Oxidative Stress Attenuates Lipid Synthesis and Increases Mitochondrial Fatty Acid Oxidation in Hepatoma Cells Infected with Hepatitis C Virus*

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Cytopathic effects are currently believed to contribute to hepatitis C virus (HCV)-induced liver injury and are readily observed in Huh7.5 cells infected with the JFH-1 HCV strain, manifesting as apoptosis highly correlated with growth arrest. Reactive oxygen species, which are induced by HCV infection, have recently emerged as activators of AMP-activated protein kinase. The net effect is ATP conservation via on/off switching of metabolic pathways that produce/consume ATP. Depending on the scenario, this can have either pro-survival or pro-apoptotic effects. We demonstrate reactive oxygen species-mediated activation of AMP-activated kinase in Huh7.5 cells during HCV (JFH-1)-induced growth arrest. Metabolic labeling experiments provided direct evidence that lipid synthesis is attenuated, and β-oxidation is enhanced in these cells. A striking increase in nuclear peroxisome proliferator-activated receptor α, which plays a dominant role in the expression of β-oxidation genes after ligand-induced activation, was also observed, and we provide evidence that peroxisome proliferator-activated receptor α is constitutively activated in these cells. The combination of attenuated lipid synthesis and enhanced β-oxidation is not conducive to lipid accumulation, yet cellular lipids still accumulated during this stage of infection. Notably, the serum in the culture media was the only available source for polyunsaturated fatty acids, which were elevated (2-fold) in the infected cells, implicating altered lipid import/export pathways in these cells. This study also provided the first in vivo evidence for enhanced β-oxidation during HCV infection because HCV-infected SCID/Alb-uPA mice accumulated higher plasma ketones while fasting than did control mice. Overall, this study highlights the reprogramming of hepatocellular lipid metabolism and bioenergetics during HCV infection, which are predicted to impact both the HCV life cycle and pathogenesis.

With ~200 million people infected worldwide, hepatitis C virus (HCV)† is a global health problem and a major cause of viral hepatitis. Persistent infection occurs in ~70% of infected patients leading to inflammation, insulin resistance, steatosis, fibrosis, and hepatocellular carcinoma (1). Current direct-acting antivirals are predicted to be a cure for most patients, but the high cost of this treatment means that the pathological consequences of persistent HCV infection will remain a concern. Although the pathology associated with chronic HCV infection was initially thought to be due to HCV-specific immune responses (2), the current opinion is that direct cytopathic effects in virally infected cells also contribute to HCV-associated liver injury (3, 4). The cellular mechanisms by which HCV replication might mediate liver injury are unclear, but there is no doubt that oxidative/nitrosative stress in HCV-infected cells plays an important role in the initiation and progression of liver damage (3, 5, 6). Oxidative/nitrosative stress essentially arises when the production of reactive oxygen (and nitrogen) species (ROS and RNS, respectively) exceeds cellular antioxidant defenses. HCV-induced oxidative/nitrosative stress has been observed in vivo and in vitro (5–9) and has been assigned to almost all HCV proteins (i.e. core, E1, E2, NS3/4A, NS4B, and NS5A) (5), with core being the most potent inducer (10, 11). Several mechanisms have been identified by which HCV infection can lead to the induction of ROS/RNS, including mitochondrial alterations (12–16); redistribution of calcium between the ER, cytoplasm, and mitochondria (17–23); increased expression of NAPDH oxidases (24, 25); enhanced expression of CYP2E1 (26–29); as well as ER stress and the unfolded protein response (10, 18, 22, 30, 31). Oxidative stress also impacts the HCV life cycle at the level of replication and translation and can lead to viral genome heterogeneity, possibly facilitating viral escape from immune detection (32–36). A better understanding of the cellular events that accompany oxidative/nitrosative stress is likely to contribute to our understanding of the pathogenesis of HCV, as well as provide insight into the HCV life cycle.
Oxidative/nitrosative stress has recently emerged as a key activator of the AMP-activated protein kinase (AMPK) signaling system in several cell types, including hepatocytes (37). AMPK is a ubiquitously expressed heterotrimeric serine/threonine kinase complex, consisting of a catalytic α-subunit and two regulatory β- and γ-subunits. Once activated, AMPK serves as a “metabolic master switch,” promoting cellular adaptation and survival in response to environmental or nutritional stressors (38). Full activation of AMPK requires specific phosphorylation (Thr-172) within the activation loop of the catalytic domain of the α-subunit by upstream kinases (39). Regardless of the stimulus, activated AMPK turns on ATP-producing processes, such as fatty acid oxidation, and turns off ATP-consuming processes, such as de novo lipogenesis (DNL) (40). Thus, the conservation of ATP is the net effect of AMPK activation.

Disturbances in lipid metabolism have long been associated with chronic HCV infection (41–47), and the discovery of a specific HCV strain based on genotype 2 (JFH-1; Japanese fulminant hepatitis-1) that efficiently infects and replicates in cultured Huh7.5/7.5.1 hepatoma cells (48–51) has provided considerable insight regarding the intimate link between host cell lipids and HCV infection, at virtually each stage of the HCV replication cycle (45). Providing there are sufficient levels of viral replication, an HCV-induced cytopathic effect is readily observed with this system and is characterized by massive cell death due to apoptosis, which strongly correlates with cell cycle arrest and the induction of numerous genes involved in antioxidative stress response (7, 9, 52–54). Rapidly proliferating cells require a constant supply of lipids for membrane biogenesis and protein modifications (55), and HCV replication is expected to increase this demand further (45). However, the demand for lipid synthesis is expected to be lower in growth-arrested cells (55), albeit with predictable consequences to the viral life cycle. This study examines the hypothesis that reprogramming of hepatic lipid metabolism occurs in infected cells undergoing HCV-induced cell cycle arrest in response to oxidative/nitrosative stress through the activation of AMPK. The link between oxidative/nitrosative stress and altered lipid metabolism is predicted to impact both the HCV life cycle and pathogenesis.

