Enhanced hepatitis C virus genome replication and lipid accumulation mediated by inhibition of AMP-activated protein kinase

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Hepatitis C virus (HCV) infection is a major cause of chronic liver disease affecting an estimated 3% of the world’s population (1). HCV is a positive-stranded RNA virus with a 9.6-kb genome that encodes a large polyprotein, translated in a cap-independent fashion and processed by cellular and viral proteases to produce 10 mature proteins: core, envelope proteins E1/E2, a cation channel p7, and six nonstructural proteins, NS2 to NS5B (2). HCV infection is associated with accumulation of intracellular lipid, which manifests as steatosis (fatty liver) in patients and is a predictor of serious liver disease (3). Furthermore, data from cell-culture studies have shown that inhibition of cellular lipid biosynthesis is detrimental to virus replication (4, 5), consistent with a role for lipid droplets in both viral genome replication and assembly of infectious particles (6). Although HCV infection has been shown to activate genes such as peroxisome proliferator-activated receptors (PPARα/γ/δ) and sterol regulatory element-binding protein-1 (SREBP-1) (7–9) that increase lipid biogenesis and inhibit mitochondrial β-oxidation, the mechanisms underpinning this regulation remain obscure. Intriguingly, HCV infection also is associated with the development of insulin resistance and type 2 diabetes. This association may result in part from dysregulation of lipid metabolism, but recent data also have pointed to direct effects of HCV on hepatic glucose uptake, again by uncharacterized mechanisms (10).

A key regulator of both lipid and glucose metabolism is AMP-activated protein kinase (AMPK). AMPK is a heterotrimeric complex consisting of α, β, and γ subunits and has been referred to as a metabolic “master switch,” because its activity is regulated by the energy status of the cell. AMPK responds to ATP depletion by detecting changes in the AMP:ATP ratio (11). AMPK is active only after phosphorylation of the α subunit at a threonine residue within the kinase domain (T172) by upstream kinases, most important of which is a heterotrimeric complex between serine/threonine kinase 11 (LKB1), sterile 20 protein-related adaptor, and mouse protein 25 (12) (Fig. S1). T172 also can be phosphorylated by the Ca²⁺ sensing kinase, CAMKKβ (13, 14). AMP promotes T172 phosphorylation by inhibiting dephosphorylation at this residue, an effect antagonized by ATP (15, 16). Once activated, AMPK phosphorylates multiple substrate proteins to effect general inhibition of ATP-consuming metabolic pathways and simultaneous activation of ATP-generating pathways, restoring ATP levels (11). These effects are particularly important in regulating liver metabolism, where activation of AMPK augments fatty acid oxidation and decreases glucose output and cholesterol and triglyceride synthesis (11, 17).

Here we demonstrate that in cells harboring HCV subgenomic replicons or infected with HCV, AMPK T172 phosphorylation was concomitant AMPK activity are dramatically reduced. We demonstrate that this effect is mediated by activation of the serine/threonine kinase, protein kinase B, which inhibits AMPK by phosphorylating serine 485. The physiological significance of this inhibition is demonstrated by the observation that pharmacological restoration of AMPK activity not only abrogates the lipid accumulation observed in virus-infected and subgenomic replicon-harboring cells but also efficiently inhibits viral replication. These data demonstrate that inhibition of AMPK is required for HCV replication and that the restoration of AMPK activity may present a target for much needed anti-HCV therapies.

