

Reactive Oxygen Species Suppress Hepatitis C Virus RNA Replication in Human Hepatoma Cells

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Hepatitis C virus (HCV) is a positive-stranded RNA virus that causes severe liver diseases, such as cirrhosis and hepatocellular carcinoma. HCV uses an RNA-dependent RNA polymerase to replicate its genome and an internal ribosomal entry site to translate its proteins. HCV infection is characterized by an increase in the concentrations of reactive oxygen species (ROS), the effect of which on HCV replication has yet to be determined. In this report, we investigated the effect of ROS on HCV replication, using a bicistronic subgenomic RNA replicon and a genomic RNA that can replicate in human hepatoma cells. The treatment with peroxide at concentrations that did not deplete intracellular glutathione or induce cell death resulted in significant decreases in the HCV RNA level in the cells. This response could be partially reversed by the antioxidant *N*-acetylcysteine. Further studies indicated that such a suppressive response to ROS was not due to the suppression of HCV protein synthesis or the destabilization of HCV RNA. Rather, it occurred rapidly at the level of RNA replication. ROS appeared to disrupt active HCV replication complexes, as they reduced the amount of NS3 and NS5A in the subcellular fraction where active HCV RNA replication complexes were found. In conclusion, our results show that ROS can rapidly inhibit HCV RNA replication in human hepatoma cells. The increased ROS levels in hepatitis C patients may therefore play an important role in the suppression of HCV replication. (HEPATOLOGY 2004;39:81–89.)

Hepatitis C virus (HCV) is a positive-sensed, single-stranded RNA virus of the *Flaviviridae* family.¹ Currently, it is estimated that there are more than 170 million people who are infected by HCV worldwide.² About 80% of HCV infection results in chronic infection that can lead to severe liver diseases such as cirrhosis and hepatocellular carcinoma. HCV genome is about 9.6 kilobase (kb) in length and consists of the 5'

untranslated region (UTR), the structural (C, E1, E2), and the nonstructural (NS) (p7, NS2, NS3, NS4A/B, NS5A/B) protein-coding regions, and the 3' UTR. Translation is mediated by the internal ribosomal entry site (IRES), located at the 5' end of the genome, and it produces a polyprotein that is subsequently cleaved by viral and host proteases to generate individual viral proteins. Some of the HCV proteins can also be synthesized from alternate reading frames through ribosomal frameshift.^{3–6}

HCV infection is associated with elevated levels of circulating reactive oxygen species (ROS) in patients.^{7–14} ROS are normal products of cell metabolism,¹⁵ and their syntheses can be heightened during inflammation.¹⁶ Recent studies have revealed a complex relationship between redox chemistry and various viral infections. For example, ROS can negatively regulate hepatitis B virus replication in liver cells without affecting the cell metabolism¹⁷ but enhance the replication of human immunodeficiency virus by activating nuclear factor kappa B.¹⁸ Viral proteins such as hepatitis B virus X protein and human immunodeficiency virus tat protein have also been shown to regulate the cellular redox status.^{19–22} HCV NS3 protein can also activate the ROS generation by activating NADPH oxidase of monocytes.²³ In addition, HCV core and NS5A proteins have been found to induce oxidative stress

Abbreviations: HCV, hepatitis C virus; UTR, untranslated region; NS, nonstructural; IRES, internal ribosomal entry site; ROS, reactive oxygen species; HIV, human immunodeficiency virus; PCR, polymerase chain reaction; NT, nucleotides; PBS, phosphate-buffered saline; RER, rough endoplasmic reticulum; WGA, wheat germ agglutinin; tBOOH, *t*-butylhydroperoxide; TBHQ, 2-tert-butyl(1,4)hydroquinone; NAC, *N*-acetylcysteine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; ER, endoplasmic reticulum.

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Received July 2, 2003; accepted October 6, 2003.

Supported by Research Scholar Grant #PF-01-037-01-MBC to J.C. from the American Cancer Society, and grants from the National Institutes of Health.

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Published online in Wiley InterScience (www.interscience.wiley.com).

DOI 10.1002/hep.20001

in cells.^{24,25} Interestingly, transgenic mice carrying the HCV core protein gene show signs of increased oxidative stress in the absence of inflammation.²⁶ Such elevated ROS concentrations are thought to play an important role in the pathogenesis of HCV.²⁶ In spite of these observations, the effect of ROS on HCV replication has yet to be determined.

In this report, we have used a subgenomic HCV RNA replicon as well as a self-replicating HCV genomic RNA to investigate the effect of ROS on HCV RNA replication. Our results indicate that ROS, at concentrations that do not affect cell viability, significantly decreased both subgenomic and genomic HCV RNA levels. This decrease occurred rapidly at the level of RNA replication and was associated with a disappearance of NS5A from the subcellular fraction that contained the HCV replication complex.

Materials and Methods

Subgenomic and Genomic HCV Replicon Constructs. The HCV subgenomic replicon of genotype 1b²⁷ (Genbank accession No. AJ242652) was constructed by serial ligation of DNA oligonucleotides and by polymerase chain reaction (PCR). *Hind*III/*Not*I and *Sca*I/*Xba*I sites were engineered at the 5' and 3' ends of the subgenomic replicon sequence for cloning into a pUC19-derived plasmid vector that contained a modified T7 promoter. This replicon carried an S1179I adaptive mutation in the NS5A region that enhanced HCV RNA replication in cell culture.²⁸ The final sequences were confirmed by DNA sequencing.

