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Peroxisome Proliferator-Activated Receptor α Antagonism Inhibits Hepatitis C Virus Replication

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Summary

Hepatitis C virus (HCV) is a global health problem and a leading cause of liver disease. Here, we demonstrate that the replication of HCV replicon RNA in Huh-7 cells is inhibited by a peroxisome proliferator-activated receptor (PPAR) antagonist, 2-chloro-5-nitro-N-(pyridyl) benzamide (BA). Downregulation of PPARγ with RNA interference approaches had no effect on HCV replication in Huh-7 cells, whereas PPARα downregulation inhibited HCV replication. Fluorescence and coherent anti-Stokes Raman scattering (CARS) microscopy demonstrate a clear buildup of lipids upon treatment with BA. These observations are consistent with the misregulation of lipid metabolism, phospholipid secretion, cholesterol catabolism, and triglyceride clearance events associated with the inhibition of PPARα. The inhibition of HCV replication by BA may result from disrupting lipidation of host proteins associated with the HCV replication complex or, more generally, by disrupting the membranous web where HCV replicates.

Introduction

Hepatitis C virus (HCV) infection is a rapidly increasing global health problem, with approximately 3% of the global population infected. It is responsible for 40%–60% of chronic liver disease worldwide [1]. Clinical efficacy of modern therapeutics is limited, and no vaccines are currently available [2–8]. Central to HCV's life cycle are host-virus interactions that support infection, replication, and viral particle assembly on a molecular level. HCV is a positive strand RNA virus of the family *Flaviviridae* and has an ~9.6 kb genome that is translated into a large polyprotein. This ~3,000 amino acid polyprotein [1] is cleaved into at least 10 mature viral proteins. They include the core protein and envelope proteins (C, E1, and E2), which are the structural proteins of the virus; a small hydrophobic protein of unknown function (p7); and the rest of the proteins indicated as nonstructural proteins (NS2–NS5B) [1]. HCV can induce changes in host cell structure and function. For example, HCV is known to induce changes in lipid metabolism [9, 10], and it induces the formation of ER-derived membranous webs on which HCV replicates [11, 12]. HCV also induces steatosis, the accumulation of lipid droplets on which HCV structural proteins are known to reside [13–16]. HCV replication requires localization of HCV NS5B and the associated complexing proteins to membranous structures of the host cell [17, 18]. Peroxisome proliferator-activated receptors (PPARs) play an important role in controlling gene expression of lipid metabolism [19]. Therefore, PPAR-mediated alterations in lipid homeostasis are likely to have an influence on HCV replication.

Peroxisomes are organelles bound by a single membrane and are involved in various metabolic functions (fatty acid oxidation, peroxide-derived respiration, cholesterol catabolism, for example) within the cell. PPARs are the nuclear receptors responsible for the effects of ligands, collectively known as peroxisome proliferators, that alter both the number and size of peroxisomes [19]. First cloned from mouse liver in 1990, PPARs are ligand-dependant transcription factors that share common structural features, including a highly conserved central DNA binding domain, C-terminal ligand binding domain, and a variable N-terminal domain. There are three identified PPAR isoforms in mammals: α, β/δ, and γ [20–26]. These isoforms are encoded by separate genes and differ in their localization, function, and ligand specificity. PPARα is primarily expressed in tissues with high lipid metabolism, including brown adipose tissue and liver, as well as the kidney, heart, and skeletal muscle [20–22]. PPARβ/δ has the highest expression in the gut, kidney, and heart, although it is expressed to a lesser extent in many other tissues [20]. PPARγ is primarily expressed in adipose tissue, and it is also expressed in the colon, immune system, and the retina [20].

PPAR receptors represent important pharmaceutical targets for the treatment of diseases, including diabetes, that involve the misregulation of glucose, cholesterol, and fatty acid metabolism [27]. PPAR receptors naturally bind to a number of fatty acids and metabolites as well as synthetic ligands [28–30]. Glitazones, including thiazolidinediones (TZD) and fibrates, are classes of drugs that activate PPAR receptors and show either glucose- and/or lipid-lowering effects [28–30]. Rosiglitazone and pioglitazone are two examples of TZD drugs that are effective in lowering glucose levels by targeting PPARγ and also have a modest effect on lipid levels through crossreactivity with other isoforms [31, 32]. It is common that agonists or antagonists of PPAR activity show crossreactivity between isoforms. Farglitazar has robust effects on glucose, high-density lipoproteins, and triglycerides in diabetic patients [27]. Crossreactivity of ligands between PPARα and PPARγ is most common primarily because the ligand binding pockets are closest in size and shape to each other [29, 30]. Also,

