Direct imaging of the disruption of hepatitis C virus replication complexes by inhibitors of lipid metabolism


**Abstract**

Here we have simultaneously characterized the influence of inhibitors of peroxisome proliferator-activated receptor α (PPARα) and the mevalonate pathway on hepatocyte lipid metabolism and the subcellular localization of hepatitis C virus (HCV) RNA using two-photon fluorescence (TPF) and coherent anti-Stokes Raman scattering (CARS) microscopy. Using this approach, we demonstrate that modulators of PPARα signaling rapidly cause the dispersion of HCV RNA from replication sites and simultaneously induce lipid storage and increases in lipid droplet size. We demonstrate that reductions in the levels of cholesterol resulting from inhibition of the mevalonate pathway upregulates triglyceride levels. We also show that the rate of dispersion of HCV RNA is very rapid when using a PPARα antagonist. This occurs with a faster rate to that of direct inhibition of 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase) using lovastatin in living cells, demonstrating the potential therapeutic value of modulating host cell pathways as part of a strategy to eliminate chronic HCV infection.

**Keywords:**
HCV  
Lovastatin  
PPARα  
Viral replication  
CARS imaging

**Introduction**

Hepatitis C virus (HCV) infection is a rapidly increasing global health problem, with approximately 3% of the global population infected (Reed and Rice, 2000; Thomson and Finch, 2005). The majority of acute HCV infections become chronic, often resulting in severe liver disease including hepatitis steatosis, cirrhosis, and hepatocellular carcinoma (Adinolfi et al., 2001; Fujie et al., 1999; Hashimoto et al., 2000; Kashireddy and Rao, 2004; Lamarre et al., 2003; Moriya et al., 1998; Patton et al., 2004). Currently, there is no vaccine available and the clinical efficacy of modern therapeutics is limited, with an unsatisfactory rate of clearance. HCV is a positive-sense RNA virus that replicates through a double-stranded (ds) RNA intermediate in the cytoplasm of host cells (Chisari, 2005; Lindenbach and Rice, 2005). Its genome encodes a ~3000-amino-acid polyprotein, which is cleaved by host and viral proteases into three structural proteins (core, E1, and E2) and seven nonstructural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B). The HCV replication complex is located on a membranous web composed of viral proteins, replicating RNA, and altered cellular membranes (Egger et al., 2002; Gosert et al., 2003; Moradpour et al., 2003, 2004, 2007).

Central to the HCV life cycle are host–virus interactions that support infection, replication, and viral particle assembly (Chisari, 2005; Lindenbach and Rice, 2005; Moradpour et al., 2007; Penin et al., 2004a, 2004b). HCV induces changes in lipid metabolism and causes the formation of endoplasmic reticulum (ER)-derived membranous webs on which HCV replicates (Adinolfi et al., 2001; Kapadia and Chisari, 2005; Sagan et al., 2006; Su et al., 2002). HCV also induces the accumulation of lipid droplets (LDs) on which the HCV core protein is known to reside (Boullant et al., 2008; Miyanari et al., 2007; Sato et al., 2006; Shi et al., 2003; Shi et al., 2002; Targett-Adams et al., 2008b; Wang et al., 2007). The viral proteins are associated with ER membranes and viral replication and particle assembly are thought to take place at the interface between LDs and the ER-derived membranous webs (Miyanari et al., 2007). Therefore, there is a direct role for cellular lipid metabolism in the life cycle of HCV, and this dependency is closely associated with lipid droplets, membranous webs, and associated ER-derived membranes, which are critical to the propagation of HCV. Hence, understanding the mechanisms that underlie the interactions between HCV and the host cell lipid storage organelles may lead to novel therapeutic strategies for the treatment of HCV infection.

Although it is well established that small molecules that target HCV proteins can behave as antiviral compounds (Lamarre et al.,...
HCV replication can also be modulated using small molecules that target the host cell environment rather than the viral genome. Depleting cholesterol with β-cyclodextrin, for example, disrupts the membranous web upon which HCV replicates (Sagan et al., 2006). Furthermore, lovastatin and fluvastatin, inhibitors of HMG-CoA reductase, the rate-controlling enzyme of the mevalonate pathway, also inhibit HCV replication (Ikeda et al., 2006; Ikeda and Kato, 2007; Wang et al., 2005; Ye et al., 2003). Other small molecule inhibitors of the mevalonate pathway, 25-hydroxycholesterol and cerulenin, also negatively affect HCV replication (Su et al., 2002). GGTI-286 is an inhibitor of downstream protein prenylation and has been demonstrated to inhibit HCV replication through the inhibition of geranylgeranylation of a host protein, FBL2 (Wang et al., 2005). Recently, we have also described a small molecule antagonist of the peroxisome proliferator activated receptor α (PPARα) pathway, 2-chloro-5-nitro-N-(pyridyl)benzamide (BA), that is able to modulate HCV replication (Rakic et al., 2006c) as well as others targeting host pathways (Rakic et al., 2006a, 2006b). PPARα receptors naturally bind to a number of fatty acids and metabolites as well as synthetic ligands like BA (Everett et al., 2000; Xu et al., 2001, 2002). PPARα acts as a sensor for the level of free fatty acids and modulates gene expression of key metabolic pathways involved in lipid metabolism (Everett et al., 2000). Activation of PPARα results in an increase in the enzymes involved in lipid metabolism, fatty acid β-oxidation, apolipoproteins, fatty acid binding proteins, medium chain acyl-CoA dehydrogenase, carnitine palmitoyl-transferase, and microsomal fatty acid ω-hydroxylase (Everett et al., 2000; Mandard et al., 2004).