The hybrid genomic HCV replicon was produced by fusing nucleotides (NT) 1–3092 of the H77c sequence²⁹ to NT 1839–7990 of the subgenomic replicon sequence via *Stu*I (NT 1839) site within the NS3 region. This fusion creates a 1a/1b hybrid HCV genome. The NS3 sequence of this hybrid differs from the subgenomic replicon sequence by a single serine-to-alanine substitution at the eighth amino acid. This hybrid sequence was cloned into the modified pUC19 plasmid, as mentioned above. The replicon RNAs were synthesized as described previously by others.²⁷ The hybrid genomic plasmid and pH77c were linearized with *Xba*I prior to RNA synthesis.

Electroporation and Cell Culture. About 5×10^6 Huh7 human hepatoma cells were rinsed with Dulbecco's Modified Eagle's Medium (DMEM) without serum, mixed briefly with 5 μ g of the subgenomic replicon RNA in 0.4 mL of this medium, and then electroporated at 220 V and 975 microfarads (μ F). Cell colonies were selected with DMEM containing 0.7 mg/mL G418. Two pooled cell clones (Sg-PC1 and 2) were obtained and subse-

quently maintained in DMEM containing 0.5 mg/mL of G418. For most of the experiments, G418 was removed from the medium one day prior to cell treatments. For transient replication experiments, 1×10^7 Huh7 cells were electroporated with 10–40 μ g of subgenomic, hybrid, or H77c RNA. Then, 1 million cells were seeded onto 60-mm cell culture dishes in DMEM containing 10% fetal bovine serum. Cells were collected at different time points, as indicated in Results.

Northern Blot Analysis. Total RNA was extracted from cells, using Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Four to 15 μ g RNA were then subjected to Northern blot analysis using a ³²P-labeled double-stranded DNA probe, prepared from NT 3669 to 6016 of the subgenomic replicon or NT 279 to 3092 of H77c. The latter was used to detect the hybrid and H77c RNA. For the detection of the negative strand RNA, a sense riboprobe, containing the 5' UTR and the neomycin region of the subgenomic replicon construct, was used. For these and all other experiments, RNA and protein bands were quantified by densitometry, using SigmaScan (Jandel Scientific, San Rafael, CA). The intensities of the bands of interest were normalized against the control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) RNA.

Real-Time PCR. HCV RNA was quantified by real-time reverse transcription (RT) PCR, as described by Lanford et al.,³⁰ except the reverse primer sequence was 5' CGGGTTGATCCAAGAAAGGA 3' (NT 210 to 191) and the fluorogenic probe, labeled with 6-FAM and BHQ-1 (Biosearch Technologies, Novato, CA). ABI 7900 Sequence Detector and Taqman Gold RT-PCR Kit (Applied Biosystems, Foster City, CA) were used. For the analysis of negative-stranded HCV RNA, reverse transcription was carried out with the forward primer prior to the PCR amplification.

³H Labeling of Newly Synthesized HCV RNA. One million Huh7 or Sg-PC2 cells were labeled with 100 μ Ci of ³H-uridine (35–50 Ci/mmol; ICN, Costa Mesa, CA) either in the presence or absence of 200 μ g/ml actinomycin D (SigmaAldrich, St. Louis, MO) for 5–6 hours and then lysed for the RNA isolation. RNA was analyzed on a 1% formaldehyde agarose gel, which was then treated with 1M sodium salicylate for 20–30 minutes for fluorography. To analyze the effect of hydrogen peroxide (H₂O₂) on nascent HCV RNA, cells were treated with 0, 20, 50, or 100 μ M H₂O₂ for the duration of ³H-labeling. To investigate the effect of H₂O₂ on HCV RNA stability, cells were pulse-labeled with ³H-uridine for 5–6 hours as described above, rinsed twice with phosphate-buffered saline (PBS), and chased with a hundredfold excess of non-

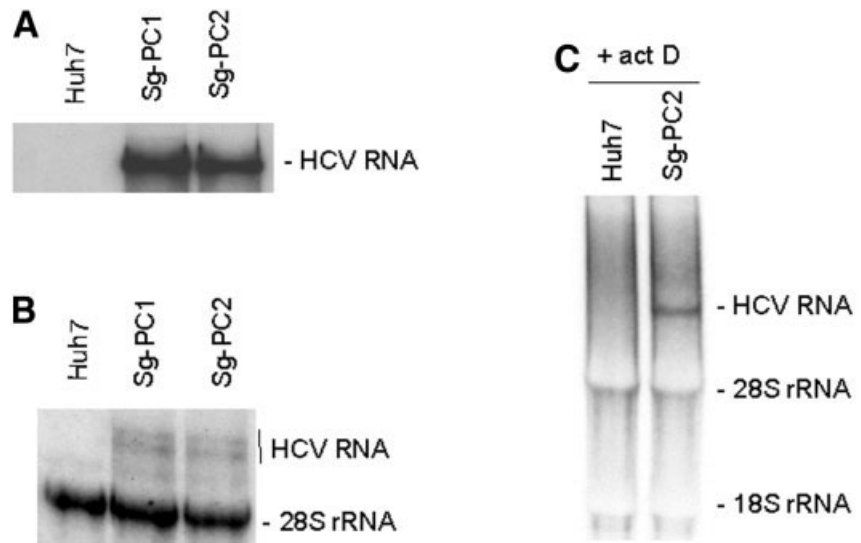


Fig. 1. Replication of HCV subgenomic RNA in Huh7 cells. (A) and (B) Northern blot analyses of total RNA from Sg-PC1 and Sg-PC2 cells using (A) ^{32}P -labeled, double-stranded HCV complementary DNA probe and (B) the minus strand-specific riboprobe. (C) ^3H -uridine incorporation assay. Naïve Huh7 cells and Sg-PC2 were labeled with ^3H -uridine in the presence of actinomycin D for 5–6 hours. RNA samples were isolated and separated on a 1% agarose gel. ^3H -RNA bands were visualized by autoradiography.

