Hepatitis C Virus Core Protein Inhibits Mitochondrial Electron Transport and Increases Reactive Oxygen Species (ROS) Production*

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Hepatitis C infection causes a state of chronic oxidative stress, which may contribute to fibrosis and carcinogenesis in the liver. Previous studies have shown that expression of the HCV core protein in hepatoma cells depolarized mitochondria and increased reactive oxygen species (ROS) production, but the mechanisms of these effects are unknown. In this study we examined the properties of liver mitochondria from transgenic mice expressing HCV core protein, and from normal liver mitochondria incubated with recombinant core protein. Liver mitochondria from transgenic mice expressing the HCV proteins core, E1 and E2 demonstrated oxidation of the glutathione pool and a decrease in NADPH content. In addition, there was reduced activity of electron transport complex I, and increased ROS production from complex I substrates. There were no abnormalities observed in complex II or complex III function. Incubation of control mitochondria with core protein also caused glutathione oxidation, selective complex I inhibition, and increased ROS production. Proteinase K digestion of either transgenic mitochondria or control mitochondria incubated with core protein showed that core protein associates strongly with mitochondria, remains associated with the outer membrane, and is not taken up across the outer membrane. Core protein also increased Ca\textsuperscript{2+} uptake into isolated mitochondria. These results suggest that interaction of core protein with mitochondria and subsequent oxidation of the glutathione pool and complex I inhibition may be an important cause of the oxidative stress seen in chronic hepatitis C.

Hepatitis C virus (HCV)\textsuperscript{2} infection produces acute and chronic hepatitis, cirrhosis, and hepatocellular carcinoma (1). Severity and rate of progression of the disease are highly variable and may reflect both host and viral factors (2) but the mechanisms of pathogenesis are incompletely understood. Because current antiviral treatment can only eliminate the virus in about 50% of patients (3–5), therapies to reduce disease progression in chronically infected individuals would be of great benefit. Thus, understanding the mechanisms of HCV pathogenesis is an important goal of HCV research. Numerous studies have shown that oxidative stress is present in chronic hepatitis C to a greater degree than in other inflammatory liver diseases (6, 7) and a prospective study showed improvement in liver injury in chronic hepatitis C with antioxidant treatment (8). Previous studies from our laboratory and others (9–12) have shown that HCV core protein induces the production of reactive oxygen species (ROS) but the mechanism of the core-associated increase in ROS production is not understood.

HCV core protein localizes to ER (13, 14), fat droplets (15, 16), and nucleus (17) as well as mitochondria (9, 18, 19). It has been shown to produce multiple cellular effects including changes in gene transcription, signal transduction, immune presentation, cell cycle regulation, and apoptosis (20–25). Despite this evidence, it is not known whether HCV core protein has a direct functional effect on mitochondria and whether this accounts for its ability to increase ROS. To clarify these questions, we investigated the interaction of HCV core protein with mitochondria in transgenic mice and by direct interaction of recombinant core protein with isolated mitochondria.

HCV protein expression caused an increase in mitochondrial ROS production, an oxidation of the mitochondrial glutathione pool, inhibition of electron transport, and an increase in ROS production by mitochondrial electron transport complex I. Direct incubation of isolated mitochondria with HCV core protein resulted in an increase of Ca\textsuperscript{2+} influx and ROS production and reproduced glutathione oxidation and the reduction in complex I function. These results suggest that direct interaction of core protein with mitochondria is an important cause of the oxidative stress seen in chronic hepatitis C.

MATERIALS AND METHODS

Generation of Transgenic Mice—The transgene, pAlbSVPA-HCV-S, containing the structural genes (core, E1, E2, and p7, nucleotides 342–2771) of hepatitis C virus genotype 1b, strain N, under the control of the murine albumin promoter/enhancer was described in detail by Lerat \textit{et al.} (26). This construct was injected into 1-cell F\textsubscript{2} zygotes of C57BL/6J × C3H/HeJ mice. Fourteen transgene-positive pups were obtained, out of 121 live births, as screened by polymerase chain reaction, using two pairs of primers spanning the promoter to the core gene for one pair and the E2 gene for the other pair. Positive results were confirmed by Southern blot analysis. Each of these transgenic founder mice were backcrossed to C57BL/6J. One transgenic line, designated SL-139 was selected for subsequent experiments. Female HCV transgenic mice were used at 5–7 months of age and age matched non transgenic littermates were used as controls. All animal procedures were performed according to the National Institutes of Health Guidelines and were approved by the Institutional Animal Care and Use Committee.
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**RNA Extraction and RT-PCR**—Liver samples were collected from transgenic mice of the first backcross (N1) generation, following carbon dioxide euthanasia. RNA was extracted from the liver using TRIzol (Sigma). Contaminating DNA was removed by brief treatment with DNase, which was removed by phenol/chloroform extraction. Reverse transcription was performed using Omniscript (Qiagen, Valencia CA), followed by PCR amplification of the E2 region using RedTaq (Sigma) according to manufacturers’ protocols. The PCR products were analyzed by electrophoresis on 2% agarose gels.

