Circulation Research is available at http://circres.ahajournals.org

DOI: 10.1161/CIRCRESAHA.116.310498

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Novelty and Significance

What Is Known?
- Endothelial cells are constantly exposed to lipids derived from dietary fat and lipoproteins.
- Capillary endothelial cells metabolize chylomicron and very low-density lipoprotein-derived triglycerides to free fatty acids for energy utilization by the heart and skeletal muscle.
- Lipid droplets are intracellular hubs for lipid metabolism but never studied in endothelial cells.

What New Information Does This Article Contribute?
- Endothelial cells can actively incorporate fat into triglyceride-rich lipid droplets, suggesting that fatty acids have an intermediate fate and are not only passively transported across the endothelium.
- Lipid droplet metabolism protects endothelial cells from lipotoxicity and provides fatty acids for mitochondrial function and transport to adjacent cells.

Here we document that lipid droplets are a new lipid storage organelle in vascular endothelial cells regulating fatty acid metabolism and flux. Abnormalities in lipid droplet metabolism may contribute to endothelial dysfunction during conditions of hyperlipidemia.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGPAT</td>
<td>1-acylglycerol-3-phosphate O-acyltransferase</td>
</tr>
<tr>
<td>ATGL</td>
<td>adipose triglyceride lipase</td>
</tr>
<tr>
<td>CPT</td>
<td>carnitine palmitoyltransferase-1</td>
</tr>
<tr>
<td>DGAT</td>
<td>diacylglycerol 0-acyltransferase</td>
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<tr>
<td>EC</td>
<td>endothelial cells</td>
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<tr>
<td>ECAR</td>
<td>extracellular acidification rate</td>
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<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>ETOX</td>
<td>etomoxir</td>
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<tr>
<td>FA</td>
<td>fatty acids</td>
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<td>FAO</td>
<td>fatty acid oxidation</td>
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<tr>
<td>GPAT</td>
<td>glycerol-3-phosphate acyltransferase</td>
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<tr>
<td>HSL</td>
<td>hormone-sensitive lipase</td>
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<tr>
<td>LD</td>
<td>lipid droplet</td>
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<tr>
<td>MGL</td>
<td>monoglycerol lipase</td>
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<td>OA</td>
<td>oleic acid</td>
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<td>PA</td>
<td>palmitic acid</td>
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<td>PLN</td>
<td>perilipin</td>
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<td>TG</td>
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LD Detection by BODIPY 493/503
Neutral lipids in ECs lining aortae or in cultured ECs were detected using the fluorescent dye BODIPY 493/503 (Invitrogen). Intact vessels from mice gavage with olive oil or aortae from mice incubated with oleic acid (OA; 1 mmol/L) overnight were fixed and en face immunostained as described in detailed methods. Cells were grown to confluence on coverslips precoated with 0.1% gelatin in PBS solution. After designated treatments, cells were washed 3× with PBS and fixed with 4% paraformaldehyde in PBS solution for 15 minutes. Fixed samples were washed 3× with PBS and stained with BODIPY 493/503 diluted in PBS at the final concentration of 0.1 mg/mL for 15 minutes to delineate LDs and with DAPI (4′,6-diamidino-2-phenylindole; Sigma; 0.1 ng/mL) to highlight nuclei. Coverslips were mounted with Fluoromount Aqueous Mounting Medium (Sigma) and imaged by laser-scanning confocal microscopy (Leica SP5) in the sequential scan mode with HCX PL APO lambda blue 63×/1.40 oil objective lens at room temperature.

LD Purification
LDs in ECs were purified based on published methods16,17 with modifications and detailed in Methods in the Online Data Supplement.

FA Release Assay
Human dermal microvascular endothelial cells (passage 10–14) were cultured on 0.4 μm pore transwell inserts (Corning CLS3460) for 4 days until the cells formed compact monolayers. C2C12 myoblasts were cultured in a separate 12-well plate until confluent and differentiated into myotubes by 2% horse serum in DMEM for 4 days. One day prior to the experiment, human dermal microvascular endothelial cells were loaded with 1 mmol/L OA added to the upper and lower chambers overnight to induce LD formation. On the day of experiment, transwell inserts with LD-rich human dermal microvascular endothelial cells were placed into the 12-well plate with or without differentiated C2C12 myotubes. Equal volume of EBM-2 medium containing FA-free albumin (50 μM) was added into both upper and lower chambers. Two percent of medium was collected from both chambers after 6 and 24 hours. Free fatty acid (FFA) concentration was determined using a fluorometric-based assay kit (Cayman Chemicals) according to instructions. To normalized FFA release, human dermal microvascular endothelial cells were lysed by protein lysis buffer (see detailed Methods), and total protein amount was determined by DC Protein Assay Kit (Bio-Rad).