labeled uridine (0.1 mmol/L) with either 0 or 100 μM H_2O_2 for 3 or 6 hours.

Subcellular Membrane Fractionation. Four 100-mm dishes of Sg-PC2 cells with or without the H_2O_2 treatment were rinsed with PBS and then with the hypotonic buffer (25 mmol/L HEPES, pH7, 0.2 M sucrose) twice. Cells were scraped off the plate in 0.5 mL hypotonic buffer, sat on ice for 10 minutes and lysed by passing through a 26-gauge needle four times. The cell lysates were centrifuged at 16,000 $\times g$ for two minutes, and the cytoplasmic supernatants were then fractionated on a discontinuous sucrose gradient, which contained 1.5 mL 2 M sucrose, 3.4 mL 1.3 M sucrose, 3.4 mL 1 M sucrose, and 2.2 mL 0.6 M sucrose in 25 mmol/L HEPES, pH 7, at 40K rpm for two hours using a Beckman SW40Ti rotor following our previous procedures.^{31,32} The interface between 2 and 1.3 M sucrose solutions, which contained Golgi membranes, and that between 1 and 0.6 M sucrose solutions, which contained the membranes derived from the rough endoplasmic reticulum (RER), were isolated (approximately 1 mL each), treated with Nonidet P-40 to a final concentration of 0.2%, diluted with 1 mL of 25 mmol/L HEPES, pH 7, and then concentrated with Centricon (Amicon Corp., Billerica, MA). The samples were then analyzed by Western blot using mouse anti-NS5A (Bioscience, Saco, ME), mouse anti-NS3 (Austral Biologicals, San Ramon, CA), goat anti-GRP78 (Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-human albumin primary antibodies (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The horseradish peroxidase-conjugated wheat germ agglutinin (WGA) (ICN ImmunoBiologicals, Costa Mesa, CA) was also used to analyze the proteins associated with the Golgi membranes.

For the analysis of the HCV RNA replication complex, the membranes isolated from the interfaces were dialyzed at 4°C twice against 25 mmol/L HEPES, pH7 and pelleted at 16,000 $\times g$ for 30 minutes. The membrane pellet was then resuspended in 50 μL of the following reaction mixture: 50 mmol/L HEPES, pH7, 5 mmol/L MgCl_2 , 0.5 mmol/L MnCl_2 , 10 mmol/L KCl, 1 mmol/L each of adenosine triphosphate, guanosine triphosphate and uridine triphosphate, 10 μM cytidine triphosphate (CTP), 5 μg actinomycin D, and 30 μCi α - ^{32}P -CTP; it was then incubated at 30°C for 90 minutes.³³ The reaction was stopped by extraction with phenol and chloroform, and the ^{32}P -labeled HCV RNA was precipitated with ethanol in the presence of transfer RNA carrier and analyzed by gel electrophoresis and autoradiography.

Results and Discussion

Sg-PC1 and Sg-PC2 were two different pooled cell clones that contained HCV subgenomic RNA replicon. As shown in Figures 1A and 1B, both positive- and negative-stranded HCV RNAs of approximately 8 kb were readily detected by Northern blot analysis in these two cell lines. The HCV RNA could be labeled by ^3H -uridine in the presence of actinomycin D, indicating that its synthesis was independent of the host RNA transcription (Fig. 1C).

To test the effect of ROS on HCV replication, one of the pooled cell clones, Sg-PC2, was treated with various concentrations of H_2O_2 , *t*-butylhydroperoxide (tBOOH), or 2-*tert*-butyl(1,4)hydroquinone (TBHQ) for 24 hours, and the HCV RNA was analyzed by Northern blot. H_2O_2 exposure induced significant decreases in the HCV RNA level (Fig. 2A). The results were confirmed by real-time