Herein, we describe the detailed phenotypic changes in LD density and size after treatment with the lipid metabolism inhibitors BA and lovastatin in Huh-7.5 hepatoma cells. Molecular imaging was completed by using coherent anti-Stokes Raman scattering (CARS) microscopy, which allows for the visualization of lipids in living cells without staining or the need for labels (Chen et al., 2002; Cheng and Xie, 2004; Evans et al., 2005; Evans and Xie, 2008; Freudiger et al., 2008; Nan et al., 2003; Nan et al., 2006; Potma and Xie, 2003; Rakic et al., 2006c). We used CARS to first quantify the effects of BA and lovastatin on lipid metabolism and storage on naive cells. This was first performed to understand the effects of the inhibitors on cellular processes in the absence of HCV. We then sought to evaluate how these changes influence the ability of HCV replicons to actively replicate. We simultaneously combined two-photon fluorescence (TPF) and CARS microscopy to capture changes in localization and phenotype of LDs and subgenomic HCV replicon RNA in live cells upon treatment with lipid metabolism inhibitors, providing insight into the dynamics and mechanics of the antiviral effects of these two small molecule modulators of host cell lipid metabolism.

### Results

**PPARα antagonism with BA results in rapid hyperlipidemia in Huh-7.5 cells**

To characterize the effects of inhibitors of lipid metabolism on cellular lipid storage and phenotype, CARS microscopy was used to analyze the lipid phenotype of human hepatoma cells (Huh-7.5) upon treatment with the PPARα antagonist, BA or the HMG-CoA reductase inhibitor, lovastatin (Figs. 1a, b). The CARS microscope was selectively tuned to image LDs by adjusting the frequency difference between the two input laser pulses derived from one light source combined with a photonic crystal fiber (Pegoraro et al., 2009) to match the inherent Raman vibration of C–H bond stretching at 2850 cm⁻¹. A high C–H concentration within lipid droplets provides high contrast for selectively imaging lipid droplets. The lipid phenotype observed after treatment showed dramatic hyperlipidemia induced by 75 μM BA (Fig. 1c), a concentration known to significantly reduce HCV replication in cell culture (Rakic et al., 2006c).

Significant lipid accumulation was observed after a 6-h treatment with BA and was mainly localized around the perinuclear regions of the cell appearing as larger LD aggregates (Fig. 1c). Voxel analysis was used to calculate the percent lipid values by volume per cell (Hellerer et al., 2007; Kennedy et al., 2009; Nan et al., 2006). Voxel analysis determines the number of 3-dimensional pixels (voxels) that are

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Fig. 1. CARS images showing changes in lipids observed in Huh-7.5 cells treated with BA and lovastatin. (a) Structure of 2-chloro-5-nitro-N-(pyridyl)benzamide (BA). (b) Structure of lovastatin. (c) The effects of 75 μM BA on Huh-7.5 cells incubated in culture for 6 h. (d) The effects of 50 μM lovastatin treated Huh-7.5 cells for either 24 or 48 h. Values on the bottom left corner of the CARS images represent voxel analysis indicating the lipid droplet volume per cell (average of 5 cells per sample). Scale bar: 10 μm.
Beza observed similar results. In cells as observed with BA treatment. Again we limited exogenous hyperlipidemia in hepatocytes, resulting in a similar lipid phenotype. This suggests that drugs used to reduce cholesterol levels can also induce those used clinically to lower serum cholesterol levels and demonstrate detectable changes in lipid density at the 24-h time point. Since BA is a transcriptional antagonist of the PPARα pathway, treatment with an agonist of this pathway should have no effect on lipid droplets and the cellular lipid levels should remain similar to the mock-treated samples. As expected, with BF treatment alone lipid levels did not change significantly and the cellular lipid phenotype remained similar to that of the mock-treated samples (Fig. 2b). In order to confirm that the effect seen with BA was due to PPARα antagonism, we carried out a complementation experiment in which cells were first induced by BA to a state of hyperlipidemia, followed by treatment with BF, which should reverse the effects of BA. Since the IC50 of BF is similar to BA (Haubenwallner et al., 1995), we evaluated the effects of 75 μM BF on Huh-7.5 cells previously treated with BA (Fig. 2c).

Next we investigated whether lovastatin would induce similar changes in LDs as BA, although the mechanisms of action are quite different. Huh-7.5 cells treated with lovastatin displayed similar total levels of lipid density at 48 h as did BA-treated cells at the 6-h time point. Increases in lipids were first observed at 24 h and continued to increase through to the 72-h time point (Fig. 1d and Fig. S1). In addition, in cells treated with lovastatin, LD complexes localized in the perinuclear region of the cell were first observed at 24 h. These complexes then diffused from the perinuclear region and adopted a more diffuse cytoplasmic pattern of lipid aggregates at 48 h. Although changes of lipid density were observed as early as 3 h for BA-treated cells, this was not the case for lovastatin treatment, which first demonstrated detectable changes in lipid density at the 24-h time point in Huh-7.5 cells. Since BA is a transcriptional antagonist of the entire PPARα pathway (Anderson et al., 2004; Auboeuf et al., 1997; Berger and Moller, 2002), while lovastatin only inhibits a single enzyme in the mevalonate pathway (Guzman et al., 1993), this may account for the large difference in kinetics observed for these two molecules for the induction of hyperlipidemia. Lovastatin treatment took approximately 8-fold longer to induce the same hyperlipidemic effect as BA treatment, and continued exposure resulted in subcellular reorganization of the LDs that was not observed with BA at any time point. The levels of lovastatin used were significantly higher than those used clinically to lower serum cholesterol levels and demonstrate that drugs used to reduce cholesterol levels can also induce hyperlipidemia in hepatocytes, resulting in a similar lipid phenotype in cells as observed with BA treatment. Again we limited exogenous lipids in cell culture media by depleting the serum concentration and observed similar results.

