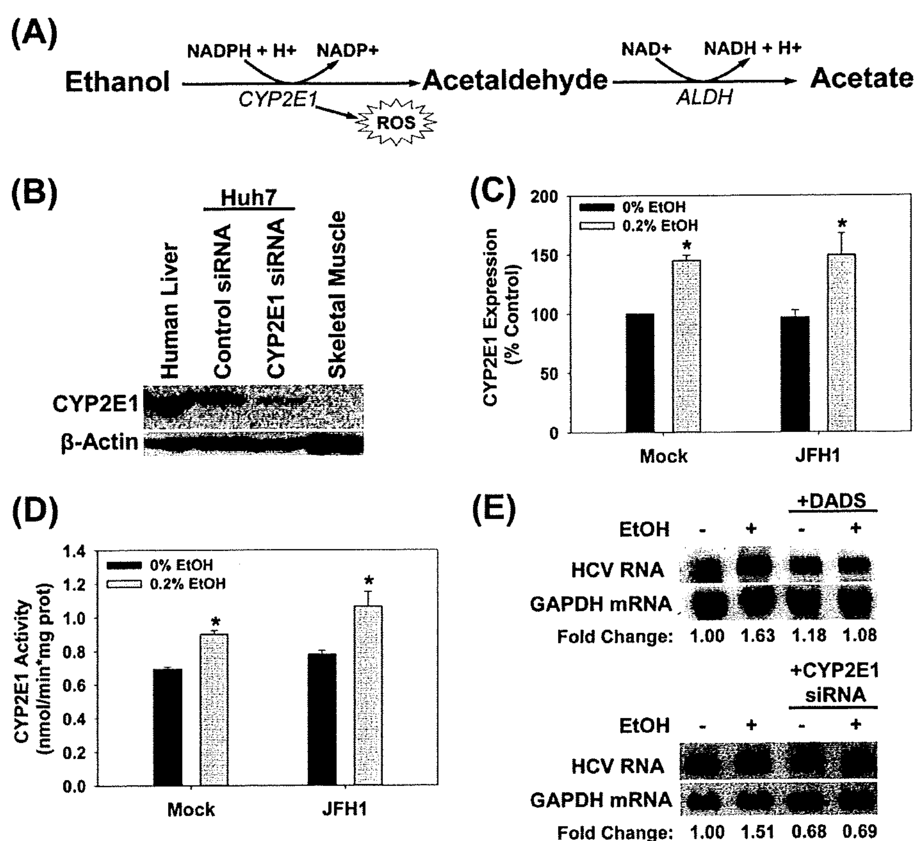

FIGURE 3. **CYP2E1 expression in Huh7 cells.** *A*, CYP2E1-dependent ethanol metabolism. *B*, human liver tissue, Huh7 cells transfected with 50 μM non-targeting control or CYP2E1 siRNA, and skeletal muscle tissue were analyzed for CYP2E1 protein content by Western blot ($n = 3$). *C* and *D*, mock- or JFH1-transfected Huh7 cells were incubated with or without 0.2% (v/v) ethanol for 48 h and analyzed for (*C*) CYP2E1 expression by Western blot ($n = 3$) and (*D*) CYP2E1-dependent *p*-nitrophenol hydroxylation activity ($n = 3$). *E*, SgPC2 cells were exposed to 0.2% ethanol ± 25 μM DADS for 24 h or transfected with 50 nM control or CYP2E1 siRNA for 24 h and then incubated with ethanol for 24 h and analyzed for HCV RNA by Northern blot ($n = 3$). *, indicates statistically significant difference for indicated sample sizes ($p < 0.05$).

Adding extracellular GSH, which is broken down into its constituents, then taken up for intracellular *de novo* GSH synthesis, and does not bypass the BSO-inhibited step, could not restore the HCV RNA level in these cells, as expected. The data suggest that BSO decreases HCV replication specifically by decreasing GSH.

To examine the effects of the exogenous ROS, JFH1 RNA-transfected cells were incubated with 0.25 milliunits/ml of glucose oxidase (GO), which produces H_2O_2 extracellularly through an enzymatic reaction in the presence of glucose, mimicking ROS generation during inflammation. GO decreased the intracellular JFH1 RNA by 30 ± 8% ($p < 0.05$) and exacerbated the suppression of HCV RNA by BSO (Fig. 4*C*). In addition, JFH1 RNA levels decreased with 25, 50, and 100 μM H_2O_2 (Fig. 4*D*). Treating cells with BSO plus GO or either agent alone likewise suppressed the subgenomic JFH1 RNA replication (Fig. 4*E*). BSO and H_2O_2 also countered the enhancement of HCV replication by ethanol (Fig. 4*F*). Furthermore, *N*-acetylcysteine (NAC) and Trolox, a water soluble vitamin E, either increased or had no significant effect on the ethanol-induced enhancement of HCV replication (Fig. 4*G*). These cell treatments did not induce cytotoxicity, as determined by the ATP assay (data not shown). Thus, ROS were not likely to be responsible for the potentiation of HCV RNA replication by ethanol. These data are consistent with the suppression of HCV RNA replication previously observed with HCV genotype 1 (10, 11).

Acetaldehyde Increases the Replication of HCV—We next evaluated whether another major product of ethanol metabolism, acetaldehyde, had similar effects on HCV as ethanol. Acetaldehyde, at physiologically relevant concentrations (25), significantly increased the HCV RNA content in both non-virus producing and virus-producing JFH1 cells (Fig. 5, *A* and *B*). Infecting naïve cells with virus-containing medium and then treating with 5 μM acetaldehyde also led to significant increases in HCV replication (Fig. 5*C*). To examine whether acetaldehyde had similar effects on genotype 1b HCV, SgPC2 cells were also incubated with acetaldehyde and analyzed for changes in HCV replication. Acetaldehyde likewise elevated the HCV RNA level in these cells (Fig. 5*D*). Another Con1 HCV subgenomic replicon cell clone, Clone B, derived in another laboratory (17), responded similarly to ethanol and acetaldehyde, indicating that the response is not specific to our cell clone (Fig. 5*D*). Thus, acetaldehyde is sufficient to potentiate HCV replication of both genotypes 1b and 2a.

Isopropyl Alcohol and Acetone Also Potentiate HCV Replication, the Role of NADH/NAD$^+$—We continued to investigate whether acetaldehyde itself or products of acetaldehyde metabolism are critical for the potentiation of HCV replication by ethanol by inhibiting aldehyde dehydrogenase with cyanamide (see Fig. 3*A*). Cyanamide suppressed the potentiation of HCV replication by ethanol just as inhibiting the first step of ethanol metabolism with 4-methylpyrazole (4MP) and DADS did, suggesting that it is not acetaldehyde itself but a downstream product of acetaldehyde metabolism that increases HCV replication (Fig. 6*A*, *left panel*).

Acetaldehyde metabolism by aldehyde dehydrogenase generates NADH and acetate (Fig. 3*A*). To determine the potential role of NADH, we first evaluated the effects of isopropyl alcohol. Isopropyl alcohol (0.2%, v/v) increases the levels of NADH like ethanol but generates acetone instead of acetaldehyde. To our surprise, isopropyl alcohol also increased the HCV RNA level (Fig. 6*B*, *left panel*) (26). Both isopropyl alcohol and ethanol increased NADH/NAD$^+$ ratio in these cells, as expected

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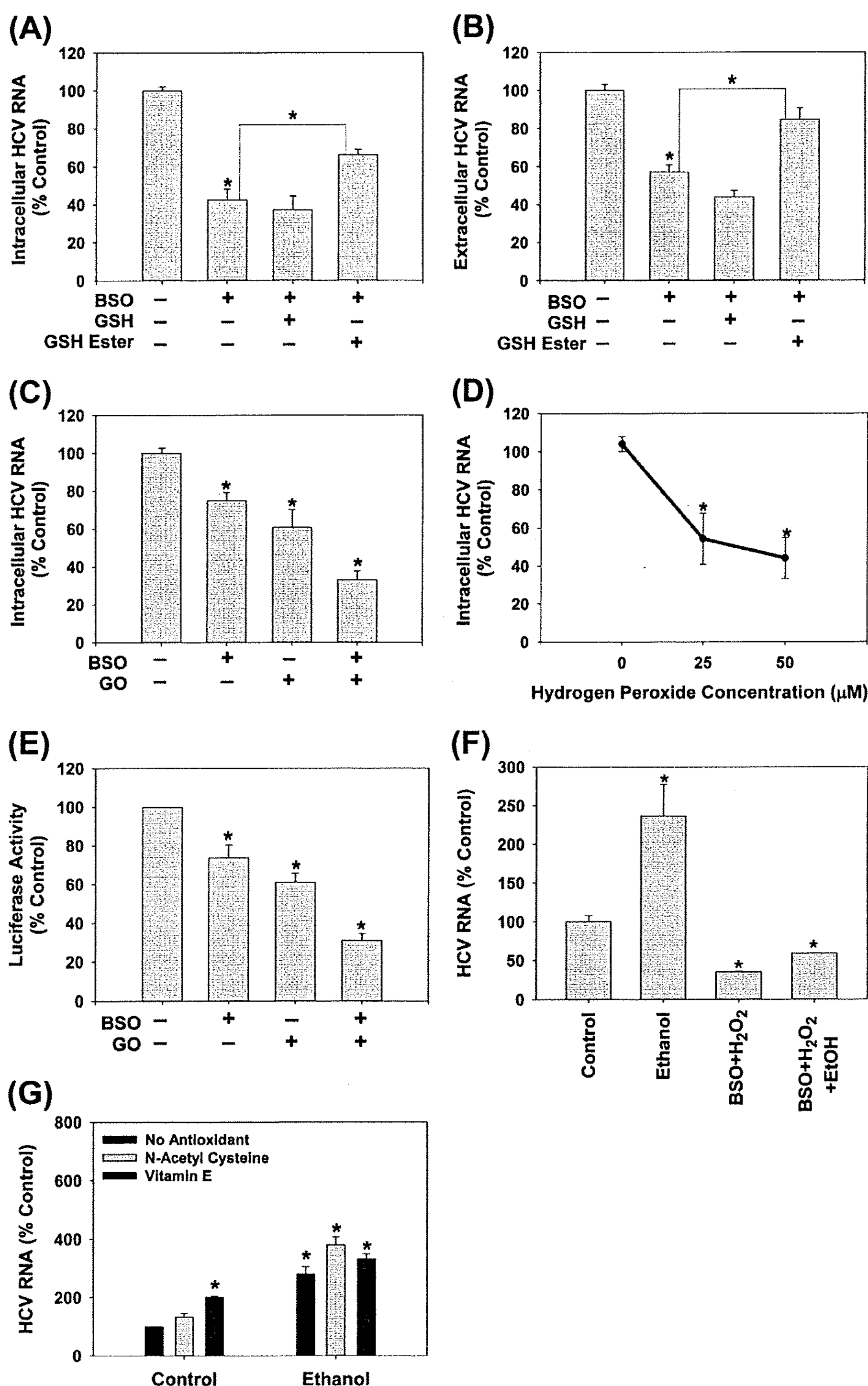

FIGURE 4. **Endogenous and exogenous ROS suppress HCV replication.** JFH1-transfected Huh7 cells were treated with BSO with and without 2 mM GSH or GSH ester (*A* and *B*) ($n = 3$), GO + glucose with and without 16 h of pretreatment with 20 μM BSO (*C*) ($n = 4$), or bolus H_2O_2 (*D*) ($n = 4$) for 24 h. Then, JFH1 intracellular (*A, C, D*) and extracellular (*B*) HCV RNA levels were analyzed by qRT-PCR. *E*, Huh7 cells transfected with SgJFH1-Luc RNA were assayed for luciferase activity after 24 h treatment with 0.25 milliunits/ml glucose oxidase + glucose with and without the BSO pretreatment ($n = 3$). *F*, SgPC2 cells were treated with 0.2% ethanol ± H_2O_2 plus BSO for 24 h, and analyzed for HCV RNA and GAPDH mRNA by Northern blot. *G*, SgPC2 cells were treated for 24 h with ethanol ± 5 mM NAC or 0.5 μM Trolox (water-soluble vitamin E). Then, HCV RNA and GAPDH mRNA levels were monitored by Northern blot and quantified by densitometry ($n = 3$). *, indicates statistically significant difference for indicated sample sizes ($p < 0.05$).