C2C12 myotubes were washed 3× with PBS, fixed with 4% paraformaldehyde in PBS for 15 minutes, and stained by BODIPY 493/503 diluted in PBS at the final concentration of 0.1 mg/mL for 15 minutes to delineate LD and with DAPI (Sigma; 0.1 ng/mL) to highlight nuclei. Fixed C2C12 samples were imaged by epifluorescence microscope (Zeiss, Axio Vert 200 mol/L) with 10× objective lens at room temperature.

for TG synthesis during LD formation and ATGL for lipolysis of LDs. Mechanistically, LD formation in ECs provides a protective mechanism from lipotoxic ER stress. Moreover, FA hydrolyzed from LDs during lipolysis can be used as a source of energy or can be released extracellularly and esterified into LDs by skeletal muscle in coculture experiments. These findings document for the first time the dynamics of LDs and implicate an active role of LDs as key organelles regulating intracellular lipid homeostasis in ECs.

Methods
Detailed expanded Methods are included in the Online Data Supplement.

Gavage Experiments
C57BL/6 mice of 8 to 12 weeks were fasted for 16 hours. Blood plasma samples for TG measurement were collected by retro-orbital bleeding prior to gavage (0 time point). Next, mice were orally gavaged with olive oil (10 mL/kg body weight). Thoracic aortae and blood plasma samples were collected at designated times for future examination of LDs in vessels and TG measurements.
Results

Characterization of LD Dynamics in EC

To determine whether ECs form LDs in response to elevated TG levels in blood, we gavaged a single bolus of olive oil to wild-type (C57BL/6) mice that were fasted overnight. Maximum TG accumulation in blood occurred 90 minutes post-gavage, and TG levels returned to baseline after 180 minutes (Figure 1A). Despite TG levels peaking at 90 minutes, prominent LD formation in ECs of thoracic aortae were observed at 180 minutes post-gavage by en face imaging of esterified neutral lipids in EC using the dye BODIPY 493/503. At 270 minutes post-gavage, LDs were no longer detectable, implying active degradation of LDs in ECs (Figure 1B and 1C). This result suggests that ECs of large blood vessels can dynamically form and degrade LD in EC after transient changes of TG levels in blood.

Next, LD formation was characterized in cultured mouse lung endothelial cells (abbreviated as EC) by supplementation of the monounsaturated (18:1) FA, OA, into the medium. OA induced LD formation in ECs in a time- (Figure 2A and 2B) and dose-dependent (Figure 2C and 2D) manner, reaching maximum cellular TG content (285.86±41.05 nmol TG/mg protein) after 24 hours (1 mmol/L OA loading). OA supplementation increased the mRNA levels of enzymes involved in TG synthesis, including GPAT4, AGPAT2, Lipin2, DGAT1, and DGAT2, whereas the expression of sterol ester synthetic enzymes, ACAT1 and ACAT2, did not change (Figure 2E). In addition to the synthetic pathway, ECs express lipases for TG degradation, including ATGL, HSL, and MGL (Figure 2F, lane 1). In response to OA supplementation, the protein levels of ATGL and HSL were increased (Figure 2F), implicating active lipolysis during LD formation. These data indicate that ECs store excessive FA into TG, and baseline lipolysis is also activated to maintain TG homeostasis.

Because LD-associated proteins regulate LD dynamics, we examined LD-associated proteins in EA.hy 926 cells preincubated with OA overnight. Reverse transcriptase quantitative polymerase chain reaction analyses showed that of the 5 known perilipins (PLN), only PLN2 and PLN3 are expressed in EA.hy 926 cells (Figure 2G). Subcellular fractionation of LDs from other cellular constituents using sucrose gradient fractionation demonstrated that LD fractions are indeed enriched with PLN2, PLN3, and lipolytic enzymes, including ATGL and its coactivator, CGI-58 (comparative gene identification-58; Figure 2H). As a control, the lipid raft protein Flot-1 (flotillin-1), ER localized Grp94 (94 kDa glucose-regulated protein), and abundant cytoplasmic protein Hsp90 (heat shock protein 90 kDa) were not detected in purified LDs. We also observed an enrichment of caveolin-1 in LD fractions similar to previous work in adipocytes.18,19 These biochemical data show that purified endothelial LD are decorated with several known LD-associated proteins that can regulate LD dynamics.

