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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Abstract

We evaluated the participation of oxidative stress in the negative regulation of HCV-RNA induced by acetylsalicylic acid (ASA). We used the HCV subgenomic-replicon cell system that stably expresses HCV-nonstructural proteins (Huh7 HCV-replicon cells) and the parental cell line. Cells were exposed to 4mM ASA at different times (12-72h) and pyrrolidine dithiocarbamate (PDTC) was used as an antioxidant control. Reactive oxygen species (ROS) production and oxidized proteins levels, cytosolic superoxide dismutase (Cu/Zn-SOD) and glutathion peroxidase (GPx) activity were measured to evaluate oxidative stress. In addition, viral RNA and Prostaglandin (PGE2) levels were determined. We observed that ASA treatment decreased ROS production and oxidized protein levels in a time-dependent fashion in both parental and HCV-replicon cells with a greater extent in the latter. Similar results were found with PDTC exposure. Average GPx activity was decreased, while a striking increase was observed in average cytosolic SOD activity at 48 and 72h in both cells exposed to ASA, compared with untreated cells. HCV-replicon cells showed higher levels of Cu/Zn-SOD expression (mRNA and protein) with ASA treatment (48 and 72h), whereas NS5A protein levels showed decreased expression. In addition, we found that inhibition of SOD1 expression reversed the effect of ASA. Interestingly, PDTC downregulated HCV-RNA expression (55%) and PGE2 (60%) levels imitating ASA exposure. These results suggest that ASA-treatment could reduce cellular oxidative stress markers and modify Cu/Zn-SOD expression, a phenomenon that may contribute to the mechanisms involved in HCV downregulation.

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**Key words:** Hepatitis C Virus (HCV), Oxidative stress, Antioxidants, Pyrrolidine dithiocarbamate (PDTC), Glutathione peroxidase (GPx).

**Abbreviations:** ASA, acetylsalicylic acid; COX-2, cyclooxygenase-2; Cu/Zn-SOD, superoxide dismutase; GPx, Glutathione peroxidase; HCV, hepatitis C virus; NF-κB, nuclear factor kappa B; NSAIDs, nonsteroidal anti-inflammatory drugs; PDTC, pyrrolidine dithiocarbamate; PGE₂, prostaglandin E₂; RAU, relative absorbance unit; RT-PCR, reverse transcription polymerase chain reaction; ROS, reactive oxygen species, 2′,7′-dichlorofluorescein diacetate (DCF-HDA).

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Introduction

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 (HCC) over a period of 20 to 30 years (7). Current therapeutic strategies for chronic hepatitis C have been restricted mainly to a combination of pegylated interferon-alpha 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-alpha (TNF-a), a cytokine that can produce oxidative stress by stimulating the generation of reactive oxygen species (45) such as superoxide ion (\( \cdot O_2^- \)) and hydrogen peroxide (H\(_2\)O\(_2\)). Cells are protected against oxidative insults by natural antioxidant products, notably glutathione, and diverse antioxidant enzymes such as
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Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (22). Previous studies have reported the beneficial effect of antioxidants such as N-acetyl-L-cystein (17) and Vitamin E on decreasing HCV replication (27).

Aspirin (acetylsalicylic acid, ASA), a nonsteroidal anti-inflammatory drug (NSAID), 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). Since its mechanism consists in inhibiting cyclooxygenase (COX) 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, pyrrolidine dithiocarbamate (PDTC), was used as a positive control due to 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.
EXPERIMENTAL PROCEDURES