**Experimental Procedures**

**Materials**—Fatty acid-free bovine serum albumin (BSA), oleic acid (OA), glycerol, leupeptin, phenylmethanesulfonyl fluoride (PMSE), protease inhibitor mixture, cholesteryl oleate, l-α-phosphatidylcholine, heptane, isopropyl ether, GW7647, α-tocopherol, N-acetylcysteine (NAC), and digitonin were obtained from Sigma. Thin layer chromatography plates (catalog no. WHT4860-820) were from Fisher. Dulbecco’s modified Eagle’s medium (DMEM), TRIzol, Platinum Quantitative PCR SuperMix-UDG, Lipofectamine 2000, 2′,3′-dichlorodihydrofluorescein diacetate (H2DCFDA), Click-iT EdU Alexa Fluor 488 flow cytometry kit, and penicillin/streptomycin mixture 2000 were purchased from Invitrogen. OxiSelect hydrogen peroxide/peroxidase assay kit was from Cell Biolabs, Inc. (San Diego). The antibodies were obtained from the following sources: rabbit anti-human CPT1A and rabbit anti-human ACADL polyclonal antibodies were from GeneTex Inc. (Irvine, CA); mouse anti-core C7-50 monoclonal antibody and mouse monoclonal IgG1 (isotype control, ab170190) were from Affinity BioReagents (Rockford, IL); mouse anti-NS5A NE10 monoclonal antibody (NE10) was a gift (Timothy L. Tellinghui, Dept. of Infectious Diseases, The Scripps Research Institute); rabbit anti-human PPARα polyclonal antibody was purchased from Applied Biological Materials Inc. (Richmond, British Columbia, Canada); rabbit anti-histone H2B (ab18977) was from Abcam (San Francisco, CA); rabbit anti-AMPKα and anti-phospho AMPKα (Thr-172) monoclonal antibodies (D5A2 and 40H9, respectively), acetyl-CoA carboxylase rabbit monoclonal antibody (C83B10), and phospho-acetyl-CoA carboxylase (Ser-79) (D7D11) rabbit mAb were from Cell Signaling Technology, Inc. (Danvers, MA); anti-human actin (clone C4) mouse monoclonal antibody was from Millipore (Billerica, MA); HRP-conjugated anti-mouse IgG antibody was from MP Biomedical (Solon, OH); HRP-conjugated donkey anti-rabbit IgG was from Jackson ImmunoResearch (West Grove, PA); Alexa Fluor goat anti-rabbit IR dye 680 and goat anti-mouse IR dye 750 were from Invitrogen. Micro BCA protein assay reagent kit was obtained from Pierce; [9,10-3H]oleic acid, [1-14C]oleic acid, and [3H]acetate were obtained from PerkinElmer Life Sciences. Western blotting detection reagents and Hyperfilm™ ECL were from GE Healthcare. PPRE Cignal™ Reporter system was from SABiosciences via Qiagen (Toronto, Ontario, Canada). ENLITEN ATP assay system bioluminescence detection kit and Dual-Luciferase reporter assay system were from Promega (Madison, WI). qScript™ cDNA SuperMix was from Quanta Biosciences (Gaithersburg, MD). All DNA primer synthesis was performed by IDT (Coralville, IA). Cell proliferation kit I (MTT) was from Roche Applied Science.

**Cells and Cell Culture**—Huh7.5 cells, a mutant line of Huh7 cells that support HCV replication with high efficiency (56, 57), and pFH-1, used to transfect Huh7.5 cells to generate infectious virus stocks, were generously provided by Dr. Charles M. Rice (Rockefeller University, New York). Unless otherwise indicated, cells were maintained in DMEM containing 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in humidified air containing 5% CO2.

**Generation of Viral Stocks and Infected Cell Cultures**—Viral stocks were collected from Huh7.5 cells transfected with Japanese fulminant hepatitis (JFH)-1 HCV RNA between 3 and 40 days post-transfection essentially as described (53, 58). Infected cell cultures were generated by inoculating low passage Huh7.5 cells with viral stocks at a multiplicity of infection (m.o.i.) = 0.01 infectious virions/cell essentially as described (53, 58). The percentage of HCV-infected cells and the production of viral RNA and infectivity titers were determined periodically by immunocytochemistry with anti-HCV core immunostaining, quantitative RT-PCR, and limiting dilution assays essentially as described (58, 59). No split was necessary after 10 days due to cytopathic effects (53). Instead, these were enrolled into experiments as “infected” cells. “Naive” cells used in experiments were derived from the same Huh7.5 cell stocks as infected cells (58). Sequencing of the viral genome (Molecular Biology Services Unit, Dept. of Biological Sciences, University of Alberta) confirmed the absence of adaptive mutation G451R previously
HCV Infection and Oxidative Stress-mediated AMPK Activation

demonstrated to confer enhanced infectivity (53) from all infected cells enrolled into experiments.

Flow Cytometry Analysis—The number of cells progressing through S-phase was determined by flow cytometry using Click-iT EdU Alexa Fluor 488 flow cytometry kit according to the supplied protocol. Flow cytometry was performed on a BD FACSCalibur machine (BD Biosciences) with analysis done using FlowJo software (version 8.7.1).

Cellular Viability—Cellular viability was estimated using cell proliferation kit I (MTT) according to the manufacturer’s instructions.

Detection of ROS/RNS—Production of ROS/RNS was monitored using the cell-permeant H2DCFDA according to the manufacturer’s instructions. Upon cleavage of the acetate groups by intracellular esterases and oxidation by intracellular H2O2, low molecular weight peroxides, peroxynitrites, and groups by intracellular manufacturer’s instructions. Upon cleavage of the acetate

Thus, fluorescence intensity is proportional to the amount of H2O2 levels were determined using the OxiSelect hydrogen peroxide/peroxidase assay kit according to the manufacturer’s instructions. In the presence of HRP, the 10-acetyl-3,7-dihydroxyphenoxazine reagent reacts with H2O2 in a 1:1 stoichiometry to produce highly fluorescent 2',7'-dichlorofluorescein. Thus, fluorescence intensity is proportional to the amount of oxidants produced by the cells. Cellular H2O2 levels were determined using the OxiSelect hydrogen peroxide/peroxidase assay kit according to the manufacturer’s instructions. In the presence of HRP, the 10-acetyl-3,7-dihydroxyphenoxazine reagent reacts with H2O2 in a 1:1 stoichiometry to produce highly fluorescent 2',7'-dichlorofluorescein.

Quantitative Immunoblot Analysis—Whole cell lysates were obtained by directly lysing cell pellets with RIPA buffer followed by sonication. Post-nuclear supernatants and nuclear extracts were prepared essentially as described (65). Whole cell lysates, post-nuclear supernatants, or nuclear extracts were separated on SDS-polyacrylamide gel, transferred to nitrocellulose membranes, and blocked with 3% BSA in PBS. Membranes were then incubated for 1 h at room temperature with appropriate dilutions of primary antibodies followed by 1:10,000 appropriate IR dye-conjugated IgG secondary antibodies. All immunoreactive bands were quantified using the Odyssey CLx Infrared Imaging System from LI-COR Biosciences (Lincoln, NE).

Malonyl-CoA Assay—Reversed phase high performance liquid chromatography was used for the determination of malonyl-CoA levels. Details of this method are supplied elsewhere (66).