In the final step of TG synthesis, DGATs add the last fatty acyl group to diacylglycerides, forming TG. Mammalian cells express 2 DGAT isoforms, DGAT1 and DGAT2.20 To assess the function of individual DGAT in EC, we used
specific chemical inhibitors that block DGAT1 (A922500) or DGAT2 (PF-06424439) during LD formation. Co-treatment of OA with A922500 blocked the majority of TG synthesis in EC with only 16.9±8.1% of LD remaining, whereas PF-06424439 inhibited TG synthesis to a lesser extent. Combining both inhibitors almost completely abolished LD formation in ECs (Figure 3A and quantified in Figure 3B), indicating that DGATs are essential for TG synthesis in ECs with a greater dependency on DGAT1 than on DGAT2.

ATGL has been identified as the key lipase initiating TG hydrolysis in adipose tissue. Because ATGL levels increased in response to OA supplementation in ECs, we further explored whether ATGL levels also increased in response to OA treatment in MLEC. Western blotting analyses showed that adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) levels increased in response to OA treatment, whereas monoglyceride lipase (MGL) expression remained unchanged in MLEC (n=3). MLEC express perilipin (PLN) family proteins (PLN2 and PLN3) shown by agarose gel of quantitative polymerase chain reaction (qPCR) products (n=3). LD fractionation from EA.hy 926 cells untreated (“−” lanes) or treated with OA (“+” lanes) show that PLN2, PLN3, ATGL, CGI-58 (comparative gene identification-58), and caveolin-1 (Cav-1) are enriched in LD fraction, whereas other proteins examined, FLOT-1 (flotillin-1), Grp94 (94 kDa glucose-regulated protein), and Hsp90 (heat shock protein 90 kDa), are not (n=3). Scale bars, 25 μm. Data in B, D, and E are expressed as mean±SEM (n=3, 5 images per experiment in B and D) and analyzed by 2-way analysis of variance (ANOVA). *P<0.05, **P<0.01, ***P<0.005.
enriched in LDs, we tested whether ATGL plays a similar role in ECs as in adipocytes. After loading of OA, inhibition of ATGL activity by Atglistatin delayed LD degradation in comparison to nontreated cells, with 56.9±7.8% of LDs remaining after 48 hours (Figure 3C and 3D), and also significantly inhibited FA release from LD-rich ECs (Online Figure IA). In addition, cotreatment with Atglistatin during OA supplementation increased TG accumulation (Online Figure IB). Inhibition of HSL (with CAY10499) and MGL (with URB602) critical lipases during subsequent steps of TG hydrolysis reduced FA release from ECs but had no effects on TG accumulation (Online Figure IA and IB). These data suggest that ATGL acts as the rate-limiting step for TG hydrolysis.

To validate these findings in intact blood vessels, isolated aortae were supplemented OA for 12 hours in the presence of inhibitors of either DGAT1 or ATGL, and EC neutral lipids were imaged en face. Indeed, DGAT1 inhibition by A922500 abolished a majority of OA-induced LDs in ECs, whereas blocking lipolysis with Atglistatin increased TG accumulation (Figure 3E and 3F). Collectively, these results in cultured ECs and ex vivo blood vessels indicate that ECs form LDs largely via DGAT1 and degrade LDs via ATGL.

Examination of LD Functions in EC

Next, we examined several potential functions of LDs in ECs, including (1) LD formation as a mechanism to protect against lipotoxicity; (2) LD metabolism of TG as a source for FA for mitochondrial function; and (3) LD metabolism as a mechanism to transiently store and liberate FA for subsequent metabolism by skeletal muscle. To test if LD formation protected cells from lipotoxicity, ECs were treated with saturated FA, including palmitic acid (PA; 16:0) and stearic acid (SA; 18:0) in the absence or presence of OA. In contrast to OA loading, equimolar concentrations of PA and SA weakly induced LD formation (Figure 4A). Instead, the saturated FA promoted ER stress assessed by BiP (binding immunoglobulin protein) and CHOP (C/EBP homologous protein) induction (Figure 4B, lanes 5 and 9), indicating that these saturated FAs were used differently than OA in ECs. When saturated FAs (PA or SA) were mixed with equimolar amounts of OA and supplemented to ECs, LD formation was delayed (Figure 4B).