Results

AMPK Activity Is Inhibited in HCV Subgenomic Replicon-Harboring Cells. Viral infection might be expected to lead to an increase in AMP concentration because of increased energy demands upon the host cell. Cellular AMP concentrations are very low and therefore difficult to measure, but the ADP:ATP ratio changes in concert with AMP:ATP ratio (because of the adenylate kinase reaction) and can be used as a surrogate for AMP:ATP (17). Surprisingly, when we compared the ATP:ADP ratios in Huh-7 cells and in cells stably harboring an HCV genotype 1b culture-adapted subgenomic replicon (18) (hereafter termed “replicon cells”), we observed no significant difference between the two cell populations (Fig. 1A). We speculated that this observation might result from the activation of AMPK in replicon cells, thereby restoring the energy balance in these cells. Active AMPK phosphorylates a large number of targets, including the two isoforms of acetyl-CoA carboxylase (ACC1/2). Importantly, phosphorylation of ACC by AMPK inhibits enzymatic activity...
AMPK Activation Inhibits HCV Genome Replication and Abrogates Lipid Accumulation in Replicon Cells. Because HCV mediated an inhibition of AMPK activity, we asked whether this effect might be important for virus replication. We treated replicon cells with AICAR, metformin, and A769662 and analyzed levels of the viral nonstructural protein NS5A at various time points following treatment. Treatment of replicon cells for 72 h dramatically reduced NS5A levels, most effectively in AICAR-treated cells (Fig. 2A). NS5A levels in replicon cells are an accepted indirect measure of genome replication, but it was possible that the decrease in NS5A abundance was caused by degradation or inhibition of translation. Therefore, to investigate directly if AMPK activation inhibited genome replication, we transiently transfected Huh-7 cells with luciferase-based genotype 1b or 2a (JFH-1) replicons, allowing direct correlation of HCV replication to luciferase activity. AICAR, A769662, and metformin treatment significantly decreased luciferase activity (Fig. 2B and Fig. S2D) in a dose-dependent manner (Fig S2D) as compared with untreated controls, suggesting that inhibition of AMPK is required for HCV genome replication. None of these compounds had any effect on cell viability at the highest concentrations used (Fig S2C).

HCV replication is associated with an intracellular accumulation of lipid, and drugs that block cholesterol and fatty acid biosynthesis have been shown to regulate replicon replication in Huh-7 cells (4, 5). AMPK inhibits lipogenesis by modulating the activity of transcription factors required for lipogenic gene expression (e.g., PPARγ/δ and SREBP-1). We postulated that the lipid accumulation induced by HCV might be mediated by inhibition of AMPK. To test this possibility, we treated replicon cells with AICAR, A769662, or metformin and visualized cellular lipid content using BODIPY. This analysis revealed that replicon cells displayed higher levels of BODIPY fluorescence than parental Huh-7 cells (Fig. 3A and Fig. S2D). Upon short-term (4-h) treatment with AMPK agonists, cellular lipid content was reduced rapidly and dramatically (Fig. 3A), implying that inhibition of AMPK activity was in part responsible for the HCV-induced increase in cellular lipid abundance. This short-term treatment did not affect NS5A levels (compared with 72-h

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**Fig. 1.** Inhibition of AMPK activity in cells harboring the HCV subgenomic replicon. (A) ATP/ADP ratio was measured as described to assess AMPK activity indirectly (n = 3). NS, not significant. (B) Cell lysates resolved by SDS/PAGE were immunoblotted with the indicated antibodies (Left). Levels of phosphorylated AMPK or ACC were quantified by densitometry in comparison with GAPDH (n = 3) (Right). All error bars indicate mean ± SEM. **Significant difference from Huh-7 (P < 0.05). (C) Replicon cells were left untreated (Control) or treated with AICAR (1 mM), A769662 (100 μM), or metformin (1 mM) for 4 h before immunoblot analysis with the indicated antibodies.