De Novo Lipogenesis—Cells were seeded in 60- mm culture dishes and allowed to adhere for 4 h prior to incubating for 4 h in 2 ml of DMEM containing 10 μCi of [3H]acetate and 100 μM sodium acetate. Cells were washed twice with PBS and collected. Lipids were extracted and resolved by TLC in neutral lipid solvent, and the bands corresponding to triacylglycerol (TAG), free cholesterol (FC), cholesteryl ester (CE), free fatty acids (FFAs), and phospholipids (PL) were scraped and analyzed as described below under “Lipid Analysis.”

Release of [3H]H2O from the β-Oxidation of [9,10-3H]Oleic Acid—These experimental conditions were adapted from studies conducted by Hansson et al. (67). Cells were seeded in 60-mm culture dishes and allowed to adhere for 4 h prior to pulse labeling for 4 h with 2 ml of serum-free DMEM containing 5 μCi of [9,10-3H]OA and 0.4 mM OA complexed to 0.4% BSA (pulse) to stimulate neutral lipid synthesis. After 4 h, the pulse media were removed by aspiration and retained for analysis, and cells were washed three times with serum-free DMEM containing 0.4% fatty acid-free BSA and then incubated with 2 ml of serum-free DMEM for an additional 4 h (chase) prior to collecting the chase media for analysis. Pulse and chase media samples were centrifuged at 2,500 × g for 5 min to remove cell debris. Labeled fatty acids were precipitated out from media samples by the addition of 30 μl of 20% BSA and 16 μl of 70% perchloric to 200 μl of culture media. Media were then centrifuged at 25,000 × g for 5 min before an aliquot of the supernatant was counted for radioactivity. The cells were washed with ice-cold PBS, and cell homogenates were prepared in the same buffer, and protein concentration was measured.

14CO2 production from the complete β-oxidation of [1-14C]oleic acid was performed as described previously (68). Briefly, [1-14C]oleic acid stock (2.5 μCi) bound to 0.4 mM OA, 0.5% BSA in 10 ml DMEM was placed in T150 flasks containing cell cultures and fitted with a center vial with a filter paper moistened with 1 ml KOH to absorb CO2. The flasks were sealed with rubber septa, and the cells were incubated at 37 °C. After 4 h of incubation, the cells were lysed by the injection of 1 ml of perchloric acid through the flask seal, which also results in the liberation of CO2. Then 0.4 ml of 1 M KOH was added to a center well suspended in the sealed flask. The incubations were continued for a further 1 h at room temperature to allow the CO2 to be absorbed onto the filter paper and KOH. The filter papers and KOH were removed, added to 4 ml of scintillation fluid, and counted.

Fatty Acid Uptake—Cumulative uptake of oleic acid was measured as described previously (69). Adherent cells were incubated with [9,10-3H]oleic acid (2.5 μCi/ml) mixed with 0.4 mM OA, 0.5% FFA-free BSA at 37 °C for 1, 5, 10, 20, or 30 min. Cells were washed with ice-cold 0.5% BSA in PBS three times and then in PBS alone three times. Cells were lysed with 1 M NaOH, and aliquots were used for scintillation counting and protein determination.

ATP Assay—ATP was measured in cells using the Promega ENLITEN ATP assay system bioluminescence detection kit according to manufacturer’s instructions.

PPRE Reporter Assay—PPRE reporter assay was performed according to the CignalTM reporter assay handbook. Briefly, cells were transfected with tandem PPRE-firefly luciferase and control Renilla luciferase using Lipofectamine 2000. GW7647 was added 24 h after the start of transfection for an additional 24 h, at which time the cells were harvested and processed according to the Dual-Luciferase reporter assay manufacturer’s instructions. Luminescence was measured with the Enspire 2300 multilabel reader (PerkinElmer Life Sciences), and firefly luciferase signal was normalized to internal control Renilla luciferase signal.

Quantitative RT-PCR— Primer sequences used for quantitative RT-PCR are immediately below. Total RNA was isolated using TRIzol reagent and reverse-transcribed with a mixture of random hexamers and oligo(dT) using qScript™ cDNA SuperMix according to manufacturer’s instructions. The transcripts
were detected by real time PCR using a Qiagen Rotorgene 3000 instrument. Reaction mixtures contained Platinum Quantitative-PCR SuperMix-UDG with 400 nM concentrations for each primer in a total volume of 20 μl. Following a 3-min initial denaturation at 94 °C, PCR amplification of all genes proceeded for 40 PCR cycles of 95 °C for 20 s, 56 °C for 20 s, and then 72 °C for 20 s. Data analysis was performed with the Corbett Rotorgene software using the standard curves method. All transcripts values were normalized to β-actin.

The primers used for quantitative RT-PCR are as follows:

- CPT1 F, 5'-CGCTTTTGGGATCCACATT-3' and CPT1 R, 5'-TGTCAGGATTTTCTCAGT-3';
- VLCAD F, 5'-AATTTCACTTGGGGTCA-3' and VLCAD R, 5'-CCCTGTTCAAGTGATTA-3';
- LCAD F, 5'-TGCCATGGTATTAGCCTTT-3' and LCAD R, 5'-CAAAGAATGTGCTCCGATCC-3';
- MCAD F, 5'-GAGGACAGAGCCCTTGGAT-3' and MCAD R, 5'-CAGCATATAATGTATGTCCTTGAT-3';
- ACO F, 5'-GCAACACTGTCGACAGAAG-3' and ACO R, 5'-AGCCTGAAGGTTGTCCTT-3';
- PPARα F, 5'-ACTGAGAAGCTGTCACCACAGT-3' and PPARα R, 5'-GCCAGCTCCAAGGACTC-3'.

Animal Care and Procedures—Severe combined immunodeficiency disorder, (SCID)/albumin (Alb)-urokinase plasminogen activator (uPA) (SCID/Alb-uPA) mice were housed in a virus- and antigen-free facility supported by the Health Sciences Laboratory Animal Services at the University of Alberta. The mice were selected for infection studies and were segregated into naïve (n = 4) and infected (n = 4) treatment groups. Four mice were inoculated with infectious serum obtained from a genotype 1A HCV-infected patient. Naïve mice did not receive the inoculation. After 2 weeks, all experimental mice were fasted for 16 h prior to blood draw for serum analysis of ketone bodies (see below), hAAT, and HCV RNA essentially as described (72–74).

Plasma Ketone Bodies—Plasma ketone body levels were measured via the cyclic enzymatic method using Autokit Total Ketone Bodies (Wako Chemicals, code no. 415-73301).