Figure 3. Diacylglycerol O-acyltransferases (DGAT)1 and adipose triglyceride lipase (ATGL) are key enzymes regulating lipid droplet (LD) dynamics in endothelial cells (ECs). A, DGAT1 inhibition by A922500 (5 μM) has a greater effect on oleic acid (OA)–induced LD formation in mouse lung endothelial cell (MLEC) than in DGAT2 inhibition by PF-06424439 (10 μM). Coincubation with both inhibitors completely abolishes LD formation, as quantified in B. C, Basal lipolysis is significantly delayed over time by Atglistatin (10 μM) in MLEC pretreated with OA (1 mmol/L) overnight, as quantified in D by triglyceride (TG) content. E, Thoracic aortae incubated with OA (1 mmol/L) overnight show LD formation in EC layer via en face immunostaining, where DGAT1 inhibitor, A922500 (5 μM), abolishes LD formation, and ATGL inhibition by Atglistatin (10 μM) enhances LD accumulation, as quantified in F by BODIPY intensity (n=3). Scale bars in A and C, 10 μm. Scale bar in E, 50 μm. Data in B, D, and F are expressed as mean±SEM (n=3 and 5 images per experiment) and analyzed by unpaired student t test. *P<0.05.
promoted (Figure 4A, bottom), and ER stress levels were diminished (Figure 4B, lanes 7 and 11). Inhibition of DGAT1 and DGAT2 promoted ER stress induced by OA and abrogated the protective effect of OA on PA/SA-induced ER stress (Figure 4B, lanes 4, 8, and 12). Interestingly, inhibition of DGAT1, but not DGAT2, was sufficient to promote ER stress induced by OA alone (Online Figure IIA), but not by combinations of OA and PA/SA (Online Figure IIB, lanes 4, 8, and 12), implying the differential substrate utilization between DGAT1 and DGAT2. Collectively, these data suggest that
the capability of ECs to form LDs is critical for their protection from lipotoxic ER stress.

The release of FA from TG via lipolysis can provide FA for energy production in oxidative tissues, such as skeletal muscle and the heart. Because LDs in ECs can be hydrolyzed via ATGL, we tested whether FA release from TG lipolysis can be used as a substrate for cellular energy. To address this, oxygen consumption rate was measured as an index of mitochondrial respiration. LD-enriched ECs (+OA) exhibited normal ATP production, maximal respiration, and nonmitochondrial respiration compared with cells treated with vehicle (−OA; Figure 4C). However, suppression of FA oxidation (FAO) by inhibition of CPT-1 (carotid palmitoyltransferase-1), with etomoxir (ETOX), baseline oxygen consumption rate, and respiratory capacity, was decreased only in LD-enriched EC (−OA + ETOX), suggesting that EC can use the LD-derived FA for energy production in the resting state (Figure 4C). As normal EC rely primarily on glycolysis for energy, we tested whether increased FAO in LD-enriched EC altered the glycolytic flux critical for ATP generation as measured by extracellular acidification rate reflecting glucose metabolism to lactate. LD-enriched ECs (+OA) have reduced extracellular acidification rate when exogenous glucose is provided (Figure 4D; +OA after glucose addition). This difference in extracellular acidification rate was normalized in EC after oligomycin to induce maximum glycolytic capacity and after 2-deoxyglucose incubation to assess nonglycolytic acidification rate (Figure 4D, after oligo and 2-deoxyglucose addition). To substantiate that the reduction in glycolysis is because of increase of FAO in LD-rich EC, we measured extracellular acidification rate when FAO is inhibited by ETOX. Indeed, the impaired glycolytic flux is partially rescued by blocking FA oxidation (Figure 4D, +OA + ETOX after glucose addition), demonstrating that increased FAO decreases glycolytic flux in LD-enriched ECs. Additionally, direct measurements of lactate are reduced in LD-enriched ECs and could be partially rescued by ETOX treatment (Online Figure III). Together, our data indicate that the metabolized LDs could provide FA as an energy resource and modulate EC metabolic flux and suppress glycolysis.

FA released from LD lipolysis could also provide extracellular FA for adipose or parenchymal tissues. Therefore, the release of free FA from LDs (generated by pretreatment with OA) was assessed in ECs cultured on a transwell insert. The EC monolayers were postconfluent as indexed by the lack of paracellular leakage of fluorescein isothiocyanate–conjugated dextran over 24 hours of experimentation (Online Figure IV). Under these conditions, FA release was monitored on both apical and basolateral sides of ECs, and similar amounts of FA accumulated ≤24 hours on either side of the transwell chamber (Figure 4E, CTRL). The release of FA was attenuated by inhibition of ATGL and HSL and enhanced by forskolin-mediated activation of lipolysis (Figure 4E). To further determine whether FA released from ECs can be taken up by parenchymal cells, we performed the transwell assay with C2C12 myotubes cultured in the bottom chamber. In this coculture system, free FA were detectable only in the apical chamber (Figure 4F), and the C2C12 myotubes were laden with LDs imaged with BODIPY (Figure 4G). The accumulated FA were released from LD-rich EC because no LDs were detected in C2C12 myotubes cocultured with normal ECs that were not pretreated with OA (Online Figure V).