**Determination of Intrahepatic Core Protein Concentration**—SL-139 mice were sacrificed by CO2 asphyxiation, and liver protein was extracted in cold RPMI 1640. Quantity of HCV core protein was determined with an HCV core ELISA kit (trak-C™, Ortho Clinical Diagnostics, Raritan, NJ). Briefly, samples were mixed with a pretreatment buffer containing detergents and incubated at 56 °C for 30 min. A set of six standards, supplied by the manufacturer, which contained 100, 50, 15, 5, 1.5, and 0 pg/ml of HCV core antigen, was prepared. Standards, pretreated samples, and controls were transferred to a microwell plate coated with capturing monoclonal antibodies against HCV core protein, and incubated at 25 °C for 60 min. After washing, monoclonal antibody F(ab’)2 fragments conjugated to horseradish peroxidase were added and incubated at 25 °C for 30 min. After a final wash step, wells were incubated in the dark with substrate for 30 min. To stop color development, sulfuric acid was added, and absorbance was measured with a microwell plate reader at a wavelength of 490 nm with a reference wavelength of 620 nm. The concentration of HCV core antigen in each sample was determined from the standard curve.

**Isolation of Mitochondria**—Liver mitochondria were isolated by a modification of the method of Johnson and Hardy (27–29). In brief, liver (400 mg) was minced on ice and transferred (10% w/v) to isolation buffer (250 mM sucrose, 10 mM HEPES, 0.5 mM EGTA, 0.1% BSA, pH 7.4). The sample was gently homogenized by 3–4 strokes with a Dounce homogenizer and loose fitting pestle. The homogenate was centrifuged for 10 min at 4 °C to obtain a crude mitochondria pellet. The mitochondria pellet was resuspended in isolation buffer (without EGTA and BSA) and centrifuged again. The combined supernatant fractions were centrifuged at 7800 g for 5 min at 4 °C to obtain a crude mitochondria pellet. The mitochondria pellet was resuspended in isolation buffer (without EGTA and BSA) and centrifuged again at 7800 g for 10 min. An aliquot was removed for determination of protein concentration by the Bio-Rad assay kit, using bovine serum albumin as the standard.

**Determination of Glutathione Content**—Liver tissue samples (50–75 mg) and mitochondrial samples (2 mg) were sonicated using a Branson Sonifier 450 (VWR Scientific Products, West Chester PA) for 15 s at power setting 3 in ice-cold 5% trichloroacetic acid and centrifuged at 3000 × g at 4 °C for 10 min. The concentration of reduced GSH was measured by the thioester method using the GSH-400 kit (Oxis International Inc., Portland, OR). Total glutathione content of samples was measured by the glutathione reductase-DTNB recycling assay (30) using a commercial kit (GSH-412, Oxis International).

To measure the effect of recombinant core protein (amino acids 1–179, kindly provided by S. Watowich) on mitochondrial glutathione, freshly isolated mitochondria were suspended in phosphate-buffered saline and incubated at 25 °C for 5 min with or without core protein. Proteins were precipitated and thiols stabilized by subsequent addition of sulfosalicylic acid to a final concentration of 5%. To confirm that decreases in reduced GSH measured by the thioester method were indeed a result of oxidation, parallel mitochondrial samples were either further oxidized by exposure to 0.2 mM tBOOH for 5 min, or reduced by freeze-thaw followed by incubation with glutathione reductase (4.1 units/ml) and NADPH (1 mM) for 5 min at 25 °C. Following reduction, samples were precipitated with sulfosalicylic acid and processed as described. Control experiments showed that this tBOOH treatment fully oxidized the glutathione pool under these conditions and it was used to determine the background value for the assay.

**NADPH and Glutathione Reductase Measurement**—NADPH was measured in isolated mouse liver mitochondria by the method described by Zhang et al. (31). Mitochondrial pellets were suspended in 0.1 M Tris, 10 mM EDTA, 1% Triton X-100, pH 7.6, and then centrifuged at 20,000 × g for 10 min to remove membrane debris and obtain clear supernatant. Absorbance at 340 nm was determined in untreated supernatants (A0), and after specific oxidation of NADPH to NADP+ with glutathione reductase and GSSG (A1). A0 – A1 represented the amount of NADPH in the sample (31). Glutathione reductase (GR) activity was measured as the rate of decrease in absorbance at 340 nm caused by the oxidation of NADPH (GR assay kit, Sigma). A reaction with assay buffer instead of mitochondrial sample was run as a blank.

