Hepatitis C Virus Stimulates the Expression of Cyclooxygenase-2 via Oxidative Stress: Role of Prostaglandin E₂ in RNA Replication

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Hepatitis C virus (HCV) infection is a major cause of chronic liver disease, which can lead to the development of liver cirrhosis and hepatocellular carcinoma. Recently, the activation of cyclooxygenase-2 (Cox-2) has been implicated in the HCV-associated hepatocellular carcinoma. In this study, we focus on the signaling pathway leading to Cox-2 activation induced by HCV gene expression. Here, we demonstrate that the HCV-induced reactive oxygen species and subsequent activation of NF- κ B mediate the activation of Cox-2. The HCV-induced Cox-2 was sensitive to antioxidant (pyrrolidine dithiocarbamate), Ca²⁺ chelator (BAPTA-AM), and calpain inhibitor (*N*-acetyl-Leu-Leu-Met-H). The levels of prostaglandin E₂ (PGE₂), the product of Cox-2 activity, are increased in HCV-expressing cells. Furthermore, HCV-expressing cells treated with the inhibitors of Cox-2 (celecoxib and NS-398) showed significant reduction in PGE₂ levels. We also observed the enhanced phosphorylation of Akt and its downstream substrates glycogen synthase kinase-3 β and proapoptotic Bad in the HCV replicon-expressing cells. These phosphorylation events were sensitive to inhibitors of Cox-2 (celecoxib and NS-398) and phosphatidylinositol 3-kinase (LY294002). Our results also suggest a potential role of Cox-2 and PGE₂ in HCV RNA replication. These studies provide insight into the mechanisms by which HCV induces intracellular events relevant to liver pathogenesis associated with viral infection.

Hepatitis C virus (HCV) is a significant cause of morbidity and mortality, infecting >170 million people worldwide (15). Chronic infection with HCV can lead to serious liver disease, including cirrhosis and hepatocellular carcinoma (HCC) (15). HCV is an enveloped single-stranded positive-sense RNA virus, approximately 9.6 kb in length, which encodes a polyprotein of about 3,000 amino acids (4, 16). This polyprotein is posttranslationally cleaved by a combination of host cell signal peptidases and viral proteinases into structural (core, E1, and E2) and nonstructural (NS2 and NS3- to NS5A/B) proteins (4). Recently, the production of an additional viral protein by a ribosomal frame shift has been reported (68). The single open reading frame is flanked by 5' and 3' nontranslated regions, which have been shown to be essential in both initiation of translation and viral replication (4, 55).

The development of subgenomic HCV RNA replicons has opened the prospects to study HCV gene expression and its effects on intracellular events (35). The HCV subgenomic replicon is a bicistronic RNA, containing a neomycin resistance gene under the translational control of HCV internal ribosome entry site, followed by the HCV nonstructural proteins encompassing NS3 through NS5B and the 3' nontranslated region under the translational control of the encephalomyocarditis virus internal ribosome entry site. G418 selection is used to maintain the replication of subgenomic replicon in the Huh7 cells (35). Several adaptive mutations in the HCV NS proteins of replicons have been identified which confer higher efficiency of replication of subgenomic replicons (5, 6, 36). HCV RNA is

translated on the rough endoplasmic reticulum (ER) and replicates within the RNP complexes in the ER membrane (16, 63). The association of RNA replication with lipid rafts has been reported (47). We have previously shown that the association of HCV nonstructural proteins with the ER membrane induces ER stress, activating an unfolded protein response (56). Depletion of Ca²⁺ stores in the ER and its uptake by mitochondria lead to generation of reactive oxygen species (ROS) (see Fig. 6) (21, 61, 62). ROS, which act as second messengers, activate transcription factors such as STAT-3, NF- κ B, and others (21, 46, 62, 64).

In response to viral infection, multiple signaling pathways are activated which participate in the regulation of gene expression related to inflammation such as cyclooxygenase-2 (Cox-2), inducible nitric oxide synthase, and interferons (10, 38, 48, 52, 70). Cox-2 expression has been found to be elevated in various cancers, including colorectal, pancreatic, gastric, lung, and head and neck (9, 44, 60, 69). Recently, increased Cox-2 expression has been documented in HCC including HCV-positive HCC (1, 43). Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit Cox-2 and block the growth of cultured HCC cells (1, 31). Cox-2 is the rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H₂ (PGH₂), the precursor of various compounds including PGE₂ (48, 65). Two Cox genes, the Cox-1 and Cox-2 genes, have been identified (23). Cox-1 is constitutively expressed in a number of cell types, whereas Cox-2 is inducible by a variety of stimuli, including oncogenes, mitogens, cytokines, growth factors, inflammatory molecules, endotoxins, and tumor promoters (17, 65). A variety of transcription factors including AP-1, NF- κ B, nuclear factor of activated T cells, and nuclear factor interleukin-6 mediate the induction of Cox-2 (28, 33). Overexpression of Cox-2 leads to increased levels of proinflammatory