Discussion

Our knowledge of lipid metabolism in ECs is derived primarily from the role of lipoprotein lipase on the surface of capillary ECs. Lipoprotein lipase hydrolyzes very low–density lipoproteins and chylomicrons, generating FFA that are taken up via a CD36-dependent mechanism for energy utilization in skeletal and cardiac muscle or re-esterified into TG in liver. In contrast to this well-studied process, how ECs respond to changes in intracellular FA levels and the biogenesis of LDs have not been described. Previous studies in vivo have reported LDs in ECs in mammalian atheromas and in arteries from a patient with cardiomyopathy homozygous for a loss of function point mutation in ATGL, highlighting the significance of lipid storage in ECs. Hence, the central focus of this article is to characterize LD biogenesis and degradation in ECs because little is known.

Here, we show that LD can be readily formed in intact ECs lining blood vessels, both in vivo and ex vivo. The transient presence of LDs in ECs after a gavage of olive oil reflects the dynamic nature of LD synthesis and metabolism, a function clearly demonstrated in vessels treated with a DGAT1 inhibitor to block synthesis or an ATGL inhibitor to block metabolism. With respect to LD synthesis, DGAT1 is primarily responsible for TG synthesis in ECs based on data using specific inhibitors of DGAT1 and DGAT2. Inhibition of DGAT1 during OA treatment accounted for over 80% of TG synthesis. On the other hand, inhibition of DGAT2 activity had modest but significant effect on TG synthesis (≈20%). This is different from LD formation in adipocytes differentiated from mouse embryonic fibroblasts lacking either DGAT1 or DGAT2, where the deletion of either DGAT did not influence TG accumulation because of compensation by the remaining enzyme. Alternatively, it is possible that this discrepancy is because of different TG synthesis pathways involved in DGAT1 and DGAT2. Previous studies have indicated that DGAT2 is responsible for incorporating endogenously synthesized monounsaturated FA into TG, whereas DGAT1 may be involved in esterifying exogenous FA taken up by cells. Given our assays provide exogenous monounsaturated FA as substrate to ECs, it is likely that DGAT1-dependent TG synthesis is favored. Nevertheless, the in vivo functional significance of DGAT1 and DGAT2 in EC is not known and is interesting to investigate using conditional knockout approaches.

In addition to the characterization of LD formation in EC, we developed a simple assay to assess the time-dependent lipolysis of TG in L, a process mediated by the canonical lipases ATGL, HSL, and MGL. Blockage of ATGL markedly reduces TG degradation and FA release from LD-loaded ECs, and the subsequent metabolism by HSL and MGL is consistent with ATGL-mediated hydrolysis of TG to diacylglycerides as the rate-limiting step in LD degradation. Isolated primary ECs lacking ATGL exhibit spontaneous LD accumulation under normal culture conditions, implying endogenous activity of ATGL in TG turnover (unpublished observations).
Indeed, ex vivo loading of ECs lining intact aortae with OA to generate LDs is augmented by inhibition of ATGL, confirming the importance of this lipase in vivo.

Finally, we show that LD formation in ECs has at least 3 clear functions: to prevent lipotoxicity, provide FA to reduce glycolytic flux, and for the release of FA from ECs to adjacent cell types as shown in EC/myotubes coculture experiments. Interestingly, PA and SA do not promote overt LD formation in ECs, but OA esterification into TG-rich LDs neutralizes the lipotoxic actions of these saturated FA. This may occur via enhanced metabolism of saturated FA or by esterification of PA and SA into OA enriched LD as previously described in CHO cells use mass spectrometry measurements of TG acyl chains. Moreover, we show that in ECs enriched with LDs, loading does not influence ATP production and oxygen consumption rate unless FA oxidation is blunted by ETOX during conditions of basal and maximal respiration (in the presence of FCCP [carbonic anhydrase p-trifluromethoxyphenylhydrazone]). Moreover, the rate of glycolysis is attenuated by OA loading, in a manner reversible by ETOX, suggesting that LD lipolysis can attenuate glycolysis and impact glycolytic reserve in ECs. Recent data have shown that the loss of CPT-1A in ECs, the target of ETOX, does not affect energetics in ECs, but reduces angiogenesis because of the loss of carbon intermediates required for DNA synthesis. However, these studies were not performed in ECs laden with LDs. Finally, in transwell assays, we show that LD-derived FA can be released non-directionally from ECs, and the release is attenuated by inhibition of ATGL and HSL and augmented by the adenylate cyclase activator, forskolin, providing evidence that LD metabolism can generate FA for utilization by other cell types. Co-culture of LD-laden ECs with C2C12 myotubes results in undetectable FA on the basolateral side of ECs and the accumulation of BODIPY-positive LD in myotubes only in ECs preloaded with OA. These data imply that FA not only can passively diffuse across ECs to provide FA for skeletal muscle oxidative metabolism, but the LD-derived FA also can regulate the flux of FA critical for metabolically active tissues.