**Inhibition of HMG-CoA reductase with lovastatin results in hyperlipidemia**

The gene expression of PPARα has been exemplified as a global regulator for directly or indirectly controlling lipid metabolism through a complex network of transcriptionally regulated pathways (Anderson et al., 2004; Auboeuf et al., 1997; Berger and Moller, 2002). After demonstrating through CARS microscopy that the PPARα antagonist, BA, increases lipid storage by altering cellular lipid metabolism, we hypothesized that bezafibrate (BF, Fig. 2a), a PPARα agonist, should reverse the effects of BA treatment by inducing PPAR binding to the peroxisome proliferators response elements (PPREs) that control the transcription of PPRE-regulated genes. Since BA is an antagonist of the PPARα pathway, treatment with an agonist of this pathway should have no effect on lipid droplets and the cellular lipid levels should remain similar to the mock-treated samples. As expected, with BF treatment alone lipid levels did not change significantly and the cellular lipid phenotype remained similar to that of the mock-treated samples (Fig. 2b). In order to confirm that the effect seen with BA was due to PPARα antagonism, we carried out a complementation experiment in which cells were first induced by BA to a state of hyperlipidemia, followed by treatment with BF, which should reverse the effects of BA. Since the IC50 of BF is similar to BA (Haubenwallner et al., 1995), we evaluated the effects of 75 μM BF on Huh-7.5 cells previously treated with BA (Fig. 2c).

CARS imaging and voxel analysis confirmed an increase in cellular lipid volume after 6 h of BA treatment (Fig. 1 and Fig. S1). Following the 6-h BA incubation, the cells were washed and treated with BF for a subsequent 6-h treatment to capture its effects on BA-induced hyperlipidemia. We then measured the effects of treatment with BF (Fig. 2 and Fig. S4) by CARS microscopy. Voxel analysis shows that the lipid density significantly decreased and returned to normal levels, ~2% lipid volume per cell, which is akin to naive cells only fixed at 6 and 12 h (Fig. 2b and Fig. S4). The results from BF treatment clearly demonstrate a reversal of BA’s effect on lipid metabolism. Control cells

**Bezafibrate reduces hyperlipidemia by activating PPARα in Huh-7.5 cells**

**Fig. 2.** Bezafibrate rescues Huh-7.5 cells from hyperlipidemia after the treatment with BA. (a) Structure of bezafibrate. (b) Huh-7.5 cells were fixed without any treatment at 12 h (left panel) and treated with 75 μM BF for 6 h (right panel). (c) Huh-7.5 cells were treated with BA for 6 h and washed with PBS buffer followed by the addition of regular medium (left panel) or 75 μM of BF treatment (right panel) for 6 h and was fixed. Values on the bottom left corner of the CARS images represent voxel analysis indicating the lipid droplet size per cell (average of 5 cells per sample). Scale bar: 10 μm.
treated with BA and then with culture media resulted in a persistent high lipid volume over the same duration for which BF was applied (Fig. 2c and Fig. S4).

**Larger LDs formed in Huh-7.5 cells after BA treatment can result from LD fusion events**

To further characterize lipid aggregate formation and, at the same time, utilize the advantage of CARS microscopy for live cell imaging, we scanned regions containing multiple cells during the first 6 h of treatment with BA (Fig. 3). The hyperlipidemia previously observed in BA-treated cells can either result from de novo lipid synthesis, increases in TG loading into LDs or LD fusion events where smaller LDs combine to form larger LDs. While monitoring the same cell over a 6h time course, we observed fusion events including one involving three independent LDs in close proximity that fused over several hours during BA treatment. This suggests that lipid fusion proteins may play a role in facilitating this process. Since BA treatment results in a dramatic increase in both the size and number of LDs and many LDs become closely associated, it is impossible to quantify the contribution of LD fusion to the formation of larger LDs. Thus, we cannot rule out LD fusion as a contributing mechanism to hyperlipidemia in Huh-7.5 cells treated with BA.

**Imaging of HCV replicon harboring cells treated with BA and lovastatin**

Once we established that CARS microscopy could be used to quantify changes in lipid metabolism and that this can be used to observe LD fusion events, we next wanted to apply this technique to examine the effects of BA and lovastatin on cell culture models for HCV. For this study, we used cells stably harboring a subgenomic HCV replicon (Fig. 4a). Since BA and lovastatin are both known to inhibit HCV replication, we wanted to test whether HCV influences the ability of either BA or lovastatin to induce hyperlipidemia in human hepatoma cells. As observed in Figs. 4b and c, replicon harboring cells treated with BA and lovastatin responded similarly to naive Huh-7.5 cells. This suggests that HCV does not prevent hyperlipidemia, but rather is inhibited by the results of the changes to cellular lipid metabolism, consistent with previous findings (Rakic et al., 2006c; Wang et al., 2005).

Having demonstrated that CARS can effectively monitor changes in lipid phenotypes upon treatment with known lipid metabolism inhibitors, we next sought to probe the effects of these compounds on HCV RNA localization while simultaneously observing their effects on lipid metabolism. Upon treatment with BA or lovastatin, changes in LD accumulation and localization may affect HCV replication complexes and modulation of neutral lipid levels may also influence viral replication. To explore the direct effect of these compounds on HCV RNA, we used two-photon fluorescence (TPF) microscopy to visualize labeled HCV RNA simultaneously with CARS microscopy in order to monitor changes in lipid phenotype. Live cells were electroporated with subgenomic HCV replicon RNA that was covalently labeled at the 5′-end with an Alexa Fluor-488 fluorescent tag (Nan et al., 2006; Noestheden et al., 2007). Although this approach does not give any information about progeny RNA replicated from the template, it is a very useful technique to track RNA localization while simultaneously evaluating the levels of lipid content and storage in the form of LDs.

