# The Significance of Lipid Droplets for the Replication of Rotaviruses and Other RNA Viruses

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## Abstract

The replication of species A rotaviruses (RVAs) and other RNA viruses involves recruitment of and interaction with the cellular organelles lipid droplets (LDs), both physically and functionally. Thus, the inhibition of enzymes of the cellular fatty acid biosynthesis pathway or the activation of cellular lipases degrading LDs reduce the functions of 'viral factories' (viroplasms for rotaviruses) or replication compartments of other RNA viruses and decrease the infectivity titers of progeny virus. In the context, rotavirus research has significantly progressed by the development of human intestinal enteroids (HIEs) as alternative for *in vitro* culture in permanent cell lines, and by the availability of plasmid only-based reverse genetics systems for RVAs, permitting exact structure-function correlations. Involvement of LDs has been found to be essential for the replication of flaviviruses (mainly hepatitis C virus), picornaviruses and noroviruses, and for the growth of some intracellular bacteria.

**Keywords:** Lipid droplets • Fatty acid biosynthesis • Lipolysis • Viral replication • Rotavirus • Hepatitis C virus • *Flaviviridae* • Picornaviruses • Noroviruses.

Species A rotaviruses (RVA) are a major cause of acute gastroenteritis in infants and young children worldwide [1,2]. While there are effective RVA vaccines in use in >100 countries which have reduced associated disease significantly, there is still a mortality of 128,000 children under the age of 5 years per annum worldwide, mainly in countries of low socio-economic conditions [3,4]. During their replication, RVAs form intracellular inclusion bodies, termed viroplasms (vpls) or 'viral factories', in which early viral morphogenesis (subviral particle assembly) and viral RNA replication (to synthesize genomic double-stranded [ds] RNAs) take place. In a classical electron microscopic (EM) study, the presence of newly synthetized RVA particles and 'deposition of lipid droplets' (LDs) in infected cells have been noted [5]. Decades later it was observed by confocal microscopy (CM) that viroplasms form complexes with LDs, as demonstrated by co-localization of lipids and LD-associated proteins (perilipin A, adipophilin, TIP-47 a.o.) with the viral NSP2 or NSP5 proteins, both virus-encoded components essential for vpl formation [6]. Interaction of LDs with vpl-like structures (VLS) in uninfected cells co-expressing viral NSP2 and NSP5 also occurs [6,7].

Lipid droplets are spherical cellular organelles of various sizes and are a major contributor to lipid homeostasis in the cell. They act as depots of neutral fats (as energy sources) and sterol esters and are surrounded by a phospholipid monolayer into which >100 proteins (most notably perilipins) are inserted [8,9]. Beyond functioning as an energy source, LDs have recently been recognized as dynamic cell organelles involved in signal transduction, membrane trafficking and modulation of immune and inflammatory responses [10-13].

Viroplasms appear to recruit LD components early during the RVA replication cycle, and NSP5-specific siRNA was found to block the complex formation of perilipin A with NSP5 as a vpl component [6]. Viral dsRNA, NSP5, and perilipin A were shown to co-sediment in low-density gradient fractions of rotavirus-infected cell extracts [6]. The lipidomes of RV-infected and uninfected MA104 cells were compared by equilibrium ultracentrifugation of cell lysates through iodixanol gradients. The concentrations of virtually all lipids were elevated in RV-infected cells. Fourteen different classes of lipids were differentiated by mass spectrometry. In detail, gradient fractions of low density (1.11-1.15 g/ml), in which peaks of the RV dsRNA genome and lipid droplet- and viroplasm-associated proteins were observed, contained elevated amounts of those lipids which are typically found in LDs, further confirming the close interaction of LDs with viroplasms [14].

Several chemical compounds or their derivatives are known to interfere with enzymes involved in fatty acid and triacyl glyceride (TAG) biosynthesis preceding LD formation: TOFA [5-(Tetradecyloxy)-2-furoic acid] is an inhibitor of the enzyme acetyl-CoA carboxylase 1 (ACC1), C75 an inhibitor of the fatty acid synthase enzyme complex (FASN), and triacsin C an inhibitor of the long chain acyl-CoA synthetase (ACSL). A combination of isoproterenol (a beta-adrenergic stimulator) and isobutylmethylxanthine (IBMX, a phosphodiesterase inhibitor) raises the intracellular cAMP level, activating a cellular hormone-dependant lipase, which leads to dispersion and degradation of LDs (lipolysis) [6,15-17]. Treatment of cells with these compounds (at non-toxic concentrations) decreased the number and size of viroplasms and inhibited dsRNA replication and the production of infectious progeny virus. This effect correlated with significant protection of cells from virus-associated cytopathicity [16,18,19]. Knockdown of FASN and ACC1 by specific siRNAs produced findings similar to those observed by inhibiting these proteins with the chemical compounds [16]. Interestingly, in TOFA-treated cells, the decrease of RVA dsRNA production was disproportionally smaller than the decrease in infectivity, suggesting that TOFA treatment may also affect the lipid composition of the endoplasmic reticulum (ER), where RVA dual-layered particles (DLPs) mature to the infectious triple-layered particles (TLPs), and inferring a role of cellular lipids in virus assembly and/or egress [18]. Of the different treatments, TOFA had the highest chemotherapeutic index. Treatment of mice with agonists of farnesoid X receptors the activation of which decreases intracellular triglyceride contents was found to decrease rotavirus replication [20]. Specific inhibitors of the fatty acid biosynthesis or lipolytic agents may be promising for the development of antiviral therapies [21].