In conclusion, this is the first study characterizing LD formation in ECs. Indeed, ECs in vivo, ex vivo, or in culture readily generate and metabolize LD, demonstrating the dynamic nature of unsaturated FA storage and utilization (see model in Online Figure VI). Moreover, we define new functions of LD-derived FA in ECs and imply that abnormal LD metabolism may contribute to vascular disease. Recent data supporting this concept show that silencing of ATGL in human ECs increases TNF-α-induced ICAM-1 (intercellular adhesion molecule) expression and nuclear factor-xB activation, and the loss of ATGL promotes micro- and macrovascular EC dysfunction and inflammation. Additional experiments testing the link between vascular LDs and inflammatory vascular diseases are clearly warranted.

Acknowledgments

We thank Roger Babitt for his invaluable technical assistance and Dr Tobias Walther for experiments on lipid droplets. The sponsors had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Sources of Funding

This work was supported by Grants R01 HL64793, R01 HL61371, and P01 HL1070295 from the National Institutes of Health, the American Heart Association (Innovative Research Grant and MERIT Grant), and the Leducq Fondation (MIRVAD network) to W.C. Sessa.

Disclosures

None.

References


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Circ Res. 2017;120:1289-1297; originally published online January 24, 2017;
doi: 10.1161/CIRCRESAHA.116.310498

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Supplemental Material

Reagents and Antibodies

Oleic acid, palmitic acid, stearic acid, A922500, Atglistatin, CAY10499, URB602, Forskolin (FSK), 3-isobutyl-1-methylxanthine (IBMX) were purchased from Cayman Chemical. BODIPY 493/503 and FITC-Dextran (70kDa) were obtained from Life Technologies. PF-06424439, Etomoxir, Oligomycin, Rotenone, Antimycin A, and 2-deoxy-glycose (2-DG), and Carbonyl Cyanide-4-(Trifluoromethoxy) Phenylhydrazone (FCCP) were obtained from Sigma. Antibodies used in this study were from the following resources with the indicated dilutions for Western Blotting: anti-ATGL (Cell Signaling, #2138, 1:1000), anti-HSL (Cell Signaling 4107, 1:500), anti-MGL (Cayman Chemical, 1:500), anti-β-actin (Sigma, A5441, 1:5000), anti-PLN2 (BioVision Inc. 3837-100, 1:1000), anti-PLN3 (Novus Biological, NB110-40764, 1:1000), anti-CGI58 (Santa Cruz, sc-100468, 1:250), anti-Cav-1 (BD Biosciences, 610060. 1:5000), anti-FLOT-1 (Santa Cruz sc-25506, 1:100, anti-Grp94 (Santa Cruz, sc-11402, 1:1000), anti-Hsp90 (BD Biosciences, 610419, 1:2000), anti-CHOP (Santa Cruz, sc-7351, 1:2000), anti-BiP (BD Biosciences, 610987, 1:1000).

Animals

WT C57BL/6 mice (Stock number: 000664, Jackson Laboratory) were maintained on pelleted rodent chow diet with free access to water at 25°C steady temperature under a fixed 12-hour light/dark cycle. All procedures were approved by the Institutional Animal Care and Use Committee of Yale University.

TG Measurements

TG samples from mice blood plasma were measured by The Yale Mouse Metabolic Phenotyping Center (MMPC) using a colorimetric based assay (Raichem). TG samples from cultured cells were determined by a colorimetric assay (Cayman) through the instructions.

Cell Lines

Mouse lung endothelial cells (MLEC, abbreviated as EC) from C57BL/6 mice were isolated and immortalized by middle-T antigen as described. Human Dermal Micovascular Endothelial Cells (HDMEC) were provided by Dr. Jordan Pober’s Laboratory using
published methods. MLEC and HDMEC were cultured in EGM-2 medium (Lonza) containing 20% FBS and supplemented with SingleQuots™ Kit (Lonza), Penicillin/Streptomycin, and 2mM L-glutamine. EAhy.926 cells (ATCC) were cultured in DMEM with 10% FBS, supplemented with Penicillin/Streptomycin, and 2mM L-glutamine, and selected with HAT (hyoxanthine-aminopterin-thymidine). C2C12 myoblasts (ATCC) were cultured in DMEM with 10% FBS, supplemented with Penicillin/Streptomycin, and 2mM L-glutamine.

**Preparation of FA-BSA complex**

The FA-BSA complex used in the experiments was prepared using the following procedure: FAs were dissolved in 100% ethanol at a concentration of 0.17M. In the case of PA and SA preparation, FA-ethanol solution was heated to 60°C and vortex for complete dissolution. Aliquots of stock solutions were conjugated with 10% FA-free BSA in Tris pH 8.0 to a concentration of 12mM. The solutions were further diluted in culture medium to the final concentrations.

