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Mechanisms of Liver Injury.
III. Oxidative stress in the pathogenesis of hepatitis C virus

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Choi, Jinah, and J.-H. James Ou. Mechanisms of Liver Injury. III. Oxidative stress in the pathogenesis of hepatitis C virus. *Am J Physiol Gastrointest Liver Physiol* 290: G847–G851, 2006; doi:10.1152/ajpgi.00522.2005.—Hepatitis C virus (HCV) is a major cause of viral hepatitis that can progress to hepatic fibrosis, steatosis, hepatocellular carcinoma, and liver failure. HCV infection is characterized by a systemic oxidative stress that is most likely caused by a combination of chronic inflammation, iron overload, liver damage, and proteins encoded by HCV. The increased generation of reactive oxygen and nitrogen species, together with the decreased antioxidant defense, promotes the development and progression of hepatic and extrahepatic complications of HCV infection. This review discusses the possible mechanisms of HCV-induced oxidative stress and its role in HCV pathogenesis.

reactive oxygen species; reactive nitrogen species; antioxidant; hepatic fibrosis; hepatocellular carcinoma

HEPATITIS C VIRUS (HCV) is a major cause of viral hepatitis. There are ~170 million people in the world who are chronically infected by this virus. In the U.S., approximately four million people have been infected by HCV, and 35,000 new HCV cases are estimated to occur every year. The infection by this virus frequently does not resolve, and ~80% of the infected individuals become chronic carriers who may then progress to severe liver diseases. Approximately 10–20% of chronically infected hepatitis C patients will develop severe liver cirrhosis, and 1–5% will develop hepatocellular carcinoma (HCC) within two to three decades of infection. HCV infection is also the single major cause of liver transplantation in the U.S.

The HCV genome was first cloned in 1989. Since then, more than 20,000 HCV sequences have been deposited into the HCV database. On the basis of the nucleotide sequences, HCV has been grouped into six major genotypes and more than 50 subtypes. There is no vaccine available yet for HCV. The current therapy for HCV patients uses interferon-α and a nucleoside analog, ribavirin. These two drugs produce severe side effects, and their combined usage generates sustained response in only ~55% of the patients. These two drugs are less effective against genotypes 1a and 1b of HCV, which unfortunately account for 70–75% of HCV infections in the U.S.

HCV is a positive-stranded RNA virus with a genome size of ~9.6 Kb. This genome encodes a polyprotein, which is translated in a cap-independent manner. This translation requires an internal ribosomal entry site (IRES) that encompasses most of the 5'-untranslated region (UTR) and the first nine codons of the polyprotein coding sequence. The HCV polyprotein is cleaved by cellular and viral proteases to generate 10 mature viral gene products, including the core protein that forms the viral capsid, NS3, which has the protease and helicase activity, NS5A, and the viral RNA polymerase NS5B. In addition to the proteins derived from the polyprotein coding sequence, the HCV RNA codes for another protein termed the F protein or the alternative reading frame protein (ARFP) using an open reading frame that overlaps with the core protein coding sequence. The research on the replication of HCV has made significant progress in recent years with the development of bicistronic HCV RNA replicons and cell culture systems that support the infectious HCV formation. These model systems will further accelerate HCV research in the near future.

OXIDATIVE STRESS DURING HCV INFECTION–THE MECHANISMS

As mentioned, HCV infection frequently leads to severe liver diseases including liver cirrhosis and HCC. The molecular mechanism of HCV pathogenesis remains unclear. However, oxidative stress has emerged as a key player in the development and the progression of many pathological conditions, including HCV-induced pathogenesis of liver. The following section describes the increased oxidative stress during hepatitis C and the possible mechanisms of its increase.

HCV infection is characterized by increased markers of oxidative stress (for a list of references, see Ref. 3). Lipid peroxidation products are increased in serum, peripheral blood mononuclear cells (PBMC), and liver specimen from hepatitis C patients. 4-Hydroxynonenal (HNE) and 8-hydroxyguanosine, a marker of oxidative DNA damage, are elevated (15). In addition, there is a significant reduction of hepatic, plasmatic, and lymphocytic GSH levels in patients chronically infected by HCV, particularly with the 1b genotype (15). The percentage of oxidized GSH (GSSG) was increased, suggesting an increased GSH turnover.