Rotavirus research experienced significant progress with the establishment of Human Intestinal Enteroids(HIEs), derived from stem cells of small intestine epithelium. HIEs are non-transformed, maintain the cellular differentiation of the natural gut epithelium, and can be passaged *in vitro* and frozen for later use [22]. In initial experiments it was shown that infection of HIEs with human RVAs resulted in viroplasm formation and LD stimulation. In addition, RVA infection caused lumenal swelling of HIEs indicating the secretory component of clinical diarrhea, thus making them an attractive model for pathophysiological studies. As an aside, HIEs also enable the replication of non-murine noroviruses which had until recently resisted propagation in cell culture [23].

Rotavirus vpls arise from a complex interplay of virus-encoded and cellular proteins, with the viral proteins NSP2 and NSP5 interacting in conjunction with complex phosphorylation cascades. The advent of plasmid only-based reverse genetics (RG) systems for RVA [24,25] has made a molecular dissection of these events possible. The hyperphosphorylation of NSP5 was found to be essential for its function, since several mutants with impaired hyperphosphorylation (created by RG) exhibited aberrant NSP5-containing structures and impaired viral replication [26]. Mutation of phosphorylation sites in NSP5 and NSP2 as well as silencing of the cellular kinase CK1alpha blocked vpl formation and the production of infectious viral progeny [26,27]. In detail, the vpl-associated NSP2 did not interact

with NSP5, nor was NSP5 hyperphosphorylated [27]. A phosphomimetic NSP2 mutant (changed in amino acid position 313 by RG) co-localized with LDs without NSP5, and vpl formation and virus replication were reduced [28].

Lipid droplets are also essential for the replication of members of the *Flaviviridae* family, e.g. hepatitis C Virus, GB Virus-B, Dengue virus and others [29-33]. HCV mutants, in which the viral core protein cannot associate with LDs, produce less infectious virus,confirming that LDs are essential for HCV replication [30,31]. In detail, ER membranes around lipid droplets where structural proteins and the HCV replicase co-localize are recruited. The ER membranes were found to be decorated with double membrane vesicles, providing a topological map of how HCV might coordinate the steps of viral replication and virion assembly [34]. The HCV lipidome is most similar to that of very low and low-density lipoproteins and dissimilar from that of organelles of the host cell [35]; perilipin 2 is critical for efficient lipoprotein and HCV particle production [36]. Thus, HCV maturation appears to be tightly connected with the biogenesis of lipoproteins [37]. Interestingly, synthetic inhibitors of diacylglycerolacyltransferases (DGATs) catalysing the formation of TAGs, inhibited HCV genome replication and particle maturation [38].

Lipid droplets were also shown to be recruited for the development of replication compartments (RCs) of enteroviruses [39,40]. The synthesis of phospholipids driven from LDs is required for the biogenesis of poliovirus RC [41]. For noroviruses, single, double and multi-membrane vesicles in association with LDs were induced by ORF1 (non-structural) proteins of the virus and found to be similar to those seen in murine norovirus (MNV)- and in picornavirus-infected cells [42].

Lipid droplet involvement was mainly observed for the replication of RNA viruses. However, Marek's disease virus (MDV), an alphaherpesvirus causing lymphoma in infected chickens, was also found to induce an increase of fatty acid biosynthesis (FAB) and of the number of LDs, following infection of primary chicken embryo fibroblasts (CEFs). Chemical inhibitors of the FAB pathway (TOFA and C75) reduced MDV infectivity titers by approximately 30-fold. Interestingly, the addition of downstream metabolites of the inhibitors, such as malonyl-coenzyme A (in the case of TOFA) and palmitic acid (in the case of C75), completely antagonized the effects of the FAB inhibitors [43].

Cellular lipids in general may have different roles in the replication of viruses, such as enabling cell entry, viral replication complex formation, provision of energy for virus replication, and signaling for virus assembly [44].

Intracellular bacteria such as Chlamydia and Mycobacterium tuberculosis also interact with LDs. For Chlamydia it was found that treatment of infected cells with triacsin C reduced bacterial proliferation [45]. For Mycobacterium tuberculosis cellular TAGs appear to have nutritional functions [46]. Nutritional requirements for LDs appear to be a general feature for intracellular bacterial pathogens [47].

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