**En Face Immunostaining**

Thoracic aortas isolated from 8-12 weeks old C57BL/6 mice were dissected and cut longitudinally for whole mount analysis. Samples were pinned down with EC facing upward, washed with PBS 3 times and fixed with 4% paraformaldehyde in PBS for 15 mins. Fixed samples were further blocked with TNB blocking buffer (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl, and 0.5% (w/v) blocking reagent (PerkinElmer FP1020) overnight at 4°C. Aortas were incubated with anti-PECAM-1 antibody (EMD Milipore, MAB1398Z, 1:250) diluted in TNB blocking buffer overnight at 4°C. Samples were washed 3 times with PBS and incubated with fluorescence conjugated secondary antibody (Cy™3 AffiniPure Goat Anti-Armenian Hamster, Jackson Immunoresearch Laborarories, 127-165-099, 1:200) at room temperature for 3 hours. BODIPY 493/503 diluted in PBS at the final concentration of 4mg/mL was applied for 30mins to delineate LD, and DAPI (Sigma, 0.1ng/ml) were used to highlight nuclei. Samples were mounted and obtained z-stack images by laser-scanning confocal microscopy (Leica
SP5) in the sequential scan mode with HCX PL APO lambda blue 63×/1.40 oil objective lens at room temperature.

**Quantitative RT-PCR**

Total RNA from EC was extracted by RNeasy kit (Qiagen). 500ng of RNA was used for cDNA synthesis by Superscript III reverse transcriptase (Invitrogen). Quantitative PCR was performed using iQ™ SYBR® Green Supermix (Bio Rad) and analyzed with iCycler system (Bio-Rad). Gene-specific PCR primers were designed using Primer3 tool and described in the table below.

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Western Blotting Analysis

Cells were washed 3x with PBS and lysed with lysis buffer (50 mM Tris-HCl pH 7.4, 0.1% SDS, 0.1% sodium deoxycholate, 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, 1.5 mg/ml protease inhibitor tablet (Roche), 0.25 mg/ml Pefabloc (Roche) and 50mM sodium fluoride.) Lysates were incubated at 4 °C for 20mins and centrifuged at 14,000 rpm for 10 min. Supernatants were collected and protein concentration measured with DC™ Protein Assay Kit (Bio-Rad.) Equal amounts of protein in each experiment was resolved by SDS-PAGE, and transferred to nitrocellulose membranes for 2 h at 90 V using Mini Trans-Blot® Cell (Bio-Rad.) Membranes were blocked using 0.1% (w/v) casein (Bio-Rad) in TBS for 50mins and incubated with primary antibodies overnight at 4 °C. Membranes were washed with TBS-T (TBS solution containing 0.1% Tween 20) and incubated with conjugated secondary antibodies for 30mins at room temperature. Membranes were washed with TBS-T and developed using the Odyssey system (Li-Cor.) Densitometry of membranes were analyzed with ImageJ software (NIH).

Lipid Droplet Purification

LD in EC were purified based on published methods with modifications4,5. Ten 150mm plates of confluent EAhy 926 cells were used for one purification sample. Cells were treated with or without 1mM OA overnight to induce LD formation. Treated cells were harvested with cell scrapers, washed twice with ice-cold PBS and lysed in hypotonic buffer (20mM Tris/HCL, pH 7.5, 1mM EDTA, 10mM NaF and pefabloc (Roche) and Complete Protease Inhibitor (Roche). Cell lysates were further dounced 40 times with tissue grinder using a “loose” pestle (Wheaton), followed by centrifugation at 600g at 4 °C for 5mins to removed cell debris and unbroken cells. 2.5% volume of the lysate was collected and labeled as “whole cell lysate fraction”. The remaining lysate were centrifuged with 3,000g at 4 °C for 30mins to spin down nuclei. 2.5% volume of the post-nuclear lysates were collected and labeled as “cytoplasmic fraction”. The remaining lysate was centrifuged with 20,000g to remove Golgi, rough ER, mitochondria and peroxisomes. The supernatant was collected and adjusted to 1 M sucrose in hypotonic buffer, and layered on a sucrose gradient with 1 ml of each 0.75, 0.5,
0.25, 0.125, 0 M sucrose. The sucrose gradient tube was centrifuged at 200,000g at 4 °C overnight with no break deceleration step. 1mL of the floating white LD fraction was collected with a pipette, and the remaining fractions were collected every 1mL. Pellet was re-suspended with protein lysis buffer (See Western Blotting Analysis) and labeled as “microsomal fraction. LD fraction was further delipidated with 10mL acetone on dry ice for 4 hrs and centrifuged for 1 hr at 4,300g, 4°C. Pellet was dried under a gentle stream of nitrogen and re-suspended in protein lysis buffer. Protein concentration of whole cell lysate, cytosolic, microsomal, and LD fractions were determined by DC™ Protein Assay Kit (Bio-Rad). Equal amounts of total protein were subjected to SDS-PAGE for Western Blotting Analysis.

