Cu/Zn superoxide dismutase (SOD1) induction is implicated in the antioxidative and antiviral activity of acetylsalicylic acid in HCV-expressing cells

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hepatitis C virus; oxidative stress; antioxidants; pyrrolidine dithiocarbamate; glutathione peroxidase

Hepatitis C virus (HCV) is one of the most important causes of chronic liver disease in the world (10). Chronic liver disease is the main cause of cirrhosis, liver failure, and liver cancer. Chronic hepatitis C can progress to cirrhosis and eventually to hepatocellular carcinoma over a period of 20 to 30 yr (7).

Current therapeutic strategies for chronic hepatitis C have been restricted mainly to a combination of pegylated interferon-α and ribavirin. Unfortunately, this is effective in less than 50% of patients infected with genotype 1 (18).

The mechanisms by which HCV causes cell damage are poorly understood, and different mechanisms have been suggested in its pathogenesis with oxidative stress having a major role in chronic HCV infection (6). HCV infection is associated with increasing levels of several oxidative stress markers, such as chronic inflammation, iron overload, and mitochondrial injury, all of those presumably induced by HCV proteins (2, 6, 24, 30). Several lines of evidence support this contention, including the existence of activated glutathione turnover, the presence of increased levels of lipid peroxidation products, augmented iron stores in the liver, and the finding of low reduced glutathione values in peripheral blood mononuclear cells and erythrocytes (2, 17, 24). Moreover, it has been shown that patients with chronic hepatitis C exhibit an increased production of tumor necrosis factor-α (TNF-α), a cytokine that can produce oxidative stress by stimulating the generation of reactive oxygen species (ROS) (45) such as superoxide ion (O₂⁻) and hydrogen peroxide (H₂O₂). Cells are protected against oxidative insults by natural antioxidant products, notably glutathione, and diverse antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (22). Previous studies have reported the beneficial effect of antioxidants such as N-acetyl-L-cysteine (17) and vitamin E on decreasing HCV replication (27).

Aspirin (acetylsalicylic acid, ASA), a nonsteroidal antiinflammatory drug, is now widely used for the prevention of cardiovascular disease (4, 16) for reducing the risk of Alzheimer’s disease (19, 33), and for colon cancers (3, 36). Because its mechanism consists in inhibiting cyclooxygenase activity, most studies have focused on studying this mechanism of action. Also, few new therapeutic applications have been reported (5, 28, 38, 41).

We previously reported that ASA reduces HCV-RNA and viral protein levels; however, this mechanism remains poorly defined. Our findings indicate that ASA could be used as an antioxidative, anti-inflammatory, and anti-HCV agent (13, 43). However, there is no information on whether its antioxidant properties could be implicated in the ASA-mediated downregulation of HCV expression reported previously by our group. Our aim in this study was to highlight information on the mechanisms involved in the antiviral effect of ASA by measuring oxidative stress markers and cellular antioxidant...
systems using an HCV subgenomic replicon cell culture system. Furthermore, a potent antioxidant, pyrroline dithiocarbamate (PDTC), was used as a positive control because of its effect as an antioxidant (14). Our findings suggest that the antiviral activity of ASA could be mediated at least in part by its antioxidant properties and the induction of Cu/Zn-SOD expression.

**MATERIALS AND METHODS**

**Cell culture and aspirin and PDTC treatment.** We used a genotype 1b HCV subgenomic replicon cell culture system described previously (26). Huh7 cells were maintained in advanced DMEM (ADMEM; GIBCO, Grand Island, NY) supplemented with 2% heat-inactivated FBS (GIBCO), 1% nonessential amino acids, 100 U of penicillin G, and 100 μg of streptomycin per milliliter at 37°C in a humidified atmosphere with 5% CO₂. Cells containing the HCV replicon were maintained in culture in the presence of 500 μM H9262.