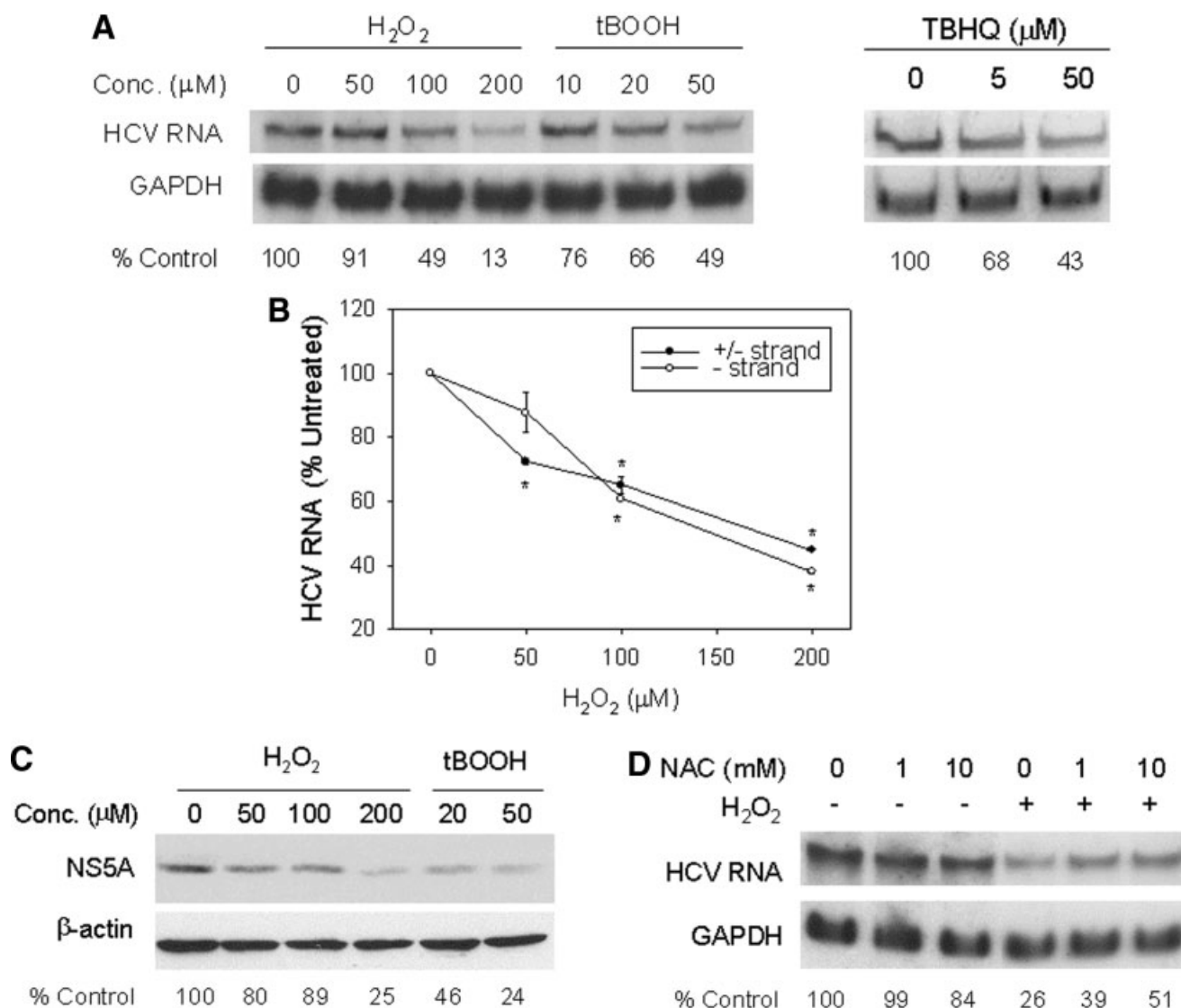


Fig. 2. Peroxide-induced suppression of the HCV subgenomic RNA level in Sg-PC2 cells. (A), (B), and (C) Sg-PC2 cells were treated with various concentrations of H_2O_2 , tBOOH, or TBHQ for 24 hours. Then, the HCV RNA level was analyzed by Northern blot (A) or real-time RT-PCR (B), and NS5A by Western blot (C). GAPDH messenger RNA and β -actin were also analyzed in (A) and (C), respectively, to serve as the controls. *Statistically significant difference from the control ($P < 0.05$). (D) Sg-PC2 cells were treated with 0, 1 or 10 mmol/L NAC either in the presence or absence of 200 μM H_2O_2 for 24 hours. The RNA and protein bands were quantified by densitometry. mM, millimole.

RT-PCR for both positive and negative stranded HCV RNAs (Fig. 2B), and these changes in the viral RNA level were accompanied by similar decreases in the HCV protein level (Fig. 2C). The tBOOH induced similar reduction of the viral RNA level (Fig. 2A). The TBHQ is a redox-cycling hydroquinone, which generates a low level of ROS in cells.³⁴ It also decreased the HCV RNA level in these cells (Fig. 2A). These results indicated that ROS could reduce HCV RNA levels in Sg-PC2 cells. The negative effects of ROS could be reversed by *N*-acetylcysteine (NAC), a thiol antioxidant compound, as the concurrent treatment of Sg-PC2 cells with NAC partially alleviated the negative effect of H_2O_2 on the HCV RNA level (Fig. 2D).

In all of the experiments shown in Figure 2, the expression levels of the GAPDH RNA were not affected, indicating that the effect of ROS on HCV RNA was not caused by the nonspecific cytotoxicity of ROS. The lack of cytotoxicity of ROS at the concentrations used was further confirmed by the trypan blue exclusion assay, by the lack of any increase in the amount of lactate dehydrogenase released into the medium, and by the lack of changes of the intracellular redox status based on the glutathione levels (data not shown).