**OCR and ECAR Measurements by Seahorse Technology**

MLEC were plated in XF 96 plates (Agilent Biosciences) until confluent. One-day prior to the experiment, cell were treated with or without 1mM OA overnight. On the day of experiment, the medium was changed to XF assay medium containing 25 mM glucose, 1 mM sodium pyruvate, and 2 mM glutamine for OCR measurement or containing no glucose, no sodium pyruvate, and 2 mM glutamine for ECAR measurement for 2 hrs at 37°C incubator without CO₂ supplementation. Cells were pretreated with or without ETOX (40µM) 30mins before measurements. OCR and ECAR were measured with an XF96 extracellular flux analyzer (Seahorse Bioscience). For mitochondrial function analyses, OCR was measured when the following compounds were injected: oligomycin (1µM), FCCP (1µM), and a mixture of rotenone and antimycin (0.5µM each). For glycolytic capacity measurements, ECAR was measured when the following compounds were injected: glucose (10mM), oligomycin (1µM), and 2-DG (1mM).

**Lactate Measurements**

MLEC were cultured in 6-well plates until confluent. One day prior to the experiment, cells were treated with or without 1mM OA overnight. On the day of experiment, the medium was replaced with EBM-2 medium with or without ETOX (40µM) for 6 hrs. Conditioned medium was collected and lactate levels were measured by a fluorometric-based L-Lactate Assay Kit.
(Cayman Chemical) according to the manufacturer’s instructions. Cell number in each condition was calculated to normalize lactate production.
Online Figure I. Inhibition of individual TG lipases reduces FA release while only ATGL inhibition increases TG accumulation during LD formation. (A) Time dependent FA release into the medium from LD-rich MLEC. This process can be inhibited by blockade of individual lipases in TG hydrolysis, including ATGL by Atglistatin (10µM), HSL by CAY10499 (10µM), and MGL by URB602 (10µM). (B) Co-incubation of individual lipase inhibitors used in (A) with 1mM OA showed that only ATGL inhibition increases TG accumulation. Data are expressed as mean ± SEM (n=4). Statistical analysis in (A) was determined by two-way ANOVA, comparing with control at each time point. Statistical analysis in (B) was determined by unpaired student-t test. *p<0.05. N.S., non significant.
Online Figure II. OA, but not mixtures of OA and PA/SA, induces ER stress in EC when DGAT1 is inhibited. (A) DGAT1 inhibition (5µM, A922500) in MLEC made OA lipotoxic as assessed by BiP and CHOP immunoblotting, whereas DGAT2 inhibition (10µM, PF-06424439) had no effect. (B) MLEC treated with combinations of OA and PA/SA (0.5mM each) did not induce ER-stress, where DGAT1 inhibition (5µM, A922500) had no effect. (n=3).
Online Figure III. Lactate production is impaired in LD-rich EC and reversed by FAO inhibition. LD-rich MLEC showed a reduction in lactate accumulation in the medium, and this reduction was reversed when FAO was inhibited via ETOX (40µM) for 6hrs. Data are expressed as mean ± SEM (n=2) and analyzed by unpaired student-t test. *p<0.05. N.S., non significant.
Online Figure IV. Lack of transendothelial transport of FITC-Dextran in EC transwell experiments. FITC-Dextran (70kDa) was added to LD loaded HDMEC cultured on a transwell insert and FITC transport across HDMEC layer over time was measured by FITC intensity (A.U.). Data are expressed as mean ± SEM (n=4).
Online Figure V. LD are not detected in C2C12 cells co-cultured with non OA-loaded EC in the transwell system. In the transwell co-culture system of HDMEC and C2C12 myotubes, LD were not detected in C2C12 myotubes if HDMEC were not pre-loaded with OA. (n=3, 5 images per experiment) Scale Bar, 50µm.
Online Figure VI. Schematic model of LD metabolism in EC. EC have the machinery to incorporate excessive intracellular FA into TG mainly via DGAT1, forming cytosolic LD. (1) The capability of LD formation protects EC from FA-induced lipotoxicity. EC also utilize canonical lipolytic lipases, including ATGL, its co-activator CGI-58, HSL and MGL, to degrade TG in LD. FA liberated from LD in EC can either (2) serve as an energy resource for FAO while down-regulating glycolytic flux or (3) release to adjacent cells for subsequent utilization.
Supplemental References