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Trypanosoma cruzi epimastigotes store cholesteryl esters in lipid droplets after cholesterol endocytosis

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ABSTRACT

The Chagas disease agent *Trypanosoma cruzi* proliferates in the insect vector as highly endocytic epimastigotes that store nutrients, including lipids in reservosomes (lysosome related compartments). Although nutrient storage is important for epimastigote transformation into infective metacyclics, the epimastigote lipid droplets (LDs) remain uncharacterized. Here, we characterized the epimastigote LDs and examined their relationship with the endocytic pathway. Fluorescence microscopy using BODIPY showed that LDs have high neutral lipid content and harbor Rab18, differently from other lipid-rich organelles (such as reservosomes). Using transmission electron microscopy (TEM), we observed a close relationship between LDs and the endoplasmic reticulum, mitochondria and glycosomes. We developed a reproducible protocol to isolate LDs, and showed (by HTPLC and GC/MS analyses) that they have 89% neutral lipids and 11% phospholipids, which are likely to form the LD monolayer seen by TEM. The LD neutral lipids were mostly sterols, although triacylglycerol, diacylglycerol, monoacylglycerol and free fatty acids (FFA) were also found. Endocytosis of ^{3}H-labeled cholesterol-BSA showed that internalized cholesterol is stored in LDs mostly in the cholesteryl ester form. Together, these results suggest that exogenous cholesterol internalized by endocytosis reaches the reservosomes and is then stored into LDs after esterification.

1. Introduction

American trypanosomiasis is a protozoan disease caused by *Trypanosoma cruzi*, and which affects 6–7 million people mostly in South and Central America, and in parts of North America (Mexico and Southern United States) [1]. *T. cruzi* has a complex life cycle, involving an invertebrate vector and intracellular replication in mammalian cells. Epimastigote forms found in the digestive tract of the insect vector have high proliferation rates sustained by active endocytosis, which is responsible for the acquisition of proteins, lipoproteins, sterols, fatty acids, sugars and other macromolecules via the flagellar pocket and the cytostome-cytopharynx complex [2,3]. Internalized lipoprotein particles, such as LDL or albumin carrying cholesterol, are delivered to 'reservosomes', the last compartment in the endocytic route of epimastigotes [4]. Reservosomes accumulate cholesterol and cholesteryl esters from the culture medium or from the digestive tract of the invertebrate vector [5]. Exogenous sterols are rapidly redistributed to the plasma membrane when parasites are subjected to lipid stress conditions, and under regular lipid availability, cholesterol is esterified by an enzyme with Acyl-CoA cholesterol acyltransferase (ACAT) activity, which is sensitive to the inhibitor Avasimibe [6]. In higher eukaryotes, ACAT is often responsible for controlling intracellular cholesterol levels by esterification and subsequent storage into lipid droplets (LDs) [7].

LDs have been described in the cytoplasm not only of mammalian cells, but also of plants, green algae, insects, yeast, protozoan and bacteria [8]. They are round compartments delimited by a phospholipid monolayer (containing basically proteins and sterols) surrounding a core formed mainly by sterol esters and triacylglycerols [9], although retinyl esters, waxes and ether lipids are also found [10]. LDs vary in diameter from 100 nm to 100 µm, depending on the cell type and

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physiological conditions [11]. Lipids stored in LDs play an important role in cell metabolism, since they are implicated in energy production, membrane biogenesis and traffic, and in the production of eicosanoids, bile salts, hormones and other intermediates involved in lipid signaling and physiology [12,13].

In pathogenic parasites, studies on LDs have focused on their involvement in the interaction between the vertebrate host and the parasite. *Toxoplasma gondii* diverts cholesterol from low density lipoproteins (LDL) internalized by the host cell for its own cholesteryl ester synthesis - via two isoforms of ACAT - and posterior storage in LDs [14]. During *Leishmania* infection, LDs accumulate in the parasite's cytoplasm, in the lumen of the parasitophorous vacuole and/or around the vacuole [15]. Structural changes and intracellular interactions of LDs in macrophages were observed, such as number, size and level of saturation of fatty acids after infection with *T. cruzi* [16].

While *T. cruzi* epimastigote forms are known to have LDs [17], there are not studies on the LDs from parasite developmental forms found in insect vectors. The epimastigotes have to withstand a decrease in nutrient availability after a blood meal, and the competition for nutrients with the microbiota present in the invertebrate gut. When epimastigotes reach the hindgut and transform into metacyclic trypomastigotes, the uptake and accumulation of lipids in LDs that occurred in the highly endocytic epimastigote form may contribute for the survival and nutrition of the low-endocytic metacyclic trypomastigotes, the forms that transmit the infection to humans. However, the cell biology and metabolic routes of LDs in *T. cruzi* epimastigotes are yet to be explored. Also, the composition of epimastigote LDs and their relationship with the compartments of the endocytic pathway have not been examined.

In this work, we established a reproducible protocol to isolate for the first time for parasitic protozoa LDs from *T. cruzi* epimastigotes, which allowed us to perform a detailed biochemical characterization of the lipids found in these compartments. Also, we described the ultrastructure and distribution of epimastigote LDs in more detail, using probes that allowed us to clearly distinguish these structures from the other lipid-rich compartments in the cell (reservosomes).