Studies using the subgenomic replicons shown in Figure 2 are limited because the translation of the HCV nonstructural proteins is driven by the encephalomyocarditis virus IRES instead of the HCV IRES and because

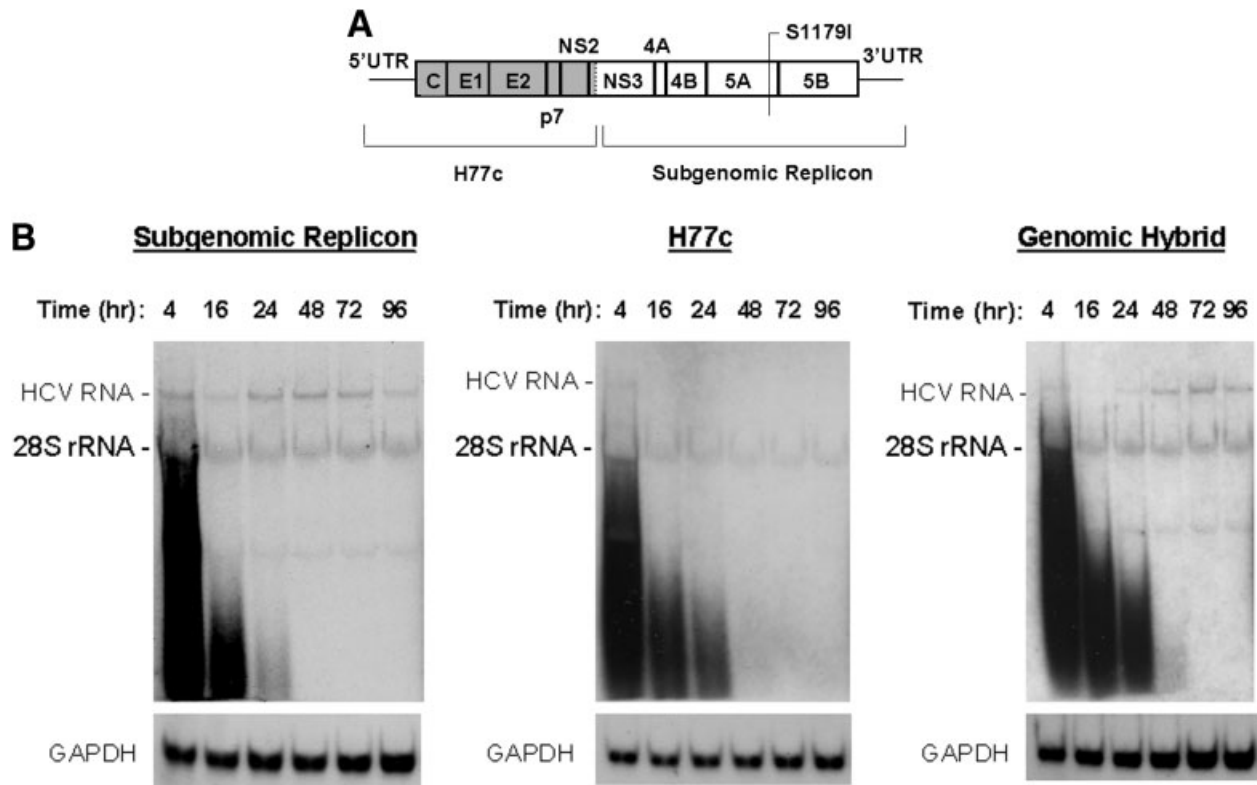


Fig. 3. Replication of the HCV genomic hybrid in Huh7 cells. (A) Schematic diagram of the full-length, HCV hybrid RNA. Shaded area represents the H77c sequence, and the white area, the subgenomic replicon sequence. The location of the S1179I adaptive mutation is indicated. (B) Huh7 cells were transiently transfected with the *in vitro* synthesized subgenomic replicon RNA (left panel), the H77c RNA (middle panel), or the full-length HCV hybrid RNA (right panel). RNA samples were collected at different time points and analyzed for their HCV RNA and GAPDH mRNA by Northern blot.

none of the structural protein genes are present. Therefore, we sought to determine the effect of H_2O_2 on the genomic HCV RNA replication. The H77c sequence, an infectious HCV clone,²⁹ from the beginning of the NS3 coding sequence to the 3' end, was replaced with the corresponding sequence of the subgenomic replicon sequence to generate a full-length, hybrid HCV sequence (Fig. 3A). This hybrid RNA was synthesized *in vitro* and electroporated into Huh7 cells. The total cellular RNA was then isolated at different time points and analyzed by Northern blot for HCV RNA.

As shown in the left panel of Figure 3B, the subgenomic HCV RNA replicated transiently in Huh7 hepatoma cells, as previously demonstrated by others. The RNA level dropped transiently at about 16 hours and rose at 24 hours, indicating initial degradation of the input RNA, followed by the active HCV RNA replication.³⁵ The RNA concentration peaked at around 48 hours and then gradually decreased. The full-length genomic H77c RNA did not replicate, as expected,²⁸ and served as a negative control (Fig. 3B, middle panel). The H77c/replicon genomic hybrid replicated transiently in cell culture (Fig. 3B, right panel). Its RNA level once again dropped

transiently at 16 hours, started increasing at 24 hours, and peaked at around 72 hours. The NS5A and core protein were also detected in cells that were transfected with the hybrid RNA four days after the transfection (data not shown).

To examine whether H_2O_2 could also decrease the full-length HCV RNA level, Huh7 cells were transiently transfected with the hybrid HCV genomic RNA. After 48 hours, these cells were treated with various concentrations of H_2O_2 , and the amount of the HCV RNA was analyzed after 24 hours. The H_2O_2 at 50 or 100 μM significantly reduced the genomic HCV RNA level to the undetectable level (Fig. 4).