**Expression of SOD and GPx activity.** The antiviral activity of ASA could be mediated at least in part by its antioxidant properties and the induction of Cu/Zn-SOD expression. Our findings suggest that the antiviral activity of ASA could be mediated at least in part by its antioxidant properties and the induction of Cu/Zn-SOD expression.

**Immunofluorescence to detect SOD and NS5A protein.** Huh7 HCV replicon cells were grown on glass coverslips and treated with 4 mM ASA at the time points indicated. They were then washed twice with trypsin-EDTA and PBS and fixed in 4% paraformaldehyde/PBS for 10 min at room temperature. Fixed cells were incubated with 15 mM NH₄Cl for 5 min and permeabilized in 0.25% Triton X-100 in PBS for 30 min and blocked with PBS 3% BSA for 30 min. After this time, cells were fluorescently labeled with an anti-Cu/Zn SOD polyclonal antibody (1:500; Novus Biologicals, Littleton, CO) and anti-NS5A monoclonal antibody (1:1,000; Biodisign International, Saco, ME). The next day, coverslips were washed with PBS, incubated with AlexaFluor 594 anti-rabbit (1:2,000 dilution), and Alexa Fluor 488 anti-mouse antibodies (Molecular Probes, 1:1,000 dilution, 1 h, at room temperature). Finally, cells were mounted onto glass slides in ProLong Gold antifade reagent with 4’,6-diamino-2-phenylindole (DAPI) (Molecular Probes) for counterstaining of cell nuclei. Confocal images were collected using a Nikon Eclipse E600 upright confocal microscope (Nikon, Kanagawa, Japan) and a Zeiss AxioCam color camera (AxioVision software) (Carl Zeiss, Göttingen, Germany). All the confocal images shown were single optical sections.

**RNA extraction.** Total RNA was extracted from Huh7 HCV replicon cells using Trizol (Life Technologies, Carlsbad, CA) according to the manufacturer’s specifications. RNA precipitates were then washed once in 75% alcohol and resuspended in 30 μl of RNase-free water.

**RT-PCR for cytosolic SOD-mRNA semiquantification.** Huh7 cells were treated with 4 mM ASA at the time points indicated and then harvested. Total cellular RNA was extracted and subjected to reverse transcription (RT). Two hundred nanograms of each cDNA were amplified by PCR for 30 cycles, each consisting of 2 min at 95°C followed by cycles of 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final incubation at 72°C for 3 min. A set of primers, upper (sense: 5’ATATTTATG-GATCTTATGGCGACGAAGGC- CCGTGTGC3’; HCV Forward (5’-GGTTCCGCAGAC- CTGCACGACACTCATAC-NFQ-3’), lower (antisense: 5’ATATATATG-GATCTTATGGCGACGAAGGC- CCGTGTGC3’) and lower (antisense: 5’ATATATATG-GATCTTATGGCGACGAAGGC- CCGTGTGC3’), were used to amplify a fragment of 465 base pairs (bp) of the Cu/Zn SOD gene.

**Real-time RT-PCR for HCV-RNA quantification.** Total RNA extracted was subjected to RT-PCR with a high-capacity complementary DNA (cDNA) archive kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s specifications. cDNAs (200 ng) were subjected to real-time PCR for HCV and GAPDH mRNA quantification. Amplifications were conducted in triplicate with the following primers: HCV Forward (+75-93 nt), 5’-GGTCTCAGAGGCTTCAGTTG-3’; HCV Reverse (−138–157 nt), 5’-GTTTCCGCAGAC-CCTACATGG-3’. A set of primers for GAPDH (5’-FAM- CTGCAGCACTACTCATA-NFQ-3’) was used to amplify a fragment of 483 base pairs (bp) of the GAPDH gene. A standard curve ranging from 0.156 to 20 U/ml was generated. The colorimetric assay was done measuring formazan dye on reduction with a superoxide anion. Samples were tested and a standard curve ranging from 0.156 to 20 U/ml was generated. The colorimetric assay was done measuring formazan dye on reduction with a superoxide anion.
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Aspiration, we used GAPDH (20×) assay (Applied Biosystems) according to the manufacturer’s specifications.