**Measurement of Oxygen Consumption**—Oxygen consumption of isolated mitochondria was measured at 25 °C using a model 782 oxygen meter system and model 1302 Microcathode oxygen electrode (Strathkevin, Glasgow, UK). Mitochondrial pellet (1–1.5 mg/ml) was added to the 1-ml sample chamber filled with respiration buffer (130 mM sucrose, 50 mM KCl, 5 mM MgCl2, 5 mM KH2PO4, 0.05 mM EDTA, and 5 mM HEPES, pH 7.4) and allowed to equilibrate with magnetic stirring. Complex I-supported state 4 respiration was initiated by addition of 5 mM glutamate and 5 mM malate to the sample chamber. Subsequent addition of 100 nmol of ADP initiated complex I-supported state 3 respiration. After returning to state 4 respiration, maximum oxygen consumption (uncoupled respiration) was measured by adding 5 μM carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP). Similarly, complex II-supported state 3 and 4 respiration was measured using 5 mM succinate.

**Effects of tBOOH and HCV Core Protein on Mitochondrial Respiration**—Isolated hepatic mitochondria were incubated with 100 μM tBOOH and/or 1, 10, or 100 ng of recombinant HCV core protein per mg of mitochondrial protein at 25 °C for 5 min. Aliquots of the mitochondrial suspension were added to the sample chamber for analysis of rates of oxygen consumption. P.O ratio and FCCP-induced consumption rates were calculated as described by Estabrook (32).

**Measurement of Complex I and III Activity**—Enzyme activity assays were performed at 25 °C by previously established methods (33, 34). Submitochondrial particles (SMPs) were prepared from mitochondria by incubation for 3 min at 37 °C followed by sonication in a microcentrifuge tube immersed in ice water. Submitochondrial particles were pelleted at 15,000 × g for 10 min, and 50 μg were used for each assay. In some instances SMPs were reduced by incubation with dithiothreitol (100 μM) for 10 min at 0 °C. Complex I activity (NADH-decyldiubiquinone oxidoreductase) was measured as the initial (5 min) rate of decrease of A340 using the acceptor 2,3-dimethoxy-5-methyl-6-n-decyl-1,4-benzoquinone (DB 80 μM) and 200 μM NADH as the donor in 10 mM Tris (pH 8.0) buffer containing 1 mg/ml BSA, 0.24 mM KCN, and 0.4 μM antimycin A. Complex III activity (ubiquinol cytochrome c reductase) was measured at 550 nm using 40 μM oxidized cytochrome c as the acceptor and 80 μM decylubiquinol as the donor in 10 mM KH2PO4 (pH 7.8), 1 mg/ml BSA, 2 mM EDTA, in the presence of 0.24 mM KCN, 4 μM rotenone, 0.2 mM ATP for 2 min. The addition of 1 μM antimycin A allowed us to distinguish between the reduction of cytochrome c catalyzed by complex III and the nonenzymatic reduction of cytochrome c by the reduced quinone. Extinction coefficients were 6200 liters/mmol cm for NADH and 2.11 × 104 liters/mmol cm for oxidized cytochrome c.
Measurement of ROS Production in Mitochondria—Mitochondrial ROS production was determined with the oxidation sensitive fluorescent probe dihydrodichlorofluorescein diacetate (DCFDA, Molecular Probes) (35). Briefly, each well of a 96-well microtiter plate was filled with respiration buffer containing 1 μM DCFDA and 0.5 mg/ml of mitochondrial particles (final volume, 0.2 ml). The reaction was started by addition of 5 mM glutamate or 5 mM succinate and then incubated at 30 °C in a shaker for 30–60 min. Fluorescence was measured with a CytoFluorII fluorescence plate reader (PerSeptive Biosystems, Inc., Framingham, MA) at excitation of 485 nm and emission of 530 nm. Some experiments included inhibitors, 5 μM FCCP, 1 μM rotenone, or 10 μM BAPTA-AM (Molecular Probes). In some experiments mitochondria from control mice were incubated for 5 min with HCV core protein. For Ca2+-induced ROS production, the mitochondrial suspension was first exposed to 125 μM Ca2+ for 30 min on ice.

Measurement of Mitochondrial Ca2+—For Ca2+ determination, mitochondria (0.5 mg/ml) were incubated for 1 h at 4 °C with the mitochondrial Ca2+ indicator Rhod-2 AM (4 μM, Molecular Probes), washed twice in 0.25 M sucrose, 2 mM K-Hepes buffer, and diluted to a buffer for 30 min on ice. The red fluorescence of Rhod-2 was measured with a CytoFluorII fluorescence plate reader (PerSeptive Biosystems, Inc., Framingham, MA) at excitation of 485 nm and emission of 530 nm. Some experiments included inhibitors, 5 μM FCCP, 1 μM rotenone, or 10 μM BAPTA-AM (Molecular Probes). In some experiments mitochondria from control mice were incubated for 5 min with HCV core protein. For Ca2+-induced ROS production, the mitochondrial suspension was first exposed to 125 μM Ca2+ for 30 min on ice.