To determine the mechanism by which peroxide decreased the HCV RNA level in hepatoma cells, Sg-PC2 cells were incubated with 3H -uridine and actinomycin D (see Fig. 1C) with and without H_2O_2 for 5–6 hours. As shown in Figure 5A, H_2O_2 dose-dependently decreased the amount of the HCV RNA that was synthesized during this time. The viral RNA fell to an almost undetectable level with 100 and 200 μM H_2O_2 treatments. The 3H -labeling of ribosomal RNA was not affected (Fig. 5A, left panel), again indicating the specificity of this suppression.

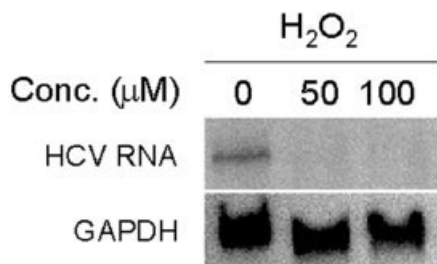


Fig. 4. Reduction of the HCV genomic RNA level in Huh7 cells by peroxide. Huh7 cells were transiently transfected with the HCV genomic hybrid RNA and treated with 0, 50 μM or 100 μM of H_2O_2 for 24 hours. GAPDH mRNA and the HCV genomic RNA levels were analyzed by Northern blot.

Next, we examined whether this suppression, observed within 5–6 hours of H_2O_2 exposure, occurred at the level of RNA stability. The HCV RNA was labeled for 5–6 hours with ^3H -uridine in the presence of actinomycin D and then chased with a hundredfold excess of nonlabeled uridine in the presence of 0 or 100 μM H_2O_2 for 3 or 6 hours. There was no appreciable decline in the control (*i.e.*, 0 μM H_2O_2) HCV RNA signal after 3 hours of chase, although some reduction was observed after 6 hours (Fig. 5B). Most importantly, however, there was no apparent change in the ^3H -RNA signal with 100 μM H_2O_2 treatment (Fig. 5B). Therefore, the rapid decline in