(Fig. 6*B*, *right panel*). In contrast, *tert*-butanol did not elevate HCV replication or the $NADH/NAD^+$ ratio (Fig. 6*B*).

Moreover, we found that acetate itself increased the level of HCV RNA as treating cells with acetone also did. In addition, ethanol, acetaldehyde, acetate, isopropyl alcohol, and acetone all showed corresponding increases in $NADH/NAD^+$ ratios (Fig. 6*B*, *right panel*) (4, 27). The $NADH/NAD^+$ ratios were positively correlated with HCV RNA content in all of these treatments ($r = 0.95$, $p < 0.001$) (Fig. 6*B*). The suppression of HCV replication by cyanamide, 4MP, and DADS in Fig. 6*A* (*left panel*) was also associated with corresponding decreases in the $NADH/NAD^+$ ratios (Fig. 6*A*, *right panel*). Therefore, changes in HCV replication paralleled the changes in the $NADH/NAD^+$ ratio, produced by these treatments.

Then, we examined whether increased $NADH/NAD^+$ ratio was required for the potentiation of HCV replication by ethanol and these other agents. Pyruvate, which re-oxidizes cytosolic NADH to NAD^+, completely abrogated the increases in HCV replication and NADH levels during ethanol, acetaldehyde, acetate, isopropyl alcohol, and acetone treatments (Fig. 6*C*). Methylene blue, which also oxidizes NADH, had similar effects on HCV as pyruvate (data not shown). In contrast, lactate, which produces NADH in the cytosol independent of ethanol, increased NADH levels to 235.9 ± 11.9% ($p < 0.05$) of the control level but had little to no effect on HCV replication (Fig. 6*D*). Together, these data indicate that whereas an alteration of cellular $NADH/NAD^+$ levels seems necessary for the ethanol-induced increases in HCV replication, elevated $NADH/NAD^+$ may not be sufficient to increase HCV replication.

The Potentiation of HCV Replication by Ethanol Requires Lipogenesis—NADH has diverse functions in the cell, and one of these functions includes modulation of lipid metabolism. For example, NADH can

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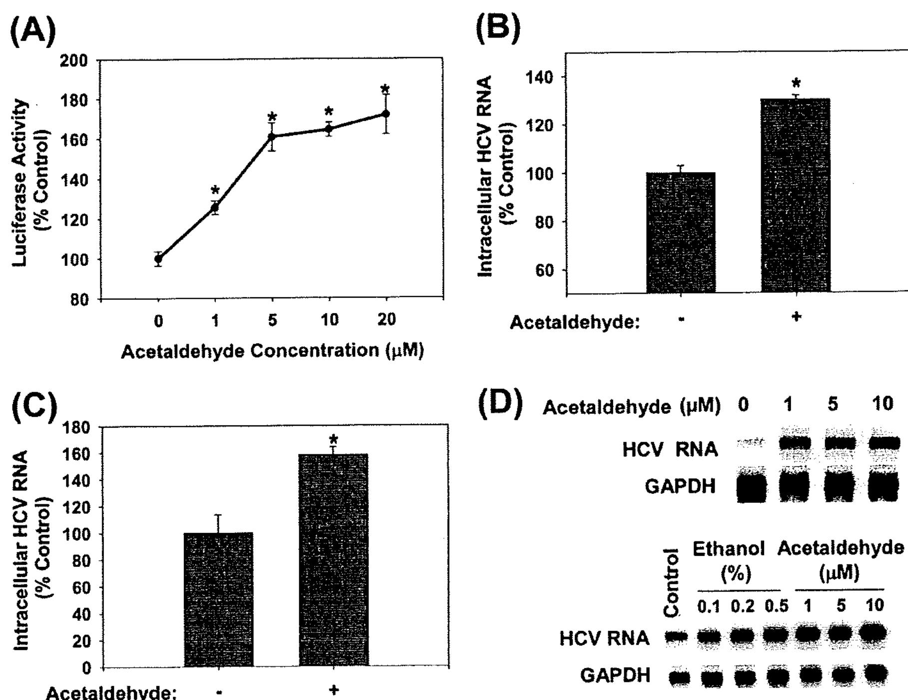

FIGURE 5. **Acetaldehyde increases intracellular HCV RNA.** SgJFH1-Luc (*A*) and JFH1 RNA-transfected cells (*B*), Huh7.5 cells inoculated with JFH1 virions (*C*), SgPC2 and Clone B cells (*D*) were incubated with acetaldehyde for 24 h and analyzed for HCV RNA by Northern blot or qRT-PCR ($n = 3$). *, indicates statistically significant difference for indicated sample size ($p < 0.05$).

inhibit mitochondrial β-oxidation and increase fatty acid synthesis (28). It is well-established that ethanol modulates fatty acid metabolism in part through NADH, and that this plays an important role in the development of steatosis in the alcoholic liver (28). Acetate and acetone would generate acetyl-CoA, which also drives lipogenesis (27, 28). Furthermore, cholesterol metabolism and fatty acid biosynthesis are important in HCV RNA replication (29). Lovastatin and fluvastatin, which are competitive inhibitors of 3-hydroxy-3-methyl-glutaryl-CoA reductase, and 5-(tetradecyloxy)-2-furoic acid (TOFA) and cerulenin, which inhibits fatty acid biosynthesis, have been shown to suppress the basal level of HCV replication (29, 30). Therefore, we next examined whether the potentiation of HCV RNA replication by above agents might be inhibited by modulators of lipid metabolism.

Lovastatin, fluvastatin, TOFA, and cerulenin almost completely inhibited the potentiation of HCV RNA replication by ethanol, acetaldehyde, isopropyl alcohol, acetone, and acetate (Fig. 7, *A* and *B*). In addition, inhibiting β-oxidation of fatty acids with β-mercaptopropionic acid caused a 15.2 ± 1.7-fold ($p < 0.01$) increase in HCV replication in these cells (Fig. 7*C*). Furthermore, ethanol, acetaldehyde, acetone, and acetate treatments increased the total intracellular cholesterol content, which was attenuated by lovastatin (Fig. 7*D*). Lactate, which increased NADH/NAD$^+$ without increasing HCV replication, had no significant effect on cholesterol levels (Fig. 7*D*). The data suggest that the elevation of HCV replication by ethanol, acetaldehyde, acetone, and acetate is mediated by increases in intracellular cholesterol and can be abrogated by the inhibition of cholesterol or fatty acid biosynthetic pathways.

DISCUSSION

High HCV titer is associated with the development and progression of liver diseases (31). In addition, ethanol consumption, high BMI, and high viral titer are strongly associated with poor response to anti-HCV therapy (32). Therefore, the increased HCV replication we saw with physiological levels of ethanol and acetaldehyde is likely to contribute to the pathogenesis and at least partly explain the negative effects that ethanol has on interferon-α therapy. Ethanol has been shown to suppress the antiviral function of interferon-α by interfering with the JAK-STAT signaling pathway (33); however, this is not likely to explain the potentiation of HCV replication we saw with ethanol because HCV effectively suppresses the type I interferon response in Huh7 cells. Additionally, ethanol and acetaldehyde could increase HCV replication in RIG-I-defective Huh7.5 cells (Fig. 5*C*, also, data not shown) (18, 34). Importantly, some ethanol treatments in this study were performed while wrapping cell culture dishes with parafilm to decrease loss of ethanol due to evaporation. However, we observed similar potentiation of HCV replication by ethanol, with and without the parafilm. The use of the parafilm also did not induce hypoxia as no significant change in the expression of hypoxia-inducible factor-1α could be found (data not shown).

Previously, it has been suggested that some of the key ethanol metabolizing enzymes might not be expressed in Huh7 cells (33). Indeed, we also found that alcohol dehydrogenase I is decreased in our Huh7 cells compared with human liver. However, CYP2E1 activity of our cells were within the normal range for human liver, and CYP2E1 expression could be enhanced by ethanol (Fig. 3). In addition, ethanol and acetaldehyde elevated the NADH/NAD$^+$ ratio, indicating that ethanol is being metabolized by our cells. Also, note that even though our cells do not have all of the normal ethanol metabolizing enzymes, our discovery that acetaldehyde and acetate can enhance HCV replication is significant as they bypass these reactions.

A previous study by Zhang *et al.* (3) using various chemical inhibitors of ethanol metabolism, suggested that some downstream metabolites of ethanol were involved in the potentiation of subgenomic HCV RNA replication by ethanol. Our data are in agreement with this study and suggest that ethanol and acetaldehyde also directly enhance HCV replication in the context of the complete viral replication cycle. In terms of the mechanism, we found that isopropyl alcohol, acetone, and acetate also increase HCV replication, and increased NADH/NAD$^+$ ratio was required for the potentiation of HCV replication by etha-