Western Blotting—Samples were lysed in 625 mM Tris, pH 7.4, 2% SDS, 1% EDTA, and 1% protease inhibitor mixture (Sigma). Mitochondria and liver lysates were centrifuged at 7900 × g for 10 min, and the supernatants (30 μg of protein) were subjected to SDS-polyacrylamide gel electrophoresis. The proteins were electrotransfered to polyvinylidene difluoride membranes (Bio-Rad), blocked overnight at 4 °C with 5% nonfat dried milk, 0.1% Tween 20 in phosphate-buffered saline and subsequently incubated for 2 h at room temperature with mouse monoclonal antibody to human hepatitis C virus core protein (1:450, Anogen, Mississauga, ON), rabbit polyclonal anti-Tom20 antibody (1:2000, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse monoclonal anti-cytchrome c antibody (1:2000, R & D System, Inc., Minneapolis, MN), mouse monoclonal IgG anti-complex III core 2 subunit antibody (1:5000, Affinity Molecular Probes, Inc.) or mouse monoclonal antimitochondrial heat shock protein 70 antibody (1:2000, affinity BioReagents, Golden, CO). The membranes were washed, incubated with appropriate secondary antibodies, and detected with the ECLplus chemiluminescence system (Amersham Biosciences).

Assessment of Core Protein Localization by Proteolysis—Isolated mitochondria from transgenic liver were incubated in respiration buffer with proteinase K (50 μg/ml) for 30 min at 4 °C. After incubation, protease activity was inhibited by addition of phenylmethylsulfonyl fluoride to a final concentration of 2 μM, followed by incubating on ice for an additional 10 min. Then 10 μg of the mitochondrial suspension was subjected to Western blotting without centrifugation. 1% Triton X-100 was used in some experiments to disrupt the mitochondrial membranes.

Statistics—Results are expressed as mean ± S.E. Student’s t test was used for statistical analyses. p < 0.05 was considered significant.
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FIGURE 2. HCV core protein expression in transgenic mouse liver and mitochondria. Immunoblot demonstration of HCV core protein expression in liver mitochondria. CON, control mice; TgM, transgenic mice; L, liver lysate; M, mitochondrial lysate. A, each lane was loaded with 100 µg of either total lysate or mitochondrial protein. Two forms of core protein were detected in transgenic but not control samples. HCV core protein in mitochondria was 4-fold more abundant in mitochondria than in liver lysate. B, purified HCV core protein (1–179) was added to control mitochondrial lysate (10 ng or 5 ng/mg mitochondrial protein) and compared with two separate mitochondrial lysates.

FIGURE 3. Glutathione content in HCV transgenic mouse liver. Glutathione content was measured in freshly isolated whole liver homogenate (A and B) or mitochondrial fractions (C and D) as described under “Materials and Methods.” Total glutathione (GSH + GSSG) was expressed as GSH equivalents (A and C). Reduced glutathione (GSH only) is shown in B and D. Values in transgenic liver samples were compared with those in corresponding control liver samples (n = 3–6, **, p < 0.01). Baseline values for total glutathione in control samples were 36.6 ± 1.9 nmol/mg protein for whole liver and 8.2 ± 1.2 nmol/mg protein for mitochondria.

demonstrating a baseline oxidation of the mitochondrial glutathione pool in these animals.

To determine if capacity to maintain a reduced mitochondrial environment was altered we measured mitochondrial GR activity and NADPH. GR activity was similar in control versus transgenic mitochondria (2.58 ± 0.27 versus 2.49 ± 0.34 milliunits/mg mitochondrial protein, n = 8). However, mitochondrial NADPH content was decreased in transgenic compared with control mitochondria (0.80 ± 0.17 versus 1.32 ± 0.17 nmol/mg mitochondrial protein, n = 9, p < 0.05). Total (NADP+ + NADPH) content was unchanged. This result further confirms the oxidized phenotype of the transgenic mitochondria.

Effects of Transgene on Mitochondrial Respiration—Previous studies have demonstrated that core protein causes depolarization of the mitochondrial membrane potential (ΔΨ) (10). This depolarization could result either from decreased electron transport or increased proton leak. To distinguish between these possibilities, we measured O2 consumption in the transgenic mouse liver mitochondria. The results are presented in Fig. 4. Transgene expression significantly reduced the P:O ratio when the complex I substrates glutamate/malate were used (Fig. 4A, p < 0.05), but had no effect on P:O ratio when the complex II substrate, succinate, was used (Fig. 4C). Maximal O2 consumption in the presence of FCCP was also reduced in transgenic mitochondria, but this change was not significant (Fig. 4B). These data suggest that inhibition of electron transport at complex I and not proton leak is the primary mechanism of reduced ΔΨ after HCV core expression.