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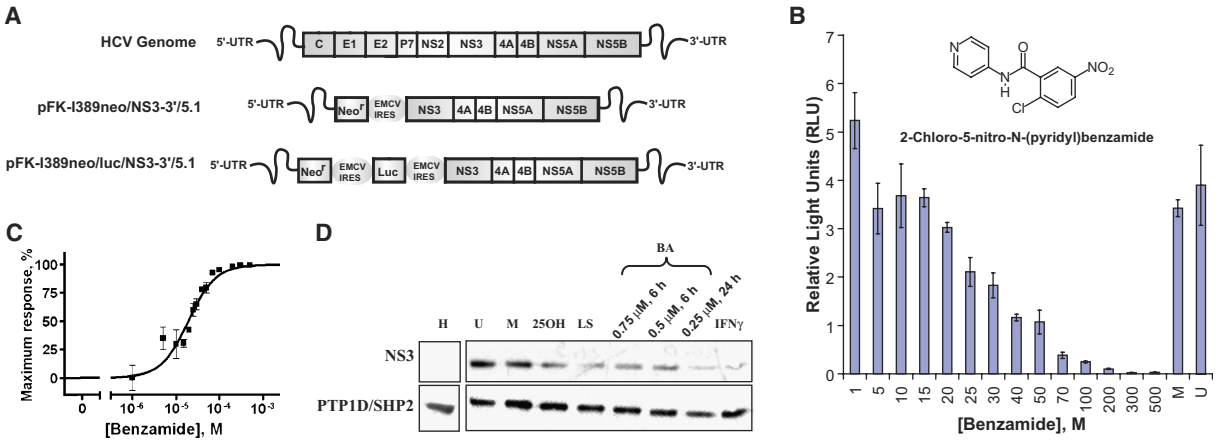

Figure 1. The Activity of Benzamide BA against Subgenomic HCV Replicons in Huh-7 Cells

(A) Schematic representation of the subgenomic replicons used in this study (see Experimental Procedures).

(B) Luciferase reporter gene activity (relative light units; RLU) of Huh7 cells stably expressing an HCV subgenomic replicon derived from pFK-I389neo/luc/NS3-3′/5.1. Note that our RLUs are divided by 1000 for ease in presentation. Activity was determined after 24 hr of exposure to increasing concentrations of 2-chloro-5-nitro-N-(pyridyl)benzamide. Data have been normalized against total protein content. M, 1% v/v MeOH; U, untreated replicon bearing Huh-7 cells. The IC_{50} of BA is expressed as a percentage of the maximal response after 24 hr of exposure to benzamide.

(C) Expression of HCV NS3 protein. Lanes are: H, Huh-7 cells; U, untreated Huh-7 cells stably harboring an HCV subgenomic replicon derived from pFK-I389/neo/NS3-3′/5.1; M, 1% v/v MeOH; 25OH, 2 μM 25-hydroxycholesterol; LS, 50 μM lovastatin; BA, 2-chloro-5-nitro-N-(pyridyl)benzamide (25, 50, 75 μM); IFNγ, 50 U interferon γ.

Data have been normalized against total protein content. Error bars were determined from statistical treatment of triplicate measurements.

a major determinant of this selectivity is thought to be due to a substitution of Tyr-314 in PPARα for His-323 in PPARγ [29, 30]. The antagonist 2-chloro-5-nitro-N-(pyridyl)benzamide inhibits PPARγ activity by covalently modifying cysteine 313 [23]. This antagonist also shows crossreactivity toward other isoforms [23]. Thus, antagonists of PPARγ/α that do not discriminate between isoforms on a molecular level can affect both lipid metabolism and glucose homeostasis.