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 Dulbecco’s Modified Eagle Medium (ADMEM GIBCO-BRL; Grand Island, NY, USA) supplemented with 2% heat-inactivated Fetal Bovine Serum (FBS; GIBCO-BRL; Grand Island, NY, USA), 1% nonessential amino acids, 100 U of penicillin G and 100 μg of streptomycin per mL at 37°C in a humidified atmosphere with 5% CO₂. Cells containing the HCV replicon were maintained in culture in the presence of 500 μg of G418/mL (Geneticin; GIBCO-BRL), which was removed two days before the experiments. For the different treatments, Huh7 HCV replicon cells were plated one day before and then media was changed and cells were treated with 4 mM ASA or 5 µM PDTC considering this moment as time zero. The cells were incubated for up to 72 hours. As previously reported, viability assays demonstrated that there are no cytotoxic effects of aspirin and PDTC at the concentrations used on parental and HCV replicon-containing cells (43). It has been reported that concentrations of salicylates between 1 and 5 mM are recommended for treatment of patient with chronic inflammatory diseases since concentrations greater than 6.5 mM are toxic for clinical use (25). To determine the site of HCV-induced intracellular reactive oxygen species production, both cell lines were plated as above and incubated with either 5µM rotenone (Sigma, St. Louis, MO), or 50 µM diphenylene iodonium (DPI) (Molecular Probes, Carlsbad, CA), and then reactive oxygen species (ROS) production was measured in cells with DCF-HAD assay upon 24 hours.
Total protein extraction. After each treatment, cells were incubated for the indicated time and total cell lysates were prepared and proteins extracted as previously described (37). Subsequently, the protein extracts were quantified using the Bradford method (BIO-RAD, Hercules, CA, USA). A standard curve was generated using bovine serum albumin (Amresco, Solon, Ohio). Total proteins were stored for further Oxiblot analysis, and SOD and GPx determinations.

Oxidative Stress Markers. To evaluate oxidative stress levels in Huh7 cells, two major indicators were measured: protein carbonylation and ROS production. Detection of oxidatively damaged proteins was done with an Oxiblot-Oxidative Protein detection kit (Chemicon International Temecula California), according to the manufacturer's recommendation. In addition, the intracellular ROS levels were assessed in total cellular extracts by using the 2',7'-dichlorofluorescein diacetate (DCF-HDA) (Molecular Probes, Carlsbad, CA) fluorescence assay, as described previously (21,32). Briefly, after preincubation with above mentioned chemicals, cells were exposed to DCF-HDA (2mM, in DMSO) for an additional 60 min to detect ROS production. DCF fluorescence of aliquots of cells was measured at room temperature using 488 nm and 525 nm as excitation and emission wavelengths, respectively.

Glutathione peroxidase (GPx) activity. GPx catalyzes the reduction of hydroperoxides, including hydrogen peroxide, using reduced glutathione and functions to protect the cell from oxidative damage. Determinations of GPx activity in Huh7 cell extracts treated or untreated with ASA and PDTC at indicated times were performed using the Total Glutathione Peroxidase Assay Kit (ZeptoMetrix, Buffalo, NY). Assays
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were performed according to the manufacturer's instructions. All determinations were performed in triplicate.

Superoxide Dismutase activity. SOD activity was determined in Huh7 cell extracts, treated or untreated with ASA and PDTC, at indicated times by a SOD Assay Kit-WST (Cayman Chemical Company, Ann Arbor, MI) according to the manufacturer's instructions. In brief, this kit allows highly sensitive SOD assay by using a highly water-soluble tetrazolium salt, WST-1 \( [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt] \), which produces a water-soluble formazan dye on reduction with a superoxide anion. Samples were tested and a standard curve ranging from 0.156 to 20 units/mL was generated. The colorimetric assay was done measuring formazan produced by the reaction between WST-1 and superoxide anion \( (·O_2^-) \). The rate of reduction with \( ·O_2^- \) was linearly related to xanthine oxidase activity and was inhibited by SOD. Absorbance was obtained with a microplate reader reading at 450 nm. This assay measures all three types of SOD (Cu/Zn, Mn and FeSOD). All determinations were performed in triplicate.

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 minutes at room temperature. Fixed cells were incubated with 15 mM \( \text{NH}_4\text{Cl} \) for 5 minutes and permeabilized in 0.25% Triton X-100 in PBS for 30 minutes and blocked with PBS 3% BSA for 30 minutes. After this time, cells were fluorescently labeled with an anti-Cu/Zn SOD polyclonal antibody (1:500; Novus
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Biologicals, Littleton, CO) and anti-NS5A monoclonal antibody (1:1000; Biodesign International, Saco, ME). The next day, coverslips were washed with PBS, incubated with AlexaFluor 594 anti-rabbit (1:2000 dilution) and Alexa Fluor 488 anti-mouse antibodies (Molecular Probes, 1:1000 dilution, 1 hour, at room temperature). Finally, cells were mounted onto glass slides in ProLong Gold antifade reagent with 4',6-diamino-2-phenylindole (DAPI)(Molecular Probes, Carlsbad, CA) 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 Corporation, 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 was amplified by PCR for 30 cycles, each consisting of 2 minutes at 95°C followed by cycles of 95°C for 1 minute, 60°C for 1 minute and 72°C for 1 minute, with a final incubation at 72°C for 3 minutes. A set of primers, upper (sense; 5´ATATTAATGGATCCTATGGCGACGAAGGCCGTGTGC3´) and lower (antisense; 5´AATATATATGCGGCCGCATTATTGGGCGATCCCAATTACACC3´), were used to amplify a fragment
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of 465 base pairs (bp) of the Cu/Zn SOD gene. RT-PCR for GAPDH mRNA (yielding a 483 bp fragment) was performed in parallel to show an equal amount of total RNA in each sample.