To confirm if HCV proteins also sensitized mitochondria to oxidative stress, we treated mitochondria with tBOOH. A 5-min exposure at 25 °C to 100 µM tBOOH had no effect on control mitochondria but it significantly reduced complex I-mediated P:O ratio and maximal O2 consumption rate in transgenic mitochondria (Fig. 4, A and B). There were no changes in the same parameters measured when succinate was used as substrate (Fig. 4, C and D).

To determine if the inhibition of O2 consumption from complex I substrates was a direct result of complex inhibition, we measured complex I and complex III activities in SMPs. Liver SMPs derived from transgenic mice had an ~25% reduction of complex I activity compared with that of SMPs from control liver (Fig. 5A, p < 0.001). Decreased activity could not be reversed by reduction of SMPs with dithiothreitol. However, complex III activity was normal (Fig. 5B). The magnitude and specificity of this effect was similar to that seen for oxygen consumption...
demonstrating that complex I inhibition was the primary cause of the reduction in respiration in transgenic mitochondria.

Effects of Recombinant HCV Core Protein on Isolated Mitochondria—To assess whether the effects seen in the transgenic mitochondria result directly from the interaction of core protein with mitochondria we measured mitochondrial respiration and enzyme activity after incubation of normal liver mitochondria with purified HCV core protein. Mitochondria were incubated for 5 min at 25 °C with varying concentrations of recombinant core protein. At concentrations of core protein present in the transgenic mitochondria (from 1–10 ng of core protein/mg mitochondrial protein), there was a specific reduction of \( O_2 \) consumption and P:O ratio from glutamate/malate with no effect on the interaction of core protein with mitochondria. Mitochondria isolated from HCV transgenic mice had two forms of core protein-associated, 23 and 21 kDa, possibly reflecting the nascent (1–191) and C-terminal-truncated processed transgene expression (Fig. 8D).

We further measured the effect of exogenous core protein on the mitochondrial glutathione pool (Fig. 7). In control mitochondria, 95% of the total glutathione pool was in the reduced form. Further reduction with glutathione reductase/NADH did not significantly increase GSH content. Incubation with 80 ng of core protein/mg mitochondrial protein did not change the total glutathione content. However it produced a significant oxidation of the glutathione pool as evidenced by a decrease in measured GSH, which could be restored by subsequent enzymatic reduction. A smaller degree of glutathione oxidation was observed at 10 ng core/mg protein and complete oxidation was produced by incubation with 0.2 mM tBOOH (Fig. 7).

**ROS Production in Mitochondria**—The effect of HCV proteins on mitochondrial ROS production was assessed using the ROS-sensitive fluorescent probe, DCFDA. Fig. 8A shows that ROS production during state 4 respiration was increased in transgenic mitochondria in the presence of complex I but not complex II substrates. ROS production was completely blocked by the complex I inhibitor, rotenone (Fig. 8B). In the presence of the complex II substrate, succinate, total \( O_2 \) consumption was greater (Fig. 4, B versus D) but ROS production was less and was not increased in transgenic mitochondria. These data indicate that complex I is the primary source of the increased ROS production in HCV transgenic mitochondria. The uncoupler FCCP reduced ROS under all conditions as previous reported (39, 40); however, the transgenic mitochondria retained increased complex I ROS production (Fig. 8C). Incubation of mitochondria for 5 min with recombinant core protein prior to addition of FCCP had similar effects on ROS production as did the HCV transgene expression (Fig. 8D). At a concentration of 10 ng/mg protein, core specifically increased complex I ROS production.

**HCV Core Protein Localization within Mitochondria**—To further determine if direct core protein interactions are a possible mechanism for in vivo effects, we next examined the nature of the interaction of core with mitochondria. Mitochondria isolated from HCV transgenic mice had two forms of core protein-associated, 23 and 21 kDa, possibly reflecting the nascent (1–191) and C-terminal-truncated processed protein HCV core protein (80 core), or 0.2 mM tBOOH (80OOG). Total glutathione was measured directly by the DTNB recycling assay as described (black bars). Reduced glutathione samples were measured either before (light gray bars) or after (dark gray bars) reduction of the glutathione pool with glutathione reductase and NADPH. *, \( p < 0.05 \); **, \( p < 0.01 \).
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FIGURE 8. ROS production in isolated mitochondria. A, ROS production was measured by the change in DCF fluorescence under conditions of state 4 respiration. B, effect of rotenone (1 μM) on ROS production. C, effect of uncoupler (5 μM FCCP) on mitochondrial ROS production. D, purified core protein (10 ng/mg protein) was incubated with control mitochondria under the same conditions as in Fig. 7C (n = 4 individual experiments. *p < 0.05; **p < 0.01). G/M, glutamate and malate; S, succinate; (-), no substrate.

form (13, 41, 42). Fig. 9A demonstrates that digestion of transgenic mitochondria with 50 μg/ml protease K removed the outer membrane protein, Tom20, without digesting the intermembrane space protein, cytochrome c, the inner membrane protein (core 2 subunit of complex III), or the matrix protein, mtHSP70. After disruption of mitochondrial membranes with Triton X-100, all proteins were digested by the protease. Incubation of control mitochondria with exogenous core protein also resulted in protease labile mitochondrial association (Fig. 9B). Therefore, under these conditions core protein behaved as an outer membrane-associated protein.