Indirect Immunohistochemistry—Engrafted human hepatocytes in paraffin-embedded liver sections were identified using the Super Sensitive ISH detection system (Biogenex, San Ramon, CA) with fluoresceinlabeled Alu probe (catalog no. HK844-2K, Biogenex, San Ramon, CA) according to the manufacturer’s instructions. For simultaneous detection of HCV antigens and human hepatocytes, the primary antibody solution provided with the ISH kit was supplemented with anti-core C7–50 and anti-NS5A NE10 antibodies (1:50 each). For detection of only HCV antigens, the primary antibody solution was replaced with anti-HCV antibodies (core and NS5A) in a PBS solution (containing 1% BSA as carrier, same solution as that provided for anti-HRP mouse monoclonal from Biogenex). Mouse monoclonal IgG1 was used as an isotype control.

Lipid Analysis—Cells were harvested in phosphate-buffered saline (PBS), sonicated on ice, and centrifuged at 1,500 rpm for 5 min in a microcentrifuge to pellet cell debris. Supernatant (cell homogenate) was collected, and lipids were extracted from cell homogenates with chloroform/methanol (2:1, v/v) in the absence (mass determination) or presence (metabolic studies) of nonradioactive lipid carriers (phosphatidylcholine, oleic acid, dioleoylglycerol, trioleoylglycerol, and cholesteryl oleate) as described previously (75). The chloroform phase containing lipids was dried under nitrogen and re-dissolved in a small volume of chloroform for scintillation counting. Alternatively, the re-dissolved lipids were applied to Silica Gel H thin layer chromatography plates. The plates were developed in heptane/isopropyl ether/acetic acid 60:40:4 (by volume) to separate neutral lipids (FA, TG, free cholesterol, and cholesteryl esters). Lipid classes were visualized by exposure to iodine vapor; bands were scraped, and the associated radioactivity was determined by scintillation counting. To analyze lipid mass, lipid extractions were carried out with cell homogenates in the absence of lipid carriers and in the presence of known amounts of tridecanoyl-glycerol (internal standard). Lipids were processed and analyzed by gas chromatography as described previously (65).

Fatty Acid Composition—For analysis of total lipid-associated FAs, total cell lipids were extracted with chloroform/methanol (2:1, v/v). The organic (chloroform) phases were dried under nitrogen and dissolved in chloroform containing 5 μg of internal standard C17:0. The FAs were converted to FA methyl esters by incubation with 1.5 ml of 6% H2SO4 in methanol for 2 h at 80 °C. Resulting FA methyl esters were analyzed by gas chromatography as described previously (75).

Statistical Analysis—Unless stated otherwise, means ± S.E. were determined for a minimum of n = 4 independent experiments. The results were statistically analyzed by the Student’s t test or two-way analysis of variance. p values less than 0.05 were considered to be significant.

Other Methods—Protein concentrations were determined using the Pierce micro BCA assay Kit according to the supplied protocol.

Results

HCV-induced Cell Cycle Arrest Is Associated with ROS Accumulation—A mutant line of Huh7 cells (Huh7.5) that supports JFH-1 (genotype 2a) HCV infection with high replication efficiency (56) and exhibits HCV-mediated growth arrest (7, 9, 53, 54) was used to examine whether oxidative stress was associated with this stage of infection. After HCV infection, the percentage of HCV-infected cells and the production of viral RNA and infectious virions were determined periodically by immunocytochemistry with anti-HCV core immunostaining,
HCV Infection and Oxidative Stress-mediated AMPK Activation

FIGURE 1. HCV-mediated cell cycle arrest. A, expression of HCV core in naive and infected Huh7.5 (at 10 days post-infection with JFH-1, m.o.i. = 0.01). HCV-positive cells were detected using an anti-core antibody (red), whereas all cell nuclei are shown using DAPI (blue). Micrographs are representative of all naive and infected cell cultures enrolled in the studies herein. B, analysis of proliferating cells in infected Huh7.5 cell cultures (at 10 days post-infection with JFH-1, m.o.i. = 0.01). Naive cells were used as controls. Following a 3-h pulse with EdU to label cells in S-phase, cells were enumerated by flow cytometry. Results are expressed as means ± S.E. for n = 4 independent experiments. C, analysis of cell viability of infected Huh7.5 cells (at 10 days post-infection with JFH-1, m.o.i. = 0.01). Naive cells were used as controls. Cell viability was determined by MTT assay. Results are expressed as means ± S.E. for n = 4 independent experiments. D, analysis of ROS levels in infected Huh7.5 (at 10 days post-infection with JFH-1, m.o.i. = 0.01). Naive cells were used as controls. Intracellular ROS/RNS and H2O2 levels were examined using H2DCF-DA and OxiSelect detection reagents, respectively. Results are expressed as means ± S.E. for n = 4 independent experiments.

quantitative RT-PCR, and limiting dilution assays essentially as described (58, 59). Infected cell cultures were enrolled into experiments as “infected cells” at 10 days post-infection (m.o.i. = 0.01), when they appeared to grow more slowly relative to their naive counterparts, with more dead cells appearing in the culture media. These cultures were characterized by high levels of HCV infection, producing >107 IU of HCV RNA per mg of cellular RNA with infectivity titers >104 focus-forming units/ml. Importantly, >95% of the infected cells were HCV core-positive (Fig. 1A), thereby minimizing the contribution of non-infected cells present in infected cultures to experimental outcomes (76). The numbers of cells progressing through S-phase were enumerated by flow cytometry (Fig. 1B). This analysis revealed that >80% of the infected cells were growth-arrested, because <20% exhibited evidence of EdU incorporation, as opposed to >75% with naive cells (Fig. 1B). Despite their reduced proliferative capacity, the surviving infected cells remained sufficiently healthy with viabilities >90% (Fig. 1C). H2DCFDA is sensitive to both H2O2 and NO-based radicals and can be oxidized to 2',7'-dichlorofluorescein by various ROS and RNS, including H2O2, although the OxiSelect reagent is specifically sensitive to H2O2 (60–64). Intracellular ROS/RNS levels were examined using H2DCFDA as the detection reagent (Fig. 1D). This analysis revealed that infected cells have ~5-fold more intracellular ROS/RNS than naive cells, indicating that HCV-induced cell cycle arrest is associated with more oxidative/nitrosative stress. At least some of this increase is due to elevated H2O2 production, because infected cells were also found to have higher H2O2 levels than naive cells.