2. Material and methods

2.1. Parasites

T. cruzi epimastigotes (Y strain) were cultivated at 28 °C in LIT (liver infusion tryptose) medium [18] supplemented with 10% FCS (Vitrocell, São Paulo, Brazil). Population growth was measured by direct cell counting in a hemocytometer. In all experiments, cells were used in the exponential phase of growth.

2.2. Isolation of lipid droplets (LDs)

LDs from epimastigotes were isolated using a protocol based on the method of Martin and Parton [19] for the isolation of lipid droplets from mammalian cells. Parasites (1×10^{10} cells) were washed in TKE buffer (20 mM Tris-HCl pH 7.3, containing 100 mM KCl and 5 mM EDTA) and resuspended in 3 mL of the same buffer. Then, cells were carefully disrupted on ice using 10 cycles of 2 s, with 1 s rest between cycles, in a GEX 600 ultrasonic apparatus (Sigma) using a standard probe (13 mm radiating diameter), operating at 10% of its total amplitude. The total homogenate (TH) was centrifuged at 2500*g* for 10 min, and the resulting supernatant (SP1) was collected and centrifuged at 12,000*g* for 10 min. The pellet (P) was discarded and the supernatant 2 (SP2) was mixed with an equal volume of TKE buffer supplemented with 1.0 M sucrose. Samples were placed at the bottom of Beckman SW 55 Ti centrifuge tubes (2 ml sample/tube) and overlaid with a discontinuous sucrose gradient in TKE buffer (1 mL steps of 0.3 M sucrose, 0.1 M sucrose and 1 mL of TKE buffer without sucrose). Tubes were centrifuged at 140,000*g* for 90 min (at 4 °C) and four fractions were collected. The top fraction (henceforth referred to as Fraction 1) of the gradient corresponded to LDs. Fraction 1 and three lower fractions (2, 3, and 4, the latter representing the pellet) were collected and washed in TKE buffer by centrifugation at 140,000*g* for 40 min. All fractions were fixed for electron microscopy analysis (see "Electron Microscopy") or frozen at – 20 °C for further analysis. A schematic representation of all gradient steps are summarized supplementary data S1.

2.3. Light microscopy

2.3.1. Identification of LDs and reservosomes in epimastigotes

Epimastigotes (1×10^7 cells) were washed in PBS and fixed in freshly prepared 4% formaldehyde in PBS for 20 min at room temperature. Then, cells were allowed to adhere to 0.1% poly-L-lysine-coated glass coverslips for 20 min, coverslips were incubated with 100 mM glycine in PBS for 10 min, and then blocked and permeabilized with 2% BSA and 0.1% saponin in PBS (pH 8) for 30 min. After that, parasites were incubated with mAb anti-cruzipain JO1 (1:1000) [20] or anti-Rab18 (1:20; SAB4200173, Sigma-Aldrich, St. Louis, Missouri, USA) antibodies in 'blocking buffer' (2% BSA in PBS, pH 8.0) for 1 h, washed in blocking buffer and incubated with goat anti-mouse (1:1000) or anti-rabbit (1:500) antibodies (for anti-cruzipain and anti-Rab-18 detection, respectively) conjugated to Alexa 488 or Alexa 546.

Alternatively, epimastigotes were incubated with transferrin coupled to CF™555 (Tf-CF555, Sigma-Aldrich) for 30 min at 28 °C in order to load reservosomes [2], washed in LIT medium, and incubated for a further 15 min (chase) at 28 °C. Then, samples were processed for anti-Rab18 immunofluorescence as described above.

To reveal lipid-rich compartments, samples were incubated with 10 μM BODIPY 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene, Sigma-Aldrich) for 30 min, washed in PBS, and mounted onto slides using Prolong Gold with DAPI (Life Technologies, Grand Island, NY, USA).

2.3.2. Number of LDs in epimastigotes

The LD number in whole parasites was evaluated using BODIPY staining. Forty cells were randomly chosen for quantification.

2.3.3. Immunolocalization of Rab-18 in isolated LDs

The LD fraction was adhered onto 0.1% poly-L-lysine coated glass coverslips for 30 min and fixed in 4% formaldehyde in PBS for 10 min, at room temperature. Samples were washed with PBS (pH 7.4), stained with 10 μM BODIPY 493/593 for 15 min and washed again with PBS. LDs were then processed for immunofluorescence for Rab18 detection as described above for whole epimastigote cells without the permeabilization step.

All light microscopy samples were observed using a Leica TCS SPE AOBS confocal laser scanning microscope (mounted on a DMI4000B inverted microscope), equipped with a 63X oil immersion objective (APO NA = 1.3). BODIPY fluorescence was detected at 488 nm excitation wavelength, while DAPI and Tf-CF555 were detected at 405 and 532 nm excitation, respectively.

2.4. Electron microscopy

2.4.1. Transmission electron microscope (TEM) of ultrathin sections

Epimastigotes were collected by centrifugation 1500*g* for 10 min, washed in PBS (pH 7.2) and fixed in 2.5% glutaraldehyde for 1 h at room temperature. Parasites were processed according to previous work [21] and embedded in Epon. Ultrathin sections were observed in Zeiss 900 transmission electron microscope operating at 80 kV.

2.4.2. Lipid cytochemistry

Epimastigotes were harvested by centrifugation at 1500 *g* for 10 min, washed in PBS (pH 7.2), and incubated in LIT medium containing transferrin coupled to 10-nm colloidal gold (Tf-Au) for 20 min,