This increased oxidative stress in hepatitis C may be explained by chronic inflammation, and the continued generation of reactive oxygen species (ROS)/reactive nitrogen species (RNS) may be explained by NAD(P)H oxidase (Nox 2 protein) of Kupffer cells and polymorphonuclear cells in the liver (Table 1) (7). NS3 protein of HCV has been found to activate Nox 2 protein of phagocytes and to trigger apoptosis and dysfunction of T cells, natural killer cells, and natural killer T cells (24). Nox 2 protein is located on phagosomal and plasma membranes, leading to increased generation of ROS and other reactive species that can exert oxidative stress to the nearby cells. Importantly, liver is a net exporter of glutathione (GSH) and supplies GSH to other tissues. Because GSH is an important endogenous antioxidant/reductant, HCV, by damaging liver, may promote systemic oxidative stress, at least in part, by

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Table 1. *Mechanism of increased oxidative stress in HCV infection*

Possible Sources of ROS/RNS
Activation of NAD(P)H oxidase of Kupffer cells and PMN cells during inflammation.
Iron overload and lipid peroxidation.
Activation of NAD(P)H oxidase by NS3 protein
Increased production of mitochondrial ROS/RNS by the electron transport chain due to core and NS5A proteins.
Decreased GSH output due to liver damage
Decreased antioxidants and antioxidant gene expression
Alcohol, drugs, and other chemicals
Increased cytokines that increase ROS
Increased expression/activity of COX-2
Increased expression of CYP2E1

ROS, reactive oxygen species; RNS, reactive nitrogen species; COX, cyclooxygenase; HCV, hepatitis C virus.

disrupting GSH export. Interestingly, however, both HCV and hepatitis B virus (HBV) cause hepatitis, and yet, HCV appears to be particularly more potent at inducing oxidative stress, suggesting mechanisms that are unique to HCV (see Ref. 3 for references). In this regard, it is interesting that excess iron deposits are found in the liver samples from some of the hepatitis C patients, which may promote the generation of free radicals in these individuals (see references in Ref. 3). The mRNAs of TNF-α and cytochrome P-450 (CYP2E1), both of which can increase ROS production, might also be elevated in hepatitis C patients (8).

Furthermore, studies have indicated that HCV can directly induce oxidative stress intracellularly in hepatocytes. HCV core gene expression has been associated with increased ROS, decreased intracellular and/or mitochondrial GSH content, and increased levels of oxidized thioredoxin and lipid peroxidation products (1, 11, 18, 20). Significant increases in the expression levels of members of the metallothioneine family, nicotinamide *N*-methyltransferase and GSH peroxidase-like protein (GPLP), were detected in the oligonucleotide microarray studies, with tightly regulated expression of the core protein in Huh7 human hepatoma cells (12). Together, these reports suggest that HCV core gene expression promotes prooxidative environment, which then induces the antioxidant defense mechanisms.

The molecular mechanism of how the HCV core protein induces oxidative stress has been investigated. The core protein has now been shown to associate with the outer mitochondria membrane via its COOH-terminal region (11, 18, 20). Previously, the increased ROS generation with HCV core protein was shown to be inhibited with diphenyliodonium (DPI) (20); on the basis of this finding, the core protein was suggested to stimulate ROS production by the electron transport chain of mitochondria (7). However, DPI inhibits Nox proteins, and it was not clear whether ROS in this study derived from the mitochondrial electron transport chain or the activation of Nox proteins. Recent studies, however, further showed evidence of increased oxidation of mitochondrial GSH and a decreased NADPH content in liver mitochondria from transgenic mice expressing the HCV structural proteins, including core (11). In addition, there was reduced activity of the electron transport complex I and increased generation of ROS from complex I substrates. Incubation of control mitochondria in vitro with recombinant core protein also caused the oxidation of GSH, complex I inhibition, and increased the ROS production. In contrast, the same experiment showed no effect of core protein on complex II and complex III activities. Core protein also increased the mitochondrial Ca^{2+} uptake. Thus it was postulated that the core protein's localization to the outer membrane results in mitochondrial dysfunction by facilitating Ca^{2+} accumulation. This increased Ca^{2+} would then inhibit electron transport and promote ROS production at complex I. These reports are consistent with the mitochondrial abnormalities found in vivo in the core-expressing animal models and hepatitis C patients (18, 19). Unfortunately, possible secondary effects of the core on the oxidative phosphorylation and ATP content have not been examined. The mechanism(s) by which the core protein affects mitochondrial calcium uptake or directly affects the electron transport chain also remain unclear and will require further studies.

