HHS Public Access

Author manuscript

Curr Opin Virol. Author manuscript; available in PMC 2017 August 01.

Published in final edited form as:

Curr Opin Virol. 2016 August ; 19: 11–15. doi:10.1016/j.coviro.2016.05.008.

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

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Abstract

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.

Introduction

Rotaviruses (RVs) remain an important cause of severe dehydrating diarrhea in infants and children under 5 years of age, and still accounting 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

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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 *ts* 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, 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 viroplasms 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 triascin 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- 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 reduction in the production of RV DLPs, but a 20-fold reduction in detectable TLPs [28], suggesting that the blockage of fatty acid synthesis may affect RV replication not only at the steps occurring within viroplasms up to DLP formation but also at the later steps of infectious virus assembly (TLPs). Reduced viral yields were also observed in rotavirus-infected cells treated with inhibitors of DGAT, A922500 or betulinic acid, or ACAT, CI-976 or PHB (for details see ref [26]). Treatment of rotavirus-infected cells with isoproterenol and IBMX, which raise cellular cyclic AMP and disperse lipid droplets into smaller microdroplets in adipocytes [12,29], resulted in reduced number and size of viroplasms, decreased production of viral dsRNA and a 120-200-fold lower yield of infectious progeny [8] (Figure 1-F). In addition, the viability of the drug-treated, rotavirus-infected cells was significantly higher at later time points post infection as compared to non-treated rotavirus-infected cells, suggesting that the reduction in rotavirus-induced cytopathicity is correlated with increased cell viability [8].

Concluding remarks and future directions

Eukaryotic cells regulate astonishingly complex homeostatic networks, yet control over this fine-tuned machinery is co-opted by viruses with expression of just a handful of proteins [17,30,31]. Here we reviewed a striking example of such viral takeover: rotavirus exploitation of lipid metabolism. Co-expression of just two rotavirus proteins, NSP2 and NSP5, is sufficient for the formation of viroplasm-like structures which co-localize with lipid droplets [7,8]. The recognition that rotavirus viroplasms require components of lipid droplets implies a critical role of lipid droplets for rotavirus biology. In this context it should be noted that lipid droplets also are crucially important for the replication of members of the *Flaviviridae* family [32-34] and of intracellular bacteria such as *Chlamydia* [35] and *Mycobacterium tuberculosis* [36].

Clinically, the findings described above may be significant because rotavirus replication occurs in mature enterocytes of the small intestine, the major site of fat absorption in the body. Stem cell-derived human intestinal enteroids (HIE) are a novel, non-transformed cell culture model that is defining new aspects of human intestinal physiology and pathophysiology. HIEs are currently being explored as a rotavirus replication system that more closely mimics the human intestinal epithelium [37]. Infection of HIEs with human rotaviruses demonstrated host range and cell type restriction and virus-induced fluid secretion; in addition, infection of HIEs with human rotaviruses has induced viroplasm and lipid droplet formation [37].

Questions to be answered by rotavirus infection of HIEs are: 1) Do lipid droplets function as a platform for rotavirus viroplasms and viral replication as shown for infected MA104 cells [8]? 2) Which of the viroplasm proteins mediates the interaction with lipid droplets? 3) In addition to interacting with preformed lipid droplets, do rotaviruses actively induce lipid droplet formation during viral replication, and what is the mechanism? 4) Do viral proteins directly interact with lipid droplet proteins or components for the formation of lipid droplets? 5) Are the neutral lipids within the lipid droplet utilized for energy production through beta oxidation in mitochondria or do lipid droplets play different roles during rotavirus infection? 6) Which gene products involved in lipid droplet formation are important for forming complexes with viroplasms? The siRNA approach [38,39] could be used to identify these factors.

Treatment of mice with chemical compounds which decrease the intracellular triglyceride content has led to a reduction of rotavirus shedding 1-3 days post infection compared to rotavirus-infected untreated animals [20]. This initial observation should be explored further to identify potential candidate therapeutic drugs against rotavirus disease. Since lipid droplets are critical for the replication of many microbes, including flaviviruses and intracellular bacteria, lipid droplet biology is wide open for future study [16,31].

Acknowledgements

We gratefully acknowledge partial support of the rotavirus research from NIH grants R01 AI080656 and P30 DK056338, Wellcome Trust grants WT082031 and RG46760, an Amgen Foundation scholarship and the Addenbrooke's Biomedical Research Centre. Many thanks are due to Drs. Jeanette Criglar and Mary K. Estes for critical comments on the manuscript.

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Highlights

- Rotavirus viroplasms and lipid droplets associate early during viral replication
- Lipid droplets may act as a platform for viral replication and assembly
- Inhibition or disruption of lipid droplets reduces rotavirus yield
- Rotavirus-infected mice shed less virus upon lipid depletion
- Lipid droplet-disrupting compounds may be developed to combat LD-requiring pathogens

Future research on the significance of viroplasm-lipid droplet complexes for rotavirus replication

- Determining the molecular mechanisms of viroplasm-lipid droplet interaction
- Defining the functions of lipid droplets in the viroplasm-lipid droplet complexes
- Exploring chemical compounds shown to disturb lipid biosynthesis and lipid droplet homeostasis as potential therapeutic agents in animal models of rotavirus infection/disease
- Assessing inhibitors of lipid droplet homeostasis as inhibitors of the replication of other microbes (flaviviruses, intracellular bacteria)