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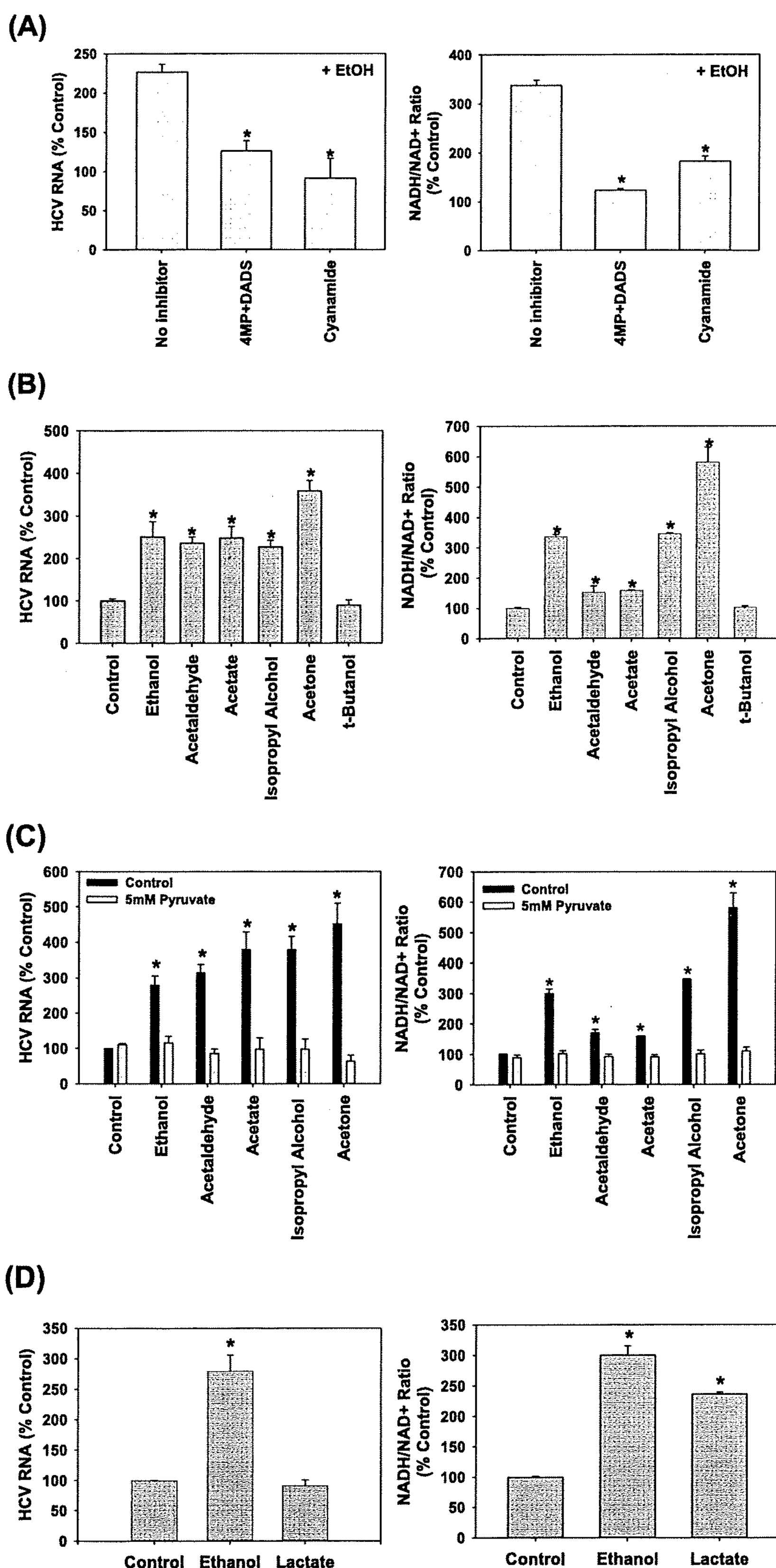
nol, acetaldehyde, as well as isopropyl alcohol, acetone, and acetate. In contrast, *t*-butanol, a tertiary alcohol that is poorly metabolized by humans and does not increase the NADH/NAD$^+$ ratio, did not elevate HCV replication, as predicted by our model (Fig. 6*B*). The NADH/NAD$^+$ ratio in ethanol-treated cells was decreased by cyanamide (Fig. 6*A*), suggesting that NADH is generated downstream of acetaldehyde (Fig. 3*A*). Acetate, the downstream metabolite of acetaldehyde, was previously considered inert but there is evidence that it can be converted to acetyl-CoA and other metabolic intermediates by mammalian cells (24, 28). Isopropyl alcohol is known to be metabolized into acetone and possibly other ketone bodies that can also be converted to acetyl-CoA (27). The mechanism by which isopropyl alcohol increases the NADH/NAD$^+$ ratio in our system is unclear and may involve residual ADH or hitherto uncharacterized enzyme activity that is induced by HCV.

In terms of how NADH increases HCV replication, NADH plays key roles in cellular bioenergetics and can modulate fatty acid synthesis as well as suppress β-oxidation (24, 28). We were interested in the potential involvement of lipids because HCV replicates in cholesterol-rich compartments in the cell, and cholesterol and fatty acid metabolism have been shown to be important for HCV replication (29). Specifically, cholesterol metabolism increases basal HCV replication by the geranylgeranylation of FBL2 (29). We found that inhibiting the host mevalonate pathway with statins and fatty acid synthesis with TOFA or cerulenin blunted the potentiation of HCV replication by ethanol, acetaldehyde, isopropyl alcohol, acetone, and acetate, whereas inhibiting β-oxidation dramatically increased HCV replication (Fig. 7). In addition, the potentiation of HCV replication by these agents was accompanied by an increase in the intracellular cholesterol content, which was attenuated by lova-



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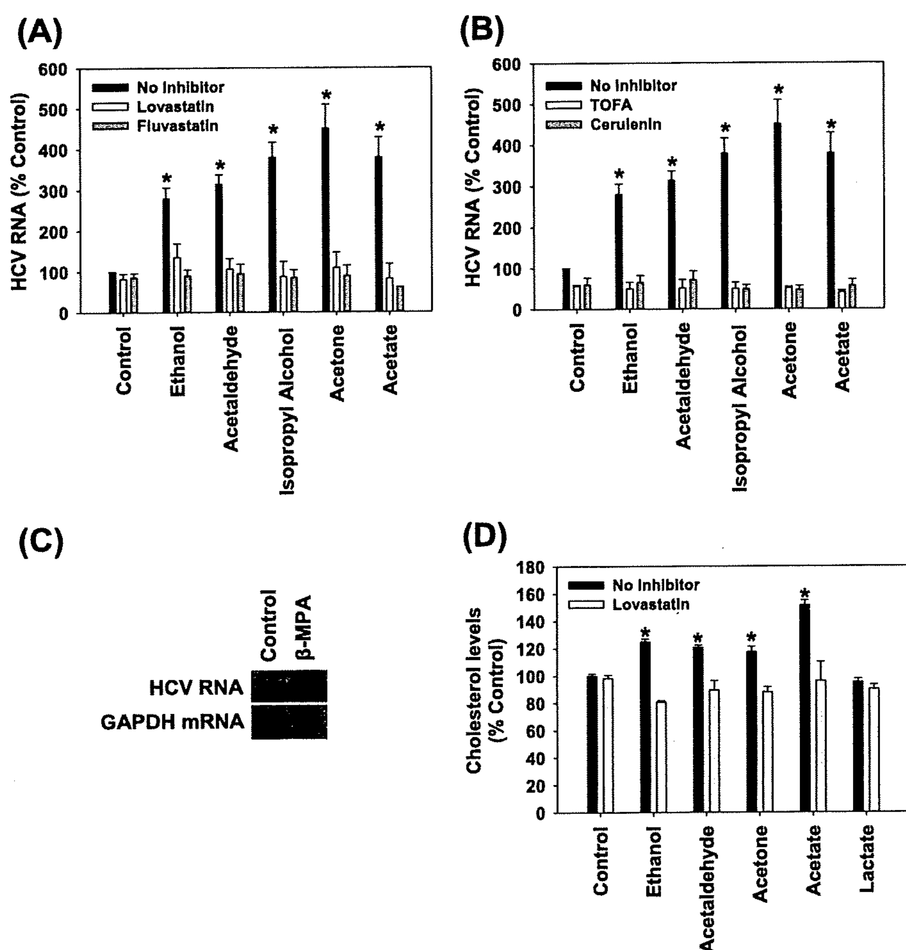

FIGURE 7. **Role of lipogenesis in the enhancement of HCV replication by ethanol, acetaldehyde, isopropyl alcohol, acetone, and acetate.** SgPC2 cells were treated for 24 h with (*A* and *B*) 0.2% ethanol, 5 μM acetaldehyde, 0.2% isopropyl alcohol, 2 mM acetone, 5 μM acetate ± 30 min pretreatment with (*A*) 5 μM lovastatin, 5 μM fluvastatin, (*B*) 5 μg/ml TOFA, 5 μg/ml cerulenin, or with (*C*) 2 mM β-mercaptopropionic acid (β-MPA). Then, HCV RNA levels were monitored by Northern blot and quantified by densitometry ($n = 3$). *D*, SgPC2 cells, treated for 24 h with ethanol, acetaldehyde, acetone, and acetate ± lovastatin, were monitored for cholesterol levels ($n = 3$). Lovastatin was activated, as described, before use (29). *, indicates statistically significant difference for indicated sample sizes ($p < 0.05$).

statin (Fig. 7*D*). Regarding potential effects of NADH on the ATP, overall ATP levels were not significantly perturbed in these cells by ethanol or other treatments (data not shown), suggesting that ATP is not likely to explain the effects that ethanol had on HCV. In fact, ethanol also increased the rate of HCV replication in the *in vitro* replication assay (Fig. 2*C* and 2*D*) which was performed in the presence of excess ATP. Taken together, these data indicate that the potentiation of HCV replication by ethanol, acetaldehyde, acetate, isopropyl alcohol, and acetone ultimately requires host lipid metabolism and is sensitive to lipid modulators, which points to potential targets for therapy. The concentrations of lovastatin and fluvastatin used here are higher than the doses used clinically to treat hypercholesterolemia. However, it is possible that statins, if used in combination with antivirals or other lipid modulators, will help control HCV replication, particularly in chronic alcoholics who show resistance to standard anti-HCV therapy (35). It is also interesting to note that the concentrations of acetone that enhanced HCV replication in this study are physiological levels that can be attained during metabolic dysfunction such as diabetes and during starvation (27), and HCV infection can lead to insulin resistance (36). In addition, acetate, which increased HCV replication at μM to mM concentrations in this study (Fig. 6*B* and data not shown), is used in hemodialysis.

Interestingly, increasing the NADH/NAD$^+$ ratio with lactate was not sufficient to increase HCV replication, suggesting that other factors may also play a role (Fig. 7*D*). Lactate also did not increase the intracellular cholesterol level. These results are consistent with an important role of cholesterol in the regulation of HCV replication. The data also indicate that even though ethanol and lactate both increase the NADH/NAD$^+$ ratio, ethanol is more lipogenic than lactate in these cells. The reason for these differences is unclear but it might be explained at least in part by the fact that ethanol can inhibit citric acid cycle as well as gluconeogenesis, which may cause acetate/acetyl-CoA produced by ethanol metabolism to be shunted more toward the lipogenic pathways, whereas these processes are likely to be stimulated by lactate (37). Ethanol can also decrease the total oxidation of fatty acids to CO_2, and increase the breakdown of glycogen, which may further drive lipogenesis in these cells (37–39). Further investigation into these effects will be beneficial to understanding how different metabolic conditions would affect HCV replication in hepatocytes.

Recently, McCartney *et al.* (7) reported an elevation of HCV RNA by ethanol in Huh7 replicon cells, transfected with

FIGURE 6. **Role of NADH/NAD$^+$ in the potentiation of HCV replication by ethanol, acetaldehyde, acetate, isopropyl alcohol, and acetone.** SgPC2 cells, supporting Con1 subgenomic HCV RNA replication, were treated with (*A*) 0.2% ethanol ± 0.1 mM 4MP plus 25 μM DADS or 0.1 mM cyanamide ($n = 3$); (*B*) 0.2% ethanol, 5 μM acetaldehyde, 5 μM acetate, 0.2% isopropyl alcohol, 2 mM acetone, or 25 mM *tert*-butanol ($n = 4$); (*C*) 0.2% ethanol, 5 μM acetaldehyde, 5 μM acetate, 0.2% isopropyl alcohol, and 2 mM acetone, with and without 5 mM pyruvate ($n = 3$); or (*D*) 0.2% ethanol or 5 mM lactate for 3 h for NADH/NAD$^+$ ratio measurement or 24 h for HCV RNA levels. HCV RNA levels were monitored by Northern blot (*A–D, left panels*). NADH/NAD$^+$ ratios were measured by an enzymatic NADH recycling assay, as described under "Experimental Procedures" (*A–D, right panels*). Northern blots were quantified by densitometry. *, indicates statistically significant difference for indicated sample sizes ($p < 0.05$).