The liver is the central organ involved in fat metabolism and lipid homeostasis [28]. It is involved in free fatty acid synthesis, esterification of triacylglycerols, and their packaging into very low-density lipoproteins (VLDL) for exportation during the fed state [28]. During the fasted state, the liver is responsible for controlling the rates of fatty acid β-oxidation and ketogenesis. The liver maintains lipid homeostasis by balancing these processes. A key mediator in maintaining this balance is PPARα [22, 24–26]. PPARα acts as a sensor for the level of free fatty acids and modulates the responses of fat-oxidizing tissues [28]. Activation of PPARα results in an increase in the enzymes involved in lipid metabolism and fatty acid β-oxidation. These enzymes include apolipoproteins, fatty acid binding proteins, medium chain acyl-CoA dehydrogenase, carnitine palmitoyl-transferase, and microsomal fatty acid ω-hydroxylase [25, 28]. PPARα knockout mice develop fatty livers and respond very poorly to fasting, resulting in hepatic lipid accumulation, hypoglycemia, and impaired ketogenesis [24, 28, 33, 34]. PPARα has also been shown to be impaired during HCV infections [35, 36], implying a role for PPARα in HCV infection and pathogenesis. Here, we show, by using Huh-7 cells harboring subgenomic HCV replicons, that PPARα antagonism results in a decrease in HCV viral replication. The decrease in viral replication is linked with an observed misregulation of lipid homeostasis that increases the intracellular lipid content.

Results

The Effects of PPARα/γ Antagonist 2-Chloro-5-Nitro-N-(Pyridyl)Benzamide on HCV Replication

In order to test the importance of PPAR activity, we first examined the effects of 2-chloro-5-nitro-N-(pyridyl) benzamide, BA [23], which targets PPARγ, and to a lesser extent, PPARα. Throughout this study, we used subgenomic HCV replicons [12, 37, 38], which are a well-established cellular model for HCV replication, in order to study host-virus molecular interactions that result from perturbation of PPAR signaling. In particular, we utilized Huh-7 cells harboring a tricistronic subgenomic HCV replicon, constructed from HCV genomic RNA of the genotype 1b, derived from the clone pFK-I389neo/luc/NS3-3′/5.1, which was previously characterized [9, 39] (schematically represented in Figure 1A), and we evaluated BA activity by utilizing the genetic reporter luciferase. After 24 hr of exposure, BA concentration dependence of the luciferase signal was observed (Figure 1B). We utilized 1% v/v methanol in complete media as the negative control or mock treatment as well as 25-hydroxycholesterol, lovastatin, and interferon γ as positive controls [9, 10, 40]. We found that higher concentrations of the benzamide were as effective at reducing HCV replicon levels as interferon treatments. Cytotoxicity assays after 24 hr treatments indicated that this compound was detrimental to cell viability only at concentrations at or above 100 μM. Furthermore, treated cells recovered from these effects after 72 hr, suggesting that the altered metabolic condition of the treated

cells may result from the misregulation of genes transcriptionally regulated by PPARα. An IC_{50} against the HCV replicon for 2-chloro-5-nitro-N-(pyridyl)benzamide was determined to be 19.1 μM (Figure 1C), consistent with the IC_{50} of this ligand for the PPARα isoform [23], which is ~800-fold lower than that for PPARγ. Higher concentrations of BA that are needed to reduce HCV replication are likely the result of PPAR isoform-specific effects on the HCV replication complex. Independent confirmation of the decrease in replication rate was obtained by performing Western blot analysis of cell lysates for the HCV NS3 protein (Figure 1D). We examined loading by using control antibodies against tubulin and the protein-tyrosine phosphatase PTP1D. PTP1D was found to be a better control for Western blots, as it was determined that BA binds to tubulin at high concentrations (ca. 100 μM). The effects of BA on PPARα signaling and lipid metabolism were confirmed by quantitative PCR analysis of mitochondrial HMG-CoA synthase mRNA levels by using the 18S rRNA levels as a control. Treatment of Huh-7 cells bearing HCV replicons with BA for 24 hr resulted in decreases in HMG-CoA synthase levels from 5- to <60-fold for BA concentrations ranging between 20 μM and 75 μM (see Supplemental Data available with this article online).

The Effects of siRNA Molecules and Mammalian Gene Overexpression on HCV Replication