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**Fig. 2.** AMPK activation inhibits HCV replication. (A) Replicon cells were left untreated (Control) or treated with AICAR (1 mM), A769662 (100 μM), or metformin (1 mM) for 0, 24, 48, and 72 h, and replicon replication was assessed by immunoblotting for NS5A (Left). NS5A immunofluorescence was performed at 72 h posttransfection (Right). Identical settings were maintained for image capture. Representative confocal images are shown. Scale bars, 10 μm.) (B) Cells transfected with in vitro transcripts of a luciferase subgenomic replicon (genotype 1b) were treated with the indicated AMPK agonists overnight. Luciferase activity was used as a measure of replication. Error bars indicate mean ± SEM. **Significant difference from control (P < 0.05).
phosphorylation of the AMPKα subunit by AKT has been reported to prevent AMPK activation in the presence of increased AMP (23), we asked whether HCV suppressed AMPK activity by activating AKT-dependent S485 phosphorylation of AMPKα.

As previously shown (22), levels of active, phosphorylated AKT were increased 2.3-fold in replicon cells compared with Huh-7 cells (Fig. 4A), and concomitantly levels of S485 phosphorylated AMPK were enhanced 1.8-fold (Fig. 4A), providing a potential mechanism for the observed inhibition of AMPK.

We reasoned that if AMPK inhibition was mediated by AKT, this inhibition could be prevented by inhibiting AKT. We therefore assessed AMPK activation in replicon cells treated with either insulin (an activator of AKT via upstream PI3K activation) or AKTVIII (a selective AKT inhibitor). Fig. 4B shows that insulin treatment of Huh-7 cells stimulated AMPK S485 phosphorylation with a concomitant loss of both AMPK T172 and ACC phosphorylation (compare lanes 1 and 2), confirming that AKT activation inhibits AMPK activation (23). This inhibition could be reversed by blocking AKT activity, because both AMPK T172 and ACC phosphorylation were restored following AKTVIII treatment (compare lanes 1 and 3). As expected, AKTVIII treatment also resulted in a loss of AMPK S485 phosphorylation. These data confirmed that AMPK activation is inhibited by AKT in Huh-7 cells. By contrast, replicon cells exhibited high levels of AMPK S485 phosphorylation which were unaffected by insulin treatment (compare lanes 4 and 5), although they were reduced following AKTVIII treatment (lane 6). Treatment of replicon cells with AKTVIII resulted in concomitant restoration of AMPK activity, as shown by increased AMPK T172 and ACC phosphorylation (compare lanes 4 and 6). Furthermore, AKT inhibition (in common with AMPK activation; Fig. 3) resulted in a rapid reduction in cellular lipid content, as indicated by a loss of BODIPY staining (Fig. 4C), further confirming that inhibition of AMPK activation via AKT was responsible for the HCV-induced increase in cellular lipid abundance. Consistent with the AKT dependence of AMPK inhibition, AKTVIII treatment also significantly inhibited replicon luciferase expression and, thus, genome replication (Fig. 4D).

To confirm further the role of S485 phosphorylation in the HCV-mediated inhibition of AMPK, we overexpressed wild-type or mutated forms of the AMPKα subunit (which will form heterotrimers with endogenous βγ subunits, displacing the endogenous α subunit). Because the exogenously expressed AMPKα was Myc-tagged, we were able to assess NS5A abundance and (therefore the levels of genome replication) by quantifying anti-NS5A fluorescence in cells that were positive for the Myc tag. This analysis revealed that NS5A abundance was unaffected by overexpression of either wild-type AMPKα or a phosphomimetic mutant (S485D) (Fig. 4E and Fig. S3). By contrast, overexpression of a nonphosphorylatable mutant (S485A) dramatically reduced the abundance of NS5A, confirming that phosphorylation of AMPKα at residue S485 is required for genome replication.