Effects of Core on Mitochondrial Ca2+ Uptake—One possible mechanism for how interaction of core protein with the outer membrane might affect mitochondrial redox state would be if it altered mitochondrial Ca2+ uptake (43). To examine this possibility we used the fluorescent Ca2+ indicator, Rhod-2, to determine the effect of core protein on intramitochondrial Ca2+. Fig. 10A demonstrates that core protein had no effect on mitochondrial Ca2+ content as long as mitochondria were incubated in standard isolation buffer without added Ca2+. Upon addition of Ca2+ to the mitochondria, however, core significantly increased total intramitochondrial Ca2+ content. As expected, Ca2+ uptake in this system was completely prevented by dissipation of the mitochondrial Δψ with FCCP.

We further measured the effect of core on Ca2+-induced ROS production in isolated mitochondria. Ca2+ addition to isolated mitochondria greatly increased ROS production, and this effect was further increased by core protein. The Ca2+ chelator, BAPTA-AM, largely prevented the Ca2+-induced increase in ROS production and diminished the effect of core protein (Fig. 10B).

DISCUSSION

Chronic HCV infection is associated with excess oxidative stress within the liver and several different experimental models of HCV protein expression reproduce this finding. HCV transgenic mice have increased hepatic lipid peroxidation (9, 11), and HCV core protein expression in hepatoma cells results in an increase in mitochondrial ROS production (9, 10). However, the details of how the core protein alters mitochondrial function are unknown.

To specifically determine the nature of the mitochondrial abnormalities, we developed a new line of HCV transgenic mice. These mice express the viral proteins core, E1, E2, and p7 on a C57BL/6 background and offered us the ability to study mitochondrial function in hepatocytes from native liver. Although these mice lack viral replication, they have the advantage of expressing viral proteins in liver at levels that are characteristic of the disease in humans. The expression of core protein (0.59
pg/μg) is well within the range of 0–2 pg/μg determined in clinical specimens with the identical ELISA kit and standards. Liver histology was normal making these mice similar to patients infected with the 1b genotype in that the presence of viral proteins alone does not result in massive steatosis (44, 45).

Our results show that HCV protein expression caused an oxidative mitochondrial phenotype characterized by oxidation of both the mitochondrial glutathione and pyridine nucleotide pools (Fig. 3). Liver mitochondria from transgenic mice displayed a specific defect in complex I-mediated electron transport and an increase in ROS production. There were no abnormalities in complex II/III-mediated oxidation of succinate. Protease digestion studies showed that core protein was directly associated with the mitochondrial outer membrane. When incubated with normal mitochondria in vitro, core protein also caused increased Ca^{2+} entry, increased ROS production, glutathione oxidation, and reduced complex I activity. It is thus likely that core protein, like multiple other viral proteins (46), has important mitochondrial effects.

Mitochondrial ROS generation can occur at either complex I or complex III (47–49), and our data demonstrate that complex I, and not complex III is the source of the core-induced ROS. Direct ROS production during state 4 respiration was increased in transgenic mitochondria only in the presence of complex I substrates, it was inhibited by the complex I inhibitor rotenone, and it was associated with decreased net electron transport activity of complex I. While complex III mediated ROS production from glutamate/malate would also be inhibited by rotenone, core protein-induced ROS production was not detected when succinate was used as substrate and thus did not originate from complex III.

Complex I ROS production has been reported to be either inhibited (50–52) or sometimes increased by rotenone (48, 53, 54). The variability of the effect of rotenone on complex I-associated ROS production could reflect the multiple possible sites within complex I at which superoxide can be formed or could reflect different ROS detoxification at these sites (55). We readily detected an increase in succinate-induced ROS after adding antimycin A (data not shown) suggesting that the ROS source primarily been generated from complex III it would have been observed. The data thus support complex I as the site of ROS generation.

Accumulating evidence now clearly demonstrates the association of core protein with mitochondria. Core protein is located in mitochondria in transgenic liver, in several different cell lines expressing this protein, and in hepatoma cells bearing genomic length replicons of HCV (9, 18, 19). Two recent studies have shown that mitochondrial core protein is specifically associated with the outer membrane (18, 19), and our studies reported here confirm that this is the case in transgenic mouse liver as well. Importantantly, Schwer et al. (18) demonstrated that core protein associates with the mitochondrial-associated membrane (MAM) fraction, a point of close contact between ER and mitochondrion. The finding that incubation of control mitochondria with recombinant core protein at concentrations similar to those associated with mitochondria in vivo oxidized the mitochondrial glutathione pool and reproduced specific complex I changes argues that it is this direct interaction that produces the effects. Separate events may be responsible for initiating and sustaining the core-induced increase in mitochondrial ROS production. The present study shows that core protein increases Ca^{2+} entry into isolated mitochondria with a subsequent increase in ROS (Fig. 10). This suggests that an increase in mitochondrial Ca^{2+} might be the initiating event.