In order to determine the molecular basis for the anti-HCV activity of 2-chloro-5-nitro-N-(pyridyl)benzamide, we examined the role of direct gene knockdown by utilizing standard RNA interference techniques. Utilizing the tricistronic HCV replicon (illustrated in Figure 1A), we tested the effects of siRNAs that target PPARα and PPARγ. Oligonucleotide duplexes that target luciferase GL2 and GL3 were used as positive and negative controls, respectively. The luciferase GL2 oligonucleotides specifically target the firefly luciferase gene contained within the tricistronic HCV replicon, whereas GL3 targets a different luciferase isoform. All of the siRNAs were tested according to manufacturer's protocols to ensure target knockdown (see the experiment described below). Downregulation of the target gene was confirmed by Western blot, although the PPARα protein levels upon treatment with siRNA were only ~50% lower than those of mock-transfected cells (see Supplemental Data). We found that knockdown of PPARα mRNA had an effect on HCV replication but that this was not as significant a decrease as was observed for the benzamide ligand (Figure 2). Knockdown of PPARγ with siRNA had no effect on HCV replication. Examination of PPARγ mRNA levels in gene expression data (unpublished data) suggests that this isoform is probably not constitutively expressed in Huh-7 cells. Overexpression of PPARα with a CMV promoter also had little effect on HCV replication, as did typical PPARα agonists such as WY-14643 ([4-chloro-6-(2,3-xylidino)-2-pyrimidinylthio] acetic acid). Overexpression with the CMV promoter was confirmed to be at least 2-fold higher than controls by Western blot (see Supplemental Data).

Next, we studied the effect of BA in the context of overexpression of PPARα using the overexpression vector pCMV-PPARα (see Experimental Procedures). We compared the effects of BA on HCV replicons in mock-transfected cells and in cells transiently transfected with the overexpression vector. PPARα expression was confirmed by Western blot. We observed that the BA compound was less effective at inhibiting HCV replication in cells overexpressing PPARα, confirming the specificity of BA for PPARα in the Huh-7 and HCV replicon cell lines. These observations provide further support that inhibition of HCV replication by BA results from antagonism of the PPARα protein.

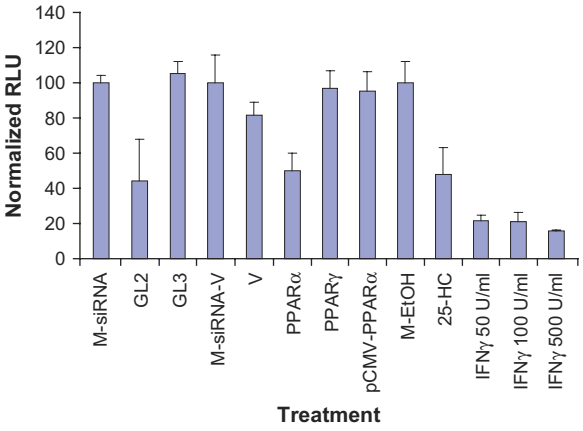

Figure 2. Luciferase Reporter Gene Activity Represented by Normalized Relative Light Units of Huh-7 Cells Stably Expressing an HCV Subgenomic Replicon Derived from pFK-I389neo/luc/NS3-3′/5.1

Activity was determined after 72 hr treatments: M-siRNA, mock siRNA treatment; GL2, siRNA molecule targeting the luciferase gene; GL3, siRNA control molecule; M-siRNA-V, control for vectors expressing scrambled siRNA; PPARα and PPARγ, siRNA vectors targeting PPARα and PPARγ; pCMV-PPARα, overexpression vector for PPARα; M-EtOH, 1% v/v ethanol; 25-HC, 2 μM 25-hydroxycholesterol; IFNγ, 50, 100, and 500 U treatments with interferon γ. Data have been normalized against total protein content. Data have been normalized against total protein content. Error bars were determined from statistical treatment of triplicate measurements.

The Effects of PPARγ/α Antagonist 2-Chloro-5-Nitro-N-(Pyridyl)Benzamide on Lipid Content in Huh-7 Cells

In order to determine the morphological effects of treating cells with the PPAR antagonist 2-chloro-5-nitro-N-(pyridyl)benzamide, we first examined cells by confocal fluorescence microscopy. Huh-7 cells were treated with various concentrations of BA for various lengths of time. These cells were then fixed and stained for lipids with oil red O dye and were counterstained with DAPI (Figure 3). We see a noticeable increase in lipid droplet accumulation in the presence of BA, and this is consistent with the observations made previously regarding the link between the PPARα receptor and lipid accumulation in clinical HCV infections [35, 36].