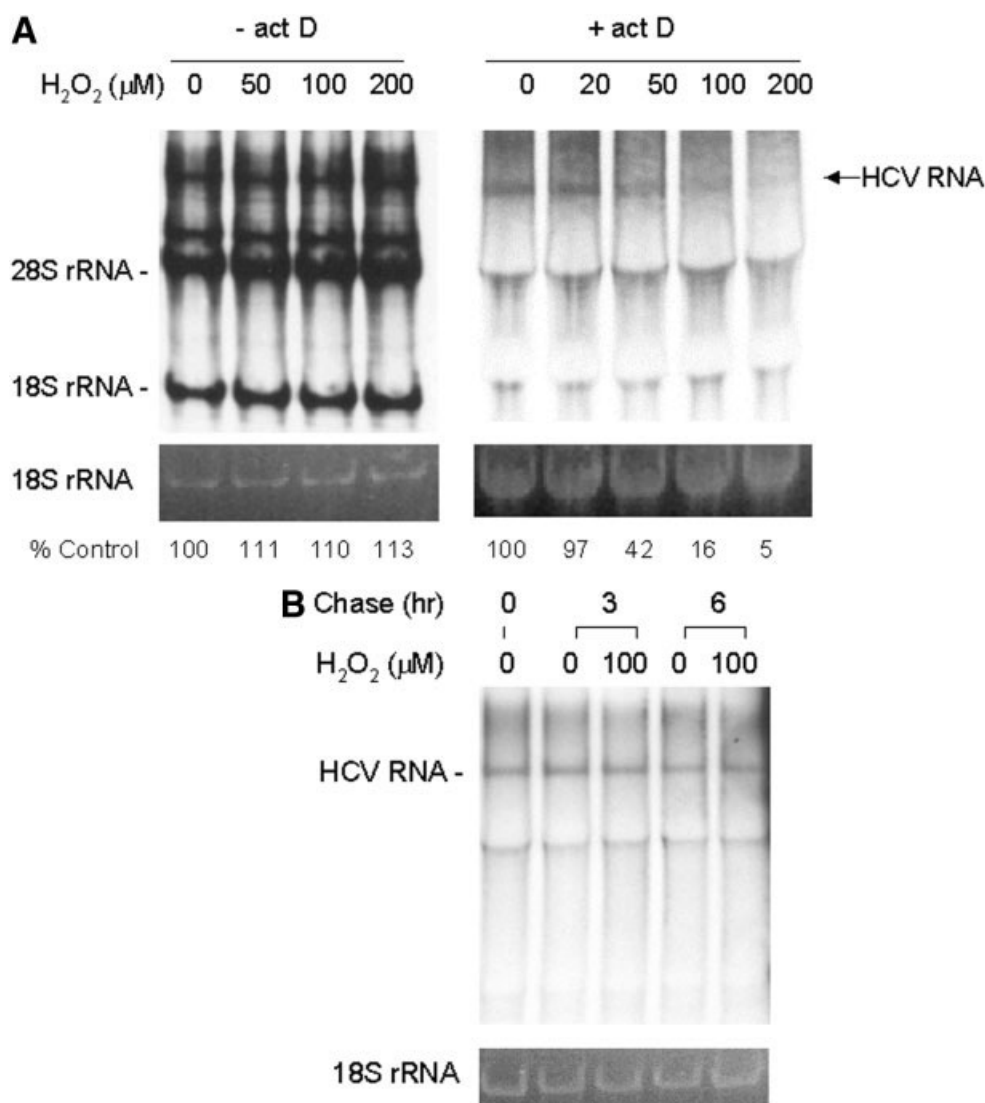


Fig. 5. Inhibition of HCV RNA replication by H_2O_2 . (A) Sg-PC2 cells were labeled with ^3H -uridine and treated with various concentrations of H_2O_2 in the presence (+ act D) or absence (– act D) of actinomycin D for 5–6 hours. Then the RNA was isolated and analyzed on a 1% RNA gel. The gel was stained with ethidium bromide to compare the amount of 18S rRNA present in each lane (lower panel), which served as the loading control. The relative levels of ^3H -HCV RNA and ^3H -18S rRNA bands were quantified by densitometry and shown at the bottom of their respective gels. (B) Sg-PC2 cells were pulse-labeled with ^3H -uridine in the presence of actinomycin D for 5–6 hours and then chased with nonlabeled uridine for 3 or 6 hours. One hundred μM H_2O_2 was also added at the beginning of chase. RNA samples were then isolated and analyzed for the stability of the ^3H -HCV RNA.

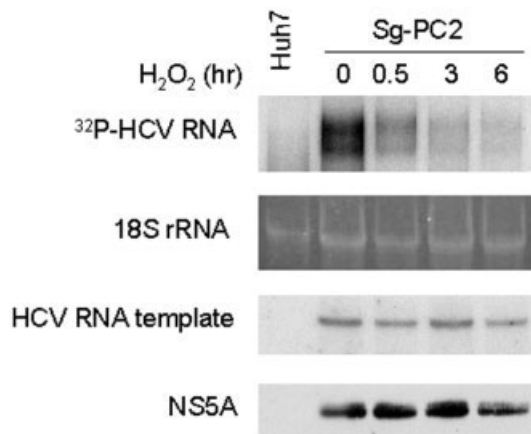


Fig. 6. Suppression of HCV RNA replication *in vitro* by H_2O_2 . Sg-PC2 cells were treated with $100 \mu M H_2O_2$ for 0, 0.5, 3, or 6 hours. Then, the cytoplasmic lysates were prepared from these and naïve Huh7 cells, and the *in vitro* replication assays were carried out at $30^\circ C$ for 1 hour with $\alpha\text{-}^{32}P\text{-CTP}$ as described by Ali et al.³⁶ The RNA products were analyzed on a 1% RNA gel, and the $^{32}P\text{-HCV RNA}$ bands were visualized by autoradiography (top panel). The gel was also stained with ethidium bromide to assess the amount of 18S rRNA (second panel), which served as the loading control. The amount of the HCV RNA template (third panel) and the NS5A protein (bottom panel), present in each cytoplasmic extract prior to the assay, was also determined by Northern blot and Western blot analyses, respectively. Note that there was a slight reduction in the amount of HCV RNA template after 30 minutes of peroxide treatment. This reduction was not observed in other experiments and could not account for the fivefold decrease in the rate of HCV RNA replication.

the rate of $^3H\text{-RNA}$ synthesis observed within 5–6 hours of the $100 \mu M$ peroxide treatment shown in Figure 5A was not due to an accelerated degradation of the viral RNA. The HCV IRES activity was not affected by H_2O_2 (data not shown).

To further investigate whether H_2O_2 directly suppressed HCV RNA synthesis, Sg-PC2 cells were first treated with $100 \mu M H_2O_2$ for 30 minutes, 3 hours, or 6 hours. Then, the cytoplasmic lysates were prepared, and the RNA replication assay was carried out *in vitro* as described by Ali et al.³⁶ All peroxide-treated lysates showed a reduction of the HCV RNA replication rate (Fig. 6, top panel). This reduction was apparent as early as 30 minutes after treatment. Neither the RNA loading, based on the amount of 18S ribosomal RNA in each lane (Fig. 6, second panel), nor the amount of the HCV RNA template, present in the lysates immediately prior to the *in vitro* RNA replication assay (Fig. 6, third panel), could account for the rapid suppression of HCV RNA replication by H_2O_2 . There was also no change in the amount of NS5A in each of the cell lysates prior to the assay up to six hours of treatment (Fig. 6, bottom panel). Note that the NS5A protein level did decrease at 24 hours after the peroxide treatment (Fig. 2C). However, this decrease was most likely a result of the decreased HCV RNA replication

rather than being the cause for the decreased replication. Therefore, the suppressive response to H_2O_2 occurred rapidly, at the level of RNA replication.

To further investigate the molecular mechanism of suppression of HCV RNA replication by ROS, we examined whether the subcellular localization of HCV NS5A protein was affected by the peroxide treatment. Sg-PC2 cells were treated with $100 \mu M H_2O_2$ for 3 hours, and then subjected to membrane fractionation experiments, as previously described.^{31,32} In the control (untreated Sg-PC2 cells), NS5A protein was detected both in Golgi and RER fractions (Fig. 7A). The peroxide treatment significantly reduced the amount of NS5A in the Golgi fraction and altered the ratio of NS5A in Golgi and RER from roughly 30:70 to about 4:96 (Fig. 7A). Similar changes were observed with NS3 protein (Fig. 7A). In contrast,