**Intracellular PGE2 measurements.** At different time points of each treatment, Huh7 replicon cells were harvested and cell membranes hydrolyzed to release intracellular prostaglandin E2 (PGE2). PGE2 levels were then assayed with a PGE2 enzyme-linked immunosorbent assay system (Amersham Biosciences, Hercules, CA) according to the manufacturer’s protocol. The minimum detectable level in this assay was 2.5 pg/well.

**RNAi assay.** A prevalidated heterogeneous mixture of 21–22-bp short interfering RNAs (siRNA) that specifically inhibit SOD1 expression was used according to the manufacturer’s specification (Ambion, Carlsbad, CA). Huh7 HCV replicon cells (5 × 10⁵) were seeded on six-well plates in ADMEM containing 2% FBS, 1% nonessential amino acids, 100 U of penicillin G/ml, and 100 μg of streptomycin/ml. The next day, cells were transfected to serum-free DMEM with siRNA directed against SOD1 (siRNA-SOD1) (100 nM). Cells were at 30%-50% confluence at the time of transfection and were incubated in the presence or absence of 4 mM ASA 30 min after transfection. siRNAs were transfected at a final concentration of 100 nM for SOD1, using siPORT Lipid Transfection Agent (Ambion). We used silencer negative control siRNA (100 nM) (AM4611; Ambion) and untransfected cells containing siPORT Lipid Agent alone as negative controls. Cells were incubated for 24–72 h. After each incubation time, total RNA was extracted and cDNA was synthesized by RT-PCR. From the cDNA, we performed real-time qPCR to quantify SOD1 mRNA and HCV-RNA, respectively. HCV-RNA levels were normalized based on the ratio of HCV/GAPDH mRNA that was amplified in the same plate by the real-time RT-PCR. Data are expressed as HCV-RNA levels relative (fold) to cells transfected with silencer negative control siRNA, which is defined as 1.0.

**Statistical analysis.** All variables were tested in triplicate, and experiments were repeated at least three times. All values were expressed as means ± SD. One-way ANOVA was used to test for differences in means, and the t-test was used for comparisons. The differences were considered significant if P < 0.05.

**RESULTS**

ASA decreases ROS levels in parental and HCV replicon cells. HCV-infected hepatocytes are known to produce ROS, which promote lipid and protein oxidation. To evaluate whether ASA treatment could have an antioxidant activity in our cell system, ROS levels were examined using the DCF-HDA assay. Parental and HCV replicon-containing cells were incubated in the presence or absence of 4 mM ASA up to 72 h, and then ROS levels were measured. First of all, we found different ROS levels when comparing both cell lines at the same conditions, observing higher levels in cells expressing HCV nonstructural proteins (Fig. 2C). Additionally, we found that ASA reduced ROS levels in a time-dependent fashion in both cell types (parental and HCV replicon cells) (Fig. 1, A and B, respectively) with a greater effect at 72 h posttreatment in HCV replicon cells (about a 45% decrease; *P < 0.05) (Fig. 1B).

As a positive control of antioxidant activity, we treated replicon cells with 5 μM PDTC. Figure 1C shows that PDTC decreased ROS levels starting at 24 h and showing a maximum decrease at 72 h posttreatment (*P < 0.05). Together, these results confirm that ASA and PDTC have an antioxidative effect.

**Aspirin diminished oxidized protein levels.** To further confirm whether ASA had an antioxidant effect on Huh7 cells, we incubated parental and HCV replicon cells in the presence or absence of 4 mM ASA. Total protein extracts were prepared, and oxidative damage of proteins was evidenced by immunoblotting the dinitrophenylhydrazone derivatives of protein carbonyls, followed by densitometric scanning as described before. Oxiblot results demonstrated that oxidized protein levels increase according to the culture time of hepatoma cells (Fig. 2). Furthermore, oxidized protein levels were higher in HCV replicon cells compared with parental cells, particularly at 24 and 48 h without treatment, demonstrating that only the presence of HCV proteins induces higher oxidative stress levels in Huh7 cells (measured in relative absorbance units; Fig. 2, A and B).