HCV-Infected Cells Exhibit AMPK Inhibition. Although the presence of the replicon was both necessary and sufficient to mediate AMPK inhibition, it was important to determine whether AMPK activity was perturbed in the context of virus infection. Huh-7 cells were transfected with in vitro transcribed full-length RNA of the cell-culture permissive genotype 2a HCV isolate, JFH-1 (24). Consistent with the replicon data, AMPK T172 and ACC phosphorylation were abrogated, whereas AMPK S485 phosphorylation was elevated in JFH-1 RNA-transfected cells (Fig. 5A). Importantly, as for replicon cells, levels of cellular lipids were elevated compared with mock-transfected cells (Fig. 5B and Fig. S4A). To demonstrate further the effects of AMPK activation on viral replication, we showed that metformin treatment reduced replication of a modified virus that expressed luciferase (J6/JFH-1Luc) (25) by 75% (Fig. 5C). To confirm that this reduction was AMPK mediated, we transfected cells with LKB1 siRNA. Although this siRNA was

**Fig. 3.** Effect of AMPK activators on lipid abundance. (A and B) Abundance of NS5A or cellular lipids (BODIPY) was evaluated in Huh-7 or replicon cells incubated in serum-free medium (A) or supplemented with sodium oleate (B). Cells were stained for lipid content with BODIPY dye for 1 h after NS5A labeling. Identical settings were maintained for image capture. Representative confocal images are shown. (Scale bars, 10 μm.) Replicon cells were treated with AMPK agonists for 4 h before processing. (C) For quantification of lipid abundance, images were captured and analyzed using Imaris software. Values were normalized to the control BODIPY levels minus oleate. Error bars indicate mean ± SEM. **Significant difference from Huh-7 (P < 0.05).

**Fig. 5.** HCV-Infected Cells Exhibit AMPK Inhibition. (A) Abundance of NS5A or cellular lipids (BODIPY) was evaluated in Huh-7 or replicon cells infected with JFH-1 or J6/JFH-1Luc. Cells were stained for lipid content with BODIPY dye for 1 h after NS5A labeling. Identical settings were maintained for image capture. Representative confocal images are shown. (Scale bars, 10 μm.) Replicon cells were treated with AMPK agonists for 4 h before processing. For quantification of lipid abundance, images were captured and analyzed using Imaris software. Values were normalized to the control BODIPY levels minus oleate. Error bars indicate mean ± SEM. **Significant difference from Huh-7 (P < 0.05).

**Fig. 6.** Treatment with AMPK activators abrogates HCV-mediated lipid accumulation. AICAR (10 mM) and Metformin (5 mM) were added to JFH-1 replicon cells for 4 h before processing. Cells were stained for lipid content with BODIPY dye for 1 h after NS5A labeling. Identical settings were maintained for image capture. Representative confocal images are shown. (Scale bars, 10 μm.) Replicon cells were treated with AMPK agonists for 4 h before processing. For quantification of lipid abundance, images were captured and analyzed using Imaris software. Values were normalized to the control BODIPY levels minus oleate. Error bars indicate mean ± SEM. **Significant difference from Huh-7 (P < 0.05).
Fig. 4. Activation of AKT in replicon cells. (A) Cell lysates were analyzed by immunoblotting with the indicated antibodies. Levels of phosphorylated AKT or AMPK were quantified by densitometry in comparison with GAPDH (n = 3). **Significant difference from Huh-7 (P < 0.05). (B) Cell lysates were analyzed by immunoblotting with the indicated antibodies. Lanes 2 and 5: insulin (Ins) treatment (100 nM) for 4 h before harvest; lanes 3 and 6: AKTVIII treatment (5 μM) for 4 h before harvest. Cont, control. (C) Abundance of N5A or lipids (BODIPY) was evaluated in untreated Huh-7 or replicon cells following treatment with AKTVIII (5 μM) for 4 h. For quantification of lipid abundance, images were captured and analyzed using Imaris software. **Significant difference from Huh-7 (P < 0.05). (D) Cells transfected with in vitro transcripts of a luciferase subgenomic replicon (genotype 1b) were treated with AKTVIII (5 μM) overnight. Luciferase activity (relative luminescence units (RLU/sec) was used as a measure of replication.”}