**Fig. 3.** Demonstration during live cell imaging of an independent lipid fusion event observed over 6 h with BA treatment. Bottom panels are high magnification representations of cropped inset shown in the upper panel. Scale bar: 10 μm.

**Fig. 4.** CARS images demonstrating lipid droplet distribution in Huh-7 cells stably harbouring a subgenomic HCV replicon were treated with BA and lovastatin. (a) Schematic representation of full-length HCV genomic RNA and the subgenomic replicon containing nonstructural proteins in the genomic RNA sequence with the addition of a luciferase reporter gene located at the 5′-region. (b) The effects of 75 μM BA on Huh-7 cells stably harbouring a subgenomic HCV replicon incubated in culture for 6 h. (c) The effects of 50 μM lovastatin on Huh-7 cells stably harbouring a subgenomic HCV replicon incubated in culture for 24 h. Values on the bottom left corner of the CARS images represent voxel analysis indicating the lipid droplet volume per cell (average of 5 cells per sample). Scale bar: 10 μm.
By combining CARS and TPF microscopies, we have observed simultaneously the development of hyperlipidemia and localization of a fluorophore-tagged HCV RNA as described previously (Nan et al., 2006). Our studies indicate that 5′-labeled HCV RNA is replication-competent (Nan et al., 2006; Noestheden et al., 2007; Tonary and Pezacki, 2006). Treatment with BA and lovastatin typically commenced 24 h after electroporation of labeled replicon RNA into cells in order to allow cells to recover and to permit viral translation and establishment of HCV replication complexes. It was generally observed that upon electroporation of Huh-7.5 cells with subgenomic HCV replicon RNA covalently labeled at the 5′-end with Alexa Fluor-488, hundreds of copies of fluorescently tagged RNA were visible following a 24-h recovery and just prior to treatment.

We observed a similar change in lipid phenotype after treatment with BA over 6 h as previously observed in naive Huh-7.5 cells. With lovastatin, changes in lipid phenotype were also observed, but not until 24 h after treatment, as seen in naive Huh-7.5 cells. Since hyperlipidemia is only observed after 24 h and the half-life of the fluorophore-conjugated RNA is approximately 12 h in Huh-7.5 cells (Nan et al., 2006; Noestheden et al., 2007), we are unable to observe significant levels of fluorophore-conjugated RNA at the time points where lovastatin is active. Accordingly, we observe weak fluorescent signals that are highly dispersed in the control cells after 48 h, irrespective of treatment. Therefore, we are unable to evaluate the effects of lovastatin on HCV RNA at the replication sites by this technique (data not shown). By contrast, the rapid kinetics of BA is compatible with the lifetime of the fluorophore-conjugated HCV RNA and thus allowed us to track changes in localization of HCV RNA and simultaneously observe changes in lipid phenotype by CARS microscopy.

**BA disrupts HCV replication complexes**

At the start time of imaging, the labeled replicon RNA was found to be localized in tightly associated bright punctate spots in distinct areas of the cells mostly localized to the perinuclear region with smaller punctate spots residing a little farther away from the nucleus. Rapid changes were observed within the first hour of treatment with BA showing a more diffuse pattern for the labeled HCV RNA (green) surrounding what originally were punctate spots (Fig. 5a). This dispersion of smaller clusters of RNA becomes more apparent with time, diffusing to the rest of the cytoplasm in a progressive manner and eventually levels off at the 5–6 h time point (Fig. 5). We hypothesize that dispersed RNA is no longer undergoing active translation or replication. This is supported by previous work that indicated a significant decrease in levels of HCV proteins and RNA upon treatment with BA at these time points (Rakic et al., 2006c). Under the same conditions but without BA treatment, fluorescently tagged HCV replicon RNA retained a punctate pattern demonstrating that the observed RNA dispersion is caused by the action of BA (Fig. 5b). Voxel analysis allowed us to quantify the dispersion of the HCV RNA as a function of cell volume. We observed that in the absence of BA, the fluorophore-labeled HCV RNA occupies roughly 6%–8% of the total cell volume. When BA is added this volume increases to ~40%. This is consistent with our hypothesis that BA treatment results in the disruption of HCV RNA replication complexes.

![Fig. 5](image-url)

**Fig. 5.** Live cell imaging of Huh-7.5 cells electroporated with a replicon HCV genomic RNA tagged with a 5′-Alexa Fluor 488 label and treated with 75 μM of BA over 6 h. (a, b) Representative images of RNA dispersion after observing more than 40 independent areas of BA-treated cells per sample with more than 6 replicates (240 independent areas). Shown here are the images of two-photon fluorescence (upper panel), CARS images (middle panel), and a magnified view indicated by the boxes of the upper panel of one cell containing labeled HCV RNA (lower panel). Scale bar: 10 μm. (a) The effects of 75 μM BA dispersing the replication complex of HCV 488-RNA. (b) Control cells containing electroporated HCV replicon RNA but no BA treatment. Values on the magnified images represent voxel analysis that measures the volume of dispersion throughout the entire cell of the 5′-fluorophore-tagged RNA from a tightly compact localization within replication complexes. Scale bar: 10 μm.
Imaging of HCV dsRNA confirms dispersion of replication complexes

