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Role of Hepatitis C virus core protein in viral-induced mitochondrial dysfunction

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SUMMARY

Hepatitis C virus (HCV) infection results in several changes in mitochondrial function including increased reactive oxygen species (ROS) production and greater sensitivity to oxidant, Ca^{2+} and cytokine-induced cell death. Prior studies in protein over-expression systems have shown that this effect can be induced by the core protein, but other viral proteins and replication events may contribute as well. To evaluate the specific role of core protein in the context of viral replication and infection, we compared mitochondrial sensitivity in Huh7-derived HCV replicon bearing cells with or without core protein expression with that of cells infected with the JFH1 virus strain. JFH1 infection increased hydrogen peroxide production and sensitized cells to oxidant-induced loss of mitochondrial membrane potential and cell death. An identical phenomenon occurred in genome-length replicons-bearing cells but not in cells bearing the subgenomic replicons lacking core protein. Both cell death and mitochondrial depolarization were Ca^{2+} dependent and could be prevented by Ca^{2+} chelation. The difference in the mitochondrial response of the two replicon systems could be demonstrated even in isolated mitochondria derived from the two cell lines with the ‘genome-length’ mitochondria displaying greater sensitivity to Ca^{2+} -induced cytochrome *c* release. *In vitro* incubation of ‘subgenomic’ mitochondria with core protein increased oxidant sensitivity to a level similar to that of mitochondria derived from cells bearing genome-length replicons. These results indicate that increased mitochondrial ROS production and a reduced threshold for Ca^{2+} and ROS-induced permeability transition is a characteristic of HCV infection. This phenomenon is a direct consequence of core protein interactions with mitochondria and is present whenever core is expressed, either in infection, full-length replicon-bearing cells, or in over-expression systems.

Keywords

HCV; JFH1; mitochondrial permeability transition; reactive oxygen species; replicons

INTRODUCTION

Hepatitis C virus (HCV) is a single-stranded RNA virus that chronically infects approximately 2% of the world’s population [1] and causes cirrhosis and hepatocellular carcinoma [2].

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DISCLOSURES

None.

Although much is known about the biology of the virus, less is understood about the mechanisms by which it causes liver disease. While there is conclusive evidence that the onset of liver disease requires a cellular immune response [3], recent studies have shown that viral infection of hepatoma cells in culture is directly cytopathic [4,5], and viral proteins themselves, particularly core, NS3 and NS5a interact extensively with the host cell proteome and have multiple direct effects on hepatocytes [6].

Oxidative stress and mitochondrial dysfunction have been widely observed in liver samples from patients with chronic hepatitis C, and the presence of oxidative protein derivatives correlates with disease severity [7–10]. Experimental studies suggest the involvement of core protein in this process [11–14]. Core protein localizes to ER [15,16], fat droplets [17,18], nucleus [19] and mitochondria [20,21]. It contributes to ER stress [22], alters mitochondrial Ca^{2+} uptake [23], and sensitizes mitochondria to permeability transition [24–26]. In transgenic mice, expression of core protein alone can be sufficient to induce development of hepatocellular carcinoma [27]. Nonetheless, the importance of these core protein effects in the context of viral RNA replication and the complete viral life cycle is unclear. Other viral proteins, particularly NS5a [28] and NS3/4a [29] have the potential to contribute to ER and oxidative stress and most of the data supporting the importance of core protein in mitochondrial dysfunction were obtained in over expression systems and transgenic mouse models that lack viral replication [13,14,30].

To determine if direct effects of core protein on mitochondria play a critical role in the context of viral infection and replication, we have examined mitochondrial function and apoptosis in JFH1 infected cells, hepatoma cells stably replicating genome-length and subgenomic HCV replicons, and incubations of isolated mitochondria from these cells with purified core protein. Our results indicate that the JFH1 infected cells and genome-length replicons cells are more sensitive to oxidant-induced cell death and mitochondrial depolarization than either subgenomic replicon cells or uninfected cells. Mitochondria obtained from subgenomic replicon cells are more resistant to permeability transition than those obtained from genome-length replicons but become similar to the genome-length mitochondria when incubated with exogenous core protein. These results indicate that core protein and its direct interactions with mitochondria play a critical role in mitochondrial dysfunction during HCV infection.

MATERIALS AND METHODS

Cell lines and reagents

Huh7 or Huh7.5 cells (obtained from Dr Charles Rice) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% FBS, 50 U/mL penicillin and 50 mg/mL streptomycin. Subgenomic and genome-length replicon-bearing cells (derived from Huh7 cells) were maintained in the same medium with the addition of G418 and/or Blasticidin for selection purposes as described in the following paragraphs. Cells with inducible and regulated expression of core protein were used as previously described [25]. All reagents were purchased from Sigma–Aldrich (St. Louis, MO, USA) unless otherwise noted.