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FIG. 1. HCV replicon induces expression of Cox-2. (A) Whole-cell lysates from cells stably expressing HCV replicons (FCA4) or Huh-7 cells transiently transfected with in vitro transcribed BM4-5 RNA or BM4-5 Pol⁻ RNA were fractionated by SDS-PAGE and immunoblotted with anti-Cox-2 monoclonal antibody. Lane 1, untransfected Huh-7 lysates; lane 2, FCA4 lysates; lanes 3 and 4, Huh-7 cells transfected with BM4-5 RNA; lane 5, Huh-7 cells transfected with BM4-5 RNA Pol⁻. (B) Induction of Cox-2 mRNA in HCV replicon-containing cells. Total cellular mRNA was analyzed by using Cox-2-specific primers as described in Materials and Methods. Bar 1, Huh-7 cells; bars 2, 3, and 4, FCA4 cells and Huh-7 cells transfected with BM4-5 and BM4-5 Pol⁻ RNA. (C) Lane 1, untransfected Huh-7 lysates; lane 2, FCA4 lysates; lane 3, FCA4 lysates treated with PDTC (100 μM) for 6 h. (D) Luciferase reporter gene assay. Huh-7 and FCA4 cells were transfected with the Cox-2 luciferase reporter gene (containing NF-κB binding sites). At 36 h posttransfection, cells were treated with PDTC (100 μM) for 6 h, BAPTA-AM (50 μM) for 2 h, and ALLM (100 μM) for 24 h before the lysates were prepared for luciferase activity.

molecule PGE₂ (65). PGE₂ is one of the most abundant lipid mediators produced during or in inflammatory reactions and modulates immune function (42). PGE₂ mediates tumor survival by several mechanisms. It inhibits tumor cell apoptosis and induces tumor cell proliferation (51). In addition to the direct effects of PGE₂ on tumor cells, it induces the production of metastasis-promoting matrix metalloproteinases and stimulates angiogenesis (20). Previous studies have shown that increased production of Cox-2 and PGE₂ modulates replication activities of cytomegalovirus, gammaherpesvirus, and hepatitis B virus (27, 53, 70).

Recently, the involvement of phosphatidylinositol 3-kinase (PI3-kinase)-Akt activation has been demonstrated in Cox-2-induced HCC (34). PI3-kinase-Akt is central to many cell signal transduction pathways (8). Virus modulation of PI3-kinase-Akt signaling has emerged as an important regulatory

mechanism of apoptotic inhibition during acute infection, long-term virus survival, and transformation (12).

In this study, we investigated the mechanism(s) of Cox-2 activation in HCV replicon-expressing cells. Here, we demonstrate that the ability of the HCV replicon to induce the expression of Cox-2 is mediated by HCV-induced oxidative stress. These events ultimately give rise to the activation of NF-κB. The activation of Cox-2 is shown to be regulated by NF-κB. We further show that Cox-2 and PGE₂ induction contributes to the regulation of HCV RNA replication.

MATERIALS AND METHODS

Plasmids, antibodies, and reagents. The Cox-2 reporter plasmid P2-1900-Luc (−1796, +104), with two copies of upstream NF-κB binding sites, and Cox-2 P2-431-NF-κB mut-Luc, with mutated NF-κB binding sites, were generous gifts of M. Lopez-Cabrera (Unidad de Biología Molecular, Madrid, Spain).

FIG. 1—Continued.

The antibodies against Akt, phospho-Akt-Ser⁴⁷³, Bad, phospho-Bad-Ser¹³⁶, and phospho-glycogen synthase kinase-3 β (GSK3 β)-Ser⁹, were obtained from Cell Signaling Technology. Pyrrolidine dithiocarbamate (PDTC), and PGE₂ were purchased from Sigma Chemicals Co. NS-398, celecoxib, LY294002, *N*-acetyl-Leu-Leu-Met-H (ALLM), and BAPTA-AM were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). The anti-Cox-2 monoclonal antibody was obtained from Cayman Chemical, Ann Arbor, MI.

Cell culture. The human hepatoma cell lines Huh-7 and FCA4 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin sulfate. FCA4 cells were generous gift of C. Seeger (Fox Chase Cancer Center, Philadelphia, PA) and were grown in 500 μ g/ml of G418 (Geneticin; Invitrogen). FCA4 cells are a Huh-7 cell line stably expressing a HCV subgenomic replicon with a single adaptive mutation, a deletion of serine residue 1176 (22). The BM4-5 RNA sequence was constructed by replacing an EcoRI (position 5083)-XhoI (position 5570) fragment of HCV genotype 1b replicon I₃₇₇/NS3-3' with the corresponding fragment, which was cloned by reverse transcription-PCR (RT-PCR) with RNA isolated from FCA4 cells (22).