Besides the core protein, HCV NS5A protein has also been reported to perturb the host redox status. NS5A was shown to significantly increase the ROS levels, as assessed by increased oxidation of dihydroethidium, in Huh7 cells (23). Huh7 cells, expressing NS5A in the context of the HCV subgenomic RNA replicon, also displayed a fivefold increase in oxidative stress (1, 23). HCV replicon was also shown to increase the amounts of MnSOD, heme oxygenase-1 (HO-1), and catalase as well as GSH content in Huh7 cells, suggesting the induction of adaptive response by the nonstructural proteins of HCV (1). The induction of MnSOD by NS5A was mediated by the activation of AP1 transcription factor by p38 MAPK and JNK signaling pathways that were inhibited with thiol antioxidants/reductants, suggesting redox signaling (23). NS5A has also been suggested to increase mitochondrial ROS generation by perturbing the cytosolic Ca^{2+} concentration indirectly through endoplasmic reticulum (ER) stress. The IRE1-XBP1 pathway in cells directs protein refolding and degradation in response to ER stress. The gene transactivation activity of XBP1 was suppressed in Huh7 cells containing the HCV subgenomic RNA replicon. Therefore, NS5A has been proposed to induce an accumulation of misfolded proteins and the induction of ER stress with the subsequent release of Ca^{2+} from the ER, followed by mitochondrial calcium uptake and the generation of ROS in the mitochondria (23).

In contrast, similar studies showed that the replicon does not alter the intracellular GSH status. Amount of reduced GSH was not decreased or increased, and GSSG remained below 1% of total intracellular GSH, suggesting that if these cells increased ROS generation, the level is not high enough to trigger adaptive increases in GSH (3). The reason for such discrepancies is unclear; however, some of them might be explained by the differences in the levels of HCV gene expression and the use of a pooled cell clone versus single cell clones in these studies. Whether NS5A protein increases ROS production or the uptake of redox-sensitive dyes into mitochondria, where ROS are generated, may be debatable. In addition, whether various HCV proteins induce ER stress at physiologically relevant concentrations remains to be resolved.

Therefore, it may be hypothesized that HCV produces oxidative stress through multiple mechanisms that include chronic inflammation, iron overload, and liver injury. Some of the HCV proteins may contribute to this process. It should also be noted that generally, the cellular redox environment is tightly

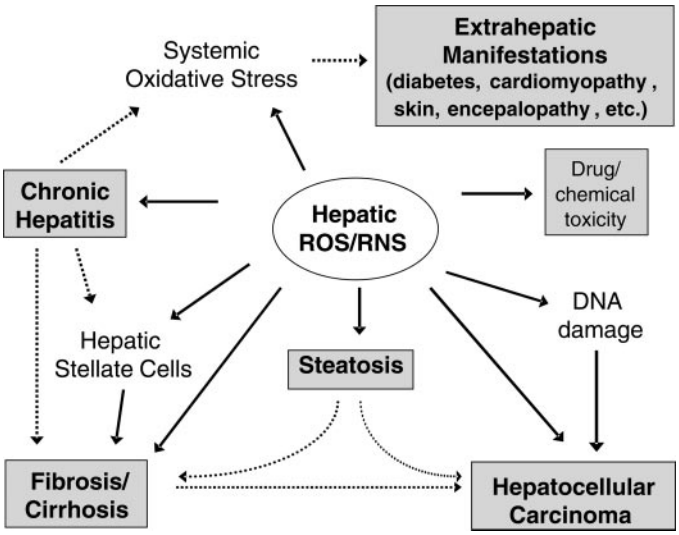

Fig. 1. Biological effects of reactive oxygen species (ROS)/reactive nitrogen species (RNS) in hepatitis C.

regulated by antioxidants/reductants as well as antioxidant enzymes that either directly remove the oxidants or reverse their chemistry. During oxidative stress, many of these antioxidant enzymes are upregulated in adaptation to stress. Therefore, any significant sustained changes in the ROS level and the ratio of reduced to oxidized GSH can indicate not only an uncontrolled production of oxidants, but also a significant dysfunction of the antioxidant defense. In this regard, it is interesting to note that core protein, unlike NS5A protein, tended to decrease, rather than increase, the GSH content (1). Similarly, despite the increased ROS production in cells that expressed the core protein, no compensatory increases in HO-1 or catalase were observed. On the other hand, both NS5A and core proteins induced MnSOD (1). The reasons for such differences in the mode of gene regulation are unclear, because both NS5A and core proteins have been suggested to modulate similar signaling pathways (e.g., MAPK), at least in cell culture (4, 23). Nevertheless, these observations raise an interesting possibility that HCV might not only increase ROS generation but also downregulate certain antioxidant genes, as previously shown with human immunodeficiency virus. Indeed, Gpx may also be decreased in the plasma of hepatitis C patients (10). The proteins that are negatively affected in this process by HCV might include Nrf2/maf transcription factors and AP-1. Nevertheless, the clinical studies have tended to show an increase, rather than decrease, in the overall antioxidant gene expression (22). Future studies aimed at determining the combined effects of core, NS5A, and other HCV proteins will be needed to answer some of these questions.

