

Lipid droplets form complexes with viroplasms and are crucial for rotavirus replication

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Recent evidence has demonstrated that a variety of pathogens target cellular lipid metabolism for their replication. Lipid droplets are a major contributor to lipid homeostasis and contain neutral fats but are also recognized as dynamic organelles involved in signal transduction, membrane trafficking and modulation of immune and inflammatory responses. Rotaviruses co-opt lipid droplets for their replication. Rotavirus viroplasms, sites of viral RNA replication and immature particle assembly, form complexes with cellular lipid droplets early in infection. Chemical compounds blocking fatty acid synthesis or interfering with lipid droplet homeostasis decrease viroplasm formation and the yield of infectious viral progeny. Lipid droplets are vital for the replication of rotaviruses as well as various members of the *Flaviviridae* family and several intracellular bacteria. Chemical compounds decreasing intracellular triglyceride content reduced rotavirus replication in an animal model and should be considered as potential therapeutic agents against disease caused by rotaviruses, flaviviruses and intracellular bacteria.

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Introduction

Rotaviruses (RVs) remain an important cause of severe dehydrating diarrhea in infants and children under 5 years of age, and still account for 200,000 deaths worldwide in 2011, even after the introduction of universal rotavirus vaccination programs in many countries [1].

Rotavirus virions are non-enveloped particles composed of three concentric, icosahedral protein layers; the viral cores contain the genome of 11 segments of double-stranded (ds) RNA and enzymes of the replication

complex. After entry into the host cell, the outer layer of the infectious triple-layered particle (TLP) is removed in endocytic vesicles. The resulting double-layered particle (DLP) is a molecular machine, which actively transcribes mRNAs from the genomic dsRNA and then extrudes them into the cytoplasm. The mRNAs are translated into six structural viral proteins (VP1, VP2, VP3, VP4, VP6, VP7) and six non-structural proteins (NSP1, NSP2, NSP3, NSP4, NSP5, NSP6), or serve as templates for dsRNA synthesis in progeny virus. The early stages of double-layered particle morphogenesis and viral RNA replication occur in discrete cytoplasmic inclusion bodies called viroplasms. DLPs interact with membranes of the endoplasmic reticulum (bound via NSP4) and acquire the outer capsid layer proteins in the cytoplasm to mature into infectious virions before being released by cell lysis or budding [2].

Rotavirus viroplasms associate with components of lipid droplets

At least 7 viral proteins (NSP2/5/6 and VP1/2/3/6) have been detected in viroplasms, but co-expression of NSP2 and NSP5 are essential for viroplasm formation. Silencing the expression of NSP2 or NSP5 by RNA interference [3,4] or intrabodies [5], or the use of specific rotavirus *t5* mutants at the non-permissive temperature [6] prevents viroplasm formation and virion production. In cultured uninfected cells, co-expression of NSP2 with NSP5 forms viroplasm-like structures (VLS) in the absence of other rotaviral proteins, but expression of NSP2 or NSP5 alone is insufficient to form VLS [7].

In addition to requiring the viral NSP2 and NSP5 proteins, viroplasms associate with cellular lipid droplet (LD) components, with the numbers of viroplasm-LD complexes increasing during the replication cycle [8•]. Lipid droplets are spherical intracellular organelles containing triacylglycerols (TAG) and sterol esters in the core, which is surrounded by a phospholipid monolayer [9]. More than 200 mammalian proteins are associated with lipid droplets [10]; most prominently, members of the PERILIPIN family of proteins (PLIN1-PLIN5) [11]. Lipid droplets are present in all eukaryotic cells and play various roles beyond neutral lipid storage. Traditionally, lipid droplets were viewed as passive storage depots for excess fat from which neutral lipids are rapidly consumed when carbon sources are depleted and additional energy supplies are required [12,13]. However, lipid droplets are increasingly recognized as dynamic organelles actively involved in diverse cellular processes including lipid homeostasis,

signal transduction and membrane trafficking [14–18]. The processes that govern the formation, composition, and different functions of lipid droplets have major significance in both basic biology and in the development of metabolic and infectious diseases [9,15].

The first evidence that rotavirus viroplasm associate with components of lipid droplets came from studies demonstrating that viroplasm-associated proteins co-localize with the lipid droplet-associated proteins PLIN1 and PLIN2 and that viroplasm-lipid droplet complexes interact with the lipophilic stain Nile red [8**]. In addition, PLIN1 (as a marker for lipid droplets) co-sedimented with NSP5 (as a marker for viroplasms) and dsRNA (as a marker of viral particles) in low-density fractions of ultracentrifugation gradients of rotavirus — infected cell extracts [8**]. The close spatial proximity of (Cy3-antibody labeled) NSP5 with PLIN1 was demonstrated by fluorescence resonance energy transfer (FRET) in rotavirus-infected cells co-expressing NSP5-EGFP [8**]. Furthermore, co-expression of NSP2 and NSP5 alone was sufficient to detect co-localization of VLS with PLIN1 on lipid droplets [8**]. Lipidome analysis demonstrated that the total cellular lipid content increases during RV infection [19*,20*] and that the lipid increase is consistent with an increase in abundance of lipid droplets that interact with viroplasms [19*]. Together, these results confirmed the close association of viroplasms with lipid droplets.