Immunoprecipitation and Western blot analysis. Exponentially growing FCA4 and Huh-7 cells transfected with BM4-5 RNA were harvested and cell extracts were prepared by incubation in radioimmune precipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 1 mM sodium orthovanadate, 1 mM sodium formate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin) for 30 min on ice. Immunoprecipitation was performed with anti-Akt and anti-Bad serum for 4 h. The immune complexes were incubated with protein A-Sepharose, washed three times with radioimmune precipitation buffer, and boiled for 5 min in SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. The samples were subjected to SDS-PAGE. Gels were electroblotted onto a polyvinylidene difluoride membrane (Amersham) in 25 mM Tris, 192 mM glycine, and 20% methanol by electrophoresis. Membranes were treated for 1 h in blocking buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.3% polyvinylpyr-

rolidone, 0.5% Tween 20 [wt/vol]), probed with antibodies against phospho-Akt-Ser⁴⁷³, phospho-Bad-Ser¹³⁶ monoclonal antibodies overnight, and washed twice for 10 min with blocking buffer, followed by incubation with secondary antibody for 45 min. After an additional washing step with blocking buffer, immunoblots were visualized with the ECL detection system (Amersham).

Reprobing immunoblots. The immunoblot membranes were submerged in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) and incubated at 50°C for 30 min with occasional shaking. The membranes were washed twice with blocking buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 0.3% polyvinylpyrrolidone, 0.5% Tween-20 [wt/vol]), and immunodetection was performed with different antibody as described above.

Intracellular PGE₂ measurements. Subconfluent Huh-7 cells stably expressing HCV subgenomic replicon (FCA4) or Huh-7 cells transiently transfected BM4-5 RNA (22) were treated with inhibitors of Cox-2 (celecoxib and NS-398) at various time periods. After the drug treatment, culture cells were washed thoroughly with cold phosphate-buffered saline, pH 7.4, and lysis reagent 1 (supplied by Amersham Biosciences) was added to the cells for 10 min. Lysis reagent 1 hydrolyzes cell membranes to release intracellular PGE₂. PGE₂ levels were then assayed with the Biotrak Prostaglandin E₂ Enzyme Immunoassay system (Amersham Pharmacia Biotech.) according to the manufacturer's protocol.

Luciferase assays. For transient transfections, Huh-7 cells were plated at a density of $\sim 5 \times 10^5$ cells/60-mm dish and maintained in Dulbecco's modified eagle's medium containing 10% fetal calf serum and penicillin (75 U/ml), streptomycin (50 U/ml) at 37°C. Cells ($\sim 50\%$ confluent) were transfected with 500 ng of luciferase reporter plasmid with Lipofectin reagent (Life Technologies). Thirty hours posttransfection, cells were serum starved overnight, followed by treatment with PDTC (100 μ M) for 6 h, ALLM (100 μ M) 24 h, and BAPTA-AM (50 μ M) for 2 h. Cells were harvested, and cellular lysates were analyzed for luciferase expression with a luminometer (14). All transfections included a β -galactosidase expression vector to serve as an internal control.

BM4-5 plasmid DNA (SP1/DS-BM4-5) (gift of C. Seeger) was linearized with ScaI and transcribed with the Ampliscribe T7 transcription kit (Epicenter Tech-

FIG. 2. HCV replicon induces production of PGE₂ accumulation in HCV replicon-expressing cells. Huh-7 cells stably expressing the HCV subgenomic replicon (FCA4) or Huh-7 cells transiently transfected with BM4-5 RNA were treated with Cox-2 inhibitors celecoxib (50 μ M) for 24 h and NS-398 (100 μ M) for 24 h. After the drug treatment, culture cells were washed thoroughly with cold PBS, pH 7.4, and lysis reagent 1 (supplied by Amersham Biosciences) was added to the cells for 10 min. The PGE₂ levels were then assayed by using the Biotrak Prostaglandin E₂ Enzyme Immunoassay system (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

nology) into RNA. BM4-5 RNA (5 μ g) was transfected into Huh-7 cells with Lipofectin reagents. At 15 h posttransfection, cells were harvested and analyzed for luciferase activity.