ROLE OF OXIDATIVE STRESS IN THE PATHOGENESIS OF HCV

The regulated production of reactive species by Nox proteins of phagocytes is believed to have a microbicidal property that is beneficial in the control of microbial infections. For example, in chronic granulomatous disease, which is characterized by a defect in the protein constituents of Nox 2 protein, the absence of the respiratory burst leads to recurrent infections and tissue granuloma formation. However, reactive molecules tend to nonspecifically oxidize essential biological macromolecules that are nearby and, consequently, lead to acute cell damage or cause gradual deterioration of important cell functions. Thus, whereas O_2 is essential for aerobic metabolism, the production of ROS, a consequence of aerobic metabolism, has been generally associated with the harmful effects. In addition, ROS (as well as RNS and reactive sulfur species) may promote pathogenesis through cell signaling (7). By altering or participating in diverse signaling pathways, these reactive species can modulate gene expression, cell adhesion, cell metabolism, cell cycle, and cell death and thereby contribute to pathogenesis. For example, transforming growth factor-β (TGF-β) is induced by ROS, and ROS and decreased GSH appear to mediate the profibrogenic effects of TGF-β (21). Today, oxidative stress is associated with diverse disease states such as atherosclerosis, neurodegenerative diseases, lung diseases, liver diseases, cancer, fibrosis, diabetes, immune dysfunctions, and Down's Syndrome among others.

Therefore, the prooxidative environment associated with HCV infection is expected to have significant pathological consequences (Fig. 1). The effects of oxidative/nitrosative stress in HCV pathogenesis most likely involves both the physically damaging effects of ROS/RNS as well as their more subtle effects on signaling. For example, oxidative stress can induce the proliferation of hepatic stellate cells, TGF-β and collagen synthesis (21). This likely plays an important role in the development of liver fibrosis associated with the HCV infection. Increased serum thioredoxin levels have also been correlated with the progression of liver fibrosis (22). Iron overload, found in some of the hepatitis C patients, was implicated in liver injury (see references in Ref. 3). Oxidative stress has also been proposed as a prognostic tool in predicting the outcome of hepatoprotective therapy as well as in monitoring the disease progression (15, 22). Furthermore, oxidative stress may participate in the development of hepatic steatosis and its progression to fibrosis in the hepatitis C patients (13, 18).

Oxidative DNA damage increases chromosomal aberrations associated with cell transformation, and oxidative stress has also been implicated in the development of HCV-associated HCC. Increased levels of ROS/RNS, for example, have been suggested to promote the development of hepatocellular carcinoma by inducing DNA damage and mutations of cellular genes (14). NS5A-induced oxidative stress has also been suggested to activate NF-κB by inducing the phosphorylation of

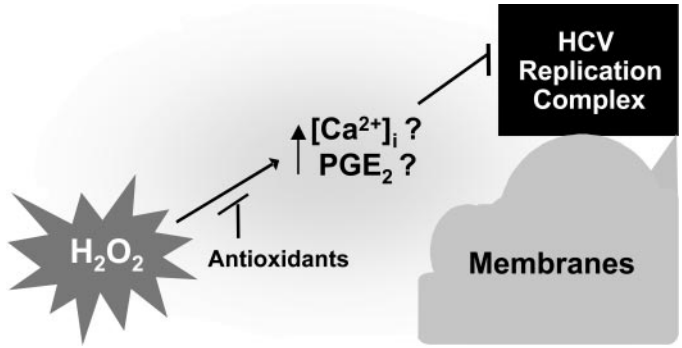

Fig. 2. Proposed mechanism of suppression of hepatitis C virus (HCV) replication by reactive oxygen species (ROS).