Discussion

Our data clearly demonstrate that HCV inhibits the activity of AMPK. Superficially, this observation seems counterintuitive, because active viral replication probably will place high energy demands upon the cell, increasing both ATP consumption and the AMP/ATP ratio. The concomitant increase in AMPK activity would switch on processes that generate ATP while switching off those that consume ATP. In the long term, this effect might benefit the virus by prolonging the life of the host cell. Why, then, does HCV mediate AMPK inhibition? Our data suggest that one answer is that loss of function of AMPK increases hepatic lipid accumulation. An increasing body of evidence shows that HCV is critically dependent on cellular lipids throughout the virus life cycle. By blocking AMPK activity, the virus can ensure that lipid biosynthesis can continue at a high level, permitting the accumulation of lipid that is required for virus replication. By overriding AMPK inhibition with AMPK agonists, not only is lipid accumulation in replicon cells abrogated, but virus genome replication also is inhibited, thereby reducing the production of infectious virus (Fig. 2 and Figs. S2B and S4B). This possibility raises exciting prospects for therapeutic approaches to HCV treatment using well-characterized AMPK agonists such as metformin, a safe and well-tolerated drug that already is used extensively for treatment of diabetes. Indeed, a recent study (26) demonstrated that inclusion of metformin with IFN/ribavirin therapy had a
Cells were lysed in Glasgow lysis buffer [GLB; 10 mM Pipes-KOH (pH 7.2), 120 mM KCl, 30 mM NaCl, 5 mM MgCl₂, 1% Triton X-100 (Sigma), 10% glycerol] (Promega) plus protease and phosphatase inhibitors (2 mM NaF, 5 mM Na₃VO₄, 5 mM NaF, 5 mM Na₂P₂O₇). Fifty micrograms of protein were resolved by SDSPAGE, transferred to a PVDF membrane using a semidry transfer apparatus, and probed with appropriate primary and secondary antibodies. ImageJ (National Institutes of Health) densitometry was used for quantification.

Adenine Nucleotide Quantification. Cells were scraped into a minimal volume of 5% perchloric acid and centrifuged (13,800 × g at 4 °C) to remove debris. The perchloric acid was neutralized, and nucleotides were extracted using an equal volume of a 1:1,1,2-trichlorotrifluoroethane:propiolactone mix. The ATP:ADP ratio was measured using capillary electrophoresis and was used as a surrogate for AMP:ATP (17).

In all experiments, statistics and effects were determined using one-way ANOVA. For comparisons between two groups, the Student’s t test was used, and for multiple comparisons, the Bonferroni correction was applied. The results were considered statistically significant at *p < 0.05, **p < 0.01. All data are presented as the means ± SEM. One asterisk denotes a statistically significant difference from untreated control (Cont) cells. Two asterisks denote a statistically significant difference from the mock (Mock) group, and three asterisks indicate a statistically significant difference from the treated group (siRNA).

Materials and Methods

Cell Culture. Huh-7 cells were cultured in DMEM with 10% FCS, 1% non-essential amino acids, 2 mM L-glutamine, 100 μM penicillin, and 100 μM streptomycin at 37 °C in a humidified 5% CO₂ incubator. Subgenomic replication-harboring cell lines (genotype 1b FKS.1) (18) were maintained in DMEM with 250 μg/mL G418.

Intriguingly, Nef also induces increases in cholesterol biosynthesis (29). The small T antigen maintains energy homeostasis during glucose deprivation by activating AMPK (37), and avian reovirus infection also has been shown to stimulate AMPK T172 phosphorylation (38). The challenge will be to dissect the precise role that AMPK activity plays in the life cycle of these viruses, allowing a better rationale for the use of AMPK agonists in antiviral therapy. In this regard, we demonstrate that reversing AMPK inhibition in HCV culture systems can reduce the accumulation of lipids, suggesting that HCV may affect one or more steps in cholesterol and/or fatty acid biosynthesis directly through AMPK inactivation. The key issue that remains to be addressed is to dissect the precise physiological interplay between HCV proteins and the control of AMPK activity.