Quantitative real-time RT-PCR. Total RNA was extracted from HCV replicon-containing cells (FCA4) as well as Huh-7 cells using RNA STAT-60 (Tel-Test, Inc., Friendswood, Tex.). HCV RNA was quantified by real-time RT-PCR with an ABI PRISM 7000 Sequence Detector (Perkin-Elmer/Applied Biosystems). Amplification was conducted in duplicate using the following primers and 6-carboxyfluorescein (6FAM)- and tetrachloro-6-carboxyfluorescein (TAMRA)-labeled probes (Perkin-Elmer): HCV Replicon Taqman probe, 5'-6FAM-CCT TCATCTCCTTGAGCAGTCCC G-TAMRA-3'; HCV Replicon RNA-FWD, CTTTGACAGACTGCAGGTCCTG; HCV Replicon RNA-REW, GCCTTAA CTGTGGACGCCTTC; 18S rRNA Taqman probe, 5'-6FAM-TGCTGGCACC AGACTTGCCCTC-TAMRA; 18S rRNA-FWD, 5'-CGGCTACCACATCCAA GGAA-3'; and 18S rRNA-REW, 5'-GCTGGAATTACCGCGGCT-3'. The sequences for the primers and probes were designed using Primer Express software (Perkin-Elmer/Applied Biosystems). Amplification reactions were performed with a 25- μ l mixture containing 8% glycerol; 1 $\times$ TaqMan buffer A (500 mM KCl, 100 mM Tris-HCl, 0.1 M EDTA, 600 nM passive reference dye ROX, pH 8.3); 300 μ M each of dATP, dGTP, and dCTP; 600 μ M dUTP; 5.5 mM MgCl₂; 900 nM forward primer; 900 nM reverse primer; 200 nM probe; 1.25 U AmpliTaq Gold DNA polymerase (Perkin-Elmer); 12.5 U Moloney murine leukemia virus reverse transcriptase (Invitrogen); and the template RNA. Reactions were performed with a 96-well spectrofluorometric thermal cycler under the following conditions: 30 min at 50°C (reverse transcription reaction), 10 min at 95°C (heat inactivation of reverse transcriptase and activation of TaqGold polymerase), and 40 cycles of 15 s at 95°C and 1 min at 60°C (PCR amplification). Fluorescence was monitored during every PCR cycle at the annealing step. At the termination of each PCR run, the data were analyzed by the automated system, and amplification plots were generated.

Cox-2 mRNA was quantified by real-time RT-PCR using an ABI PRISM 7000 Sequence Detector (Perkin-Elmer/Applied Biosystems). Total cellular RNA was extracted, and the cDNA was reverse transcribed from 1 μ g of total RNA using oligo(dT) primers. Quantitative PCR of Cox-2 was carried out by using a SYBR green kit (QIAGEN, CA) and two specific primer sets (sense, 5'-CCATGTCA AAACCGAGGTGTAT-3'; antisense, 5'-TCCGGTGTGAGCAGTTTCT-3'). The PCR cycling parameters were same as those described for HCV replicon RNA.

RESULTS

HCV stimulates Cox-2 expression. In this study, we investigated the molecular mechanism(s) of Cox-2 activation in the context of HCV translation/replication activities. To examine whether HCV replicon induces Cox-2 gene expression, lysates from cells stably expressing HCV subgenomic replicons (FCA4 cells) or those transiently transfected with replicon RNA (BM4-5) or replication defective replicon RNA (BM4-5 Pol⁻) in Huh-7 cells were fractionated by SDS-PAGE and immunoblotted with an anti-Cox-2 monoclonal antibody. Western blot analysis shows that the cells expressing either BM4-5 or BM4-5 Pol⁻ subgenomic replicons stimulated expression of Cox-2 (Fig. 1A, lanes 2, 3, and 5) compared to untransfected Huh-7 cells (Fig. 1A, lane 1). The levels of Cox-2 activation were slightly higher in BM4-5 RNA-transfected cells compared to those transfected with replication-defective replicon RNA (Fig. 1A, compare lanes 4 and 5). To determine whether HCV gene expression induces the mRNA expression of Cox-2, total cellular RNA was extracted from Huh-7, FCA4, and Huh-7 cells transiently transfected with BM4-5 and BM4-5 Pol⁻ RNA and quantified by real-time RT-PCR as described in Materials and Methods. The results showed an increase in Cox-2 mRNA expression in cells containing the HCV replicon (Fig. 1B, bars 2 to 4), suggesting that HCV gene expression regulates Cox-2 activity at the level of transcription. Cells expressing the replication-defective HCV subgenomic replicon showed a similar increase in Cox-2 mRNA levels (Fig. 1B), indicating that HCV RNA translation is sufficient to induce transcription of Cox-2 mRNA. Several studies have previously documented that the induction of Cox-2 is mediated by oxidative stress (3, 32). We