HCV infection

DNA coding for the Japanese Fulminant Hepatitis 1 (JFH1) sequence, genotype 2a, was obtained from Dr T. Wakita. The plasmid was propagated, reversed transcribed and the resulting RNA used to transfect (by electroporation) Huh7.5 cells for the production of intact viral particles as described [31]. For the experiments described in this paper, Huh7.5 cells (3×10^5) were seeded onto T-25 flasks and infected the following day with HCV at a multiplicity

of infection of 0.5–1.0. At 48 h postinfection, control and infected cells were seeded on six-well plates and treated as described in the following sections.

Construction of HCV replicon bearing cells

A subgenomic selectable dicistronic HCV replicon was constructed from a genotype 1a H77c infectious molecular clone as reported previously [32]. Similarly, a genome-length version of this same HCV replicon was constructed as indicated in Fig. 1a. Translation of the downstream cistron was driven by the encephalomyocarditis virus IRES, leading to the production of NS3-NS5B alone (subgenomic) or C, E1, E2, p7, NS2, NS3-5B (genome-length) transcripts. *In vitro* transcripts derived from these replicon constructs were electroporated into Huh-7 cells, and cell clones were selected based on G418 resistance. Both, subgenomic and genome-length HCV replicon cells express and replicate a stable and at high level HCV RNA but do not produce viral particles. The subgenomic replicon cells express the nonstructural viral proteins including NS3 to NS5B [32,33], while the genome-length replicon cells express the full range of HCV proteins including core protein.

Preparation of mitochondria

Cells (1×10^7) were rinsed with PBS, scraped, resuspended in HEPES isotonic mannitol (HIM) buffer (200 mM mannitol, 70 mM sucrose, 1 mM ethylene glycol-bis (β -amino ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 10 mM HEPES, pH 7.5) and homogenized by 12 strokes in a tight-fitting Dounce homogenizer. Cell lysates were clarified by centrifugation at 500 *g* for 5 min and subjected to a second centrifugation at 10 000 *g* for 10 min to collect a crude mitochondrial pellet. For proteinase K accessibility assays, crude mitochondria were resuspended in HIM buffer without EGTA.

Hydrogen peroxide measurements

Hydrogen peroxide levels were measured in control and HCV-infected cells using Amplex Red Reagent (Invitrogen, Eugene, OR, USA) following a modified version of the protocol described by Mohanty *et al.* [34]. Control and infected cells were plated on 96-well plates and incubated overnight. The following day medium was replaced with 100 μ L of Krebs Ringer Phosphate [145 mM NaCl, 5.7 mM NaH₂PO₄, 4.9 mM KCl, 0.5 mM CaCl₂, 1.2 mM MgSO₄, 5.5 mM Glucose (pH 7.3)] containing 50 μ M Amplex Red reagent and 0.1 U/mL Horseradish peroxidase. Cells were incubated for 30 min to 1 h, and fluorescence was read at 544 nm excitation and 590 nm emission wavelengths. Cell viability was calculated in parallel using Cell Titer-Blue Reagent (Promega, Madison, WI, USA) following manufacturer instructions. Amplex Red fluorescence was normalized using cell viability measurements obtained from Cell Titer Blue.

Cell viability assays

Cells (1×10^4) were seeded onto 48-well culture plates and incubated overnight prior to the addition of 30 μ M propidium iodide (PI). Total fluorescence was measured at various time points after the addition of PI (excitation, 530 nm; emission, 625 nm) in a CytoFluorII fluorescence plate reader. Baseline fluorescence (A) was determined immediately after the addition of PI. Treatment fluorescence (B) was determined after overnight t-butyl hydroperoxide (tBOOH) exposure, and maximal fluorescence (C) at the conclusion of the experiment following the addition of digitonin (0.4 mg/mL). The percentage of cell death was calculated as $(B - A)/(C - A) \times 100$. Alternatively, control and infected cells were seeded on six-well plates ($\sim 1 \times 10^5$ cells/well) and either left untreated or treated with 0–200 μ M tBOOH. After overnight (12–16 h) incubation with tBOOH, cells were collected and stained with 7-amino-actinomycin D (7-AAD; BD Biosciences, San Diego, CA, USA) and analysed by flow cytometry.

Proteinase K accessibility assay

Freshly isolated mitochondria were aliquoted and incubated alone, in the presence of proteinase K (50 $\mu\text{g/mL}$) or in the presence of proteinase K and Triton X-100 (1%). All the mixtures were kept at room temperature for 30 min before addition of phenylmethylsulfonyl fluoride (2 mM) to terminate proteolysis. Samples were then incubated on ice for an additional 10 min, boiled in SDS sample buffer and analysed by SDS-PAGE and western blotting.