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 glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA quantification. Amplifications were conducted in triplicate with the following primers: HCV Forward (+75-93 nt), 5'-GCGTCTAGCCATGGCGTTA-3'; HCV Reverse (+138-157 nt), 5'-GGTTCCGCAGACCACATGG-3'; and the TaqMan probe (+94-110 nt), 5'-FAM-CTGCACGACACTCATA-NFQ-3'. Thermal cycling conditions were as follows: initial setup at 50°C for 2 minutes, then 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s and 60°C for 60s. For each PCR reaction, 12.5 µL of TaqMan PCR Master Mix, 1.25 µL of 20X Assay Mix, and 11.25 µL of cDNA diluted in RNase-free water were added. Fluorescence was monitored at the annealing step and the amplification plots were generated. GAPDH-RNA expression was used to normalize the RNA concentration. For GAPDH-RNA quantification, we used GAPDH (20X) assay (Applied Biosystems, Foster City, CA) according to the manufacturer's specifications.

Intracellular Prostaglandin E$_2$ (PGE$_2$) Measurements. At different time points of each treatment, Huh7 replicon cells were harvested and cell membranes hydrolyzed to release intracellular PGE$_2$. PGE$_2$ levels were then assayed with a PGE$_2$ enzyme-linked
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215 immunosorbent assay system (Amersham Biosciences, Hercules CA) according to the
216 manufacturer's protocol. The minimum detectable level in this assay was 2.5 pg/well.

217 **Statistical Analysis.** All variables were tested in triplicate, and experiments were
218 repeated at least 3 times. All values were expressed as mean ± SD. One-way analysis
219 of variance was used to test for differences in means, and the *t* test was used for
220 comparisons. The differences were considered significant if *P* < 0.05.

221 **RNAi assay**
222 A pre-validated heterogeneous mixture of 21-22 bp short interfering RNAs (siRNA) that
223 specifically inhibit SOD1 expression, was used according to the manufacturer's
224 specification (Ambion, Carlsbad CA). Huh7 HCV replicon cells (5 × 10^5 ) were seeded
225 on 6-well plates in Advanced DMEM containing 2% FBS, 1% nonessential
226 aminoacids, 100 U of penicillin G per mL and 100 μg of streptomycin per mL. The next
day, cells were transfected to serum-free DMEM with siRNA directed against superoxide
227 dismutase 1 (siRNA-SOD1) (100nM). Cells were at 30%-50% confluence at the time of
228 transfection and were incubated in the presence or absence of 4 mM ASA 30 minutes
229 after transfection. siRNAs were transfected at a final concentration of 100 nM for SOD1,
230 using siPORT Lipid Transfection Agent (Ambion; Carlsbad CA). We used silencer
231 negative control siRNA (100 nM) (Ambion: Part number AM4611, Carlsbad CA) and
232 untransfected cells containing siPORT Lipid Agent alone as negative controls. Cells
233 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
234 quantify SOD1 mRNA and HCV-RNA respectively. HCV-RNA levels were normalized based
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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.