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CYP2E1; the effect could be suppressed by NAC, leading to the conclusion that the increase was due to ROS generation by CYP2E1. In contrast, we have consistently found that ROS suppresses HCV replication whereas antioxidants tend to counter this suppression (10–14) (Fig. 4). In particular, our BSO studies clearly demonstrate that endogenous ROS are sufficient to suppress HCV replication in cell culture (10, 11). Also, NAC and vitamin E either enhanced or had no significant effect on the potentiation of HCV replication by ethanol (Fig. 4*G*) as well as acetaldehyde, isopropyl alcohol, acetone, and acetate (data not shown). The reason for this discrepancy is unclear. However, CYP2E1 generates acetaldehyde as well as ROS, both of which can react with thiols, such as cysteine and GSH, which are generated from NAC, and the study by McCartney *et al.* did not differentiate whether the potentiation of HCV replication by ethanol was due to ROS, acetaldehyde, or other variables (7, 28). NAC can also have other effects on cells, including alteration of the pH and acting as a pro-oxidant, and careful monitoring of the pH and comparison with other antioxidants and pro-oxidants, therefore, are necessary. Indeed, our findings have been recently corroborated by other studies that show that HCV RNA replication is enhanced by antioxidants (*e.g.* vitamins E and C) and suppressed by lipid peroxidation products and ROS (12–14, 40). The mechanism by which ROS suppresses HCV replication is still not completely clear but it is likely to involve calcium and the dissociation of HCV replication complex from the membranes (10, 11). Detailed understanding of the mechanism by which ROS suppresses HCV replication and how acetaldehyde, NADH, acetyl-CoA, and ROS affect HCV *in vivo* will require additional *in vitro* and animal studies.

Therefore, we show that physiological levels of ethanol, acetaldehyde, and acetone promote HCV replication in the context of the complete HCV replication, and that the response is likely mediated by the modulation of host lipid metabolism requiring elevated $NADH/NAD^+$. Further study into the precise mechanisms of this regulation may lead to the development of novel treatments that target both the virus and its pathogenic interactions with ethanol in chronic hepatitis C patients.

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ORIGINAL ARTICLE

HCV genotype 1b chimeric replicon with NS5B of JFH-1 exhibited resistance to cyclosporine A

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Abstract Cyclosporine A (CsA) is a well-characterized anti-HCV reagent. Recently it was reported that the genotype 2a JFH-1 strain was more resistant than genotype 1 HCV strains to CsA in a cell culture system. However, the JFH-1 responsible region for the resistance to CsA remains unclear. It was also demonstrated that in genotype 1b HCVs, NS5B interacts with cyclophilin (CyP). To clarify whether or not NS5B of JFH-1 is significant for CsA resistance, we developed a chimeric replicon with NS5B of JFH-1 in the genotype 1b backbone. The chimeric replicon was more resistant to CsA than the parental genotype 1b replicon. Furthermore, reduction of CyPA had a greater effect on HCV RNA replication and sensitivity to CsA than reduction of CyPB. Here, we demonstrated that NS5B of JFH-1 contributed to this strain's CsA-resistant phenotype. NS5B and CyPA are significant for determining HCV's sensitivity to CsA.

Introduction

The combination of a pegylated interferon (IFN) with ribavirin (RBV) is the current standard therapy for chronic hepatitis C and yields a sustained virological response (SVR) rate of about 55% [6]. This means that about 45% of patients with chronic hepatitis C are still threatened by the progress of the disease to cirrhosis and hepatocellular carcinoma. To find a more effective therapy, several anti-HCV reagents have been reported using HCV replicon systems [11, 14]. Especially, cyclosporine A (CsA), which is widely used as an immunosuppressive reagent, and its derivatives, which lack immunosuppressive activity, possess anti-HCV activity [8, 18, 19]. These reagents will help to improve the SVR rate.

Cyclophilins (CyPs), CsA ligands, are a family of cellular enzymes possessing peptidyl-prolyl isomerase activity. CyP family members play significant roles in numerous cellular processes, including transcriptional regulation, immune response, protein secretion and mitochondrial function [7]. CsA possesses three major cellular targets: CyP, the calcineurin-nuclear factor of activated T-cells pathway and P-glycoprotein [7]. The mechanism of anti-HCV activity of CsA is through disassociation between CyP and HCV nonstructural protein 5B (NS5B), an RNA-dependent RNA polymerase [20]. Fernandes et al. [5] also reported that NS5A was significant in the sensitivity of HCV to CsA. However, the role of CyPs as a cellular target of CsA in HCV RNA replication remains controversial [17, 20, 21]. While genotype 1a and 1b HCV strains were highly sensitive to CsA, a genotype 2a strain, JFH-1, was less sensitive to CsA [12, 21]. Moreover, in genotype 1b HCV, interaction between CyPB and HCV NS5B is required for robust HCV RNA replication [10].

To investigate whether or not NS5B of JFH-1 is an important factor for determining sensitivity to CsA, we engineered a 1b/2a chimeric HCV subgenomic replicon derived from genotype 1b HCV-O RNA, in which NS5B and a 3′-untranslated region (UTR) were replaced with

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those of HCV JFH-1 RNA. This replicon system enables to investigate the direct effect of NS5B on the CyPs.

We report here that NS5B of JFH-1 contributes to the CsA-resistant phenotype of this strain. Furthermore, CyPA but not CyPB is essential for HCV RNA replication in 1b and 1b/2a chimeric replicon-harboring cells. Finally, supplementation with vitamin E (VE) negates the anti-HCV activity of CsA in the presence or absence of CyPs. These results contribute to our understanding of the mechanism(s) that mediate the efficacy of CsA's anti-HCV activity.

Materials and methods

Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum. The HuH-7-derived OR6c cells were cultured as previously described [10]. The cells harboring the subgenomic replicon were maintained in the culture medium containing G418 (0.3 mg/ml; Promega, Madison, WI).

Reagents

IFN-α, IFN-γ, and VE were purchased from Sigma-Aldrich (St. Louis, MO). CsA was purchased from Calbiochem (San Diego, CA). Pitavastatin (PTV) was purchased from KOWA Co., Ltd. (Tokyo, Japan). Mizoribine (MZB) and RBV were kindly provided by Asahi Kasei Pharma (Tokyo, Japan) and Yamasa Corporation (Choshi, Japan), respectively.

Plasmid construction

The plasmid of pORN/3-5B/QR,KE,RS/5B(J) was based on pORN/3-5B/QR,KE,RS [1] and was constructed by replacing the NS5B coding region and 3′UTR with the corresponding JFH-1 sequence. The NS5A/NS5B junction was set after amino acid 2419 of HCV-O and generated by polymerase chain reaction (PCR). The sequence numbering for coding and non-coding regions was based on a sequence from GenBank: HCV-O (accession no. **AB191333**) and JFH-1 (accession no. **AB047639**). Retroviral vector pCX4bsr [2] was used as an expression vector. To obtain full-length CyPA and CyPB cDNAs, reverse transcription (RT)-PCR with KOD-plus DNA polymerase (Toyobo, Osaka, Japan) was performed as previously described [4]. The pCX4bsr/Myc-CyPA and pCX4bsr/Myc-CyPB plasmids expressing Myc-tagged CyPA and CyPB, respectively, were obtained by inserting the PCR products of full-length CyPA and CyPB into the MluI-NotI sites of the pCX4bsr/Myc vector. Expression plasmids for HA-tagged NS5B (HCV-O and JFH-1) were generated by insertion of PCR fragments encoding each HCV protein into the MluI-NotI sites of the pCX4bsr/HA vector. The sequences of all constructed plasmids were confirmed by the sequencing analysis as described previously [1].

RNA synthesis, RNA transfection, and selection of G418 cells

Plasmid DNAs were linearized by XbaI and used for the RNA synthesis with the T7 MEGAScript kit (Ambion, Austin, TX). In vitro transcribed RNA was transfected into OR6c cells by electroporation [9]. The transfected cells were selected in culture medium containing G418 (0.3 mg/ml) for 3 weeks.

RNA interference, lentiviral vector construction

A detailed description of methods of RNA interference and lentiviral vector construction is available in Supplementary Materials.

Western blot analysis

Western blot analysis was performed as described previously [1]. The antibodies used in this study were those against NS3 (Novocatra Laboratories, UK), NS5A (a generous gift from Dr. A Takamizawa, Research Foundation for Microbial Diseases, Osaka University), NS5B (1b, a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), NS5B (1b/2a) [15], CyPA (BIOMOL, Plymouth Meeting, PA), CyPB (Affinity BioReagents, Rockford, IL), and β-actin (AC-15; Sigma).

Immunoprecipitation

Immunoprecipitation was performed as described previously [10]. Briefly, pre-cleared cell lysates were incubated with an anti-Myc antibody (PL14; MBL, Nagoya, Japan). Immunocomplexes were recovered by adsorption to protein G-Sepharose resin (GE Healthcare Bioscience, Uppsala, Sweden). After three washes with lysis buffer, the immunoprecipitates were analyzed by immunoblot analysis using anti-Myc and anti-HA (3F10; Roche, Mannheim, Germany) antibodies.

Evaluation of sensitivity of anti-HCV reagents

The cells were plated onto 24-well plates (1.5–2 $\times$ 10^4 cells/well). After 24 h culture, the culture medium was replaced with anti-HCV reagent containing medium. After 72 h additional culture, the cells were washed with

phosphate-buffered saline once, harvested with Renilla lysis reagents (Promega), and subjected to Renilla luciferase (RL) assay according to the manufacturer's protocol.

Statistical analysis

The luciferase activities were statistically compared between the various treatment groups using Student's t test. P values of less than 0.05 were considered statistically significant.

Results

The 1b/2a chimeric replicon is less sensitive to CsA than the 1b replicon

To investigate the mechanism(s) underlying CsA's anti-HCV activity, we engineered a 1b/2a chimeric HCV subgenomic replicon derived from genotype 1b HCV-O RNA, in which the NS5B and 3′UTR regions were replaced with those of HCV JFH-1 RNA (Fig. 1a). These RNAs were transfected into OR6c cells. After 3 weeks' selection of G418, we successfully obtained a 1b replicon or 1b/2a chimeric replicon-harboring cells as polyclones (see Supplementary Material). The colony forming efficiencies of the 1b replicon and the 1b/2a chimeric replicon were 5150 ± 361 and 62 ± 10 colonies/μg RNA, respectively. Sequence analysis of HCV RNA in 1b replicon or 1b/2a chimeric replicon-harboring cells revealed that there was no conserved amino acid substitution (data not shown). The RT-quantitative PCR analysis revealed that intracellular HCV RNA copies were $3.8 \pm 0.1 \times 10^7$ and $1.1 \pm 0.1 \times 10^7$ copies/μg total RNA in 1b replicon and 1b/2a chimeric replicon-harboring cells, respectively.

