Mechanisms of Liver Injury.

III. Oxidative stress in the pathogenesis of hepatitis C virus

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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 susceptibility of the polyprotein coding sequence. The HCV polyprotein is cleaved by cellular and viral proteases to generate 10 mature protein 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 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 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.

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 HCV infection 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 (NAD(P)H oxidase) of Kupffer cells and polymorphonuclear cells in the liver (Table 1) (7). NS3 protein of HCV has been found to activate NAD(P)H oxidase and to trigger apoptosis and dysfunction of T cells, natural killer cells, and natural killer T cells (24). NAD(P)H oxidase 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
**Table 1. Mechanism of increased oxidative stress in HCV infection**

<table>
<thead>
<tr>
<th>Possible Sources of ROS/RNS</th>
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<tr>
<td>Activation of NAD(P)H oxidase of Kupffer cells and PMN cells during inflammation.</td>
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<td>Iron overload and lipid peroxidation.</td>
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<td>Activation of NAD(P)H oxidase by NS3 protein</td>
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<td>Increased production of mitochondrial ROS/RNS by the electron transport chain due to core and NS5A proteins.</td>
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<td>Decreased GSH output due to liver damage</td>
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<tr>
<td>Decreased antioxidants and antioxidant gene expression</td>
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<tr>
<td>Alcohol, drugs, and other chemicals</td>
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<td>Increased cytokines that increase ROS</td>
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<tr>
<td>Increased expression/activity of COX-2</td>
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<td>Increased expression of CYP2E1</td>
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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 cytome B-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 metallothionein 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 Ca2+ uptake. Thus it was postulated that the core protein’s localization to the outer membrane results in mitochondrial dysfunction by facilitating Ca2+ accumulation. This increased Ca2+ 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 API 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 Ca2+ 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 XBPA 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 Ca2+ 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

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**Themes**

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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 O2 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
Oxidative stress; increased generation of reactive species then trig-
hepatitis C patients. Hyperglycemia is known to induce oxida-
tions 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 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.

CONCLUDING REMARKS
Hepatitis C virus infection is associated with severe alter-
ations 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.

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