Fig. 5. Inhibition of AMPK activity is observed in cells transfected with full-length JFH-1 RNA or infected with JFH-1 virus. (A) Cells were electroporated with full-length JFH-1 RNA and analyzed for AMPK activation status as described in Fig. 1B. (B) Abundance of NSSA or cellular lipids (BODIPY) was analyzed as described in Fig. 3. JFH-1 transfected cells are outlined in white; untransfected cells in the same field are outlined in red. For quantification of lipid abundance, images were captured and analyzed using Imaris software. **Significant difference from transfected (Mock) cells (p < 0.05). C) Cells were electroporated with either control (Cont) or LKB1–specific siRNA, incubated for 72 h to allow silencing of the target gene, and then transfected with J6/JFH-1 luc RNA. Samples at 48 h posttransfection were analyzed for luciferase activity. Metformin was added to cells at 24 h posttransfection. Cell lysates were analyzed by immunoblotting with the indicated antibodies to confirm LKB1 silencing. Results are expressed as mean ± SEM (n = 3). **Significant difference from untreated control (p < 0.05). (D) Huh-7 cells were mock-infected or infected with JFH-1 virus at a multiplicity of infection of 0.5 focus-forming units per cell. Cell lysates were prepared at 48 h posttransfection and analyzed by immunoblotting with the indicated antibodies.
are displayed as single optical sections of 50-μm thickness. For detection of exogenous AMPKαs, cells were transfected with plasmids expressing Myc-AMPKα1, fixed 48 h posttransfection, permeabilized, and probed with a mouse monoclonal anti-Myc antibody (1 μg/mL) followed by an Alexa Fluor 488 anti-mouse secondary. For quantification cells were serum-starved overnight ≥ 20 μg/mL sodium olate and AMPK agonists as described. Images were captured and analyzed using Imaris (Bitplane AG) or Imagej software. Threshold of each channel were set at 10% of the maximum intensity. Vesicles of a diameter of 0.5 μm were counted and divided by the cell number for each image.

**Translucent Subgenomic Replicon Luciferase Assays.** T7 transcripts were generated from linearized DNA templates of JFH1 (SGR-Luc-JFH-1) (40) or genotype 1b (FKS.1 Luc) luciferase subgenomic replicons. Then 4 × 10⁴ cells were washed in diethylpyrocarbonate (DEPC)-treated PBS, resuspended in 400 μL PBS, and electroporated with replicon RNA (5 μg) in 0.4-cm cuvettes at 950 μF, 270 V. Then 1 × 10⁵ cells were seeded into each well of 96-well plates. Cells were lysed directly in 96-well plates at 4 and 24 (JFH-1) or 4 and 48 h posttransfection (FKS.1) in 1× passive lysis buffer (PLB) (Promega). Luciferase activity was measured using luciferase assay reagent (LAR; Promega) on a BMG plate reader. AMPK agonists were added at 8 or 32 h posttransfection for JFH-1 and FKS.1, respectively. Statistical significance of differences was determined using the paired Student’s t test. P < 0.05 was accepted as significant.

**Virus Assays.** Cells were washed in DEPC-treated PBS and resuspended at 2 × 10⁶ cells/mL. Then 8 × 10⁴ cells were electroporated with 10 μg of JFH-1 RNA. For replication assays, cells electroporated with siRNA were incubated for 72 h to allow silencing of the target gene before transfection with 1 μg JFH-1 LUC RNA using Lipofectin (Invitrogen) following the manufacturer’s instructions. Samples were harvested in 100 μL PLB at 4/48 h posttransfection. For infection experiments, virus inoculum was titrated by focus-forming assay (41) and used to infect Huh-7 cells at a multiplicity of infection of 0.5 in complete medium. For Western blotting, cells were lysed in GLB as described above at 48 h posttransfection. Virus harvest and titrations were performed as described (41).

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