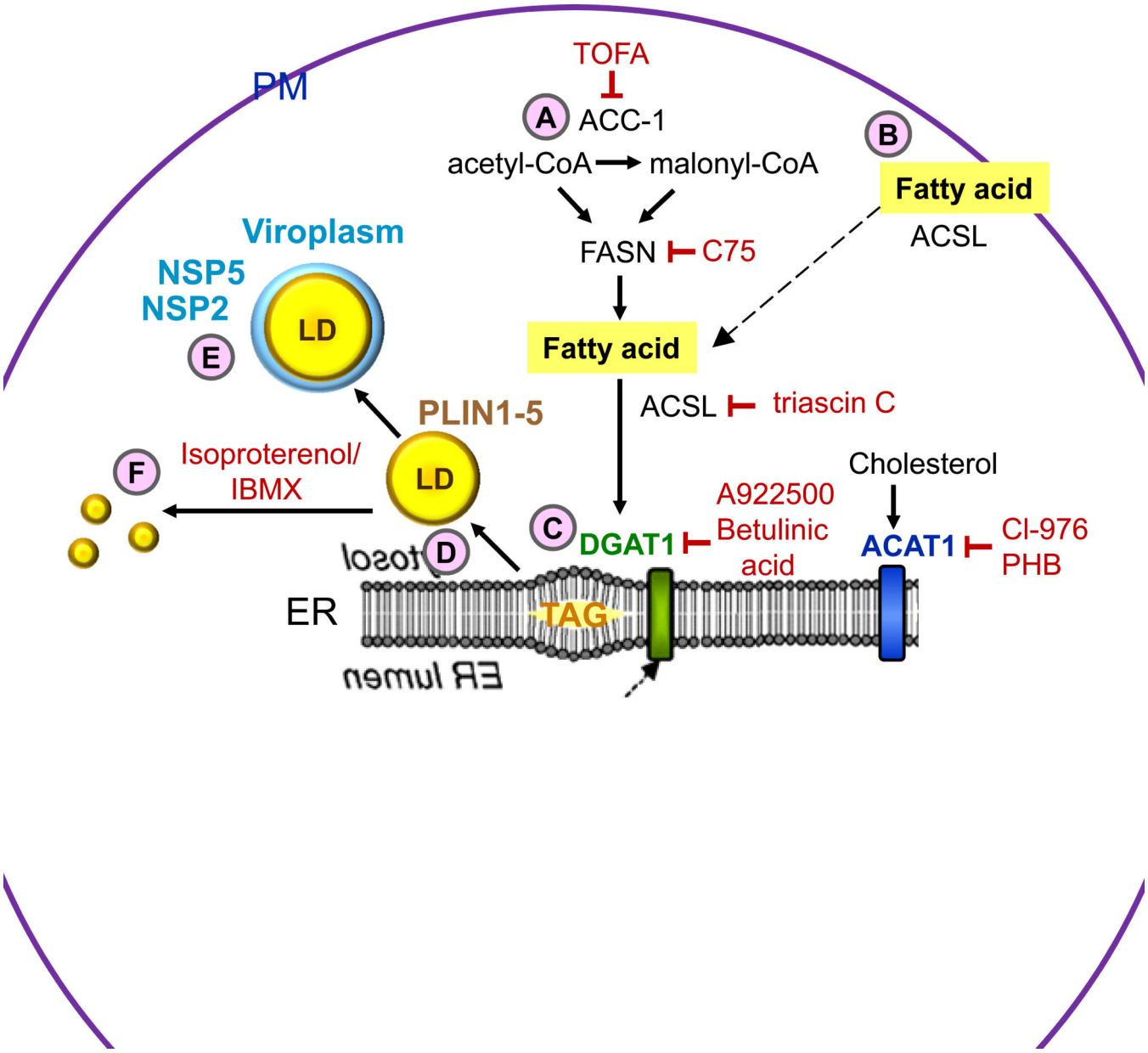

Figure 1.

Processes of lipogenesis and lipid droplet biogenesis, and interventions that disrupt viroplasm formation. **(A)** *De novo* fatty acid synthesis involves the conversion of acetyl-CoA into malonyl-CoA by acetyl-CoA carboxylase 1 (ACC-1). Fatty acid synthase (FASN) catalyzes the synthesis of the fatty acid palmitate from acetyl-CoA and malonyl-CoA. **(B)** Long chain acyl-CoA synthetase (ACSL) facilitates extracellular fatty acid uptake and converts fatty acids into their corresponding CoA esters for oxidation or esterification into complex lipids (e.g. triglycerides, phospholipids and cholesterol esters). **(C)** The ER-localized enzymes diacylglycerol acyltransferases (DGAT1 and DGAT2), and acyl-coenzyme A (CoA):cholesterol acyltransferases (ACAT1 and ACAT2) synthesize triacylglycerol (TAG) from fatty acids and sterol esters from cholesterol, respectively. These

products are stored in the lipid bilayer of the ER. **(D)** Lipid droplets bud from the ER into the cytoplasm and acquire lipid droplet-associated proteins (PLINs 1-5). **(E)** Rotavirus viroplasms associate with lipid droplets. Inhibitors (shown in red) that block lipid droplet formation or disperse lipid droplets significantly decrease the number and size of viroplasms and the amount of infectious viral progeny. **(F)** Treatment of cells with isoproterenol and IBMX fragment lipid droplets into smaller microdroplets.