We used coherent anti-Stokes Raman scattering (CARS) microscopy to examine the effects of BA on living Huh-7 cells. CARS microscopy is a powerful imaging modality that is derived from the inherent vibrations of molecules. CARS microscopy can image samples with molecular specificity, without the need for labeling, by tuning the frequency difference between the two laser beams into a specific molecular vibration. CARS offers the ability to image live cells [41, 42] and tissues [43] with high spatial resolution in real time. In this

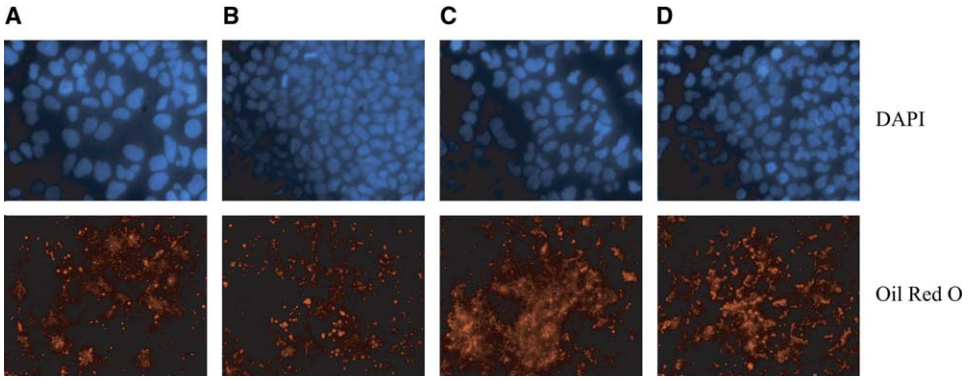

Figure 3. Fluorescence Microscopy of Oil Red O and 4′,6-Diamidino-2-Phenylindole (DAPI)-Stained Huh-7 Cells Stably Expressing an HCV Subgenomic Replicon Derived from pFK-I389neo/NS3-3′/5.1

(A–D) Cells were imaged after 24 hr under the following conditions: (A) untreated; (B) 1% v/v MeOH; (C) 25 μM BA; (D) 50 μM BA.

experiment, the CARS system was tuned to the strong lipid CH_2 vibrational resonance at 2845 cm^{-1} in order to selectively image lipids.

We performed a time course examination of Huh-7 cells upon treatment with the PPAR antagonist BA by using CARS microscopy tuned to illuminate lipids (Figure 4). A clear increase in cellular lipids after treatment with BA was observed. The effects on these live cells were clearly more dramatic and not limited to lipid droplet accumulation (Figure 4A). An increase in lipid content in Huh-7 cells treated with BA was observed compared to untreated and mock-treated cells that were also imaged in CARS microscopy experiments for the duration of the time course. The observed increase in lipid content within the Huh-7 cells can be attributed to decreases in fatty acid activation, transport, β-oxidation, secretion, triglyceride clearance, and cholesterol catabolism, which is consistent with what is known about PPARα signaling in the liver [21, 25, 28, 44].

Immunostaining for the HCV polymerase gene NS5B in the replicon-bearing Huh-7 cells was conducted in the presence of BA. Treatments were conducted for 6 hr at concentrations ranging from 0 to 75 μM (Figures 4B–4F). These experiments were conducted to