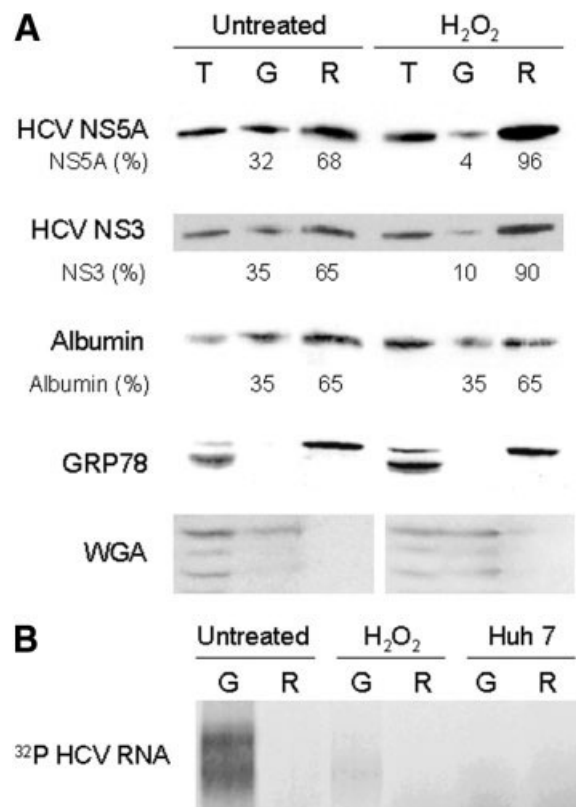


Fig. 7. Subcellular membrane fractionation for analysis of HCV replication complex. Sg-PC2 cells were treated with 0 or $100 \mu M H_2O_2$ for 3 hours. These and naïve Huh7 cells were then subjected to membrane fractionation experiments, as described in Materials and Methods. (A) Western blot analysis for NS5A, NS3, albumin, GRP78, and proteins containing complex-type glycans. T represents total cell lysates prior to fractionation, and G and R represent the Golgi and RER fractions, respectively. Proteins containing complex-type glycans were analyzed using WGA. Each of the protein bands was quantified by densitometry. (B) *In vitro* HCV RNA replication analysis. Golgi and RER membrane fractions were subjected to *in vitro* HCV RNA replication assays as described in Materials and Methods. $^{32}P\text{-labelled}$ HCV transcripts were analyzed by RNA gel electrophoresis and autoradiography.

peroxide did not affect the subcellular localization of albumin. To ensure that this gradient faithfully separated Golgi and RER membranes, each fraction was also analyzed with the anti-GRP78 antibody and WGA. GRP78 is an endoplasmic reticulum (ER)-associated protein and WGA binds to proteins with complex-type glycans in the *trans*-Golgi. As shown in the same figure, GRP78 was found in the RER fraction while WGA was primarily bound to proteins in the Golgi fraction.

To understand how the loss of NS5A and NS3 from the Golgi fraction might affect HCV RNA replication, each of the fractions in Figure 7A was subjected to *in vitro* HCV replication assays. Although the majority of these proteins were found in the ER fraction in these experiments (Fig. 7A), HCV RNA replicating activity was found mostly in the Golgi fraction (Fig. 7B). H₂O₂ treatment suppressed the HCV RNA replication in this fraction (Fig. 7B). As H₂O₂ also reduced the HCV protein level in the Golgi fraction, the suppression of HCV RNA replication by H₂O₂ in this fraction was likely due to the loss of active HCV RNA replication complexes.

The finding that active HCV RNA replication complexes were mostly identified in the Golgi fraction may be due to the association of the HCV replication complexes with the Golgi membranes as suggested by Serafino et al.³⁷ Alternatively, lipid rafts and membrane webs, which have also been reported to contain HCV replication complexes,^{38–40} might cosediment with the Golgi membranes in our experiments. It should be noted that lipid rafts are also known to form in the Golgi complex.⁴¹ The close association of NS5A and NS3 with the HCV RNA replication activity is in support of the previous observations that these proteins are important components of the HCV replication complexes.^{42,43} The reduction in the amount of NS5A/NS3 in the Golgi fraction indicates a possible disruption of the replication complexes or the perturbation of intracellular transport of these proteins by ROS. If it is the latter, the effect was highly specific, as the transport of albumin as well as proteins containing the complex glycans was not affected (Fig. 7).

In this report, we showed that ROS, within the biologically relevant concentration range, could suppress HCV RNA replication in Huh7 cells. The rapid response of HCV RNA replication to ROS is suggestive of a mechanism that involves signaling. ROS are known to affect diverse signaling pathways.⁴⁴ These signaling events could either rapidly disrupt existing active HCV replication complexes or suppress the formation of new complexes in Huh7 cells, to result in a reduced amount of NS5A and NS3 and, hence, HCV RNA replicating activity in the Golgi fraction. It will be interesting to identify the molecular targets of ROS that eventually affect HCV replica-

tion complexes. Finally, an important corollary to our findings might be that the antioxidants, which are currently being investigated as potential adjunct therapy for various liver diseases,⁴⁵ might in fact facilitate HCV replication by counteracting ROS in these patients, as suggested in Figure 2D. Due to the large number of HCV patients, further investigation in this research area is warranted.

Acknowledgment: The authors acknowledge Anne Strohecker for her assistance with the construction of the HCV subgenomic replicon, and Dr. Stanley Lemon for pRL-HL construct. The authors thank Dr. Henry Jay Forman and Dr. Rui-Ming Liu for critical reading of this manuscript.

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