RESULTS

ASA decreases ROS levels in parental and HCV replicon cells. HCV-infected hepatocytes are known to produce reactive oxygen species, which promote lipid and protein oxidation. To evaluate if ASA treatment could have an antioxidant activity in our cell system, ROS levels were examined using the DCF-DA assay. Parental and HCV replicon-containing cells were incubated in the presence or absence of 4 mM ASA up to 72 hours 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. 1A and 1B, respectively) with a greater effect at 72 hours post-treatment 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 hours and showing a maximum decrease at 72 hours post-treatment (*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
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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
hours without treatment, demonstrating that only the presence of HCV proteins induces
higher oxidative stress levels in Huh7 cells (measured in relative absorbance units
(RAU) (Fig. 2A and 2B). Interestingly, we found that ASA treatment dramatically
decreased oxidized protein levels in both cell types (parental and replicon cells) at 24
(at about 28%), 48 (about 50%) and 72 (about 40%) hours showing an antioxidant effect
in this cell system (Fig. 2A and 2B). 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 increase in both parental and HCV replicon-containing
cells, to a greater extent in the latter.

Inhibition of mitochondrial electron transport by rotenone and diphenylene iodonium
(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-
HAD assay upon 24 hours. In both cell lines the endogenous ROS production was
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inhibited by DPI and rotenone (Fig. 2C). Normalizing levels of mitochondrial reactive oxygen species 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 glutathion peroxidase and superoxide dismutase activity. Additionally, we were interested in determining whether ASA could influence cellular antioxidant systems, such as glutathione peroxidase (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 (GR). Parental and HCV replicon-containing cells were incubated in the presence or absence of 4 mM ASA until 72 hours, 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 figure 3, reaching maximum inhibition at 12 and 48 hours (*$P< 0.05$) in both parental and HCV replicon cells compared with their control (non-treated cells) (Fig. 3A and 3B). This inhibitory effect of ASA was not likely due to its cytotoxic effect since total protein synthesis did not differ significantly among treated and non-treated 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 were evaluated. We observed a striking increase in average cytosolic superoxide dismutase activity in both parental and HCV replicon cells exposed to ASA, observing a statistically significant difference ($p<0.05$) at 48 and 72 hours compared with untreated cells (Figs. 3D and 3E). Using the same statistical analysis, similar results were observed when cells were treated with 5μM PDTC (12 and 48 hours) as a control under the same conditions. Together these results suggest that ASA treatment could induce cytosolic SOD activity.

**Increased SOD expression versus 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. In order 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 down-regulation (48 and 72h) (Fig. 4A and 4B). HCV replicon cells were untreated or stimulated with 4 mM ASA for 48 and 72 hours, 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
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Cu/Zn SOD with ASA treatment compared with untreated cells in the same time frame (48 and 72h), whereas NS5A protein levels showed reduced expression in ASA-treated cells (Fig. 4A and 4B). 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 hours); 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. While Huh7 parental cells showed equal/decreasing levels of Cu/Zn SOD-mRNA at 48 and 72 hours with ASA treatment, replicon cells showed a dramatic increase in cytosolic SOD-mRNA levels at the same times points of exposure (Fig. 5A 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 showed) 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 non-sense control siRNA) (Fig. 5C). However, when we downregulated SOD1 expression in cells treated with 4mM
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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 down-regulating 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). Based on this, we next explored the effect of PDTC (5 µM) on HCV-RNA expression in HCV replicon cells at 3 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 4mM 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 hours 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 4mM ASA under the same conditions. Interestingly, we found that PDTC exposition also reduced PGE₂ levels up to 60% at 72 hours in HCV replicon-containing cells compared with untreated cells (Fig 7) (*P < 0.05). ASA-treated cells showed similar results (Fig. 7B).
DISCUSSION

Reactive oxygen species (45) and 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 (43). Results from literature along with our own suggest that ASA could be a good antiviral candidate most probably due to its antioxidative and anti-inflammatory properties (21, 31, 42, 43). There are previous reports suggesting antiviral activity of ASA. Spier et al. published the first report demonstrating that aspirin has an antiviral effect (42). They reported a diminished replication of human cytomegalovirus (HCMV) infecting coronary artery smooth muscle cells with ASA treatment (42). Recently, it has been reported that salicylates [sodium salicylate and acetylsalicylic acid (ASA)] inhibit flavivirus replication, such as Japanese encephalitis virus (JEV) and Dengue virus (DENV), in addition to other viruses such as influenza virus, but the mechanisms involving this effect have not been clearly elucidated (25, 29). Liao et al. reported that the mechanism by which salicylates suppress flavivirus infection may involve p38-MAPK activity, being independent of blocking NF-κB (25). In addition, Mazur et al. suggested that ASA efficiently blocks influenza virus replication in vitro and in vivo in a mechanism involving expression of proapoptotic factors, inhibition of caspase activation and blocking nuclear export of viral ribonucleoproteins (29).
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In this study, we demonstrated first that aspirin treatment reduces oxidative stress marker generation, an effect assessed by reactive oxygen species and protein oxidized measurements (Figs. 1 and 2) in a time-dependent fashion and correlating with a previously observed decrease in HCV-RNA expression in a hepatoma cell line containing HCV subgenomic replicon. Aspirin is known to cause a multitude of pharmacologic actions through inhibition of cyclooxygenase(s) and reduced production of prostaglandins. Recently, novel cytoprotective and antioxidant mechanisms of aspirin have been identified which independent of cyclooxygenase inhibition (40).

