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Cytoskeleton disruption in J774 macrophages: Consequences for lipid droplet formation and cholesterol flux☆

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ABSTRACT

Macrophages store excess unesterified cholesterol (free, FC) in the form of cholesteryl ester (CE) in cytoplasmic lipid droplets. The hydrolysis of droplet-CE in peripheral foam cells is critical to HDL-promoted reverse cholesterol transport because it represents the first step in cellular cholesterol clearance, as only FC is effluxed from cells to HDL. Cytoplasmic lipid droplets move within the cell utilizing the cytoskeletal network, but, little is known about the influence of the cytoskeleton on lipid droplet formation. To understand this role we employed cytochalasin D (cyt.D) to promote actin depolymerization in J774 macrophages. Incubating J774 with acetylated LDL creates foam cells having a 4-fold increase in cellular cholesterol content (30–40% cholesterol present as cholesteryl ester (CE)) in cytoplasmic droplets. Lipid droplets formed in the presence of cyt.D are smaller in diameter. CE-deposition and -hydrolysis are decreased when cells are cholesterol-enriched in the presence of cyt.D or latrunculin A, another cytoskeleton disrupting agent. However, when lipid droplets formed in the presence of cyt.D are isolated and incubated with an exogenous CE hydrolase, the CE is more rapidly metabolized compared to droplets from control cells. This is apparently due to the smaller size and altered lipid composition of the droplets formed in the presence of cyt.D. Cytoskeletal proteins found on CE droplets influence droplet lipid composition and maturation in model foam cells. In J774 macrophages, cytoskeletal proteins are apparently involved in facilitating the interaction of lipid droplets and a cytosolic neutral CE hydrolase and may play a role in foam cell formation. This article is part of a Special Issue entitled Advances in High Density Lipoprotein Formation and Metabolism: A Tribute to John F. Oram (1945-2010).

1. Introduction

Macrophage-derived foam cells, both in culture and in vivo, have a large fraction of accumulated excess cholesterol stored as CE in cytoplasmic lipid droplets. It is generally thought that the rate limiting step of cellular cholesterol clearance is the hydrolysis of lipid droplet-CE to FC, a form of cholesterol which can be exported from the cell. Therefore, when viewed in the context of reverse cholesterol transport (RCT), the first step of RCT may not be the efflux step but rather hydrolysis of stored CE followed by transport of the generated FC to the plasma membrane, where it can be removed by an extracellular acceptor such as HDL or HDL apoprotein through various pathways, as studied extensively by John Oram and colleagues [1–4]. Thus CE metabolism at the site of the lipid droplet is integral to RCT.

Macrophages, like many other cells, convert excess cholesterol (free cholesterol, FC) into cholesteryl ester (CE) through the actions of acyl coenzyme A:cholesterol acyl transferase (ACAT) and store the CE in cytoplasmic lipid droplets. These lipid droplets give the classic "foamy" appearance to atherosclerotic macrophage foam cells. Macrophage lipid droplets were once thought of as the cell's lipid storage locker, floating in the cellular milieu as the site where FC is converted to CE and back to FC establishing a CE cycle [5]. Since the discovery of the PAT (Perilipin (perilipin 1), Adipophilin (perilipin 2), and TIP47 (perilipin 3)) family of lipid droplet proteins in the 1990s [6,7], research on lipid droplet composition and function, in adipocytes and steroidogenic cells, has dramatically increased. We now know that cytoplasmic lipid droplets are intimately involved in cellular processes including lipid and endosomal trafficking (for reviews see [8,9]).

Abbreviations: CE, cholesteryl ester; FC, free cholesterol; TG, triglyceride; acLDL, acetylated low density lipoprotein; BSA, bovine serum albumin; cpm, counts/min; FBS, fetal bovine serum; PBS, phosphate-buffered saline; nCEH, neutral cholesteryl ester hydrolase; ACAT, acyl coenzyme A:cholesterol acyl transferase; cyt.D, cytochalasin D; PL, phospholipid; DGAT, acyl CoA:diacylglycerol acyltransferase

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Cytoplasmic CE lipid droplets are thought to originate in the endoplasmic reticulum (ER) [10–12], the cellular location of ACAT [13], through accumulation of CE in the ER bilayer, forming a lens, which eventually buds off from the ER. Lipid droplets then travel on the cell's microtubule skeleton [12,14]. Proteomic studies have indicated that proteins associated with stability (perilipin, ADRP, TIP47) are present on lipid droplets and have identified cytoskeletal proteins (actin, tubulin, vimentin) as candidate droplet proteins in many cells types [15–18]. The cytoskeleton has been implicated in the initiation and progression of atherosclerosis through its involvement in endothelial cell-promoted monocyte recruitment [19,20] and uptake of oxidized LDL, the probable lipid source for macrophage foam cell formation [21,22]. In fact, recent work from Oram and Heinecke's laboratories implicates cytoskeletal proteins as part of a sterol-responsive network in macrophages [23]. They found that cytoskeletal proteins are significantly up-regulated in response to cholesterol deposition in macrophages [23].

Tabas et al. demonstrated that disrupting the cytoskeleton in macrophages reduces cholesterol esterification [24]. Although cytoskeletal proteins are proposed to be present on lipid droplets, it is presently unclear if the cytoskeleton is involved in the formation, metabolism or stability of these organelles. We therefore examined the effects of cytoskeletal disruption on macrophage CE droplet formation and metabolism.