To confirm the dispersion of HCV RNA that was actively replicating in its replication complexes, we then used an antibody specific for dsRNA in cells stably replicating subgenomic HCV replicons. Imaging was performed to capture changes in dsRNA intermediates as a function of time. To observe a change in localization, from punctate to diffuse localization, we treated the cell line stably harboring an HCV subgenomic replicon with BA (Fig. 6). By using a monoclonal antibody that has been shown to specifically recognize dsRNA in replication complexes based on previous studies (Schonborn et al., 1991; Targett-Adams et al., 2008a), we observed a typical punctate fluorescence pattern in HCV replicon cells detected by immunofluorescence. Our observations using this approach were consistent with results seen in live cell imaging of Alexa Fluor-tagged HCV RNA (no BA treatment) as the labeled HCV RNA was highly concentrated in the replication complexes known to reside in the perinuclear region of the cell (Targett-Adams et al., 2008a). We observed that after a corresponding treatment with BA for 6 h (Figs. 6c, d) the detection of HCV dsRNA was much more diffuse confirming our results with labeled HCV replicon RNA. Voxel analysis indicated a greater than 5-fold increase in the dispersion of the RNA relative to control cells, with voxel analyses indicating that the HCV RNA occupied from 30% to 60% of the cell volume after BA treatment. We do not see appreciable staining in naive Huh-7.5 cells with the same staining protocol confirming the specificity of the antibody to dsRNA intermediates of HCV over cellular RNA (data not shown). Thus, the dsRNA imaging experiments confirm our hypothesis that BA is dispersing HCV RNA from functional replication complexes.

Upregulation of triglycerides compensates for a decrease in cholesterol

Next we sought to understand the effects of these two inhibitors on cholesterol, cholesterol ester, and triglyceride levels in cells supporting HCV replication that contain subgenomic replicons. To evaluate the changes in neutral lipid components in the treated cells, we measured the cellular levels of cholesterol and cholesterol esters in HCV replicon harboring cells with or without BA or lovastatin treatment (Table 1). Given that lovastatin is a well-known inhibitor, which acts by blocking HMG-CoA reductase, the rate determining enzyme in the cholesterol pathway, it is not surprising that a significant decrease of cellular levels of cholesterol and cholesterol esters is observed upon treatment with lovastatin after 48 h (Table 1). BA-treated cells also show a decrease in cholesterol levels; however, cholesterol ester levels, a major LD component, remain relatively constant throughout the course of treatment (Table 1). This is consistent with the observed upregulation in LD biogenesis and the induction of hyperlipidemia. Although cholesterol ester levels are significantly decreased by lovastatin yet remain unchanged with BA treatment, with CARS microscopy a hyperlipidemic phenotype is consistently observed when treated with either of the small molecule inhibitors. Because LDs primarily contain a core of cholesterol esters and triglycerides, triglyceride levels must increase to compensate for the decrease in cholesterol ester levels. To confirm this hypothesis, we measured cellular levels of triglycerides from the same cellular extracts used to measure cholesterol ester levels. Upon treatment with BA (6 h), or lovastatin (48 h), an increase in triglyceride levels is observed compared with the levels in untreated cells (Table 1). These observations validate the hyperlipidemic effects observed under CARS microscopy and confirm that high levels of lipid aggregates observed are consistent with an upregulation of triglyceride levels induced by both small molecules.

Table 1

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<th>Cholesterol1</th>
<th>Cholesterol esters1</th>
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<td>6 h HCV + BA</td>
<td>−1.65 ± 0.06</td>
<td>−1.09 ± 0.09</td>
<td>+ 1.37 ± 0.11</td>
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<tr>
<td>48 h HCV + lovastatin</td>
<td>−2.72 ± 0.02</td>
<td>−3.04 ± 0.07</td>
<td>+ 1.93 ± 0.05</td>
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1 (−) Fold changes represent reductions, whereas (+) fold changes represent increases.

Expression of HCV core protein does not modulate the effects of BA

The HCV core protein is a key building block for the viral nucleocapsid and is an essential protein for HCV infectivity (Boula et al., 2007, 2008; McLauchlan, 2000, 2009; McLauchlan et al., 2002). The core protein is known to colocalize with LDs and components of the very low density lipoprotein (VLDL) assembly pathway (Boula et al., 2008; Gao et al., 2004; McLauchlan, 2009; Sato et al., 2006; Shi et al., 2002). The core protein has also been established to induce fatty acid synthase (FAS) activity in cell culture and induce hyperlipidemia in both cells and animal models (Moriya et al., 1997; Oem et al., 2008; Yang et al., 2008). The HCV genotype 3a core protein is known to exert a much stronger influence on lipid metabolism (McLauchlan, 2009). Here we tested the ability of core proteins from either genotype 1a or 3a to modulate the effects of BA on HCV RNA dispersion. We used bicistronic plasmids expressing GFP and HCV core protein (Fig. 7a) so that we could track individual cells expressing the core protein by GFP and observe changes in the cell by confocal microscopy. We chose to overexpress the core protein rather than use a genomic replicon because we wanted to test whether core has any influence on BA activity at maximal levels of core overexpression.

Hepatoma cells stably harboring an HCV subgenomic replicon were transfected with either the genotype 1a or 3a core protein (Abid et al., 2005; Pazienza et al., 2007) and dsRNA localization was measured in cells expressing GFP using antibody staining. Expression of the core protein was confirmed by Western blot (Fig. 7b). A detailed

![Image](50x160 to 286x360)