Anti-inflammatory compounds such as acetylsalicylic acid, ibuprofen, and indomethacin were reported to be inhibitors of glycation, perhaps by preventing the oxidative stress associated with the formation of advanced glycation endproducts (AGEs) (protein-aging) (35). Aspirin contributes to the intracellular metabolism of ATP, inhibiting inducible nitric oxide synthase, modulating the activity of nuclear factor (38)-kappa B and mitogen-activated protein kinases (MAPK) (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 (non-treated cells) (Fig. 3A and 3B). These data suggest that ASA-treatment may either
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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 G et al. (23) who studied patients with chronic HCV infection who were under the influence of oxidative stress associated with lower levels of GPx. They observed that these impairments returned to the levels of healthy controls after they were treated with pegylated interferon alfa-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 in order to confirm them.

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 (Fig. 4 and 5). Since 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 experiment should be performed in order 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
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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 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 (32). In another report, Pratap et al. demonstrated that aspirin reduced TBARS levels and induced elevation of GSH, SOD and catalase levels in a middle cerebral artery occlusion model of focal cerebral ischemia in rats (34). 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 transferase, and GPx activities when male C57 BL/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. 6A and 6B). Recently, the antiviral function of PDTC against influenza virus through the
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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 reactive oxygen species 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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FIGURE LEGENDS

**Fig. 1 ASA decreases lipid peroxidation in both Huh7 cells types.** (A and B) Effect of ASA on ROS levels in Huh7 parental and HCV replicon cells. Parental and HCV replicon-containing cells (5 x 10^5 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 DCF-HAD fluorescence assay. (C) PDTC, was used as a control because of its effect as an antioxidant. HCV replicon cells (5 x 10^5 cells) were treated with a potent antioxidant (5 µM pyrrolidine dithiocarbamate, PDTC) for 0 to 72h. The ROS levels were then determined. The data are the mean ± standard deviation of triplicate cultures, and the experiment was repeated three times (*P < 0.05).

**Fig. 2 Aspirin and PDTC decreased oxidized protein levels.** (A-B) Effect of ASA on oxidized protein levels in Huh7 parental and HCV replicon cells. Parental and HCV replicon-containing cells (5 x 10^5 cells) were incubated in the presence or absence of 4 mM ASA at different time points (0 - 72h). At the end of incubation, cell lysates were prepared and equal amounts of protein extracts (50 µg) were quantified by Oxiblot-Oxidative Protein detection kit. Band intensities were quantified with Phoretix 1D v2003.02 software and are showed as relative absorbance units (RAU). (C) To determine the site of HCV-induced intracellular ROS production, both cell lines were plated at a density of 15,000 cells/well in 96-well culture plates, and incubated with either 5µM rotenone or 50 µM DPI, and then ROS levels were measured in cells by
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using DCF-HAD assay upon 24 hours, using a Fluorometer at a wavelength of 503 nm (excitation) and 529 nm (emission).