HCV-induced Cell Cycle Arrest Is Associated with ROS/RNS-mediated AMPK Activation—Full activation of AMPK requires specific phosphorylation (at Thr-172) within the catalytic domain of the α-subunit by upstream kinases (38), and this signaling system is activated by oxidative stress in various cell types, including liver cells (37). The possibility that AMPK might be activated in infected cells was examined by immunoblot analysis (Fig. 2A). The levels of AMPK were found to be similar between naive and infected cells, but infected cells contained substantially more Thr-172 phosphorylated AMPK (p-AMPK) than naive cells, indicating that more of the AMPK is activated in these cells. To examine the contribution of ROS/RNS to AMPK activation, two different antioxidants were used (Fig. 2B). α-Tocopherol is membrane-soluble and functions primarily as an inhibitor of lipid peroxidation by virtue of its chain-breaking and free radical scavenger actions. In contrast, NAC is a small water-soluble molecule directly providing –SH groups for adduction or oxidation, and it is a precursor of glutathione (77). Pre-treatment of infected cells with either α-tocopherol or NAC led to a reduction in cellular ROS/RNS production by ~30% (Fig. 2B). The cellular levels of p-AMPK were also reduced by ~30% when infected cells were pre-treated with α-tocopherol, whereas total AMPK levels remained unchanged (Fig. 2C). This is an indication that oxidative/nitrosative stress plays some role in the activation of AMPK in
Huh7.5 cells during HCV-induced cell cycle arrest. Although the levels of p-AMPK were also found to be reduced by ~30% in infected cells pre-treated with NAC, these reduced levels did not reach statistical significance (data not shown).

**HCV-induced Cell Cycle Arrest Is Associated with Attenuated DNL Activity**—Acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl-CoA, which is the first committed intermediate formed during fatty acid synthesis. Two separate genes encode different ACC isoforms in hepatocytes as follows: ACC1 is localized in the cytosol and is committed to DNL, and ACC2 is anchored to the mitochondrial membrane and is involved in the control of fatty acid oxidation via allosteric inhibition of CPT1, which is also present on the mitochondrial surface, by locally produced malonyl-CoA (38). Both ACC isoforms are phosphorylated by AMPK (at Ser-79 and Ser-220 for ACC1 and ACC2, respectively) in hepatocytes (including Huh7 cells (78)), thereby inhibiting malonyl-CoA production in both compartments (79, 80). Because HCV-mediated growth arrest was associated with activated AMPK, the expression of ACC1/2 and the presence of their AMPK-phosphorylated derivatives were examined by immunoblot analysis using antibodies that were selected based on their ability to detect both ACC1 (265 kDa) and ACC2 (280 kDa) isoforms and their AMPK-phosphorylated derivatives (Fig. 3A). However, only a single ACC species (~265 kDa) was detected in naive and infected Huh7.5 cells when these antibodies were used. Although this is consistent with what has previously been reported with Huh7.5 cells when the same antibodies were used (81), both ACC isoforms are present in Huh7 cells and have predictable mobility on SDS-PAGE (78). The reason for this disparity is not understood, but because alternative promoter usage and exon splicing can result in several ACC1 and ACC2 variants ranging from 253 to 280 kDa (82), we also performed immunoblot analysis using ACC1- and ACC2-specific antibodies (data not shown). This analysis revealed that both ACC species are present in naive and infected cells, albeit with similar mobility on SDS-PAGE (i.e. ~265 kDa). Furthermore, there was no apparent difference in the levels of ACC1 or ACC2 between naive and infected cells. In agreement with having higher p-ACC levels, infected cells had just 50% of the malonyl-CoA present in naive cells (Fig. 3B).

HCV replication has been shown to suppress glucose uptake through the down-regulation of cell surface glucose transporters (83). To rule out the possible contribution of altered sub-
strate supply to the drop in malonyl-CoA levels, infected cells were supplied with [3H]acetate. This offers an alternate pathway of producing acetyl-CoA via acetyl-CoA synthetase instead of from pyruvate via the pyruvate dehydrogenase complex. Thus, any change in the incorporation of [3H]acetate into cellular lipids is due to altered catalytic efficiency, for which ACC activity has major influence. As expected with higher p-ACC levels, infected cells incorporated less [3H]acetate into FFA and their esterified derivatives (TAG, PL, and CE) than naive cells (Fig. 4). In addition, infected cells incorporated less [3H]acetate into free cholesterol (FC) than naive cells, indicating that these cells also have reduced cholesterol biosynthetic activity. These results demonstrate unequivocal association between HCV-induced cell cycle arrest and attenuated DNL activity.

**HCV-induced Cell Cycle Arrest Is Associated with Enhanced β-Oxidation Activity**—A drop in malonyl-CoA is expected to increase β-oxidation activity through the disinhibition of CPT1 (79, 80, 84, 85). To examine this possibility, mitochondrial β-oxidation was monitored by the release of 3H2O from [9,10-3H]oleic acid (Fig. 5A), an assay for long- and medium-chain fatty acid oxidation (86). By the end of the labeling period (pulse), infected cells had released significantly more 3H2O into the media than had naive cells. Naive and infected cells released substantially less 3H2O into the media after oleic acid was withdrawn from the culture media (chase), indicating that exogenous fatty acids enter a pool that is tightly coupled to mitochondrial β-oxidation. However, infected cells continued to release more 3H2O into the media than did naive cells even after fatty acids were withdrawn from the culture media. This indicates that the mobilization of TAG in preformed storages pools (e.g. lipid droplets) for β-oxidation occurs more readily with the
infected cells. Infected cells also released considerably more $^{14}$CO$_2$ due to the complete $\beta$-oxidation of [1-14C]oleic acid than naive cells (Fig. 5B), indicating that the complete oxidation of long-chain fatty acids is more efficient in the infected cells (86). It is noteworthy that infected and naive cells had taken up similar levels of labeled oleic acid within the first 10 min of the labeling period (Fig. 5C), ruling out the possibility that enhanced $\beta$-oxidation observed with the infected cells was due to altered cellular uptake of labeled oleic acid. Reduced levels of labeled oleic acid in infected cells observed at later time points (10–20 min) is likely a reflection of enhanced $\beta$-oxidation rather than reduced uptake. As expected with enhanced mitochondrial $\beta$-oxidation, infected cells exhibited higher cellular ATP levels than their naive counterparts (Fig. 5D). These results demonstrate an unequivocal association between HCV-induced cell cycle arrest and enhanced $\beta$-oxidation activity.