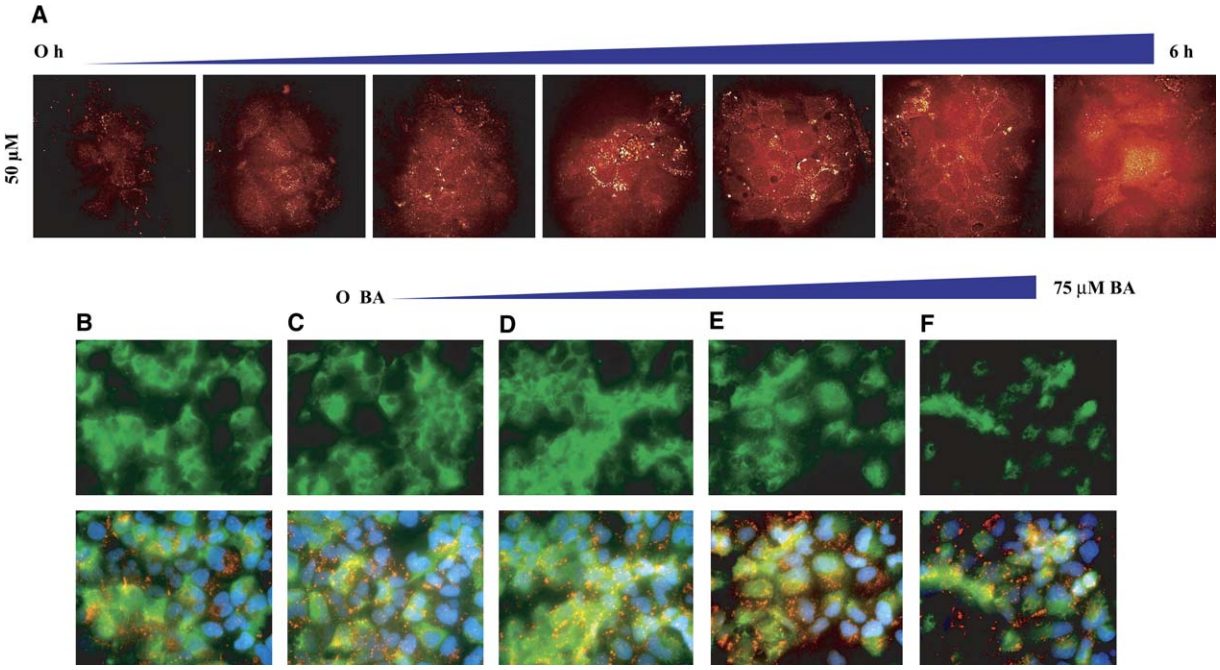

Figure 4. CARS and Fluorescence Microscopy of the Effects BA Elicits in Lipid Homeostasis

(A) Huh-7 cells were incubated at 37°C and 5% CO_2 after treatment with 50 μM BA. The CARS microscope was tuned to the lipid stretch at 2845 cm^{-1}, and images were acquired at 60 min intervals over 6 hr.

(B–F) Fluorescent microscopy of NS5B (green) immunostained Huh-7 cells bearing subgenomic HCV replicons, counterstained with oil red O (red) and DAPI (blue). (A) Huh-7 cells bearing HCV subgenomic replicons that were (B) 1% v/v MeOH treated and (C–E) treated with 25, 50, and 75 μM BA for 6 hr.

determine if mediation of PPAR signaling affected levels and subcellular localization of the HCV protein NS5B. Consistent with previous studies [18, 45–47], we observe NS5B subcellular localization concentrating in the perinuclear region where membranous web structures are known to reside. We observe a decrease in signal intensity for NS5B with increasing doses of BA that is consistent with the NS3 Western blot data (Figure 1D). The benzamide also appears to enhance cellular permeability of the antibodies at lower doses. Fluorescence microscopy shows no evidence for an alteration of NS5B subcellular localization, although it is possible that NS5B and the associated replication complexes are dispersed upon treatment with BA.

Discussion

The PPARα nuclear hormone receptor plays a critical role in regulating liver-specific metabolic functions through obligate heterodimerization with RXR and LXR and subsequent transactivation of genes containing PPREs in their promoter regions [24, 25, 44, 48, 49]. Misregulation of this gene or its downstream targets has also been implicated in liver-specific disease states. PPARα activation over prolonged periods is linked to tumorigenesis in rats [49], the development of steatosis, and delayed liver regeneration in knockout mice after partial hepatectomy [48, 50–52]. Hepatocellular levels of PPARα and carnitine palmitoyl acyl-CoA transferase 1 (CPT1A) have been shown to be depressed in untreated patients with HCV infections [35, 36]. A link between this disruption of PPARα gene expression at the mRNA level and HCV core protein expression in HepG2 cells, and thus a link between PPARα and HCV pathogenesis, has also been made [36]. Here, we show that perturbation of PPARα with either an antagonist or siRNA also has a negative effect on HCV replication. Using a genetic reporter for HCV RNA levels, and by Western blot analyses, we have characterized the anti-HCV effects of BA and siRNA's targeting of the PPARα gene (Figures 1 and 2). Each of the latter acted to inhibit HCV replication in HCV replicons with different rates and reactivities. The PPARα/γ antagonist BA acted more rapidly and with a higher efficacy.

We have observed that time required for siRNAs to significantly affect luciferase reporter levels is much longer (48–72 hr) than that observed with BA (6–24 hr), suggesting that the disruption of HCV replication occurs more rapidly with the PPARα small-molecule antagonist. This is likely owing to the rapidity by which the small molecule can diffuse to its target, and to the fact that it can act directly on the receptor posttranslationally [23]. Antagonists of PPARα are known to downregulate HMG-CoA synthase transcriptionally, which we confirmed by quantitative PCR. Antagonists of PPARα also induce hyperlipidemia, in contrast with the action of agonists [24, 26, 44]. This phenotype was confirmed by using fluorescence and CARS microscopy. Moreover, CARS microscopy allowed for a more detailed examination of the kinetics of BA action in live cells through indirect observations of the hyperlipidimic condition resulting from its action on PPARα. We observed an increase in lipid content within 1–2 hr after treatment of Huh-7 cells with BA (Figure 4). These results are consistent with a loss of HCV replicon replication as measured by HCV NS3 (Figure 1D) and NS5B (Figures 4C–4F) levels.