2. Materials and methods

2.1. Materials

BSA (essentially fatty acid free), heat-inactivated fetal bovine serum (FBS), gentamicin, cytochalasin D, FITC conjugated anti-actin, cholesteryl methyl ether, and FC were purchased from Sigma-Aldrich (St. Louis, MO). [1,2-^{3}H]cholesterol was obtained from New England Nuclear (Waltham, MA). Organic solvents were obtained from Fisher Scientific (Pittsburgh, PA). Tissue-culture flasks and plates were from Corning (Corning, NY). Tissue culture medium was obtained from Gibco-Invitrogen (Carlsbad, CA). Human LDL ($1.019<d<1.063$ g/ml) and HDL_3 ($1.125<d<1.21$ g/ml) were isolated by sequential ultracentrifugation, dialyzed against 0.15 mol/L NaCl, and sterilized by 0.45 μm filtration. LDL was acetylated with acetic anhydride [25]. Alexa Fluor 594 phalloidin was purchased from Invitrogen-Molecular Probes (Carlsbad, CA). Latrunculin A was purchased from Cayman Chemicals (Ann Arbor, MI).

2.2. Cell culture

J774 murine macrophages were routinely grown in RPMI containing 10% FBS and 50 μg/ml gentamicin. To enrich the macrophages with cholesterol (to create foam cells), RPMI containing 1% FBS, 1 μCi/ml [^{3}H]cholesterol, acetylated LDL (acLDL, 100 μg protein/ml), +/− cytochalasin D (cyt.D, 2 μM), +/− latrunculin A (250 nM) was added to the cells for 24 h. After the enrichment period, the cells were incubated in medium containing 0.2%BSA for 18 h to allow the cellular pools of [^{3}H]sterols to equilibrate. In some incubations, cyt.D was added during the equilibration phase. At 2 μM cyt.D we did not detect cellular toxicity (measured by cellular lactate dehydrogenase release, Roche kit, Basel, Switzerland, data not shown).

2.3. CE droplet isolation

J774 macrophage cells were cholesterol-enriched by incubation with acLDL and 1%FBS for 24 h. Following this incubation the cells were incubated for 18 h in 0.2%BSA to allow the cellular pools of sterols to equilibrate. There was a 4–5 fold increase in cellular cholesterol with 30–40% of the cholesterol present as cholesteryl ester (CE). Cholesterol enrichment using this protocol results in the formation of neutral lipid droplets in the cytoplasm of the cell. To isolate cellular lipid inclusions, 10–100 mm dishes of macrophages containing inclusions were washed three times with PBS and scraped into 3 ml PBS (3 ml per 100 mm dish) containing 20 μl/ml protease inhibitor cocktail (Sigma-Aldrich, St. Louis MO). The cells were disrupted using sonication [26]. Homogenization by this procedure reproducibly produced lipid inclusions [18,26–30]. The homogenates were centrifuged at 26,000 rpm in an SW40Ti rotor for 30 min. The floating lipid layer was removed with a syringe, dispersed in 100 mM Na_2CO_3 (pH = 11) and spun again. This treatment stripped away any loosely associated proteins on the droplet [31]. The floating droplet layer was removed with a syringe, dispersed in PBS and spun again to wash. The final lipid layer containing the droplets was removed and stored on ice until use. Acid phosphatase (Sigma-Aldrich kit, St. Louis, MO) and lactate dehydrogenase activity (Roche kit, Basel, Switzerland) were determined in the floating lipid layer to determine if there was lysosomal or cytoplasmic contamination in the lipid fraction.

2.4. Droplet protein analysis

Droplets were extracted using the method of Bligh and Dyer [32]. The interface containing droplet protein which formed between the organic and aqueous layers of the extraction was collected. The aqueous layer was discarded and the organic layer containing droplet lipid was dried under nitrogen and stored at 0 °C for later analysis. The proteins were dissolved in an SDS sample buffer containing β-mercaptoethanol as a reducing agent and the proteins were separated on a 3–8% tris-acetate gel. Proteins were visualized using Coomassie brilliant blue and submitted to the Children's Hospital of Philadelphia's Protein Core Facility. The entire lane was cut into 1 mm sections. Gel pieces were digested with trypsin (Promega; 12.5 ng/μl) at pH 8.0 at 37 °C for 18–24 h, reactions were stopped by addition of 5% trifluororacetic acid. The peptide samples were spotted on a MALDI plate and then overlayed with α-cyano-4-hydroxycinnamic acid matrix. MALDI-TOF Tandem MS spectra were acquired on a Finnegan LTQ mass spectrometer equipped with a vMALDI ion source. Bioworks 3.2 employing the SEQUEST algorithm and the SWISSPROT database was used for protein identification. Samples were analyzed using Sequest (ThermoFinnigan, San Jose, CA; version 27, rev. 12). Sequest was set up to search the mouse database assuming the digestion enzyme trypsin. Sequest was searched with a fragment ion mass tolerance of 1.00 Da and a parent ion tolerance of 1.5 Da. Scaffold (version Scaffold-01_06_06, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability. Protein identifications were accepted if they could be established at greater than 99.9% probability and contained at least 2 identified peptides.

2.5. Protein and lipid determination

Lipids were extracted from cell monolayers using isopropanol or from isolated lipid droplets by the method of Bligh and Dyer. Cholesteryl methyl ether was used as an internal standard. Total and unesterified cholesterol was quantitated by GLC [33]. Total phospholipid were measured by the method of Rouser et al. [34]. Protein was measured by the method of Markwell et al. [35]. Triglyceride (TG) was determined using a commercially available kit (Sigma-Aldrich, St. Louis, MO).

2.6. Cholesterol efflux to HDL

After [^{3}H]cholesterol loading and labeling the cells, as described above, media containing HDL (20 μg protein/ml) was added for 2 h. To determine cholesterol efflux, media were sampled at indicated times, filtered and counted by liquid scintillation counting to determine [^{3}H] released. [^{3}H]-Sterols in the media were compared to total [^{3}H] at time zero to determine the percent release of [^{3}H]free-cholesterol.