**HCV-induced Cell Cycle Arrest Is Associated with Elevated Nuclear PPARα and PPARα Target Gene Expression**—PPARα is a type II nuclear receptor localized to the nucleus as part of an inhibitory complex prior to ligand-induced activation (87). Ligand-induced activation of PPARα leads to the enhanced expression of PPARα target genes, many of which have important roles in $\beta$-oxidation (including CPT1) (87). However, several kinase-dependent processes also appear to lead to the activation of PPARα, including AMPK (88). Because AMPK was activated in infected cells, PPARα and CPT1 expression levels were examined by immunoblot analysis using nuclear and whole cell extracts, respectively. PPARα was readily detected in nuclear extracts of infected cells (Fig. 6A). By contrast, very little (if any) PPARα was detected in nuclear extracts from naive cells (Fig. 6A). Infected cells contained ∼2-fold more CPT1 than naive cells (Fig. 6B). To examine the activation status of nuclear PPARα, a PPARα-selective agonist (GW7647) was administered to cells following transient transfection with a PPAR-responsive luciferase reporter gene (Fig. 6C). Without GW7647, naive and infected cells exhibited similar activities of the luciferase reporter gene. It is not known which of the three PPAR (α, β/δ, and γ) family members contribute to this baseline activity because each of these is present in liver (87). Furthermore, there is evidence that HCV infection can lead to the

**FIGURE 5. Enhanced $\beta$-oxidation in infected cells.** A, JFH-1-infected Huh7.5 cells (at 10 days post-infection, m.o.i. = 0.01) were incubated with [9,10-3H]oleic acid for 4 h (pulse), followed by incubation in DMEM (chase). Naive cells were used as controls. Media equivalent to 500 μg of cell protein were analyzed for radioactivity in the form of $^3$H$_2$O as described under “Experimental Procedures.” Results are expressed as means ± S.E. for $n = 4$ independent experiments. B, JFH-1-infected Huh7.5 cells (at 10 days post-infection, m.o.i. = 0.01) were incubated with [1-14C]oleic acid for 4 h, and the $^{14}$CO$_2$ production from the complete $\beta$-oxidation of [1-14C]oleic acid was analyzed as described under “Experimental Procedures.” Naive cells were used as a control. Results are expressed as means ± S.E. for $n = 4$ independent experiments. C, JFH-1-infected cells (at 10 days post-infection, m.o.i. = 0.01) were incubated with [9,10-3H]oleic acid for the indicated times and then lysed, and the amount of radioactivity in lysates was determined by scintillation counting. Naive cells were used as a control. Results are expressed as means ± S.E. for $n = 4$ independent experiments. D, cellular ATP levels were analyzed in JFH-1-infected cells (at 10 days post-infection, m.o.i. = 0.01). Naive cells were used as a control. Results are expressed as means ± S.E. for $n = 4$ independent experiments.
HCV Infection and Oxidative Stress-mediated AMPK Activation

**FIGURE 6. Increased nuclear PPARα correlates with increased cellular CPT1 and enhanced expression of β-oxidation genes in infected cells.**

A, nuclear PPARα levels in infected HuH7.5 cells (at 10 days post-infection with JFH-1, m.o.i. = 0.01) were analyzed by immunoblot analysis. Naive cells were used as a control. Blots were stripped and re-probed for histone (H2B, loading control). Immunoblot is representative of n = 4 independent experiments.  

B, cellular CPT1 levels in infected HuH7.5 cells (at 10 days post-infection with JFH-1, m.o.i. = 0.01) were analyzed by quantitative immunoblot analysis (right). Immunoblot is representative of n = 4 independent experiments, and results are expressed as means ± S.E. for n = 4 independent experiments. Naive cells were used as a control. Blots were stripped and re-probed for actin (loading control) and HCV core (infection control). 

C, infected HuH7.5 cells (at 10 days post-infection with JFH-1, m.o.i. = 0.01) were transfected with the PPAR reporter supplied by the CignalTM PPAR reporter (luc) kit. After 24 h of transfection, the media were changed to DMEM containing 10% FBS with (300 μM) or without (0 mM) GW7647 in DMSO. The final DMSO concentration did not exceed 0.1% (v/v). Dual luciferase assay was performed 24 h post-treatment, and reporter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization. Naive cells were used as a control. Results are expressed as means ± S.E. for n = 4 independent experiments. 

D, expression of genes encoding CPT1, acetyl-CoA carboxylase 2 (ACC2), very long-, long-, and medium-chain acyl-CoA dehydrogenase (VLCAD, LCAD, and MCAD, respectively), acyl-CoA oxidase, and PPARα were quantified by quantitative RT-PCR in infected HuH7.5 cells (at 10 days post-infection with JFH-1, m.o.i. = 0.01) and normalized to actin. Naive cells were used as a control. Results are expressed as means ± S.E. for n = 4 independent experiments.

increased expression and activation of PPARγ in hepatoma cells (41). Importantly, a 1.5-fold increase in reporter activity was observed with infected cells compared with naive cells (Fig. 6A). Immunoblot analysis of nuclear PPARα levels in infected HuH7.5 cells showed that at least some of the PPARα was unactivated prior to infection with JFH-1. Despite having substantially more nuclear PPARα than naive cells, no appreciable change in reporter activity was observed when GW7647 was administered to infected cells. This is an indication that PPARα is constitutively active in infected cells. In support of this, a significant up-regulation of several PPARα gene targets with important roles in fatty acid oxidation was observed with infected cells compared with naive cells (Fig. 6B). These included CPT1, medium- and long-chain acyl-CoA dehydrogenase (MCAD and LCAD, respectively), acyl-CoA oxidase (ACO), and PPARα genes.

HCV Infection Is Associated with Enhanced β-Oxidation in the Humanized Livers of SCID/uPA Mice—The SCID/Alb-uPA mouse model is the first small animal model capable of supporting the entire HCV life cycle. This model was extensively reviewed, and they continue to be the preferred model for in vivo studies of HCV infection (89, 90). A genomic study of the host response to HCV infection with this model has uncovered a potential link between disturbances in lipid metabolism and oxidative stress (8). Specifically, mice that showed evidence of HCV-mediated oxidative stress also exhibited induction of several genes with important roles in β-oxidation, including PPARα (8). Hepatome and bile ductular cells are present at high levels in liver repopulation with human hepatocytes (as determined by serum-based ELISA for hAAT at weaning) were selected for infection studies and then segregated into naive (n = 4 mice) and infected (n = 4 mice) treatment groups. For the infected group, mice were inoculated with serum obtained from a genotype 1A HCV-infected
Naive mice did not receive the inoculation. At 2 weeks post-inoculation, all experimental mice were fasted for 16 h prior to blood draw for serum analysis of ketone bodies, hAAT, and HCV RNA titers and liver sampling for detection of HCV antigens (core and NS5A) using immunohistochemistry (Fig. 7B). Following 16 h of fasting, the circulating levels of ketone bodies were found to be ~2-fold higher in infected mice versus naive mice (Fig. 7B). Hepatocytes expressing HCV-specific...
antigen have been detected in liver biopsies previously using immunohistochemistry or fluorescence microscopy, but HCV antigens are recognized by either technique only at or near the limits of sensitivity (93–96). Using immunohistochemistry, we identified cells that labeled positively for HCV antigens (core and NS5A) in the livers from all infected mice. This diffuse and weak labeling was localized to histologically integrated human hepatocytes (brown nuclei) and was absent when immunohistochemistry was performed using an isotype control antibody.