There are a number of possible mechanisms by which 2-chloro-5-nitro-N-(pyridyl)benzamide inhibits HCV replication in the replicon model. While it is possible that this small molecule may interact directly with HCV proteins involved in replication, siRNA experiments (Figure 2) suggest that the mechanism likely involves the downstream effects of PPARα antagonism. Hyperlipidemia can also disrupt replication complexes by altering membranous structures in which replication occurs [11, 12], or by altering the lipidation of host proteins that are necessary for replication to occur [10, 53]. Previous studies have indicated that genes associated with lipid metabolism, such as SCAP- and SREBP-regulated genes of the mevalonate pathway, are differentially regulated during the initial stages of HCV infection and that these genes may be predictors of the outcome of viremia [9]. Lovastatin, a potent inhibitor of HMG-CoA reductase that catalyzes the conversion of HMG-CoA to mevalonic acid (the rate-limiting step in the synthesis of cholesterol) [54], and GGTI-286, an inhibitor of a geranylgeranyl transferase enzyme, also inhibit HCV replication by an indirect route [10, 53]. Transcriptional regulation of hepatocellular genes, including lipid transfer genes such as HMG-CoA synthase and CPT1A, by PPARα is well-documented [20–22, 27–29]. We suggest that the most likely mechanism of anti-HCV action of the benzamide involves posttranslational antagonism of PPARα, and that this subsequently alters the transcriptional regulation of a number of gene products including those that regulate lipid metabolism and lipid transfer. The effects are likely enhanced relative to the siRNA owing to crossreactivity of the small-molecule antagonist.

Significance

We have demonstrated that both small molecules and siRNA molecules that perturb the function of PPARα also inhibit HCV replication in replicon systems. This activity is linked with a hyperlipidemic condition within the Huh-7 cells, although it is not necessarily causally related. There exists strong evidence that chronic HCV infections are associated with the impaired expression of PPARα, and the likely culprit leading to this change in the host cell is the core protein of HCV [35, 36]. Our data suggest that the impaired expression of PPARα during chronic infection is not advantageous to HCV propagation. Rather, a consequence of chronic infection may favor the host rather than the virus in controlling the levels of HCV in the infected liver. This may also be a mechanism by which HCV controls its own levels in vivo in an evolutionary attempt to evade the host immune response. Further experiments that examine the precise mechanism by which PPARα inhibits the HCV lifecycle may aid in the identification of novel targets for therapeutic intervention.

Experimental Procedures

Tissue Culture

Cell monolayers of the human hepatoma cell line Huh-7 were grown in DMEM medium supplemented with 100 nM nonessential amino acids, 50 U/ml penicillin, 50 μg/ml streptomycin, and 10% FBS

(CANSERA, Rexdale, ON). HCV replicons were maintained in the same medium plus 250 μg/ml G418 Geneticin (GIBCO-BRL, Burlington, Ontario). Huh-7 cells stably replicating HCV replicons derived from the pFK-I389neo/NS3-3′/5.1 subgenomic replicon were kindly provided by Ralf Bartenschlager (Institute of Hygiene, University of Heidelberg, Heidelberg, Germany), and the pFK-I389neo/luc/NS3-3′/5.1 plasmid is described elsewhere [9, 39].

Small-Molecule Treatments

Huh-7 cells harboring the HCV subgenomic replicon were seeded at 1×10^4 cells/well in DMEM in a 96-well plate. After 24 hr, at a confluency of 70%–80%, cells were treated with BA (1–500 μM), for 24 hr. The effect of the benzamide molecule on Huh-7 cells was analyzed by luciferase and toxicity assays. The same conditions were followed for treatments with 25-hydroxycholesterol (2.5 μM), lovastatin (50 mM), and interferon γ (50–500 U/ml).

Overexpression and siRNA Transfection in HCV Replicon Cells

The siRNA duplexes homologous to GL2 and GL3 luciferases were purchased from Dharmacon (Lafayette, CO). The siRNA vector mixes, targeting the PPARα and/or the PPARγ gene, were purchased from Panomics (Redwood City, CA). The siRNA or overexpression vector transfections were routinely done in 96-well plates according to the manufacturer's protocol, and assays were carried out at 48, 72, and 96 hr posttransfection.