Next, we examined the sensitivity of the 1b replicon and 1b/2a chimeric replicon to anti-HCV reagents (Fig. 1b). HCV RNA replication was monitored through reporter

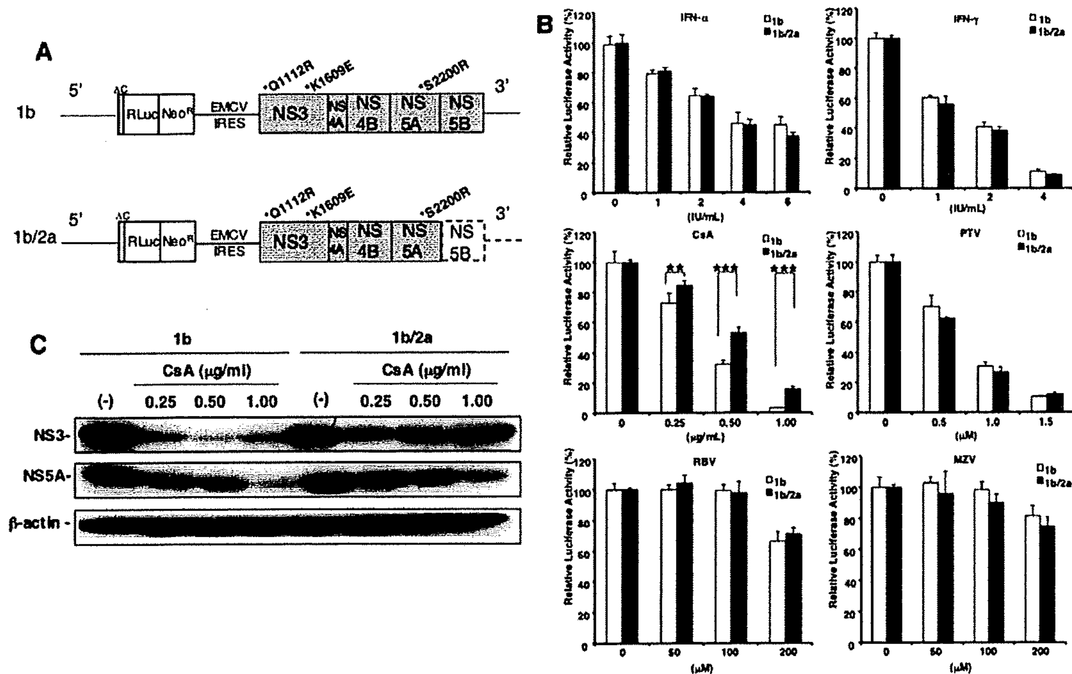

Fig. 1 1b/2a chimeric replicon-harboring cells are less sensitive to CsA. **a** Gene organization of subgenomic RNA. RLuc and DC indicate the RL gene and the 12 N-terminal amino acid residues of the core protein as a part of internal ribosomal entry site (IRES), respectively. The positions of adaptive mutations are indicated by *asterisks*. *Shaded boxes*, *dotted open boxes*, *thin lines*, *dotted lines*, *sick lines*, and *open boxes* indicate open reading frame (ORF) derived from HCV-O strain, ORF derived from JFH-1 strain, UTR of HCV-O strain, UTR of JFH-1 strain, encephalomyocarditis virus IRES, and fusion protein RL with neomycin phosphotransferase (Neo^R), respectively. **b** Effects of various anti-HCV reagents on HCV RNA replication in the 1b replicon (*open columns*) and in the 1b/2a chimeric replicon (*closed columns*) harboring cells. The cells were treated with IFN-α, IFN-γ, CsA, PTV, RBV, and MZB, respectively. After 72 h of treatment, the RL assay was performed according to the manufacturer's protocol. The relative luciferase activity is calculated when the RL activity of untreated cells was assigned as 100% (** $P < 0.01$; *** $P < 0.001$). **c** Western blot analysis of HCV proteins. The 1b replicon or 1b/2a chimeric replicon-harboring cells were treated with CsA for 72 h. After treatment, the cell lysates were subjected to Western blot analysis. The production of NS3 and NS5A was analyzed using anti-NS3 and anti-NS5A antibodies, respectively. β-Actin was used as a control for the amount of protein loaded per lane

activity encoded by replicon RNAs in stable cell lines harboring these autonomously-replicating RNAs. The results revealed that the 1b/2a chimeric replicon was less sensitive to CsA than the 1b replicon. However, there were no differences in sensitivity to other anti-HCV reagents (IFN-α, IFN-γ, PTV, RBV, and MZB) between the 1b replicon and 1b/2a chimeric replicon (Fig. 1b). We also tested the expression levels of HCV proteins (NS3 and NS5A) in CsA-treated replicon-harboring cells (Fig. 1c). CsA decreased HCV protein expression levels in the 1b eplicon-harboring cells in a dose-dependent manner. On the other hand, in the 1b/2a chimeric replicon-harboring cells those levels were not changed at the higher concentration of CsA treatment. These results suggest that NS5B of JFH-1 decreased the sensitivity to CsA in 1b/2a chimeric replicon-harboring cells.

CyPA is essential for HCV RNA replication

It has been reported that CyPs are responsible for CsA's anti-HCV activity [17, 20, 21]. Therefore, we next examined the role of CyPs in HCV RNA replication and the anti-HCV activity of CsA using short-hairpin RNA (shRNA) against CyPA or CyPB. The silencing of CyPA or CyPB by shRNA was confirmed by Western blot analysis (Fig. 2a). The silencing of CyPA significantly suppressed HCV protein expression in 1b replicon-harboring cells and in 1b/2a chimeric replicon-harboring cells. The silencing of CyPB didn't suppress HCV protein expression in the former replicon-harboring cells and slightly suppressed in the latter replicon-harboring cells. We also demonstrated that the HCV RNA levels in these cells correlated with the results of Western blot analysis (data not shown). These results suggest that CyPA is essential for replication of both 1b and 1b/2a chimeric replicon. On the other hand, CyPB might partially affect HCV RNA replication of the 1b/2a chimeric replicon.

We next evaluated the sensitivity to anti-HCV reagents (CsA and IFN-α) between control cells and CyPs-knockdown cells. The results revealed that the sensitivity to CsA was drastically enhanced in both CyPA-knockdown 1b replicon-harboring cells and 1b/2a replicon-harboring cells (Fig. 2b), while the sensitivity to IFN-α was not dramatically changed in replicon-harboring either cells (see Supplementary Material). The silencing of CyPB slightly improved the sensitivity to CsA in the 1b/2a chimeric

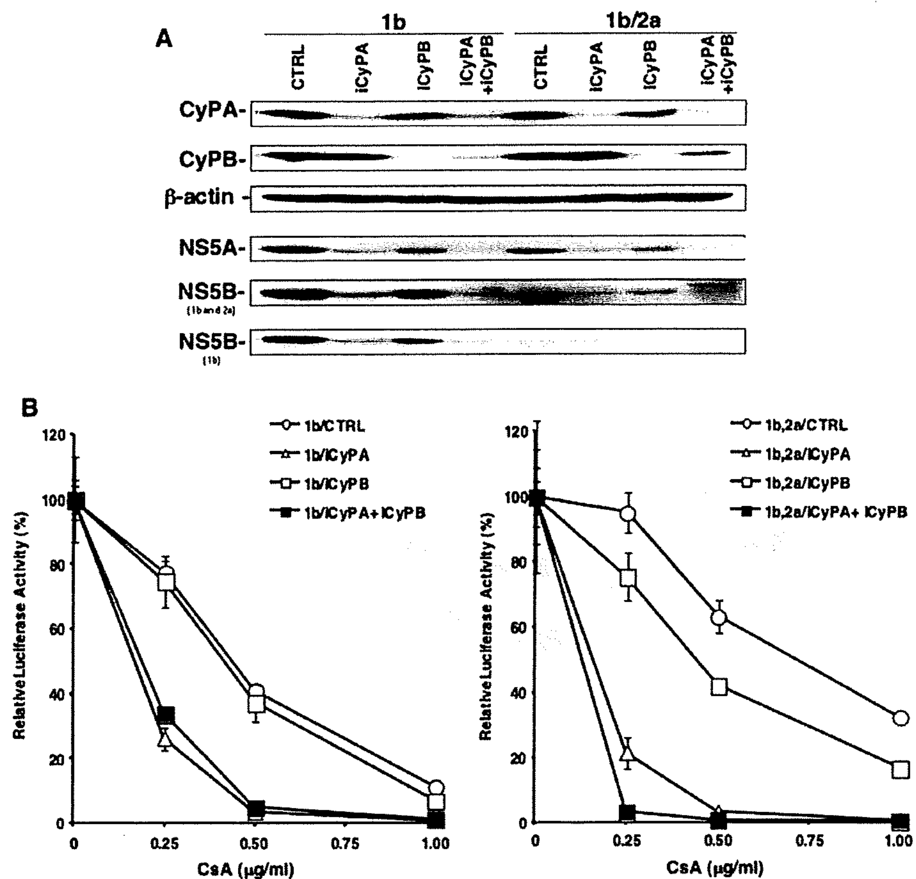

Fig. 2 CyPA is essential for HCV RNA replication and modulates the anti-HCV activity of CsA. **a** Western blot analysis of HCV proteins and CyPs. The 1b replicon and 1b/2a chimeric replicon-harboring cells were transduced with the indicated shRNA for 1 week. The cell lysates were subjected to Western blot analysis. CTRL indicates the control cells transfected with the empty vector. **b** Effects of CyPs on CsA's anti-HCV activity in the 1b replicon- (*left panel*) and 1b/2a chimeric replicon- (*right panel*) harboring cells. After 72 h treatment with CsA, the RL assay was performed. The relative luciferase activity was calculated as described in Fig. 1b. The cells transfected with CTRL, CyPA, CyPB, and both CyPA and CyPB shRNA indicate *open circles*, *open triangles*, *open squares*, and *closed squares*, respectively

replicon-harboring cells but made no improvement in the 1b replicon-harboring cells. Moreover, the silencing of CyPA and CyPB additively enhanced the sensitivity to CsA in the 1b/2a chimeric replicon-harboring cells but not in the 1b replicon-harboring cells. These results suggest that the major cellular determining factor in HCV RNA replication is CyPA rather than CyPB in the 1b/2a chimeric replicon-harboring cells.

HCV NS5B interacts more strongly with CyPB than with CyPA

Although it has been reported that the interaction between CyPs and NS5B is important for HCV RNA replication using glutathione S-transferase pull-down assay [20, 21], the binding activity of NS5B to CyPs in physiological conditions remains unclear. To evaluate the interaction between CyPs and NS5B, we performed an immunoprecipitation assay using 293FT cells transfected with the expression vectors of CyPs (CyPA or CyPB) and NS5B (HCV-O or JFH-1 strain). The obtained results revealed that both NS5Bs interacted more strongly with CyPB than with CyPA. Furthermore, NS5B of HCV-O interacted more strongly with CyPs than did NS5B of JFH-1 (Fig. 3). Since CyPA expression is important for robust HCV RNA replication, these results suggest that the interaction between CyPA and NS5B might not be important for HCV RNA replication or for the anti-HCV activity of CsA.

VE completely negates CsA's anti-HCV activity in the presence or absence of CyPs

We previously reported that VE supplementation negated CsA's anti-HCV activity [22]. To rule out the possibility that VE negates CsA's anti-HCV activity in only CyP-expressing cells, we examined whether or not VE could negate CsA's anti-HCV activity in the presence or absence of CyPs (Fig. 4). Surprisingly, VE negated CsA's anti-HCV activity in the presence or absence of CyPs. It is noteworthy that VE negated this activity more efficiently in CyPA knockdown cells than in the control or CyPB knockdown cells.

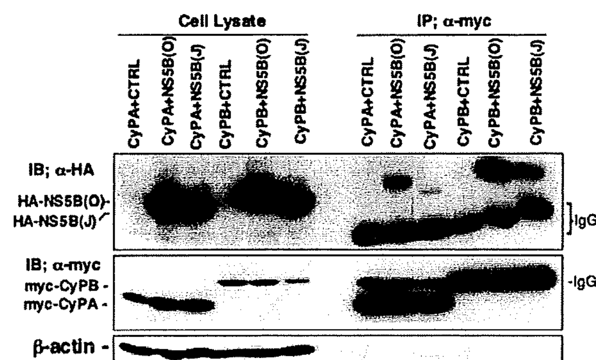

Fig. 3 CyPs interact with HCV NS5B. 293FT cells were cotransfected with plasmids expressing Myc-tagged CyP and HA-tagged HCV NS5B for 48 h. The cells were lysed and subjected to immunoprecipitation with monoclonal Myc-antibody, followed by immunoblot analysis with either anti-HA (*top*) or anti-Myc (*bottom*) antibodies. CTRL indicates empty vector

Discussion

Since it was first reported that CsA possesses anti-HCV activity, several groups have found that CsA suppresses HCV RNA replication using HCV replicon-harboring cells. In addition, the genotype 1a and 1b replicons possess high sensitivity to CsA [17, 19, 21], but the replicon of genotype 2a, JFH-1 strain, is less sensitive to CsA [12]. However, the mechanism of CsA resistance remains unclear.