**FIG. 1.** Acetylsalicylic acid (ASA) decreases lipid peroxidation in both Huh7 cells types. A and B: effect of ASA on reactive oxygen species (ROS) levels in Huh7 parental and hepatitis c virus (HCV) replicon cells. Parental and HCV replicon-containing cells (5 × 10⁵ cells) were incubated with or without ASA (4 mM) at different time points (0–72 h). At the end of incubation, ROS levels were assessed in total cellular extracts by using 2',7'-dichlorofluorescein diacetate (DCF-HDA) fluorescence assay. C: pyrrolidine dithiocarbamate (PDTC) was used as a control because of its effect as an antioxidant. HCV replicon cells (5 × 10⁵ cells) were treated with a potent antioxidant (5 μM PDTC) for 0 to 72 h. The ROS levels were then determined. The data are the means ± SD of triplicate cultures, and the experiment was repeated 3 times (*P < 0.05).
Interestingly, we found that ASA treatment dramatically decreased oxidized protein levels in both cell types (parental and replicon cells) at 24 (about 28%), 48 (about 50%), and 72 (about 40%) h, showing an antioxidant effect in this cell system (Fig. 2, A and B). These data together suggest the possibility that the time-dependent increased lipid (Fig. 1) and oxidized protein levels may reflect the cellular response to intrinsic oxidative stress in hepatoma cells, and ASA treatment prevented these unexplained increases in both parental and HCV replicon-containing cells, to a greater extent in the latter.

**Inhibition of mitochondrial electron transport by rotenone and DPI decreased ROS levels.** To further determine the site of HCV-induced intracellular ROS production, Huh7 parental and replicon cells were first incubated with either rotenone, a mitochondrial e-transport inhibitor of complex I, or DPI, a NADPH-dependent oxidase inhibitor, and then ROS production was measured in cells with DCF-HDA assay upon 24 h. In both cell lines, the endogenous ROS production was inhibited by DPI and rotenone (Fig. 2C). Normalizing levels of mitochondrial ROS with each of these agents prevents ROS accumulation. This result suggests that NADPH oxidase and the mitochondria respiratory chain are required for inducing ROS production in these cells.

**Effect of aspirin and PDTC on GPx and SOD activity.** Additionally, we were interested in determining whether ASA could influence cellular antioxidant systems, such as GPx and SOD enzymes. GPx catalyzes the reduction of hydroperoxides including hydrogen peroxides by using reduced glutathione and functions to protect the cell from oxidative damage. We measured GPx activity indirectly by a coupled reaction with glutathione reductase. Parental and HCV replicon-containing cells were incubated in the presence or absence of 4 mM ASA until 72 h, and then average GPx activity was measured. It is important to point out that, contrary to what we expected, average GPx activity was reduced in ASA-treated cells in a time-dependent fashion, as shown in Fig. 3, reaching maximum inhibition at 12 and 48 h (*P < 0.05) in both parental and HCV replicon cells compared with their control (nontreated cells) (Fig. 3, A and B). This inhibitory effect of ASA was not likely due to its cytotoxic effect because total protein synthesis did not differ significantly among treated and nontreated cells with the same amount of ASA (data not shown). These data suggest that ASA treatment may either have reduced the expression rate of the enzyme protein or reduced protein stability affecting its enzymatic activity. We observed similar
results when cells were treated with 5 μM PDTC as a positive control under the same conditions (Fig. 3C). These findings in ASA-treated cells demonstrate that antioxidant status was compromised with several important components of the antioxidant defense mechanism being significantly decreased.