Increased mitochondrial Ca^{2+} has been previously observed to increase ROS production, possibly by stimulating electron flow in the respiratory chain, or altering structure of electron transport complexes (see Refs. 43 and 56 for reviews). At the present time the mechanism of how core increases mitochondrial Ca^{2+} uptake is not known, but direct effects on mitochondria Ca^{2+} transporters or effects on ER-mitochondrial interactions are both possible.

Subsequent to the Ca^{2+}-induced increase in ROS production, complex I may play a role in sustaining and amplifying increased mitochondrial ROS production. Complex I is the site most sensitive to oxidative damage of the electron transport carriers and inhibition of complex I occurs during the early stages of mitochondrial damage (57). Specific inhibition of complex I has previously been reported to be a consequence of oxidation of the mitochondrial glutathione pool and consequent glutathionylation of complex I subunits (58). Murphy and coworkers have observed that glutathionylation decreased complex I activity and increased complex I ROS production. Glutathionylation was reversed by treatment with DTT, but there was no recovery of complex I activity. These findings are strikingly similar to the effects of HCV core protein on liver mitochondria. It is thus possible that core-induced GSH oxidation and consequent complex I glutathionylation can sustain and amplify the oxidized mitochondrial environment.

Net oxidation of the glutathione pool could result either from increased GSH oxidation or decreased GSSG reduction. Whereas we did not observe any effect on glutathione reductase activity or total nicotinamide nucleotide content, the increase in GSSG was associated with a decrease in NADPH. Because the mitochondrial NADPH pool is maintained primarily by the activity of several systems such as nicotinamide nucleotide transhydrogenase, isocitrate dehydrogenase, and malate dehydrogenase (59), it is possible that changes in the activities of these enzymes, in addition to Ca^{2+}-induced ROS production, could contribute to net oxidation of the glutathione pool.

It has previously been shown that several HCV proteins, including core protein (60), can contribute to a state of ER stress (61) and this may secondarily alter mitochondrial function via changes in Ca^{2+} homeostasis (62). Whereas we cannot rule out a contribution of ER stress in the transgenic mouse liver, the experiments with in vitro addition of core protein indicate that direct mitochondrial effects are present as well.

In conclusion, our study shows that HCV core protein localizes to mitochondria, associates with the mitochondrial outer membrane, increases mitochondrial Ca^{2+} uptake, and causes oxidation of the glutathione pool. This change in mitochondrial redox state inhibits complex I activity, further increases ROS production and can create positive feedback loop (58). Mitochondrial GSH depletion or oxidation is associated with enhanced liver injury in alcoholic, toxic, and inflammatory liver diseases (63), and thus could have significant effects on response to inflammation and the development of progressive liver disease in chronic hepatitis C.

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REFERENCES
1. Seeff, L. B. (2002) Hepatology 36, S35–S46
2. Cerney, A., and Chisari, F. V. (1999) Hepatology 30, 595–601
3. Manns, M. P., McHutchison, J. G., Gordon, J. G., Rustgi, V. K., Shiffman, M., Reid- dolar, R., Goodman, Z. D., Koury, K., Ling, M., and Albrecht, J. K. (2001) Lancet 358, 958–965
4. Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncales, F. L., Jr., Haussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J.,