Recently, Murayama et al. [16] reported that NS3 helicase and NS5B of JFH-1 were essential for robust replication using intragenotypic 2a replicon with J6 backbone. In contrast, Binder et al. [3] demonstrated that NS3 helicase from JFH-1 reduced replication efficiency of 1b/2a chimeric replicon with NS5B from JFH-1 in genotype 1b Con1 backbone. These results suggest that the effect of co-substitution of NS3 helicase with NS5B on HCV RNA replication is different between genotype 1b and 2a backbones.

In this study, we clearly demonstrated that the viral determining factor of sensitivity to CsA is NS5B, by using 1b/2a chimeric replicon-harboring cells. The homology of NS5B region between HCV-O and JFH-1 is 75% in amino acids. Fernandes et al. [5] reported amino acid change from serine to glycine at position 556 of NS5B in CsA resistant 1b replicon. Interestingly, amino acid at this position in HCV-O and JFH-1 are serine and glycine, respectively. The results indicate that the difference in amino acid sequences in NS5B between the HCV-O and JFH-1 strains contributes to the sensitivity to CsA.

Moreover, we further demonstrated that the major cellular determining factor for HCV RNA replication is CyPA rather than CyPB. CyPB is partially involved in only 1b/2a chimeric replicon RNA replication. These results suggest that decreased endogenous expression of CyPA by shRNA contributes to suppression of HCV RNA replication. Furthermore, the knockdown of CyPA enhances CsA's anti-HCV activity. Since the silencing of CyPB slightly enhanced the sensitivity to CsA in only the 1b/2a chimeric replicon-harboring cells, the expression level of CyPB might contribute to the suppression of HCV RNA replication in the case of genotype 2a, JFH-1 strain.

It has been reported that CyPB binds to NS5B and regulates its activity [20]. We also demonstrated that NS5B

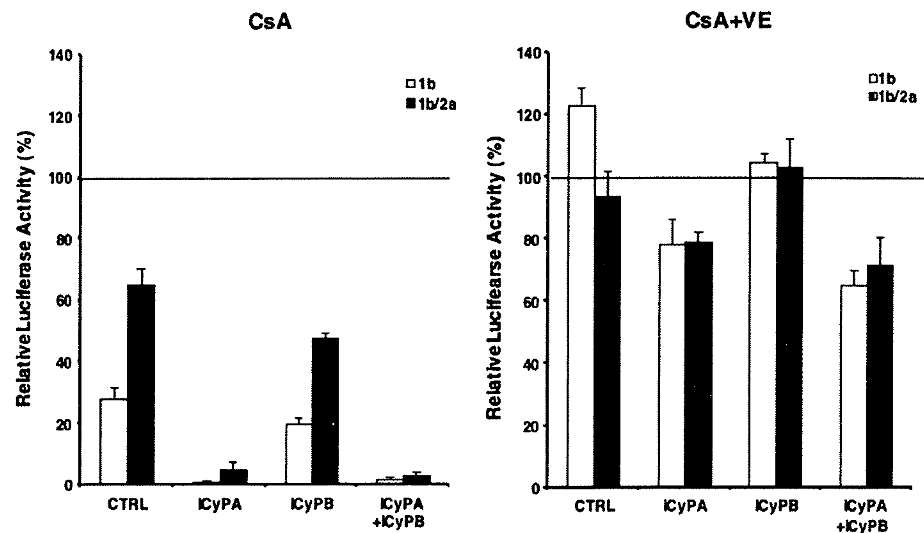

Fig. 4 The effect of CsA (0.5 μg/ml) in combination with VE (10 μM) on HCV RNA replication in the 1b replicon- (*open columns*) and the 1b/2a chimeric replicon- (*closed columns*) harboring cells. After 72 h treatment, the RL assay was performed according to the manufacturer's protocol. The relative luciferase activity was calculated as described in Fig. 1b

bound to CyPB. However, our results revealed that NS5B more strongly interacted with CyPB than with CyPA. The difference in binding activity between CyPA and CyPB may be caused by subcellular localization. It has been reported that CyPA and CyPB are localized in cytoplasm and endoplasmic reticulum (ER), respectively [7]. On the other hand, NS5B localizes with ER membranes [13]. Our data, showing that NS5B interacted more strongly with CyPB than with CyPA, might be attributable to the difference in subcellular localization between cytoplasm and ER. We also demonstrated that NS5B of HCV-O interacted more strongly with CyPs than did NS5B of JFH-1. Moreover, the expression of CyPA plays a major role in robust HCV RNA replication. On the other hand, CyPB has little impact on HCV RNA replication. Taken together, these results suggest that the interaction between CyPA and NS5B might partially affect HCV RNA replication and the anti-HCV activity of CsA.

It is noteworthy that VE can negate CsA's anti-HCV activity in the presence or absence of CyPs. We also examined whether or not the combination treatment of CsA and other antioxidants (vitamin C, sodium selenate, and coenzyme Q10) could negate CsA's anti-HCV activity. Among these antioxidants, only VE negated CsA's anti-HCV activity (data not shown). Understanding VE's involvement in CsA's anti-HCV activity may help us identify factors other than the interaction between CyPA and NS5B.

CsA derivatives that affect only CyPA and that also lack immunosuppressive function will have advantages over CsA. A combination therapy of CsA or CsA derivatives with VE should be avoided so as not to affect CsA's anti-HCV activity clinically. In conclusion, we have demonstrated that NS5B of JFH-1 contributed to the CsA-resistant phenotype of this strain using 1b/2a chimeric replicon-harboring cells and CyPA is a major cellular determining factor in HCV RNA replication.

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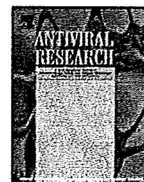

Identification of bisindolylmaleimides and indolocarbazoles as inhibitors of HCV replication by tube-capture-RT-PCR

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ABSTRACT

We devised a screening method for hepatitis C virus (HCV) inhibitors by exploiting the JFH1 viral culture system. The viral RNA released in the medium was adsorbed onto PCR plates, and real-time RT-PCR was performed by directly adding the one-step RT-PCR reaction mixture to the wells. The "tube-capture-RT-PCR" method obviates the need for labor-intensive RNA isolation and should allow high-throughput screening of HCV inhibitors. To substantiate the validity of the assay for drug screening, a pilot screen of an inhibitor library composed of 95 compounds was performed. In addition to the known inhibitors of HCV replication included in the library, the assay identified the PKC inhibitor bisindolylmaleimide I (BIM I) as an HCV replication inhibitor. BIM I was also effective in reducing the viral protein level in genotype 1b and 2a subgenomic replicon cells, indicating inhibition of HCV replication. Further assays revealed that a broad range of bisindolylmaleimides and indolocarbazoles inhibit HCV, but no correlation was found between the PKC inhibition pattern and anti-HCV activity. These series of compounds represent new classes of inhibitors that may warrant further development.

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1. Introduction

Hepatitis C virus, a major cause of chronic liver disease, has infected over 170 million people. The current mainstream anti-HCV therapy is a combination of interferon (IFN) and ribavirin. However, the therapy is not effective in approximately half of HCV-infected patients and has considerable side effects in many patients; thus, there is an urgent need for novel HCV therapies.

Various assays for HCV drug screening have been reported, many of which rely on HCV replicon systems. Although HCV replicon-based systems have greatly facilitated HCV research and drug discovery, these systems do not completely reflect the entire HCV life cycle and are not capable of identifying inhibitors of several important steps such as viral attachment, entry, and release. The recently introduced HCV cell culture systems (Wakita et al., 2005) should overcome these limitations and enable identification of inhibitors that would not be recognized by the replicon-based screens.

Here we describe a simple screening method for discovering anti-HCV drugs using the JFH1 viral culture system. Antiviral activity was determined by RT-PCR measurement of viral RNA released in the medium of infected cells. To increase efficiency, we devised a method that avoids tedious RNA isolation.

As a proof of concept, the method was used to evaluate a compound library and successfully confirmed the anti-HCV activity of cyclosporin A. In addition, a potent and selective PKC inhibitor, BIM I, was also identified as an anti-HCV agent. We found that other bisindolylmaleimides and indolocarbazoles also inhibit HCV, whereas anti-HCV activity was not associated with PKC inhibition. HCV inhibition by bisindolylmaleimides or indolocarbazoles has not been reported, and we expect that our assay will facilitate the identification of previously unrecognized HCV inhibitors. The bisindolylmaleimides and indolocarbazoles are already in clinical trials and may merit attention as HCV drug candidates.

2. Materials and methods

2.1. Cells and virus

Plasmid pJFH1, containing full-length cDNA of the JFH1 isolate, was used to generate HCV production in cell culture, as described elsewhere (Wakita et al., 2005), and the supernatant was passaged in Huh 7.5.1 cells. To prepare virus stock for screening, naïve Huh 7.5.1 cells were infected with the passaged supernatant virus, and the medium was collected 7 days post-infection and stored at

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−80 °C until use. The infectious titers of the viruses were determined by immunofluorescence analysis of the infected Huh 7.5.1 cells using anti-core antibody (2H9). The infectious titers of the stocks were generally about 3×10^5–1×10^6 ffu/ml, corresponding to about 3×10^7–1×10^8 copies of JFH1 RNA/ml. A subgenomic replicon cell, clone 4-1, which harbors the genotype 2a HCV genome (Kato et al., 2003; Date et al., 2004) and clone 5-15, which harbors the genotype 1b HCV genome (Lohmann et al., 1999), were also cultured in Dulbecco's Modified Eagle's medium (DMEM) with fetal bovine serum (FBS).

2.2. Reagents

The SCADS inhibitor kit I was provided by the Screening Committee of Anticancer Drugs supported by a Grant-in-Aid for Scientific Research on the Priority Area "Cancer" from The Ministry of Education, Culture, Sports, Science and Technology, Japan. The PKC β isozyme selective inhibitor LY333531 (Ruboxistaurin) was from Alexis Corp. (Lausen, Switzerland). Other chemicals were purchased from Merck Calbiochem (Darmstadt, Germany). Interferon-α (IFN-α) was from PeproTech, Inc. (Princeton Business Park, Princeton, NJ).

2.3. Quantitative real-time RT-PCR

Huh 7.5.1. cells were seeded in 96-well plates at a density of 20,000 cells per well in a volume of 120 μl. The next day, 15 μl of test compounds was added and the cells were infected with 15 μl of virus stock of HCV-JFH1 at a multiplicity of infection (MOI) of 0.01. After 5 days of culture, 100 μl of medium was transferred to a PCR plate, incubated on ice for 30 min, centrifuged at 3500 rpm for 15 min, and then removed. Twenty microliters of One Step SYBR PrimeScript RT-PCR Kit reaction mixture (Takara-Bio Co., Otsu, Japan) was added into the PCR plate wells, and quantitative real-time PCR was performed using an ABI Prism 7000 sequence detector (PE Applied Biosystems, Foster City, CA). The primers used were 5′-GAGTGTCGTACAGCCTCCAG-3′ (nucleotides 97–116), and 5′-AGGCCTTTCGCAACCCA-3′ (nucleotides 280–264) from the non-coding region of HCV-JFH1, at a concentration of 200 nM. Media from the control wells without drug were serially diluted to create a standard curve, which was used to determine the relative amount of HCV RNA in the media of HCV-infected cells treated with the compounds. Cell growth was monitored by MTT assay, as described previously (Fukazawa et al., 1995).