Additionally, the influence of ASA on Cu/Zn SOD from parental and HCV replicon cells treated with aspirin was evaluated. We observed a striking increase in average cytosolic SOD activity in both cell types. Parental and HCV replicon-containing cells (5 × 10⁵ cells) were incubated in the presence or absence of 5 μM PDTC for 0, 12, 24, and 48 h, and SOD activity was determined as described above. D and E: effect of ASA on cytosolic SOD activity in both cell types. Parental and HCV replicon-containing cells (5 × 10⁵ cells) were incubated in the presence or absence of 4 mM ASA at different time points (0, 12, 24, 48, and 72 h). At the end of incubation, cells were lysated, total proteins extracts were prepared, and cytosolic SOD activity was measured as described above. The above-mentioned results suggest the possibility that ASA treatment could modify the expression of SOD and exert a negative effect on HCV-RNA levels. To investigate this possibility, we first evaluated Cu/Zn SOD expression and its subcellular localization by immunofluorescence analysis in HCV replicon cells exposed to ASA, at the same time points in which we observed the highest HCV-RNA downregulation (48 and 72 h) (Fig. 4, A and B). HCV replicon cells were untreated or stimulated with 4 mM PDTC (12 and 48 h) as a control under the same conditions. Together these results suggest that ASA treatment could induce cytosolic SOD activity.

Increased SOD expression vs. diminished NS5A expression in replicon cells with ASA treatment. The above-mentioned results suggest the possibility that ASA treatment could modify the expression of SOD and exert a negative effect on HCV-RNA levels. To investigate this possibility, we first evaluated Cu/Zn SOD expression and its subcellular localization by immunofluorescence analysis in HCV replicon cells exposed to ASA, at the same time points in which we observed the highest HCV-RNA downregulation (48 and 72 h) (Fig. 4, A and B).
mRNA levels at the same times points of exposure (Fig. 5, replicon cells showed a dramatic increase in cytosolic SOD- of Cu/Zn SOD-mRNA at 48 and 72 h with ASA treatment, Whereas Huh7 parental cells showed equal/decreasing levels parental and replicon cells with ASA treatment were different. SOD-mRNA responses of tentative RT-PCR, and the results obtained were inside the linear range (Fig. 5). We observed that SOD-mRNA responses of HCV replicon-containing cells were incubated in 15 mM NH4Cl for 5 min and permeabilized in 0.25% Triton X-100 in PBS for 30 min and blocked with PBS 3% BSA for 30 min. Cells were fluorescently labeled with an anti-Cu/Zn SOD polyclonal antibody (1:500 dilution) and anti-NS5A monoclonal antibody (1:1,000). The next day, cove rsips were washed with PBS, incubated with AlexaFluor 594 anti-rabbit (1:2,000) and Alexa Fluor 488 anti-mouse antibodies (Molecular Probes, 1:1,000, 1 h, at room temperature). Finally cells were mounted onto glass slides in ProLong Gold antifade reagent with 4',6-diamino-2-phenylindole (DAPI) for counterstaining of cell nuclei. Confocal images were collected using a Nikon Eclipse E600 upright confocal Microscope and a Zeiss AxioCam color camera.

ASA for 48 and 72 h, and then cytosolic SOD1 and NS5A viral protein levels were detected by immunofluorescence using specific anti-SOD and anti-NS5A antibodies. We observed that HCV replicon cells expressed higher levels of Cu/Zn SOD with ASA treatment compared with untreated cells in the same time frame (48 and 72 h), whereas NS5A protein levels showed reduced expression in ASA-treated cells (Fig. 4, A and B). Second, we evaluated the effect of ASA on Cu/Zn SOD- mRNA levels. HCV replicon-containing cells were incubated in the presence or absence of ASA at different times (0–72 h); then Cu/Zn SOD-mRNA levels were analyzed by semiquantitative RT-PCR, and the results obtained were inside the linear range (Fig. 5). We observed that SOD-mRNA responses of parental and replicon cells with ASA treatment were different. Whereas Huh7 parental cells showed equal/decreasing levels of Cu/Zn SOD-mRNA at 48 and 72 h with ASA treatment, replicon cells showed a dramatic increase in cytosolic SOD- mRNA levels at the same times points of exposure (Fig. 5, A and B). Together, these data suggest that ASA increased Cu/Zn SOD-mRNA expression in replicon cells, a phenomenon that could contribute to the mechanism involved in the downregulation of NS5A expression in the same cells.