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3 J. Liang, personal communication.
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and Yu, J. (2002) N. Engl. J. Med. 347, 975–982
5. Davis, G. L. (2000) Gastroenterology 118, S104–S114
6. Barbaro, G., Di Lorenzo, G., Asti, A., Ribersarsi, M., Belloni, G., Gritorio, B., Filese, G., and Barbarini, G. (1999) Am. J. Gastroenterol. 94, 2198–2205
7. Valgimigli, M., Valgimigli, L., Trere, D., Giautani, S., Pedulli, G. F., Granantieri, L., and Bolondi, L. (2002) Free Radic. Res. 36, 939–948
8. Hougum, K., Venkataramani, A., Lyche, K., and Chojkier, M. (1997) Gastroenterology 113, 1069–1073
9. Okuda, M., Li, K., Beard, M. R., Showalter, L. A., Chung, A. S. M., and Weimann, S. A. (2002) Gastroenterology 122, 366–375
10. Otani, K., Korenaga, M., Beard, M. R., Li, K., Qian, T., Showalter, L. A., Singh, A. K., Wang, T., and Weimann, S. A. (2005) Gastroenterology 128, 96–107
11. Moriya, K., Nakagawa, K., Sato, T., Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Miyazawa, T., Ishii, K., Tani, T., Imai, K., Todoroki, T., Kimura, S., and Koike, K. (2001) Cancer Res. 61, 4365–4370
12. Wen, F., Abdalla, M. Y., Aloman, C., Xiang, J. H., Ahmad, I. M., Walewski, J., McCor-
13. Santolini, E., Migliaccio, G., and La Monica, N. (1994) J. Biol. Chem. 268, 3631–3641
14. Moradpour, D., Englert, C., Wakita, T., and Wands, J. R. (1996) Virology 222, 51–63
15. Moriya, K., Nakagawa, K., Sato, T., Shintani, Y., Fujie, H., Miyoshi, H., Tsutsumi, T., Miyazawa, T., Ishii, K., Tani, T., Imai, K., Todoroki, T., Kimura, S., and Koike, K. (2001) Cancer Res. 61, 4365–4370
16. Yasui, K., Wakita, T., Tsukiyama-Kohara, K., Funahashi, S. I., Ichikawa, M., Kajita, T., Moradpour, D., Wands, J. R., and Kohara, M. (1998) J. Virol. 72, 6048–6055
17. Schaff, Zs., Chapman, M. J., Miyamura, T., and Brechot, C. (1997) Gastroenterology 113, 1271–1281
18. Houglum, K., Venkataramani, A., Lyche, K., and Chojkier, M. (1997) Cancer Res. 57, 6858–6868
19. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) J. Biol. Chem. 260, 421–427
20. Becker, L. B., vanden Hoek, T. L., Shao, Z. H., Li, C. Q., and Schumacker, P. T. (1999) J. Biol. Chem. 274, 159–168
21. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) J. Biol. Chem. 260, 421–427
22. Weihofen, A., Binns, K., Lemberg, M. K., Ashman, K., and Martoglio, B. (2002) Science 296, 2215–2218
23. Brookes, P. S., Yoon, Y., Robotham, J. L., Anderson, M. W., and Sheu, S. S. (2004) Am. J. Physiol. Cell Physiol. 287, C817–C833
24. Adinolfi, L. E., Gambardella, M., Andrea, A., Tripodi, M. F., Utili, R., and Ruggiero, G. (2001) Hepatology 33, 1358–1364
25. Kumar, D., Farrell, G. C., Fung, C., and George, J. (2002) Hepatology 36, 1266–1272
26. Loguercio, C., and Federico, A. (2003) Free Radic. Biol. Med. 34, 1–10
27. Cadenas, E., Boveris, A., Wands, J. R., and Stroh, A. (2001) Arch. Biochem. Biophys. 380, 248–257
28. Turrens, J. F., and Boveris, A. (1980) Biochem. J. 191, 421–427
29. Turrens, J. F., Alexandre, A., and Lehninger, A. L. (1985) Arch. Biochem. Biophys. 237, 414
30. Vrable, A. S., Albright, C. D., Craciumescu, C. N., Salganik, R. I., and Zeisel, S. H. (2001) FASEB J. 15, 1739–1744
31. Li, Y., Stansbury, K. H., Zhu, H., and Trush, M. A. (1999) Biochem. Biophys. Res. Commun. 262, 80–87
32. Becker, L. B., vanden Hoek, T. L., Shao, Z. H., Li, C. Q., and Schumacker, P. T. (1999) Am. J. Physiol. 277, H2240–H2246
33. Takeshige, K., and Minakami, S. (1979) Biochem. J. 180, 129–135
34. Kwong, L. K., and Sohal, R. S. (1998) Arch. Biochem. Biophys. 350, 118–126
35. Chen, Q., Vazquez, E. J., Moghaddas, S., Hoppel, C. L., and Lesnefsky, E. J. (2003) J. Biol. Chem. 278, 36027–36031
36. Bianchi, K., Rimessi, A., Prandini, A., Szabadi, K., and Rizzuto, R. (2004) Biochim. Biophys. Acta 1742, 119–131
37. Higuchi, M., Prosek, R. J., and Yeh, E. T. H. (1998) Oncogene 17, 2515–2524
38. Taylor, E. R., Rurrell, F., Shannon, R. J., Lin, T. K., Hirst, J., and Murphy, M. P. (2003) J. Biol. Chem. 278, 19603–19610
39. Hanukoglu, I., and Rapoport, R. (1995) Endocr. Rev. 16, 231–241
40. Benali-Furet, N. L., Chami, M., Houel, L., De Giorgi, F., Vernejoul, F., Lagorce, D., and Barbarini, G. (1999) Biochemical and Medical Aspects. in Free Radic. Res. Oncogene 24, 4921–4933
41. Tardif, K. D., Waris, G., and Siddiqui, A. (2005) Trends Microbiol. 13, 159–163
42. Gong, G., Waris, G., and Siddiqui, A. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 9599–9604
43. Oladottor, K., Pascoe, G. A., and Reed, D. J. (1988) Arch. Biochem. Biophys. 263, 226–235