Fig. 3 Effect of Aspirin and PDTC on glutathion peroxidase and superoxide dismutase activity. (A and B) Effect of ASA on glutathion peroxidase (GPx) activity in both cell types. Parental and HCV replicon-containing cells ($5 \times 10^5$ 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, cell lysates were prepared and GPx activity was measured using the Total Glutathione Peroxidase Assay Kit (ZeptoMetrix, Buffalo, NY) with equal amounts of protein extracts. (C) PDTC was used as an antioxidant control. Huh7 replicon cells ($5 \times 10^5$ cells) were incubated in the presence or absence of 5 µM PDTC for 0, 12, 24 and 48 h, and GPx 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 ($5 \times 10^5$ 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 by a SOD Assay Kit-WST (Cayman Chemical Technologies, US.) according to the manufacturer's instructions with equal amounts of protein extracts. (F) Huh7 replicon cells ($5 \times 10^5$ 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. The data are the mean ± standard deviation of triplicate cultures, and the experiment was repeated three times (*$P < 0.05$).
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Fig. 4 SOD and NS5A expression in HCV replicon cells upon ASA treatment

HCV replicon cells (2 x 10^2) were grown on glass coverslips and treated with 4 mM ASA for 48 and 72 hours. Following incubation cells were washed and fixed in 4% paraformaldehyde/PBS for 10 min at room temperature. Fixed 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 (1:1000). The next day, coverslips were washed with PBS, incubated with AlexaFluor 594 anti-rabbit (1:2000) and Alexa Fluor 488 anti-mouse antibodies (Molecular Probes, 1:1000, 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.

Fig. 5 Effect of ASA on SOD-mRNA levels and SOD1 silencing. (A) Huh7 parental and HCV replicon cells (5 x 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) The 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
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without treatment) which is defined as 1.0. (C) Effect of SOD1 silencing on HCV-RNA expression. Huh7 replicon cells cultured in Advanced DMEM supplemented with 2% FBS were plated at a density of 500,000 cells/well in 6-well culture plates. Next day, they were transfected in serum-free ADMEM with siRNA directed against superoxide dismutase 1 (siRNA-SOD1), silencer negative control siRNA (Ambion: Part number AM4611) or with siPORT Lipid Agent alone. Total RNA was extracted at 24, 48 and 72h post transfection, 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 showed are the mean ± SD of triplicate cultures and the experiment was repeated 3 times (*P < 0.05).

Fig. 6 HCV-RNA expression upon ASA and PDTC treatment. (A) Huh7 HCV replicon cells (2 x 10⁵ cells) were incubated with (5 µM) or without PDTC for 24, 48 and 72 h. (B) Huh7 HCV replicon cells (2 x 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 non-treated control, which is defined as 1.0. The data showed are the mean ± 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 x 10^4 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 4mM ASA under the same conditions. After PDTC and ASA-treatment, intracellular PGE_2 levels were then assayed by using the Biotrak Prostaglandin E_2 Enzyme Immunoassay system (Amersham Biosciences, Hercules CA) according to the manufacturer's protocol.
Fig. 1

A

Normalized ROS levels over time in parental cells.

B

Normalized ROS levels over time in replicon cells.

C

Normalized ROS levels over time in replicon cells with PDTC.

* p<0.05
Fig. 2

A  PARENTAL CELLS

B  HCV REPLICON CELLS

RAU

- ASA
+ASA 4mM

0 6 12 24 48 72h

- ASA
+ASA 4mM

0 6 12 24 48 72h
Fig. 2

![Bar graph showing ROS levels in parental and replicon cells with and without DPI (50 μM) and rotenone (5 μM).]
Fig. 5

A

Time (h) 0 24 48 72
Huh7 Cells Parental Replicon Parental Replicon Parental Replicon Parental Replicon
ASA (4mM) - - + + - - + + - - + + - - + +

SOD
465 bp

GAPDH
483 bp

B

* p<0.05

SOD expression (fold times)

Huh7 Cells Parental Replicon Parental Replicon Parental Replicon Parental Replicon
ASA (4mM) - - + + - - + + - - + + - - + +
Time (h) 0 24 48 72
Fig. 5

C

![Graph showing the effect of different treatments on HCV-RNA levels over time.](image)

- **Non-sense siRNA:**
  - 24h: +
  - 48h: +
  - 72h: +

- **siRNA-SOD1:**
  - 24h: -
  - 48h: +
  - 72h: +

- **ASA 4mM:**
  - 24h: -
  - 48h: -
  - 72h: +
Fig. 6

A. HCV Replicon Cells

![Bar chart showing HCV RNA levels in cells treated with PDTC compared to control.](chart.png)

B. HCV Replicon Cells

![Bar chart showing HCV RNA levels in cells treated with ASA compared to control.](chart.png)