Inhibition of SOD1 expression reverses the effect of ASA. To further confirm the role of SOD1 in the antiviral action of ASA, we achieved specific inhibition of SOD1 using siRNA technology in replicon cells (SOD1-mRNA was inhibited around 50–60% at 48–72 h, respectively) (data not shown) treated and untreated with ASA, and then we measured HCV- RNA expression (Fig. 5C). When we introduced siRNA to downregulate the expression of SOD1, HCV-RNA levels were decreased at 48 and 72 h compared with control cells (untreated and cells transfected with nonsense control siRNA) (Fig. 5C). However, when we downregulated SOD1 expression in cells treated with 4 mM ASA, the HCV-RNA levels were greater than those observed in cells treated only with siRNAs and cells treated only with ASA (Fig. 5C). We found that siRNAs-SOD1 partially reversed the negative effect of ASA on HCV expression. Together, these results suggest that SOD1 activity may play a role in the modulation of HCV subgenomic replication by ASA in cultured cells.

PDTC treatment downregulates subgenomic HCV-RNA expression and PGE2 in a time-dependent fashion. The suggested antioxidant property of ASA and the parallel results with PDTC downregulating oxidative stress markers seen in this research prompted us to suspect the possibility that PDTC could exert a similar negative effect on HCV-RNA levels like that observed with ASA treatment (43). On the basis of this, we next explored the effect of PDTC (5 µM) on HCV-RNA expression in HCV replicon cells at three different time points (24, 48, and 72 h). Total cellular RNA was then extracted and subjected to real-time RT-PCR for HCV-RNA quantification. As a positive control, we incubated these cells with 4 mM ASA under similar conditions. We observed that PDTC inhibited HCV-RNA expression in a time-dependent fashion compared with untreated cells, showing the highest effect at 72 h after treatment (Fig. 6A), similar to the results observed in ASA-treated cells (Fig. 6B). These observations suggested that ASA and PDTC function as antioxidants, and perhaps inhibition of ROS overproduction by these compounds is involved in HCV-RNA downregulation.

Finally, we evaluated whether PDTC could inhibit PGE2 production in HCV replicon-treated cells compared with untreated cells. PGE2 levels were determined in Huh7 replicon cells in cultures exposed with PDTC (5 µM) (Fig. 7). As a positive control, we incubated these cells with 4 mM ASA under the same conditions. Interestingly, we found that PDTC exposure also reduced PGE2 levels up to 60% at 72 h in HCV replicon-containing cells compared with untreated cells (Fig. 7) (*P < 0.05). ASA-treated cells showed similar results (Fig. 7B).