FIG. 3. HCV replicon induces Akt phosphorylation via PI3-kinase. Huh-7 and FCA4 cells were treated with antioxidant PDTC (100 μ M) for 6 h, celecoxib (50 μ M) for 24 h, and LY294002 (50 μ M) for 12 h. Equal amounts of cellular lysates were immunoprecipitated with anti-Akt serum, fractionated by SDS-PAGE, and immunoblotted with anti-Akt Ser⁴⁷³ serum. Lanes 1, Huh-7 lysates; lanes 2, FCA4 lysates; lanes 3, FCA4 lysates treated with celecoxib (A), PDTC (B), or LY294002 (C). (Bottom) Total Akt in Huh-7 and HCV replicon-expressing cells.

have shown that the expression of HCV subgenomic replicon and nonstructural proteins activates NF- κ B via oxidative stress and Ca^{2+} signaling (21, 62). HCV gene expression induces endoplasmic reticulum-overload response (EOR), exhibiting depletion of Ca^{2+} stores in the ER and its uptake by mitochondria leading to generation of ROS (21). To examine if the Ca^{2+} release and ROS generated during HCV gene expression play a role in the Cox-2 activation process, HCV-expressing cells were treated with a Ca^{2+} chelator (BAPTA-AM) and an antioxidant (PDTC). The results showed that cells stably expressing the HCV replicon (FCA4) treated with BAPTA-AM and PDTC effectively inhibited the activation of Cox-2 (Fig. 1C, lane 3, and D, bar 4). To further confirm that the activation of Cox-2 is mediated by Ca^{2+} signaling and ROS, we also performed reporter assays using a plasmid vector in which luciferase gene was placed under the control of the Cox-2 promoter/enhancer. Cells expressing the HCV replicon (FCA4) and Huh-7 cells were transiently transfected with the Cox-2 promoter/enhancer luciferase construct and treated with the Ca^{2+} chelator (BAPTA-AM) and an antioxidant, PDTC. The results showed that the stimulation of the Cox-2 reporter gene was abrogated by the Ca^{2+} chelator (BAPTA-AM) and the antioxidant (PDTC) (Fig. 1D, bars 4 and 5), suggesting that activation of Cox-2 is mediated by Ca^{2+} signaling and ROS. The expression of Cox-2-Luc (Mut) construct in Huh-7 and FCA4 cells did not show stimulation of luciferase activity (bars 2 and 7). ROS has been shown to function as a component of signal transduction cascades, which leads to the activation of transcription factor NF- κ B (21, 39, 46, 61). Since the Cox-2

promoter/enhancer (−1796 to +104) contains NF- κ B binding sites, we examined the role of HCV-induced NF- κ B in the regulation of Cox-2 gene expression. Our results show that NF- κ B-controlled Cox-2 promoter-luciferase activity was enhanced in cells expressing the HCV replicon (Fig. 1D), whereas the Cox-2 promoter-luciferase plasmid with mutated NF- κ B binding sites (P2-431-NF- κ Bmut) displayed reduced luciferase activity (Fig. 1D, bar 7), confirming the notion that Cox-2 stimulation was mediated via NF- κ B motifs. We have recently demonstrated that the activation of NF- κ B in HCV-expressing cells involves the calpain-mediated degradation of its inhibitory subunit I κ B α that is phosphorylated at tyrosine residues 42 and 305 (62). To determine whether the activation of Cox-2 is regulated through calpain-mediated NF- κ B activation, HCV-expressing cells were treated with the inhibitor of calpain, ALLM. The results show that the stimulation of Cox-2 reporter gene was reduced in the presence of ALLM (Fig. 1D, bar 6). These results implicate a potential role of ROS in the activation of NF- κ B in the HCV replicon-induced Cox-2 activation process by the pathway described previously (62).

HCV gene expression induces the production of PGE₂. The Cox-2 enzymatic activity catalyzes the conversion of arachidonic acid into prostaglandins, the inflammatory molecules. The elevated level of Cox-2 activity in HCV replicon-expressing cells was monitored by measuring the accumulation of PGE₂ by an enzyme-linked immunosorbent assay. The results show that the intracellular levels of PEG₂ were significantly induced in cells stably expressing the HCV replicon (FCA4) as well as in Huh-7 cells transiently transfected with the BM4-5

FIG. 4. Inhibitors of Cox-2 and PI3-kinase inhibit GSK3- β ⁹ and Bad¹³⁶ phosphorylation in HCV-expressing cells. (A) Equal amounts of cellular lysates from Huh-7 cells and FCA4 cells treated with inhibitors of Cox-2 celecoxib (5 μ M) for 24 h, NS-398 (100 μ M) for 24 h, and PI3-kinase (LY294002) (50 μ M) for 12 h were subjected to SDS-PAGE and immunoblotted with anti-phospho-GSK-3 β Ser⁹. Lane 1, Huh-7 lysates; lane 2, FCA4 lysates; lanes 3 to 5, FCA4 lysates treated with celecoxib, NS-398, or LY294002, respectively. (B) Equal amounts of cellular lysates were immunoprecipitated with anti-Bad serum, fractionated by SDS-PAGE, and immunoblotted with anti-BadSer¹³⁶ serum. Lane 1, Huh-7 lysates; lane 2, FCA4 lysates; lanes 3 and 4, FCA4 lysates treated with celecoxib and LY294002, respectively.