For further analysis of the drug effect and determination of the copy number of HCV RNA in medium and cells, HCV RNA was extracted from 140 μl medium with the QIAamp Viral RNA mini kit (QIAGEN GmbH, Hilden, Germany), and eluted with 60 μl of elution buffer. Eight microliters of the viral RNA eluate was subjected to quantitative real-time PCR using Taqman EZ RT-PCR Core reagents (PE Applied Biosystems). The primers were 5′-CGGGAGAGCCATAGTGG (nucleotides 129–145) and 5′-AGTACCACAAGGCCTTTCG (nucleotides 289–271) at a concentration of 200 nM, and the Taqman probe was FAM-5′-CTGCGGAACCGGTGAGTACAC-3′-TAMRA (nucleotides 147–167) at a concentration of 300 nM (Takeuchi et al., 1999). Standard JFH1 RNA for measurement of copy number was transcribed from plasmid pSRG-JFH1-Luci, which was derived from pSRG-JFH1 (Kato et al., 2003), using the AmpliScribe T7 High Yield Transcription Kit (Epicentre Biotechnologies, Madison, WI). The transcribed RNA was purified and diluted with ribonuclease-free water containing yeast tRNA and 0.2% DTT, as previously described (Suzuki et al., 2005).

2.4. Western blotting

Cells were lysed with Radio-ImmunoPrecipitation Assay (RIPA) buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 1 mM EDTA) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 25 μg/ml of each of antipain, pepstatin, and leupeptin, and centrifuged. The amount of protein in the supernatant was then measured. Cell lysates containing equal amounts of protein were separated by SDS-PAGE, transferred onto polyvinylidene difluoride (PVDF) membranes, and probed with antibodies against core (2H9), NS5A (Austral Biologicals, San Raman, CA), α-tubulin (Merck Calbiochem), and GAPDH (Santa Cruz Biotech. Inc., Santa Cruz, CA). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies and specific proteins were visualized by chemiluminescence.

3. Results

3.1. Assay development

To establish an efficient RT-PCR-based screen for anti-HCV agents, we searched for methods that could be carried out without labor-intensive RNA isolation. We tested whether tube-trapping methods used to obtain plant viral RNAs for RT-PCR (Rowhani et al., 1995; James, 1999; Suehiro et al., 2005) could be applied to HCV. A JFH1 stock solution (3×10^5 ffu/ml, 3×10^7 copies/ml) was serially diluted fourfold, put into the wells of the PCR plate, incubated on ice for 30 min, and then centrifuged at 3500 rpm for 15 min. The supernatant was removed and quantitative RT-PCR was performed by direct addition of the one-step RT-PCR reaction mixture. We found that HCV, like plant viruses, are adsorbed onto the well wall during incubation. As shown in Fig. 1a, RNA adsorption appeared to be linear over a broad range of viral concentrations. HCV RNA could still be detected after seven fourfold dilutions, indicating that the "tube-capture" is a quantitative method that can detect less than 200 copies of HCV RNA. We compared the CT values from this "tube-capture method" with those from the conventional method of RNA extraction. The efficiency of RNA recovery by "tube-capture" was calculated to be about 9% of the conventional method. However, as shown in Fig. 1a, there was a close correlation between the CT values obtained from the two methods ($R = 0.988$), demonstrating the usefulness of this method.

3.2. Identification of BIM I as an inhibitor of HCV infection

To explore the possibility of tube-capture-RT-PCR as a simple screen for discovering anti-HCV compounds, we first tested whether the method would detect the antiviral activity of IFN-α. Huh7.5.1 cells were seeded in a 96-well plate and infected with HCV-JFH1 at an MOI of 0.01. After 5 days, HCV RNA released in the medium was assayed by the tube-capture method. Under these conditions, the CT from the control medium was usually about 18–20. As shown in Fig. 1b, a substantial reduction in the amount of HCV RNA was observed when the cells were infected in the presence of IFN-α.

For further validation of the ability of the assay to identify HCV inhibitors, we performed a pilot screen using an inhibitor kit provided by the Screening Committee of Anticancer Drugs (SCADS inhibitor kit I). This kit contains 95 inhibitors including cyclosporin A, a compound reported to inhibit HCV replication.

Cyclosporin A was identified (Fig. 1c), providing a proof of concept for screening for anti-HCV drug candidates. In addition, our assay also identified the PKC inhibitor bisindolylmaleimide I (BIM I) (Fig. 2a, black columns). The IC_{50} was about 0.1 μM, which is comparable to that of cyclosporin A and about 200-fold lower than the IC_{50} for cell growth (Fig. 2b). In addition, BIM I inhibited the cytopathic effect of HCV JFH1. Infection with HCV resulted in about a 20% reduction of cell growth. BIM I at 1 μM enhanced the growth of

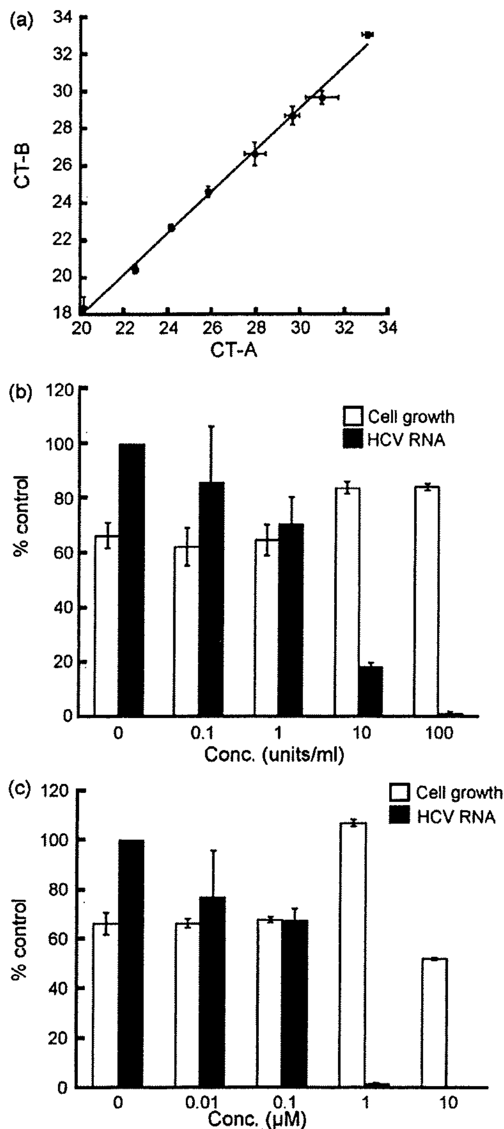

Fig. 1. RT-PCR-based screen for anti-HCV agents using the JFH1 viral culture system. (a) Correlation between CT values from "tube-capture-RT-PCR" (CT-A) and ordinary RNA extraction (CT-B). JFH1 stock solution (3×10^5 ffu/ml) was serially diluted four-fold and quantitative real-time PCR was performed as described under Section 2. CT-A was the average CT of three wells using tube-capture-RT-PCR and CT-B was the average CT of three HCV RNA eluates using a kit (QIAamp Viral RNA mini). (b) and (c) Huh 7.5.1 cells were infected with JFH1 in the presence of the indicated concentrations of IFN-α (b) or cyclosporin A (c). HCV RNA in the medium (closed (black) columns) was assessed by "tube-capture-RT-PCR" as described under Section 2. Open (white) columns represent percentage of cell growth compared with that of control cells without virus and compound. Columns, mean of triplicate wells; bars, SD.

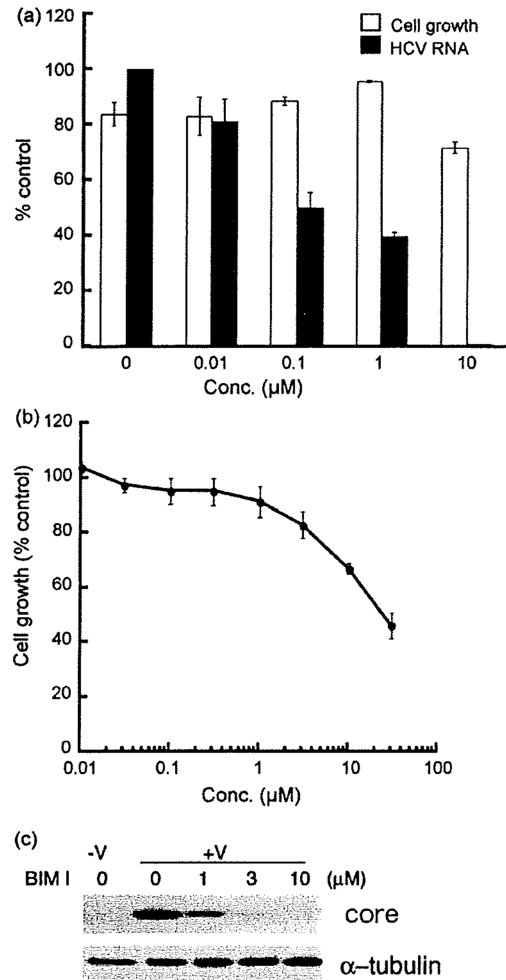

Fig. 2. BIM I inhibits HCV. (a) Effects of BIM I on HCV JFH1 RNA. Huh 7.5.1 cells were infected with JFH1 in the presence of the indicated concentrations of BIM I and assayed for HCV RNA and cell growth as in Fig. 1. Columns, mean of triplicate wells; bars, SD. (b) Effects of BIM I on growth of Huh 7.5.1 cells. (c) Effects of BIM I on HCV core protein in cells. Cells were infected with JFH1 at an MOI of 0.2 in the presence of the indicated concentrations of BIM I and cultured for 2 days. Cells were lysed and subjected to western blotting as described under Section 2. "V" indicates infection with HCV.

infected cells, almost to the level of uninfected cells (Fig. 2a, white columns). Recovery of cell growth was also observed with INF-α or cyclosporin A treatment (Fig. 1b and c). BIM I reduced cell growth of uninfected cells only at concentrations of 1 μM or higher (Fig. 2b). BIM I also inhibited the production of the HCV core protein with marginal effects on host α-tubulin levels (Fig. 2c).

3.3. *BIM I inhibits HCV replication*

To our knowledge, the anti-HCV effects of BIM I or other PKC inhibitors have not been reported. Because the majority of current HCV drug screening relies on replicon-based models, we investigated the possibility that BIM I targets a step in the HCV life cycle that is not included in the replicon systems, such as attachment, entry or release. We treated two subgenomic replicon cells with BIM I and examined the amount of NS5A protein.

As shown in Fig. 3a and b, BIM I dose-dependently reduced NS5A in both 1b and 2a subgenomic replicon cells, but not the host GAPDH. The results indicate that BIM I inhibits a process involved in the replication of HCV subgenomic replicons. However, although the NS5A level appeared to be more vulnerable, cell growth was substantially suppressed by BIM I (Fig. 3c). Whereas a significant difference between the IC_{50} for HCV RNA and cell growth was observed in the HCV cell culture system, the reduction of NS5A in replicon cells overlapped with the effects on cell growth.