IκBα at tyrosine-42 and tyrosine-305 (23). This phosphorylation of IκBα involved ZAP-70 as a probable tyrosine kinase, and the degradation of IκBα appeared to be mediated by calpain proteases. Stat3 was also activated (23). NF-κB modulates cell growth/apoptosis, and Stat3 can induce cell transformation. The activation of NF-κB has also been suggested to further activate cyclooxygenase-2 (COX-2), which can be blocked by antioxidant, Ca^{2+} chelator, and calpain inhibitor; this activation of COX-2 would lead to an increased synthesis of PGE_2 (25). Possible activation of phospholipase A2 by ROS in the posttranslational activation of COX-2 was not tested. PGE_2 can inhibit the apoptosis of tumor cells, induce their proliferation, promote metastasis, and stimulate angiogenesis (25). It should be noted that PG synthesis can also generate ROS. These observations indicate that oxidative stress induced by HCV may cooperate with other factors to promote oncogenesis. Indeed, mitochondrial localization of proteins, followed by mitochondrial dysfunction, may be a common mechanism of viral tumorigenesis.

The systemic oxidative stress found in the hepatitis C patients, which is probably secondary to the hepatic oxidative stress, may also explain some of the extrahepatic manifestations of HCV infection such as the skin problems (16). In addition, HCV infection has been associated with diabetes, and the reactive species may participate in the development of diabetes and other complications associated with diabetes in hepatitis C patients. Hyperglycemia is known to induce oxidative stress; increased generation of reactive species then triggers a signaling cascade that alters the activity of insulin receptor substrate, leading to insulin resistance (6). TNF-α was identified as the key molecule that promotes the development of diabetes during HCV infection (9), and TNF-α increases ROS production. Therefore, oxidative stress may contribute to both hepatic and extrahepatic complications of HCV infection (Fig. 1).

OXIDATIVE STRESS, ANTIOXIDANTS, AND HCV TITER

Due to the role of oxidative stress in HCV pathogenesis, antioxidants have been proposed to treat HCV patients. For example, in a recent clinical trial, normalization of liver enzymes was observed in 44% of chronic HCV patients with elevated pretreatment levels, using a combination antioxidant therapy (17). Histological improvement was also noted in 36.1% of the patients.

Nevertheless, it is unclear how the increased ROS/RNS affects HCV replication. In replicon-based cell culture studies, oxidative stress was found to suppress HCV RNA replication (3, 25). *N*-acetylcysteine inhibited this suppressive effect. ROS suppressed HCV RNA replication by disrupting the formation of the HCV-RNA replication complex that cofractionated with Golgi membranes (3). Suppression occurred within 30 min without evidence of cell toxicity. In addition, this oxidative suppression of HCV replication might be mediated by calcium and PGE_2 (Fig. 2) (25). These findings suggested that the chronic activation of Nox 2 protein might suppress HCV replication through signaling; this also implied that the microbicidal effect, previously attributed to ROS generation by Nox 2 protein, may be at least partially attributed to redox signaling.

However, in vivo studies have yielded inconsistent results. For example, in a recent study, the reduction of viral load was observed with a combination antioxidant therapy in 25% of the patients (17). Another study found that viral load positively correlated with the erythrocyte malondialdehyde (MDA), a product of lipid peroxidation, but not the plasma MDA (10). Although such results are difficult to interpret, some of these findings may be explained by the aforementioned effects that HCV proteins have on the host redox status. In addition, it is possible that antioxidants have other effects, such as on the host immune system, which leads to an overall reduction of HCV viral load in vivo. Furthermore, oxidants/antioxidants may have different effects on other steps of the HCV life cycle. The new in vitro cell culture systems that support the entire life cycle of HCV are expected to help delineate the complex relationship that may exist among oxidative stress, HCV, and its pathogenesis.

CONCLUDING REMARKS

Hepatitis C virus infection is associated with severe alterations of host redox status. The increased ROS/RNS levels play important roles in the development of HCV-associated liver diseases. Whereas the antioxidants/reductants might be useful at improving HCV-associated diseases, whether these compounds suppress, enhance, or have no effect on HCV remains to be studied further. With regard to the antioxidant therapy, it should also be noted that ascorbic acid (vitamin C) can in fact promote hydroxyl radical production in the presence of free iron (2). Thus some antioxidants can act as prooxidants rather than antioxidants in hepatitis C patients with excess iron deposition in the liver. This calls for a careful reevaluation in the choice of antioxidants and how they are currently used and being recommended to treat hepatitis C.

GRANTS

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