**DISCUSSION**

ROS (45) and the disturbance of the redox balance have been implicated in the pathogenesis of many viral diseases, including hepatitis C. We have previously reported that ASA, at a concentration compatible with the amounts used for the treatment of patients undergoing anti-inflammatory therapy (1-5 mM), decreases HCV-RNA and viral protein levels, but the mechanism involved has not yet been completely established.
Fig. 5. Effect of ASA on SOD-mRNA levels and SOD1 silencing. A: Huh7 parental and HCV replicon cells (5 × 10^5 cells) were incubated with or without ASA (4 mM) at different time points (0, 24, 48, and 72 h). At end of incubation, total cell RNA was extracted at each time point, and Cu/Zn SOD-RNA levels were analyzed by semi-quantitative RT-PCR. PCR was performed at 30 cycles. RT-PCR for GAPDH mRNA was performed in parallel to show an equal amount of total RNA in each sample. B: ratio of Cu/Zn SOD/GAPDH mRNA from RT-PCR detection was quantified with Phoretix 1D v2003.02 software. Data are expressed as relative band intensities to control (cells without treatment), which is defined as 1.0. C: effect of SOD1 silencing on HCV-RNA expression. Huh7 replicon cells cultured in Advanced DMEM (ADMEM) supplemented with 2% FBS were plated at a density of 500,000 cells/well in 6-well culture plates. The next day, they were transfected in serum-free ADMEM with siRNA directed against SOD1 (siRNA-SOD1), silencer negative control siRNA (Ambion: AM4611) or with siPORT Lipid Agent alone. Total RNA was extracted at 24, 48, and 72 h posttransfection; then cDNA was synthesized by RT-PCR, and then we performed real-time qPCR assay to quantify SOD1-mRNA and viral RNA. HCV-RNA levels were normalized based on the ratio of HCV/GAPDH mRNA that was amplified in the same plate by the real-time RT-PCR. Data are expressed as HCV RNA levels relative (fold) to cells transfected with silencer negative control siRNA, which is defined as 1.0. Reactions were run on the AB Prism 7500 platform. The data shown are the means ± SD of triplicate cultures, and the experiment was repeated 3 times (*P < 0.05).
Anti-inflammatory compounds such as ASA, ibuprofen, and indomethacin were reported to be inhibitors of glycation, perhaps by preventing the oxidative stress associated with the formation of advanced glycation endproducts (protein-aging) (35). Aspirin contributes to the intracellular metabolism of ATP, inhibiting inducible nitric oxide synthase, modulating the activity of NF-κB (38) and MAPKs (15). According to the chemical structure of ASA, it can be readily incorporated after it is solubilized to salicylate. In this way, the drug has the capacity to grasp hydroxyl radicals, performing its antioxidant effect. Perhaps, this antioxidant property of ASA could explain the antiviral effect observed against HCV in replicon cells.

In the same cell line, we further evaluated cellular antioxidant systems by monitoring the enzymatic activity of GPx and SOD enzymes. First, contrary to what we expected, average GPx activity was decreased in ASA-treated cells compared with its control (nontreated cells) (Fig. 3, A and B). These data suggest that ASA treatment may either diminish the expression rate of the enzyme protein or decrease protein stability affecting its enzymatic activity. These results suggest that the antiviral effect of ASA could be mediated by blocking of GPx or its further products. Our results are in agreement with the results published by Levent et al. (23), who studied patients with chronic HCV infection who were under the influence of interferon-α-2b plus ribavirin combination therapy. In addition, Chrobot et al. (12) studied antioxidant enzymes levels and found diminished GPx levels in serum and erythrocytes in patients with abnormal liver function. Results of our study are consistent with these findings, but further experiments should be performed to confirm them.

Fig. 6. HCV-RNA expression upon ASA and PDTC treatment. A: Huh7 HCV replicon cells (2 × 10⁵ cells) were incubated with (5 μM) or without PDTC for 24, 48, and 72 h. B: Huh7 HCV replicon cells (2 × 10⁵ cells) were incubated with (4 mM) or without ASA for 24, 48, and 72 h. Upon PDTC and ASA treatment, HCV-RNA levels were quantified by real-time RT-PCR. RNA viral levels were normalized based on the ratio of HCV/GAPDH mRNA that was amplified in the same plate by the real-time RT-PCR. Data are expressed as HCV RNA levels relative (fold) to nontreated control, which is defined as 1.0. The data shown are the means ± SD of triplicate cultures, and the experiment was repeated 3 times (⁎P < 0.05).