Experimental dissection of viroplasm-lipid droplet complex formation is challenging because the detailed mechanisms of lipogenesis and lipid droplet biogenesis as well as viroplasm formation are unknown. A time course analysis of viroplasm and lipid droplet morphogenesis by confocal microscopy revealed that small viroplasms (identified by NSP5) are detected early during infection prior to the association of viroplasms with lipid droplets [8**]. Furthermore, knockdown of NSP5 in rotavirus-infected cells by specific siRNA reduced the production of dsRNA and infectious progeny virus [4,8**]. These results suggested that viroplasms recruit and require components of lipid droplets for viral replication. It is possible that the interaction or post-translational modification of either or both NSP2 and NSP5 may induce conformational changes in these viral proteins [21,22] to allow this viral complex to associate with lipid droplets and recruit other viral proteins to form larger viroplasms. Lipid droplets can be considered as platforms for topological organization and assembly of viroplasms, enabling viral early morphogenesis and RNA replication.

Lipogenesis and lipid droplet biogenesis

Figure 1 illustrates the current understanding of lipogenesis and lipid droplet biogenesis, and delineates interventions of this process which decrease rotavirus replication. Lipogenesis involves the biogenesis of components of

lipid droplets: phospholipids, triacylglycerol and fatty acids. As a first step, acetyl-CoA carboxylase 1 (ACC-1) converts acetyl-CoA into malonyl-CoA (Figure 1-A). In a reiterative process, the fatty acid synthase (FASN) complex catalyzes the reaction of acetyl-CoA with malonyl-CoA resulting in the synthesis of the sixteen carbon fatty acid palmitate. Palmitate is the precursor molecule for synthesis of phospholipids, triacylglycerol and other fatty acids. As an alternative to lipogenesis, cells can import long chain fatty acids from extracellular media (Figure 1-B). Although the mechanism of fatty acid transport through the plasma membrane is not completely understood, it is believed that long chain acyl-CoA synthetase (ACSL) plays a key role in this process [23]. ACSL family members convert long chain fatty acids into hydrophilic acyl-CoAs that can no longer escape the cell. The mechanistic details of lipid droplet biogenesis remain poorly understood [14,24,25]. The prevalent model is that lipid droplets form from the neutral lipid within the lipid bilayer of the endoplasmic reticulum (ER) where the enzymes required for the biosynthesis of TAG, diacylglycerol acyltransferases (DGAT1 and DGAT2), and sterol ester synthesis, acyl-coenzyme A (CoA):cholesterol acyltransferases (ACAT1 and ACAT2), reside [14] (Figure 1-C). The accumulation of neutral lipids between the lipid bilayer of the endoplasmic reticulum promotes the cytosolic leaflet to release a lipid droplet into the cytoplasm (Figure 1-D). Subsequently, lipid droplets acquire numerous proteins (PLIN1-PLIN5).

Formation of viroplasm-lipid droplet complexes is vital for rotavirus replication

The formation of complexes between viroplasms and lipid droplets (Figure 1-E) appears to be vital for rotavirus replication as compounds that block lipid droplet formation or disperse lipid droplets significantly decrease the number and size of viroplasms and the amount of infectious progeny produced. Steps in lipogenesis and lipid droplet biogenesis at which intervention affects rotavirus viroplasm formation and replication are also shown in Figure 1. Treatment of rotavirus-infected cells with triastin C, an inhibitor of ASCL3, reduced viroplasm size and number with a corresponding reduction in viral yield [8**,26*]. Inhibiting key molecules in lipogenesis, ACC-1 with TOFA, or FASN with C75, yielded varying results. Treatment of rotavirus-infected cells with TOFA reduced both the infectivity of progeny virus and viral dsRNA production in a time-dependent and dose-dependent manner. Addition of TOFA at 4 hours prior to infection had the greatest effect on viral infectivity and dsRNA yield but a decrease in both these characteristics was still observed when TOFA was added at 4 hours post infection [27*]. Treatment of rotavirus-infected cells with C75 showed only a modest effect, but in combination with TOFA, a synergistic reduction in viral yield was reported [27*]. TOFA treatment of rotavirus-infected cells also had a broader effect in that it caused a 2-fold

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