replicon RNA (Fig. 2, bars 6 and 9). Cox-2-specific inhibitors (celecoxib and NS-398) were tested for their ability to block the accumulation of PGE₂ in response to HCV gene expression. Both the inhibitors used in this analysis were capable of blocking the induction of PGE₂ in FCA4 cells by about 50 to 60% and 40 to 50% in Huh-7 cells and transiently transfected cells, respectively (Fig. 2, bars 7, 8, 10, and 11). These results indicate that the accumulation of PGE₂ in HCV replicon-expressing cells was induced by the enzymatic activity of Cox-2. In this assay, we used various concentrations of the standard PGE₂ supplied by the manufacturer. The results displayed a dose-dependent increase in the absorbance at 450 nm (bars 1 to 4), reflecting the specific binding of PGE₂ with anti-PGE₂ antibody.

HCV replicon stimulates Akt phosphorylation via PI3-kinase. Previously, it has been shown that the overexpression of Cox-2 and production of PGE₂ activates Akt in human HCC via a PI3-kinase-dependent mechanism (34). Akt acts as an important signal mediator, which regulates cell survival and proliferation. We therefore tested the effect of HCV gene expression on Akt activation. Because Akt is activated by phosphorylation at Ser⁴⁷³ in human cells, the level of phospho-AktSer⁴⁷³ was examined by Western blot analysis. Lysates from Huh-7 cells and cells expressing HCV subgenomic replicons (FCA4) were immunoprecipitated with anti-Akt serum, subjected to SDS-PAGE, and electroblotted onto a nitrocellulose membrane. The membrane was then incubated with anti-phospho-AktSer⁴⁷³ polyclonal antibody. This analysis demonstrated

that the cells expressing subgenomic replicons contained activated phospho-AktSer⁴⁷³ (Fig. 3A, lane 2). To determine whether the phosphorylation and activation of Akt in the HCV replicon-expressing cells occurs through the ROS- and Cox-2-induced PI3-kinase pathway, cells expressing HCV subgenomic replicons (FCA4) were incubated with antioxidant, PDTC, and specific inhibitors of Cox-2 (celecoxib) and PI3-kinase (LY294002). This analysis indicated that the cells incubated with PDTC, celecoxib, and LY294002 displayed reduced levels of phospho-AktSer⁴⁷³ (Fig. 3A to C, compare lanes 3 to lanes 2). The inhibitory effects of celecoxib and LY294002 on Akt phosphorylation and activation were further confirmed by measuring the Akt-mediated phosphorylation of GSK-3 β and proapoptotic protein Bad, both of which are downstream substrates of Akt. As shown in Fig. 4A and B, the HCV replicon-expressing cells displayed increased phosphorylation of GSK-3 β and BadSer¹³⁶. The phosphorylation of these substrates was abolished by inhibitors of Cox-2 (celecoxib and NS-398) and PI3-kinase (LY294002) (Fig. 4A, lanes 3 to 5, and B, lanes 3 and 4). The reduction of GSK-3 β ⁹ and BadSer¹³⁶ phosphorylation correlated with a corresponding decrease in Akt phosphorylation (Fig. 3). These results suggest that ROS, Cox-2, and PI3-kinase pathways collectively mediate the activation of Akt in HCV-expressing cells.