Fig. 6. Immunofluorescence detection of HCV dsRNA in cells stably harbouring subgenomic HCV replicons after the treatment with BA. All samples were imaged as described in the Materials and methods section, to detect Cy2-tagged secondary antibody using the FITC channel on the confocal microscope. (a) A control sample of cells stained with Cy2-tagged secondary antibody in the absence of primary antibody that recognizes HCV dsRNA. (b–d) HCV dsRNA staining of cells harbouring a subgenomic replicon was labeled with a monoclonal primary antibody and Cy2-conjugated secondary antibody with and without BA treatment. Naive cells did not show any staining. (b) Cells harbouring a subgenomic replicon without treatment; (c, d) cells treated with 75 μM BA for 6 h. Values on the bottom left of the images represent voxel analysis that measures the volume of dispersion of HCV dsRNA throughout the cell (arrow) in the shown image. Scale bar: 10 μm.
Fig. 7. Immunofluorescence detection of HCV dsRNA in cells stably harbouring subgenomic HCV replicons that were also overexpressing HCV core protein and treated with BA. All samples were imaged as described in the Materials and methods section, to detect Cy3-tagged secondary antibody using the tritc channel on the confocal microscope. (a) Schematic representation of bicistronic plasmids encoding EGFP and HCV core1a or core3a under different promoters were transfected into cells stably harbouring subgenomic HCV replicons. (b) Western blot expression levels of HCV core protein. Lanes are H, Huh-7.5 cells expressing HCV core; R, Huh-7 cells stably harbouring subgenomic HCV replicons; M, mock of 10 μL of lipofectamine; C, 5.0 μg of transfected bicistronic core plasmid. (c) The effects of 75 μM BA on cells stably harbouring subgenomic replicons that are expressing HCV core protein 1a and 3a. EGFP was detected in the fitc channel to monitor core expression, and HCV dsRNA was detected in the tritc channel. Values on the bottom left of the images represent voxel analysis that measures the volume of dispersion of HCV dsRNA throughout the cell in the shown image. Scale bar: 10 μm. (d) A graph quantifying the percentage of dispersion of dsRNA in Huh-7 cells stably harbouring subgenomic HCV replicons and expressing either the HCV core protein from genotype 1a or 3a were treated with and without BA (average of at least 5 samples per data set).
study of the effects of genotype 3a core by CARS microscopy is the subject of another study (Lyn, R.K. and Pezacki, J.P., manuscript in preparation). Interestingly, HCV replicon harboring cells that were overexpressing the HCV core protein still showed a dramatic increase in RNA dispersion upon treatment with BA for 6 h (Fig. 7c, d). Neither the overexpression of core 1a nor that of core 3a had an appreciable effect on the degree of HCV dsRNA dispersion. It appears that neither protective effects nor synergistic effects were observed even with core 3a, which is well known to have a strong influence on sterol response element binding protein (SREBP) and FAS activity. Rather, the effects of BA appear to dominate over any changes to the host cell that are induced by the HCV core protein.

Discussion

Host interactions and specific host factors play important roles in HCV replication (Chisari, 2005; Sato et al., 2006; Tai et al., 2009; Vaillancourt et al., 2009). Previously, it has been established that host cell lipid metabolism plays an important role in HCV propagation and that inhibitors of the mevalonate pathway and antagonists of PPARα-signaling block HCV replication (Pezacki et al., 2009; Rakic et al., 2006c; Sagan et al., 2006; Su et al., 2002). In addition, eliminating cellular sphingolipids decreases HCV replication (Sakamoto et al., 2005) and the budding of infectious HCV particles involves association with apolipoprotein-dependent VLDL particles for viral assembly and egress (Gastaminza et al., 2008; Huang, Chen, and Ye, 2007); In the case of inhibitors of the mevalonate pathway, it has been established that a geranylgeranylated host protein, FBL2, is required for competent HCV replication complexes (Wang et al., 2005). However, the correlation of changes in cellular lipid metabolism and simultaneous effects of HCV are difficult to measure. CARS microscopy is a powerful tool for noninvasively examining changes in LDs that result from treatment with small molecules that affect host lipid biogenesis pathways. Coupled with TPF, CARS microscopy can measure effects on lipid metabolism while simultaneously tracking fluorescently tagged HCV RNA molecules. Herein we show that HCV RNA is dispersed from replication complexes upon treatment with BA. This likely prevents the association of the HCV RNA with host and viral proteins that are necessary for replication. Alternatively, membrane environments such as the membranous web that serve as a platform for HCV replication complexes may also be perturbed resulting in the observed decrease in HCV replication. In addition, it has recently been reported that LDs, thought to act primarily as storage organelles, are important for viral replication and assembly of the replication complex in LD-associated membranes (Miyanari et al., 2007). The changes we observed in viral RNA localization upon induction of a hyperlipidemic state in HCV replicon expressing cells as well as cells overexpressing HCV core protein supports these findings.

Herein, we have shown that treatment with lipid metabolic inhibitors resulted in perturbations in lipid phenotype over time, specifically changes in LD density and distribution. BA rapidly induces hyperlipidemia by 6 h after treatment while lovastatin treatment results in progressive lipid loading from 24 to 72 h. Upon these changes in lipid metabolism, we measured a significant increase in triglyceride levels, the other major component found within LDs, which shows that the overall composition of the droplets is likely changing as the hyperlipidemic state is induced since the ratios of cellular cholesterol and triglycerides are clearly altered during treatment with BA and lovastatin.

The biogenesis of triglycerides and cholesterol requires cellular acetate. As acetyl-CoA is formed, it enters either the mevalonate pathway or the malonyl-CoA pathway to form cholesterol for the former and triglycerides for the latter (Fig. 8a). Our results suggest that inhibition of the mevalonate pathway with BA does not prevent triglyceride synthesis and may actually promote it as a homeostatic response to the decrease in cholesterol levels. Lovastatin may elicit similar signaling through a homeostatic response to the inhibition of the mevalonate pathway and cholesterol synthesis.

Because the timeline for the emergence of hyperlipidemia was observed to be similar to that of HCV RNA knockdown upon BA treatment, we monitored changes in HCV RNA localization in an attempt to visualize and understand the mechanism by which HCV replication is inhibited. Employing a 5′-Alexa Fluor-tagged subgenomic HCV replicon RNA that was electroporated into Huh-7.5 cells, we observed a punctate RNA staining localized in the perinuclear region where the replication complex is known to reside (Moradpour et al., 2003, 2004, 2007; Wolk et al., 2008). Simultaneous TPF and CARS microscopy was used to capture the dynamics of RNA localization, and at the same time monitor changes in lipid droplet distribution. We have demonstrated that HCV RNA labeling is an effective tool to visualize dynamic processes and localization in living cells. In addition, this was confirmed by dsRNA antibody staining (Fig. 6). Live cell imaging studies also provided the means to understand changes in localization that are associated with changes in the host cell.