To further elucidate the stage of the HCV life cycle affected by BIM I, Huh 7.5.1 cells were inoculated with higher titers of JFH1 (MOI 2) and then treated with 3 μM BIM I, starting at different time points after infection. JFH1 appeared to complete the life cycle in about 48 h, judging from the expression profiles of viral RNA and proteins in cells (Fig. 4a). When BIM I was added at the time of infection,

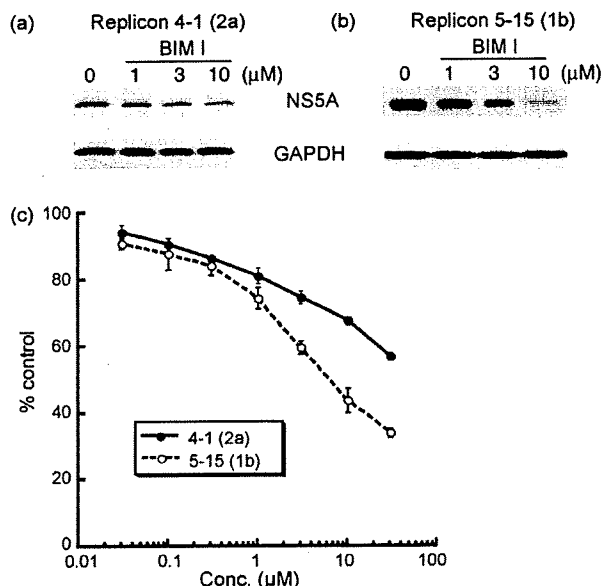

Fig. 3. Effect of BIM I on subgenomic replicon cells. (a) Subgenomic replicon cells harboring genotype 2a (4-1) (a) and genotype 1b (5-15) (b) were treated with BIM I for 2 or 4 days, respectively. Cells were lysed and analyzed by western blotting with anti-NS5A antibody or anti-GAPDH antibody. (c) Effect of BIM I on cell growth of the two replicon cells. Cells were incubated for 2 (4-1) or 4 days (5-15) with BIM I, and cell growth was measured by MTT assay.

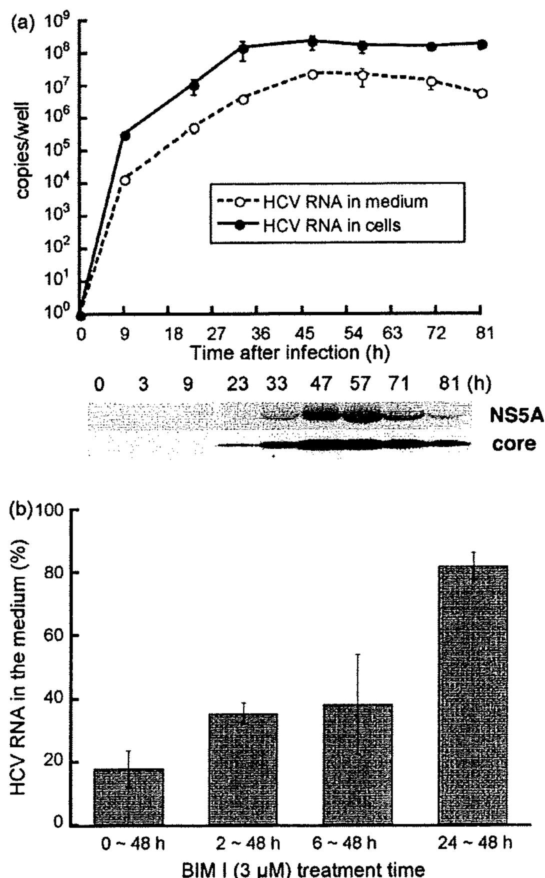

Fig. 4. (a) Expression profile of the HCV RNA and proteins. Huh 7.5.1 cells were infected with HCV as described above, but at an MOI of 2. The cells and medium were harvested at the indicated time and analyzed for HCV RNA and proteins. The core and NS5A protein were detected in the cell lysate. (b) Effect of time of addition. BIM I (3 μM) was added to Huh 7.5.1 cells just before and 2, 6, or 24 h after HCV addition at an MOI of 0.2. After 48 h of incubation following virus infection, the HCV RNA in the medium was extracted and measured with quantitative real-time RT-PCR. The indicated values represent the averages for two independent experiments.

the amount of viral RNA in the medium after 48 h decreased to less than 20% of control cells without inhibitor treatment. Addition of BIM I at 6 h post-infection still resulted in a reduction of viral RNA to 30% of control, but after 24 h the antiviral activity of BIM I was significantly diminished and only a modest decline to about 80% was observed (Fig. 4b). These results suggest that interference with RNA synthesis or translation of viral proteins accounts, at least in part, for the anti-HCV activity of BIM I.

3.4. *HCV inhibition by bisindolylmaleimide and indolocarbazole compounds does not involve PKC*

Since the discovery of staurosporine as a broad-spectrum protein kinase inhibitor, a variety of bisindolylmaleimide and indolocarbazole inhibitors with different potencies and selectivity have been developed. BIM I is one such compound that is highly specific for PKC and is broadly used to analyze PKC-mediated events. To gain insight into the relevance of the PKC inhibitory spectrum and antiviral activity, panels of different bisindolylmaleimide and indolocarbazole compounds were tested in the assays. Contrary to our expectation, no correlation was found between the ability to inhibit PKC and HCV.

Another bisindolylmaleimide PKC inhibitor without the N-dimethylaminopropyl chain, BIM IV, displayed similar significant anti-HCV activity (Fig. 5). Other structurally related pan- and isozyme-specific PKC inhibitors such as BIM II, Ro31-8220 (BIM IX), LY333,531 and 3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione (PKCβ Inhibitor, Calbiochem) or an indolocarbazole compound K252c also inhibited HCV replication (not shown). However, as shown in Fig. 5, the non-PKC-inhibitory analog BIM V (Toullec et al., 1991) and arcyriaflavin A, an indolocarbazole compound with no reported effects on PKC (Zhu et al., 2003), were also effective in reducing HCV RNA. BIM V was actually more potent than BIM I. Whereas the effect of BIM I on HCV overlapped with cytotoxicity in this particular experiment, BIM V was virtually nontoxic at a dose (1 μM) that reduced HCV RNA to less than 20% of control. The results indicate that a broad range of bisindolylmaleimide and indolocarbazole compounds inhibit HCV replication, albeit in a PKC-independent manner.

4. Discussion

HCV replicon systems have made significant contributions in HCV research and drug development. Nevertheless, as drug screening tools, replicon systems have limitations because they are not capable of identifying inhibitors of several important events in the viral life cycle. The use of HCV cell culture systems should overcome the drawbacks of the replicon systems and facilitate the identification of inhibitors with novel mechanisms of action. Actually, it has recently been shown that use of an infectious HCV system identified inhibitors that a replicon-based screen did not recognize (Zhang et al., 2008).

We developed a simple screening method for HCV inhibitors that measures the viral RNA released from JFH1-infected cells. The assay does not require specially engineered viruses. The "tube-capture-RT-PCR" method obviates the need for labor-intensive RNA isolation and significantly increases the efficiency of screening. The validity of the assay was confirmed by successful identification of known

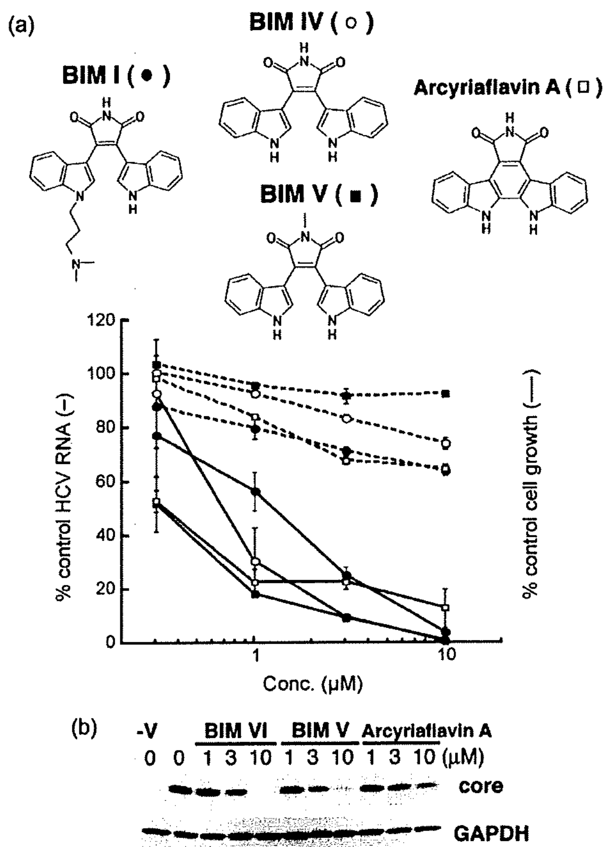

Fig. 5. Effects of bisindolylmaleimide or indolocarbazole compounds on HCV infection. (a) Effects of BIM I, BIM IV, BIM V, and arcyriaflavin A on HCV RNA in the medium. Huh 7.5.1 cells were seeded and infected with HCV at an MOI of 0.01 in the presence of drugs. After 4 days of incubation, the HCV RNA in the medium was extracted and quantified. The relative amounts of HCV RNA with BIM I (closed circle), BIM IV (open circle), BIM V (closed rectangle), and arcyriaflavin A (open rectangle) are represented by solid lines. Cell viability, represented by dotted lines, was determined by MTT assay of a parallel culture without HCV challenge. (b) Effects of BIM IV, BIM V and arcyriaflavin A on core proteins in cells. The cells were infected with JFH1 at an MOI of 0.2 in the presence of the indicated concentrations of compounds and analyzed as described in Fig. 2c. "–V" indicates control without HCV infection.

HCV inhibitors in the pilot screen. In addition, the assay identified the PKC inhibitor BIM I.

BIM I is a widely used compound, and it was somewhat surprising to us that its anti-HCV activity had not been reported. HCV replication in the cell culture system appeared to be considerably more susceptible to BIM I than in the replicon systems, and this is probably why this compound had not been identified as an HCV inhibitor.

Because virus replication is closely linked to host cell growth, HCV inhibition could occur as a result of cell growth inhibition. However, as shown in Fig. 2a, BIM I reduced the cytopathic effect of HCV infection just like interferon-α and cyclosporin A. BIM I, at 1 μM, enhanced the growth of infected cells almost to the level of uninfected cells, presumably because HCV replication, but not cell growth, was inhibited, resulting in a reversal of the cytopathic effect.

Because the PKC inhibitory properties of bisindolylmaleimides and indolocarbazoles have been characterized extensively, we tested a panel of commercially available compounds in the assay to gain insight into the role of PKC in HCV replication. However, no correlation between PKC inhibition and antiviral activity could be found. Anti-HCV activity did not involve PKC inhibition, apparently because a non-PKC-inhibitory analog, BIM V, was also active. Furthermore, PKC inhibitors with different structures, such as calphostin C and H-7, did not show specific inhibition of HCV (data not shown).

Previous studies have indicated that bisindolylmaleimide PKC inhibitors have cellular targets other than PKC. It has been shown that BIM I and Ro31-8220 inhibit p70S6K and p90RSK (Alessi, 1997; Roberts et al., 2005) and that BIM V inhibits p70S6K (Marmy-Conus et al., 2002). Although we did not monitor the activities of these enzymes in our experiments, inhibition of p70S6K is unlikely to be responsible for the anti-HCV effect of PKC inhibitors, because Ishida et al. reported that silencing of p70S6K enhanced HCV RNA abundance (Ishida et al., 2007).

Bisindolylmaleimides and indolocarbazoles have also been reported to inhibit the ATP-binding cassette (ABC) transporters P-glycoprotein and multidrug resistance-associated protein 1 (MRP1), efflux pumps that play important roles in cancer drug resistance (Merritt et al., 1999; Gekeler et al., 1995). More recently, Robey et al. reported that BIMs I, II, III, IV, and V, K252c, and arcyriaflavin A inhibit ABCG2, an ABC half-transporter that confers resistance to various antitumor agents (Robey et al., 2007). Whether ABC transporters play any role in HCV infection awaits further study. We are currently examining the anti-HCV effects of other ABC transporter inhibitors.

In conclusion, we developed a simple infectious HCV system-based assay that can be used for high-throughput screening of HCV inhibitors and identified bisindolylmaleimides and indolocarbazoles. These compounds might represent lead substances for the development of new HCV drugs. Further analysis of the mechanism of HCV-inhibition by these compounds might reveal a new mechanism of regulation of HCV infection.

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