Effect of Cox-2 inhibitors and PGE₂ on HCV replication. To evaluate the effect of Cox-2 activity and production of PGE₂ on HCV RNA replication in replicon-containing cells, FCA4 cells were treated with the inhibitors of Cox-2 (celecoxib and NS-398) and subsequently incubated with exogenous PGE₂. To determine the levels of HCV RNA, total cellular RNA was extracted from Huh-7 and FCA4 cells and quantified by real-time RT-PCR. The results show an approximate 35 to 50% increase in HCV RNA levels in FCA4 cells that were incubated with Cox-2 inhibitors (celecoxib and NS-398) (Fig. 5A, and B, bars 3 and 4), suggesting that elevation of Cox-2 enzymatic activity by HCV down-regulates RNA replication. Similarly, HCV-expressing cells incubated with exogenous PGE₂ also showed a decrease in HCV RNA levels (Fig. 5C). Moreover, the inhibitory effect of PGE₂ on HCV replication can be substantially restored by treatment of cells with Cox-2 inhibitors (celecoxib and NS-398) (Fig. 5C). To demonstrate that the effect of Cox-2 inhibitors and PGE₂ on HCV replication is not specific to the FCA4 stable cell line, we also performed transient transfections of Huh-7 cells with an in vitro-synthesized subgenomic replicon BM4-5 RNA (22) and incubated these cells in the presence of Cox-2 inhibitors (celecoxib and NS-398) and exogenous PGE₂. Similar reductions in HCV RNA levels were observed using the transient-transfection scheme of expression (Fig. 5B and C). Together, these data suggest a negative impact of Cox-2 on HCV replication. Because HCV nonstructural proteins play a critical role in HCV replication (35), we also examined the expression of one of the nonstructural proteins, NS5A, encoded by HCV subgenomic replicon RNA in the presence of Cox-2 inhibitors. We observed that the addition of Cox-2 inhibitors (celecoxib and NS-398) enhanced the levels of NS5A protein expression, suggesting that Cox-2 down-regulates HCV gene expression and replication (Fig. 5D, compare lanes 3 and 4 with lane 2). These data collectively implicate a potential role of activated Cox-2 and PGE₂ in the regulation of HCV RNA translation and replication.

FIG. 5. Effect of Cox-2 inhibitors and PGE₂ on HCV RNA replication. (A and B) FCA4 cells and Huh-7 cells transiently transfected with in vitro-synthesized BM4-5 RNA were first incubated with the inhibitors of Cox-2 (50 μ M celecoxib and 100 μ M NS-398) for 24 h. Total RNA was extracted and subjected to quantitative RT-PCR analysis. The data were expressed as relative HCV RNA levels related to the RNA level of the control cells. (C) Stably HCV replicon-expressing cells (FCA4) or Huh-7 cells transiently transfected with BM4-5 RNA were exogenously added with 0.1 μ M and 0.5 μ M of PGE₂, respectively. Quantitative RT-PCR analyses were carried out as described above. (D) FCA4 cells were treated with the inhibitors of Cox-2 (50 μ M celecoxib; 100 μ M NS-398). Equal amounts of cellular lysates were subjected to SDS-PAGE and Western blotted with anti-NS5A serum. Lanes 1 and 2, equal amounts of Huh-7 and FCA4 lysates; lanes 3 and 4, FCA4 lysates treated with celecoxib and NS-398, respectively.

DISCUSSION

HCV infection stimulates host inflammatory responses comprising cellular effectors and soluble factors. One such factor that has recently been implicated in the host response to a wide variety of viruses is Cox-2-controlled prostaglandin E₂ molecules (27, 48, 53, 70). Cox-2 expression has been found to be elevated in various cancers, including colorectal, pancreatic, gastric, lung, and head and neck cancer (9, 44, 60, 69). Recently, increased Cox-2 expression has been documented in HCC, including HCV-positive HCC (1, 43), indicating its potential role in hepatocarcinogenesis. It is unclear how Cox-2 exerts its oncogenic effect at the molecular level in these cancers. Cox-2-derived prostaglandins could signal in an autocrine or paracrine manner, triggering a wide variety of complex cellular processes that lead to tumor growth. It has been demonstrated that Cox-2 inhibition is an important target for the chemoprevention of cancer by NSAIDs through inhibition of

cell proliferation, angiogenesis, and induction of apoptosis (13, 23). In the present study, we investigated the molecular mechanism(s) of Cox-2 activation and the cell survival cascade in response to oxidative stress induced by HCV gene expression. It is well established that NF- κ B binding sites exist in the Cox-2 promoter/enhancer region, which regulates the expression of Cox-2 (49). NF- κ B, a transcription factor, regulates expression of numerous cellular and viral genes and plays an important role in inflammation, innate immune responses, tumorigenesis, and cell survival (2, 29). We have previously shown that RNA translation and replication activities associated with the HCV life cycle activate NF- κ B via Ca²⁺ signaling and ROS (21, 57, 62). In the present analysis, we show that the HCV subgenomic replicon induces the expression of Cox-2 that is mediated via NF- κ B activation (Fig. 1). Furthermore, we observed that the induction of Cox-2 expression is sensitive to Ca²⁺ chelators,

FIG. 6. Model illustrating the mechanism(s) of HCV replicon-induced cell survival cascade via oxidative stress and activation of NF- κ B and Cox-2. PGE₂ generated by Cox-2 activity stimulates the phosphorylation of PI3-kinase-Akt, Bad¹³⁶, and GSK-3 β ⁹ phosphorylation. By a mechanism not clearly understood, Cox-2 and PGE₂ negatively regulate HCV RNA replication.

antioxidants and calpain inhibitors, suggesting that HCV-induced activation of NF- κ B is involved in the Cox-2 activation process (Fig. 1 and 6) (61). This is consistent with previous observations in which numerous viral proteins have been shown to up-regulate the expression of Cox-2 that is mediated by transcription factors NF- κ B, nuclear factor of activated T cells, CCAAT/enhancer binding protein, and others (7, 10, 33, 38, 65, 70). The role of ROS and NF- κ B in viral pathogenesis and in the progression of multistage carcinogenesis has been documented (18, 39, 46, 52, 54).