A more sensitive method, with larger numbers of mice, is required to examine whether there is a correlation between the proportion of HCV-infected human hepatocytes in the chimeric livers of SCID/Alb-uPA mice and the production of ketone bodies in the starved state. The results of this experiment provide encouraging in vivo evidence that hepatic mitochondrial β-oxidation is enhanced during HCV infection.

**Cellular Lipids Accumulate in Huh7.5 Cells during HCV-induced Cell Cycle Arrest.**—HCV infection is associated with the hepatic accumulation of lipids, and this feature contributes to the development of hepatic steatosis in the clinical setting and to various aspects of the viral life cycle in vitro (41). Because the combination of attenuated DNL and enhanced β-oxidation is not conducive to lipid accumulation, cellular lipid levels were investigated in naive versus infected cells. Cellular levels of TAG, PL, CE, and FC were determined via gas chromatography (Fig. 8A). Surprisingly, each of these lipid classes was significantly elevated in infected cells when compared with naive cells. A comprehensive fatty acid profile of total lipids was also obtained (Table 1). This analysis revealed significant alterations in the fatty acid composition of cellular lipids in infected versus naive cells (boldface). The ratios of specific fatty acids, typically targeted to biological membranes via myristoylation, and this promotes phosphorylation of the Thr-172 site by upstream kinases, suggesting possible preferential activation of AMPK at membrane sites (98). This raises the possibility that local scavenging of ROS/RNS by α-tocopherol residing in biological membranes and/or the different scavenging capacities of α-tocopherol and NAC for various ROS/RNS species (99) may have contributed to this phenomenon. The attenuated DNL and enhanced β-oxidation observed during this stage of infection likely reflect AMPK-mediated lowering of both ACC1- and ACC2-derived malonyl-CoA pools (79, 80). In addition to ACC, malonyl-CoA decarboxylase plays an important role in the turnover of malonyl-CoA and is also activated by AMPK in liver, resulting in increased fatty acid oxidation (100). This raises the possibility that malonyl-CoA decarboxylase also contributed to lower malonyl-CoA levels and therefore enhanced β-oxidation in these cells.

Very little is known about the role PPARα has in HCV-induced liver disease. Although histological data provide indirect evidence for the down-regulation of PPARα (101–103), it is uncertain whether any of the liver tissue contained HCV infected hepatocytes. A direct role for PPARs in the development of hepatic steatosis and hepatocellular carcinoma has come from studies that combine mice with a homozygous deletion of PPARα with mice having hepatic expression of the HCV core protein (104, 105). Although the physiological relevance is questionable in the absence of viral replication, these phenomena were found to be associated with sustained PPARα activation. In our study, the relatively higher abundance of nuclear PPARα in infected cells was associated with increased expression of several PPARα target genes involved in β-oxidation (including PPARα and CPT1). This is a strong indication that

![Figure 8](https://example.com/figure8.png)

**Figure 8.** Lipids accumulate in infected cells with changes in fatty acid composition and derived fatty acid indices. Lipids were extracted from infected Huh7.5 cells (at 10 days post-infection with JFH-1, m.o.i. = 0.01). Naive cells were used as a control. A, cellular levels of FC, CE, PL, and TAG were determined via gas chromatography. Results are expressed as means ± S.E. for n = 4 independent experiments. B, corresponding fatty acids were converted to fatty acid methyl esters and analyzed by gas-liquid chromatography. The relative contributions of saturated and monounsaturated fatty acids and PUFA to the total fatty acids are shown. Results are expressed as means ± S.E. for n = 4 independent experiments. C, elongase activity index (18:0/16:0), SCD1 activity index (18:1n-9/18:0), and Δ6 desaturase activity index (20:4n-6/18:2n-6). Results are expressed as means ± S.E. for n = 4 independent experiments.

**Discussion.**—Here, we show that HCV-induced growth arrest is associated with ROS/RNS-mediated AMPK activation. We found that the activation of AMPK was more sensitive to α-tocopherol than to NAC. The reasons for this are not understood, but AMPK is targeted to biological membranes via myristoylation, and this promotes phosphorylation of the Thr-172 site by upstream kinases, suggesting possible preferential activation of AMPK at membrane sites (98). This raises the possibility that local scavenging of ROS/RNS by α-tocopherol residing in biological membranes and/or the different scavenging capacities of α-tocopherol and NAC for various ROS/RNS species (99) may have contributed to this phenomenon. The attenuated DNL and enhanced β-oxidation observed during this stage of infection likely reflect AMPK-mediated lowering of both ACC1- and ACC2-derived malonyl-CoA pools (79, 80). In addition to ACC, malonyl-CoA decarboxylase plays an important role in the turnover of malonyl-CoA and is also activated by AMPK in liver, resulting in increased fatty acid oxidation (100). This raises the possibility that malonyl-CoA decarboxylase also contributed to lower malonyl-CoA levels and therefore enhanced β-oxidation in these cells.
PPARα also contributed to the increase in β-oxidation activity in these cells. Furthermore, we provide evidence that PPARα is persistently activated during this stage of infection. Aside from AMPK, activators of ERK-MAPK, JNK, and p38 MAPK, PKA, and PKC enhance PPARα activity, whereas GSK inhibits PPARα activity (88). Although the mechanism remains to be determined, several opportunities exist for HCV-mediated PPARα activation because HCV can modulate each of these pathways in a manner that is expected to enhance PPARα activity (106–109). Furthermore, HCV-induced growth arrest was associated with elevated PUFA levels (see below), which serve as natural ligands for PPARα (87), representing yet another opportunity for HCV-induced PPARα activation.

This study also provided the first in vivo evidence for the up-regulation of mitochondrial β-oxidation during HCV infection because SCID/Alb-uPA mice accumulated higher plasma ketones in the fasted state when they were antecedently infected with the patient-derived genotype-1A HCV. Although the variation of ketone production within naive and infected groups warrants a more expanded study with larger numbers of mice, and more HCV genotypes, the results of this study are encouraging given that a link between oxidative stress and increased expression of several β-oxidation genes (including PPARα) has already been established in this HCV mouse model (8).