By combining the use of labeled RNA in live cell imaging, we find that modulating host metabolic processes indirectly induces changes in HCV RNA localization. Immediately after treatment with BA, TPF images show the rapid dispersion of labeled RNA in a diffuse pattern throughout the cytoplasmic space over 6 h. This coincides with changes in lipid dispersion in the cell that likely disrupts the replication complex to which the HCV RNA is coupled in LD-associated membranes. This progressive diffusion of labeled RNA directly corresponded to the time points observed under changes in lipid phenotype. This is consistent with previous results demonstrating that BA decreases HCV RNA replication over a 6-h time course (Rakic et al., 2006c). RNA dispersion from replication complexes was quantified using voxel analysis and showed a marked increase in RNA dispersion occupying greater than 40% on average, of the cell after only a 6-h BA treatment.

While we observed an increase in hyperlipidemia as demonstrated by CARS microscopy, cells treated with either inhibitor consistently displayed LD aggregates resulting by an increase in LD size and number. These large complexes are initially confined to the perinuclear region of the cells, although with lovastatin treatment, they diffuse into the cytoplasm slowly over time. LD fusion was observed to occur in live cells as demonstrated by CARS microscopy and is likely a source for the formation of larger LDs observed. Larger LDs may also arise from increases in TG loading into LDs as well as an increase in de novo synthesis of lipids. The fact that we have observed some fusion events indicate that the SNARE system, which is known to have an important role in LD fusion, involving several proteins including NSF (N-ethylmaleimide-sensitive factor), α-SNAP (soluble NSF attachment protein) and the SNAREs (SNAP receptors), SNAP23 (synaptosomal-associated protein of 23 kDa), syntaxin-5, and VAMP4 (vesicle-associated membrane protein 4) (Boström et al., 2007; Olofsson et al., 2008), may be modulated during treatment with BA. Previously it was shown that the knockdown of the genes for SNAP23, syntaxin-5, or VAMP4 decreases the rate of fusion and the size of the LDs observed in liver cells (Boström et al., 2007; Olofsson et al., 2008). The observation of LD fusion suggests that BA, and thus negative regulation of PPARα signaling, may stimulate the SNARE system to further aid in the storage of triglycerides and cholesterol esters.

The HCV core protein, in particular, the core protein originating from genotype 3a, is well known to induce hyperlipidemia largely by activating SREBP-controlled genes as well as increasing FAS activity in hepatoma cells (McLauchlan, 2009; Moriya et al., 1997; Oem et al., 2008; Yang et al., 2008). However, during overexpression of HCV core proteins, we do not observe dispersion of HCV RNA as measured using a dsRNA-specific antibody. This implies that the effects of core protein alone are not sufficient to influence replication complex localization and stability. Also, we find that core neither promotes nor attenuates
the effects of BA on HCV RNA localization. The upregulation of FAS and ultimately VLDL secretion by the HCV core protein may be controlled evolutionarily in order to maximize infectious particle secretion but not so high as to cause changes in replication complex fidelity through altered localization.

Our observations of changes in LD phenotype may provide deeper insight for the importance of the local membrane environment necessary for HCV replication. We observed punctate HCV RNA staining patterns consistent with localization at replication complexes in the interstitial space between the LD and the ER membrane forming a LD-associated membrane or membranous web as has been previously proposed (Moradpour et al., 2003, 2004, 2007; Wolk et al., 2008). When hyperlipidemia is induced with the PPARα antagonist, BA, two possible pathways may lead to the dispersion of HCV RNA (Fig. 8b.) First, BA has been shown to cause a rapid decrease in HMG-CoA reductase levels (Rakic et al., 2006c) that likely results in the reduction in geranylgeranylation of FBL2 (Wang et al., 2005), and, in turn, causes the release of HCV replicon RNA from the replication complexes. FBL2 interacts with NS5A at the site of the replication complex where the HCV nonstructural proteins reside (Wang et al., 2005). Second, it is possible that by increasing LD size via droplet fusion events there are also changes to the local environment around LDs and the membranous web that disrupts the replication complexes and this could give rise to the rapid kinetics of the RNA dispersion observed.

In conclusion, we have shown that HCV RNA replication is disrupted by BA and lovastatin by altering host cell metabolism and, specifically, by causing a dispersion of the RNA from replication complexes. CARS and TPF can be used to measure dynamics and subcellular localization of HCV RNA while at the same time imaging changes in host cell metabolism and alterations in LDs. This is particularly significant for HCV given that LDs are of such critical importance for the infectivity of the virus. CARS and TPF microscopy show a buildup of lipids and LDs upon treatment with BA, a PPARα antagonist, which is consistent with an impaired function of the PPARα receptor. Simultaneous CARS and TPF experiments indicate that BA disperses HCV RNA, and this is coincident with a decrease in HCV replication that suggests that the HCV RNA is no longer located in replication complexes in the membranous web environment.
Materials and methods

In vitro transcription

In vitro transcripts were generated using a MEGAscript kit (Ambion, Austin, Texas) according to the manufacturer's protocol. In brief, the template DNA was linearized with the restriction enzyme Scal (New England BioLabs, Pickering, Ontario), precipitated for less than 30 min, and resuspended in RNase-free water to a concentration of 0.5μg/μL. The in vitro reaction was set up and incubated at 37 °C for 2 h. To degrade the DNA template, 1μL of DNaseI was added and incubated for another 15 min at 37 °C. The in vitro transcripts were then cleaned using the MEGAclear kit from Ambion (Austin, Texas) according to the manufacturer's protocol. The concentration was determined by measurement of the absorbance at 260 nm with a ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE), and RNA integrity was verified by electrophoresis using the Agilent 2100 bioanalyzer with the RNA LabChip kit according to the manufacturer's protocol.