Previous studies have shown that elevated levels of Cox-2 in response to viral infection are linked to the production of PGE₂ (48). Prostaglandins serve as second messengers that elicit a wide range of physiological responses in cells and tissues. In the present analysis, we observed the increased levels of PGE₂ in HCV-expressing cells. Specific Cox-2 inhibitors (celecoxib and NS-398) eliminated the accumulation of PGE₂, suggesting that Cox-2 mediates the production of intracellular PGE₂ (Fig. 2). Celecoxib selectively inhibits Cox-2 activity without inhibition of Cox-1 and lacks the side effects associated with traditional NSAIDs (66). Recent studies have shown that

celecoxib inhibits the growth of several human cancers (26, 66, 67). It is well documented that overexpression of Cox-2 and the subsequent increase in prostaglandin synthesis can contribute to tumorigenesis by affecting apoptosis (41, 52, 58). Virus-mediated inhibition of apoptosis can be achieved through inhibition of the PI3-kinase-Akt pathway (12). Modulation of this pathway by the viruses provides an alternative to the expression of viral oncogenes or the direct inhibition of proapoptotic proteins. It is generally accepted that defective control of apoptosis is one of the central mechanisms of tumorigenesis because it allows cells to survive. In addition to these studies, we also observed that induction of Cox-2 and PGE₂ in HCV-expressing cells activates survival kinase, serine/threonine protein kinase B (Akt), and its direct downstream substrates GSK-3 β and Bad (Fig. 3 and 4). Furthermore, the results demonstrated that antioxidant and inhibitors of Cox-2 and PI3-kinase inhibited phosphorylation of Akt downstream substrates GSK-3 β ⁹, and BadSer¹³⁶ (Fig. 3 and 4). Bad is a member of the Bcl-2 family of proapoptotic proteins but upon phosphorylation binds to 14-3-3 proteins. This interaction neutralizes its proapoptotic activity (12). Recently, it has been shown that HCV NS5A can phosphorylate and activate PI3-kinase, which is also accompanied by downstream phosphorylation of Akt and Bad (25, 50).

The exact mechanism by which Cox-2-induced elevation of PGE₂ activates Akt in HCV-expressing cells remains to be defined. Two classes of PG receptors transduce signals upon binding of the ligands, the G protein-coupled cytoplasmic receptor class (EP receptors for PGE₂), and the nuclear receptor class of peroxisome proliferator-activated receptor; the latter acts as a transcription factor (34). Because the receptor-coupled G protein is known to transduce the signal to the PI3-kinase (30), the cytoplasmic EP receptor appears to be the preferred mediator for Cox-2-induced Akt activation.

It has been reported that Cox-2-generated PGE₂ decreases replication of adenoviruses and parainfluenza virus (37, 40), hepatitis B virus (19, 27), and human immunodeficiency virus type 1 infection (24, 45, 59). Our results demonstrate that the activation of Cox-2 and PGE₂ involves ROS (Fig. 1 and 6). The role of ROS in HCV replication has previously been demonstrated (11). Consistent with these reports, the data presented here demonstrates the effect of Cox-2 activity and PGE₂ production on HCV replication by quantitative real-time RT-PCR. Our results show enhanced levels of HCV RNA in HCV-expressing cells incubated with the inhibitors of Cox-2 (celecoxib and NS-398) (Fig. 5A and B), suggesting that Cox-2 activity negatively regulates HCV replication. Similarly, exogenously added PGE₂ displayed the downregulation of HCV RNA (Fig. 5C). The mechanism by which PGE₂ modulates viral replication remains unclear. Prostaglandins and its derivatives can potentially interfere with virus replication at multiple levels while triggering the synthesis of cytoprotective proteins by host cells (45).

These studies demonstrate constitutive activation of Cox-2 and induction of PGE₂ by the HCV subgenomic replicon, which involves oxidative stress and activation of NF- κ B (Fig. 6). Activation of Cox-2 and induction of PGE₂ in the model activates cell survival kinase PI3-K/Akt via G-protein-coupled EP receptors. Our data also provide important clues to the understanding of molecular mechanisms of chronic liver dis-

ease induced by oxidative stress and proinflammatory molecules Cox-2 and PGE₂.

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