Fig. 7. Effect of PDTC on prostaglandin synthesis. Huh7 HCV replicon cells (1 × 10⁴ cells) were incubated in presence or absence of PDTC (5 μM) and incubated at different time points (0, 12, 24, 48, and 72 h). As a positive control, we incubated these cells with 4 mM ASA under the same conditions. After PDTC and ASA treatment, intracellular prostaglandin E2 (PGE2) levels were then assayed by using the Biotrak Prostaglandin E2 Enzyme Immunoassay system (Amersham Biosciences, Hercules, CA) according to the manufacturer’s protocol (⁎P < 0.05).
Furthermore, we examined cytosolic SOD activity, mRNA, and protein expression. First, we found a striking increase in Cu/Zn SOD activity in Huh7 replicon cells upon ASA treatment. We further demonstrated by immunofluorescence analysis and RT-PCR a translational and transcriptional upregulation of Cu/Zn SOD expression in Huh7 replicon cells treated with ASA at the same time that it decreased HCV expression (Figs. 4 and 5). Because the activity of SOD is not saturable by the substrate, the only reasonable way to increase SOD activity is by increasing mRNA and protein levels, as we have shown before. It is important to note that additional experiments should be performed to elucidate the role of the major SOD product, hydrogen peroxide, which may cause chemical changes and modify several signal transduction pathways into the cells (20). In addition, we found that inhibition of SOD1 expression reversed the effect of ASA. Together, these results suggest that SOD1 activity may play a role in the modulation of HCV subgenomic replication by ASA in cultured cells. Therefore, if aspirin can modify oxidative stress levels, this effect can provide hepatocytes with the capacity to counteract the harmful effects induced by HCV infection.

There are a few reports of the effect of ASA on SOD activity and expression. Polat et al. (32) demonstrated that ASA administration decreased antioxidant enzyme activities after bile duct ligation in rats, being statistically significant for CAT and GPx, but not for SOD activity. In another report, Pratap et al. (34) demonstrated that aspirin reduced thiobarbituric acid-reactive substance levels and induced elevation of GSH, SOD, and catalase levels in a middle cerebral artery occlusion model of focal cerebral ischemia in rats. These findings are also supported by Cai et al. (9), showing that glutathione peroxidase and SOD were inhibited in mice treated with ASA. Contrary to this, there is a report by the same group where they observed a smaller increase in SOD, glutathione transerase, and GPx activities when male C57BL/6 mice were exposed to ASA. Nevertheless, it is likely that SOD plays another role in the cell regulation of oxidative stress signaling pathways although the stimuli for this are yet to be ascertained.

Finally, we compared ASA-mediated antioxidant effect with this of a potent antioxidant molecule (PDTC) (14) in HCV replicon-containing cells. Cells were treated in parallel in the presence or absence of ASA or PDTC. Surprisingly, PDTC inhibited HCV-RNA expression and prostaglandin production in the same way as ASA, in addition to its antioxidant property, in a time-dependent fashion compared with untreated cells (Fig. 6, A and B). Recently, the antiviral function of PDTC against influenza virus through the inhibition of viral gene replication and transcription has been reported (44). PDTC is known to regulate gene expression and/or activity of cellular antioxidant enzymes or transcription factors (44). PDTC could act as an inhibitor of HCV RNA-dependent RNA polymerase, as has been shown for influenza virus, but also as a modulator of cell factors associated with viral gene replication or transcription. These findings give rise to several interesting questions, about the possible immunomodulating activities of this compound, but further experiments should be performed to highlight information in this context. Several clinical trials have previously suggested a beneficial effect of antioxidants in patients with chronic HCV infections (1, 8, 11, 39, 45, 46).

Because of the critical role in both viral and cellular gene expression, HCV-induced ROS generation might constitute an excellent target for any therapeutic attempt to inhibit the cellular changes that are mediated by HCV infection and which might contribute to cirrhosis development. In addition, here we show that this increase in ROS is prevented by inhibitors of electron transport chain in mitochondria. In summary, the above-mentioned results suggest the possibility that ASA treatment could reduce cellular oxidative stress markers and modify Cu/Zn SOD expression, a phenomenon that could contribute to the mechanisms involved in the downregulation of HCV expression.

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DISCLOSURES
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AUTHOR CONTRIBUTIONS

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