5'-End labeling of RNA with Alexa Fluor 488 C5 dye

RNA in vitro transcripts were labeled with an Alexa Fluor 488 C5 maleimide dye (Invitrogen, Burlington, ON) using the 5'-EndTag Nucleic Acid Labeling System from Vector Laboratories (Burlingame, CA). Briefly, 120μg of HCV RNA was labeled with the thiol-reactive label, Alexa Fluor 488 C5 maleimide. Briefly, a thiophosphate is transferred from ATP–S to the 5' hydroxyl group of the RNA by T4 polynucleotide kinase. After addition of the thiol functional group, a thiol-reactive label was chemically coupled to the 5'-end of the RNA. Finally, the labeled RNA is purified by phenol–chloroform extraction and precipitated prior to quantification.

Simultaneous CARS and two-photon microscopy

The CARS microscopy system uses a single femtosecond Ti:sapphire oscillator (Spectra Physics Tsunami operating at 80 MHz) as the excitation source. The frequency difference between two input lasers, Stokes and pump beam, is equal to that of the Raman resonance of interest. The second longer wavelength (Stokes beam) is generated from the pump and STOKES lasers, stokes and pump beam, is equal to that of the Raman resonance of interest. The second longer wavelength (Stokes beam) is generated from the pump and STOKES lasers, sapphire oscillator (Spectra Physics Tsunami operating at 80 MHz) in the wavelength range of 1035 nm with negligible amplitude and precipitated prior to quantification.

Electroproportion of labeled RNA in Huh-7.5 cells

Huh-7.5 cells were transfected with fluorophore-labeled RNA through electroproportion using a BTX ECM830, Harvard Apparatus Inc., Holliston, MA. Briefly, cells were washed twice and resuspended with PBS. Five micrograms of labeled RNA was mixed with 80μL of washed cells with a density of 2.4×10^6 cells in a 4-mm gap cuvette and was pulsed five times at 820 V, 99μs pulse length at 1.1-s intervals. After a 10-min recovery time, cells were resuspended in prewarmed DMEM and plated on Lab-Tek Chambers.

BA and lovastatin treatment

Twenty-four hours after electroproportion of labeled RNA, at an approximate density of 70%–90% confluence, cells were treated with either 75μM BA (Calbiochem, San Diego, CA) or 50μM lovastatin (Cedarlane, Burlington, ON) in a total volume of 2 mL per Lab-Tek Chamber well. BA was diluted in prewarmed DMEM and was added to the cellular sample prior to imaging with the specified time courses.

Fixed cell protocol

At the desired endpoints, cells were washed twice with PBS, followed by a 15-min incubation at room temperature with fixing solution (4% formaldehyde, 4% sucrose, 1 mL). The fixed cells were washed twice with PBS for 3 min and then stored at 4 °C in PBS prior to imaging.

Measurement of cellular triglyceride and cholesterol content

Cells were transfected with HCV RNA as described above. At 24 h after transfection, cells were treated with BA and lovastatin for 6 and 48 h, respectively, after which lipids were extracted from 10^5 trypanosed cells. Triglyceride and cholesterol concentrations were analyzed directly by spectrophotometric analyses, using the triglyceride and cholesterol/cholesterol ester quantification kit (BioVision, Mountain View, CA) according to the manufacturer's instructions. Total protein levels were quantified with the BCA protein assay (Pierce, Rockford, IL). Triglyceride and cholesterol levels were expressed as nmol/mg protein and μg/mg protein, respectively.

Statistical analysis

Individual experiments in this study were performed in triplicate in order to confirm the reproducibility of the results. Values are represented as means±standard deviations. The statistical significance of differences between two or more means was evaluated by using analysis of variance (ANOVA); P values of less than 0.05 (indicated by asterisks) were considered to be statistically significant.
Rescue with Bezafibrate

Following 6 h of BA treatment, culture cells were washed 2× PBS, and then treated with either 75 μM BF (Cedarlane, Burlington, ON) in prewarmed DMEM (2 ml), or DMEM only (2 ml). Cells were fixed in the same manner as described.

Quantitative voxel analysis

Quantitative data from the CARS images were determined using ImageJ software. A voxel counting procedure was used to determine the number of voxels in a defined region that met a set threshold intensity. Using our software and conditions, this was set to 55, to eliminate all background. Using the ImageJ software the regions of interest (ROI) were defined by outlining the cell from autofluorescent images or by viewing z-stacks of the individual images. The voxels that met the threshold intensity were then counted in the ROI of a defined cell outline revealing the volume of lipids. In each image, 5 cells were counted for a lipid volume average.

Immunofluorescence analysis

Huh-7 cells harboring the HCV subgenomic replicon were seeded at 1.4 × 10^5 cells/well in DMEM on coverslips in a 12-well plate. After 24 h, at a confluency of 70%–80%, cells were treated with 75 μM BA for 3 and 6 h. After BA treatments, cells were washed once with 1× PBS, pH 7.4 and fixed with precooled 100% methanol for 10 min at −20°C. Cells were washed three times with 1× PBS and incubated for 1 h at room temperature with a mouse monoclonal antibody specific for dsRNA (J2, 1:300 dilution in PBS, Scicons, Hungary). After three more washes with PBS, cells were incubated with Cy2-labeled donkey anti-mouse IgG secondary antibody (1:1000 dilution in PBS, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). As a loading control, membranes were stripped and reprobed using a mouse PTP1D 1× antibody (1:2500 dilution; Sigma, Saint Louis, MO) was used with the same 2nd antibody described above. Protein bands were visualized by Western Lightning Western Blot Chemiluminescence reagents (GE Healthcare, Baie d’urfe, QC) according to the manufacturer’s protocol.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.08.022.

References