Finally, we found a significant accumulation of lipids in Huh7.5 cells during HCV-mediated growth arrest, with changes in FA composition characterized by increases in saturated and monounsaturated fatty acids and even larger increases in PUFA. Although HCV-mediated dysregulation of enzymatic fatty acid conversion steps can account for some of these changes, hepatocytes are incapable of deriving PUFA from DNL (97). Defective TAG export as VLDL has been demonstrated with JFH-1-infected Huh7 cells using the same culture conditions as those used herein (58). Because PUFAs are a constituent of TAG (110), defective VLDL assembly/secrection would account for at least some of the elevated PUFA levels associated with infected cells during this stage of infection. The peroxidation of PUFAs, regulated in part through sphingosine kinase-2, has recently been shown to severely restrict the replication of all HCV strains with a demonstrated ability to induce cell cycle arrest (32, 54). Thus the accumulation and peroxidation of PUFAs may attenuate HCV replication in stressed tissue, including cells undergoing HCV-mediated cell cycle arrest, possibly facilitating long term viral persistence and cellular survival.

In contrast with the results reported herein, AMPK inhibition has been reported with JFH-1-infected Huh7 cells at 48 h post-infection (m.o.i. = 0.5), and this promoted cellular lipid accumulation and viral replication (111). In this scenario, AMPK inhibition was mediated by the phosphorylation of AMPK at Ser-485 through activation of the (PI3K)-AKT pathway (111). Despite the possibility that ROS accumulated in these cells (11, 112, 113), this scenario does not contradict the ROS-mediated AMPK activation observed during HCV-induced growth arrest because phosphorylation of AMPK at Ser-485 has been shown to prevent subsequent phosphorylation at Thr-172, and hence full activation of AMPK (114). AMPK inhibition was also reported with JFH-1-infected Huh7 cells at 5 days post-infection (m.o.i. = 10), when replication had already been established (115). In this scenario, HCV-mediated ER stress and the unfolded protein response induced autophagy through inhibition of the AKT/mammalian target of rapamycin pathway. ROS are an important consequence of HCV-induced ER stress, and ROS inhibit mammalian target of rapamycin through activation of AMPK and inhibition of AKT, leading to apoptosis (116). A complicated issue is whether ROS contributed to ER stress-induced autophagy in this scenario because ER stress-induced suppression of mTOR excluded regulation by AMPK. Indeed, others have shown that ER stress-induced deactivation of the AKT/mTOR pathway excludes the involvement of AMPK (117). Although the precise regulatory pathway is still enigmatic, available evidence indicates that inositol-requiring ER-to-nucleus signal kinase-1 (IRE1), PRK (RNA-de-
HCV Infection and Oxidative Stress-mediated AMPK Activation

A phiên bản protein kinase)-like ER kinase, and increased [Ca\(^{2+}\)] are distinct initiators of ER stress-induced autophagy in mammalian cells (118–120). Furthermore, HCV-induced apoptosis, which strongly correlates with cell cycle arrest and viral replication (9, 54), was not observed in Huh7 cultures undergoing HCV-induced autophagy (115). Although this may have been due to the low JFH-1 replication efficiency when Huh7 cells are used, this is an indication that the severity of ER stress and ROS levels was insufficient for the induction of apoptosis, raising the possibility that ROS-mediated activation of AMPK occurs at a later stage, when more ROS accumulates.

Proteomic profiling of HCV-infected Huh7.5 cells has provided strong evidence that the delay in cell cycle progression during HCV infection is accompanied by an adaptive metabolic response aimed at channeling substrates from synthetic to energetic purposes (7). The shift from AMPK inhibition observed prior to HCV-induced cell cycle arrest to AMPK activation observed with growth-arrested infected cells agrees with this concept. In addition to AMPK inhibition, the increased expressions of sterol regulatory element-binding protein and fatty acid synthase, both important for intracellular lipid synthesis, are thought to enhance lipogenesis after HCV gains entry into cells (121, 122). Lipid droplets, which serve as important organelles for HCV assembly (123), are also targeted for degradation by HCV-induced autophagy (124, 125). Moreover, it has become increasingly evident that lipids play important roles in the autophagy process itself, both at the level of regulation of autophagy and as membrane constituents required for formation of autophagic vesicles (126). Thus, AMPK inhibition after HCV gains cellular entry would help support virion production while supplying the lipids necessary for autophagy and continued cellular proliferation. However, activation of the AMPK signaling system is essential for the survival of cells under oxidative stress, and various studies indicate that ROS promote cell survival by increasing mitochondrial biogenesis and the expression of genes involved in antioxidant defense through the AMPK pathway (127–130). Thus, as infection progresses and cell stress increases, ROS-induced AMPK activation would promote metabolic homeostasis and rebalancing of the redox tone, thereby allowing HCV to fully exploit the lipid-laden and energy-rich cell to nourish replication and virus assembly. How HCV induces cell cycle arrest in hepatoma cells is not understood, but prolonged AMPK activation in response to ROS has been shown to trigger caspase activation and onset of the intrinsic mitochondrial apoptosis pathway (57, 131, 132), which are characteristic of the HCV-mediated cytopathic effects observed with Huh7.5 cells (9, 54). Furthermore, cancer cells are dependent on ACC activity to provide sufficient fatty acids for proliferation and survival (55, 133, 134). Likewise, inhibition of ACC activity results in cell cycle arrest and triggers apoptosis (55), raising the possibility that ROS-mediated AMPK activation may have a role in HCV-induced cell cycle arrest and apoptosis (9, 54). Such a mechanism is likely to promote dissemination of the infection.

In conclusion, this study firmly establishes that ROS-induced activation of AMPK, attenuated DNL, and increased β-oxidation are associated with HCV-induced cell cycle arrest. Building on previous studies demonstrating AMPK inhibition prior to HCV-induced cell cycle arrest (111, 115), these results strongly support the HCV-induced metabolic reprogramming predicted by genomic and proteomic studies (7, 8), which are likely to contribute to the pathophysiology of HCV infection.

Author Contributions—D. N. D. conceived and coordinated the study, wrote the paper, and designed the experiments. N. M. K. coordinated the study, interpreted the data, provided study supervision and administrative support, and performed critical revision of the manuscript for important intellectual content. R. L. provided study supervision, provided interpretation and supervision for lipid analyses, interpreted the data, and performed critical revision of the manuscript for important intellectual content. C. H. P. performed cell culture experiments and data analysis and prepared figures. J. T. L. and G. L. performed experiments with mice. R. B. assisted with RT-PCRs and data analysis. A. A.-M. performed ROS determinations and performed critical revision of the manuscript for important intellectual content. M. L. and W. R. A. performed critical revision of the manuscript for important intellectual content.

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