Luciferase Reporter Assay and Protein Quantification

Huh-7 cells harboring subgenomic replicons were transfected with siRNA or treated with the benzamide compound as described previously. At assay endpoints, cells were washed once with PBS and lysed with cell culture lysis buffer (Promega, Madison, WI). Luciferase assay substrate was added, and the relative light units (RLU) were measured with an Lmax luminometer (Molecular Devices Corporation, Sunnyvale, CA). Values were normalized against total protein content by doing the Bio-Rad DC Protein Assay (Bio-Rad, Mississauga, ON) according to the manufacturer's protocol.

Immunoblot Analysis

Huh-7 cells harboring subgenomic replicons (1×10^6 cells) were seeded in 60 mm dishes for preparation of Western blot lysates. After small-molecule treatment, cells were washed and lysed with an SDS lysis buffer consisting of 50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 100 mM dithiothreitol (DTT), and 0.1% bromophenol blue (prepared without the DTT and bromophenol blue). A protease inhibitor cocktail mix (Roche Diagnostics, Penzberg, Germany) was added to each extract. The protein concentration of each sample was quantified by using the Bio-Rad DC Protein Assay according to the manufacturer's protocol. Prior to loading, 10% v/v of DTT and bromophenol blue (1:1) were added to each sample, and 60 μg/well was loaded onto a SDS-PAGE gel (10% resolving, 4% stacking gel). The resolved proteins were transferred to a Hybond-P (Amersham Biosciences, Piscataway, NJ) polyvinylidene difluoride membrane. The membrane was probed against HCV NS3 protein by using a mouse anti-NS3 1° antibody (1:500 dilution, ViroStat, Portland, ME) followed by a 2° (HRP)-conjugated goat anti-mouse IgG antibody (1:1000 dilution) obtained from Jackson ImmunoResearch Laboratories, Inc. (Westgrove, PA). As a loading control, a mouse PTP1D 1° antibody (1:2500 dilution; Sigma, Saint Louis, MO) was used with the same 2° antibody described above. Protein bands were visualized by Western Lightning Western Blot Chemiluminescence reagents (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA) according to the manufacturer's protocol.

Cytotoxicity Assays

Huh-7 cells expressing the HCV subgenomic replicon were treated as described above. After 24 hr, cells were washed and lysed with Triton X-100, and a CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) was performed according to the manufacturer's protocol. The absorbance was read with a Spectramax M2 (Molecular Devices Corporation, Sunnyvale, CA), by using SOFTmax Pro software, at 490/650 nm.

Immunofluorescence Analysis

The fluorescent imaging of Huh-7 cells harboring the subgenomic replicon was performed by using an Axiovert 200M inverted microscope from Zeiss (North York, ON) with the Axiovision 3.1 software. The cells were photographed with an AxioCam connected to the inverted microscope. Cells were probed for HCV NS5B protein and incubated with mouse anti-NS5B (5B-12B7) 1° antibody (1:100 dilution kindly provided by Darius Moradpour, Department of Medicine II, University of Freiburg, Freiburg, Germany), followed by incubation with Cy2-labeled donkey anti-mouse IgG 2° antibody (1:100 dilution, Jackson ImmunoResearch Laboratories, Inc., Westgrove, PA). Cells were stained for lipids with oil red O (0.5% in isopropanol; Sigma-Aldrich, Oakville, ON) and were counterstained with 300 nM DAPI (Molecular Probes, Eugene, OR).

CARS Imaging of Live Cells

Huh-7 cells were seeded at 5×10^4 cells/well in 4.2 cm^2 borosilicate culture vessels (VWR, Mont-Royal, PQ) and incubated at 37ºC and 5% CO_2. At 70%–80% confluency, the cells were washed once with prewarmed DMEM, and BA, previously dissolved in methanol at a concentration of 10 mM, was diluted to 100 μM in DMEM and added to the cells. CARS signals from the cells were collected in the forward direction. Typical pump (711 nm) and Stokes (892 nm) excitation powers were 9 and 3 mW, respectively, at the sample. A detailed discussion of CARS instrumentation is presented elsewhere [41, 42].

Supplemental Data

Supplemental Data including Western blots and functional data for the validation of isoform-specific activity of BA as well as protocols and a list of primers used for quantitative PCR analyses can be found at http://www.chembiol.com/cgi/content/full/13/1/23/DC1/.

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