Mitochondrial membrane potential ($\Delta\psi_m$) assay

Cells (1×10^6) were harvested by trypsinization and incubated with 10 $\mu\text{g/mL}$ JC-1 (Molecular Probes, Carlsbad, CA, USA), a dual emission lipophilic cationic dye, for 10 min at 37 °C. After washing with PBS, cells were analysed by flow cytometry on a FACS-Vantage instrument as described previously [25]. Cells with normal $\Delta\psi_m$ had high red fluorescence and low green fluorescence. Mitochondrial depolarization caused a gain of green fluorescence accompanied by a concomitant loss of red fluorescence. The percentage of cells with depolarized mitochondria was calculated. Mitochondrial membrane potential was measured in cells treated overnight with 0–200 μM tBOOH. In some cases, cells were also pretreated with 10 μM of BAPTA-AM [1,2-bis-(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid tetrakis (acetoxymethyl ester)], an intracellular Ca^{2+} chelator, for 30 min at 37 °C prior to the treatment with tBOOH.

Thapsigargin-induced mitochondrial permeability transition (MPT) measurement

Cells ($\sim 1 \times 10^5$) were seeded onto glass coverslips and allowed to grow to about 50% confluency. They were then washed with PBS, and incubated at 37 °C for 30 min with 1 μM of Rhodamine 123 (Invitrogen, Molecular Probes) in DMEM F12 50:50 medium without phenol red (Mediatech, Herndon, VA, USA). The coverslips were washed with PBS and mounted as the floor of a chamber (0.8 mL) with PBS as the bath solution. The chamber was then placed on a thermostated stage of a Nikon TE200 inverted epifluorescent microscope (Nikon Instruments, Melville, NY, USA). Cells were serially imaged at 10 s intervals with a Nikon 40 $\times$, 1.4 NA oil objective and a FITC-filter set (excitation 465–495 nm, dichroic mirror 505 nm, emission 515–555 nm). The fluorescence intensity was quantitated using Metamorph (Molecular Devices, Sunnyvale, CA, USA) software and plotted as percent of control value before thapsigargin treatment. The time point when 200–400 μM thapsigargin was added to the chamber was set as time 0.

Cytochrome c release assay

Freshly isolated mitochondria were incubated with various concentrations of CaCl_2 at room temperature for 30 min in cytochrome *c* buffer (300 mM mannitol, 10 mM HEPES, 0.3 mM KH_2PO_4 , pH 7.4). Exogenous core protein [35] at a concentration of 20 ng/mg mitochondrial protein was incubated with mitochondria along with Ca^{2+} for 30 min when indicated. To the mixture, 50 $\mu\text{g/mL}$ of proteinase K was then added to remove any cytochrome *c* that was released from the intermembrane space.

Western blotting

Western blotting was performed using anticore antibody (Affinity BioReagents, Golden, CO, USA), anti-NS3 antibody (Abcam, Cambridge, MA, USA), antimitochondrial heat shock protein 70 (Affinity BioReagents), anti-Tom20 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anticytochrome *c* antibody (R&D System, Minneapolis, MN, USA), anticomplex III core 2 subunit antibody (Molecular Probes) or anti-GRP 78 (Santa Cruz Biotechnology). All secondary antibodies were from Amersham Biosciences (Piscataway, NJ, USA). Immunoblots were detected using the ECL Plus Western Blotting Detection System (Amersham Biosciences).

Immunofluorescence

Cells grown on chamber slides were fixed with methanol: acetone (50:50) solution for 10 min at room temperature, washed with PBS and incubated with mouse anticore (Affinity BioReagents, Portland, ME, USA) or mouse anti-NS5A antibody (Maine Biotechnology Services) in immunofluorescence buffer (PBS containing 2.5 mM EDTA and 1% BSA). After washing, coverslips were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes) washed and counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 10 min at room temperature. Coverslips were washed again after DAPI staining and mounted. Prepared slides were observed in a Nikon Eclipse 800 upright epifluorescence microscope (Nikon Instruments). Images were acquired using a Nikon CoolSNAP camera.

Statistics

Results are expressed as mean $\pm$ SE. The Student *t* test or paired *t* test was used for statistical analyses. *P* < 0.05 was considered significant.

RESULTS

Mitochondrial association of core protein in cellular HCV model systems

In order to characterize the specific role of core protein in HCV mitochondrial effects, we compared JFH1-infected Huh7.5 cells, genome-length replicon cells (expressing core) and subgenomic replicon cells (without core). Infection with HCV in Huh7.5 cells and viral protein expression in replicon cells was monitored by immunocytochemistry and immunoblot. As shown in Fig. 1b (lane 4) and Fig. 1c, by day three postinfection with JFH1, expression of core protein could be detected by both methods and NS3 was present on immunoblot. Similar results for the genome-length replicon cells are also shown in Fig. 1b (Lane 3) and Fig. 1d. Infected cells express levels of core protein similar to those seen in a previously established stably transfected cell line (Fig. 1b, Lane 1) [26] and these are slightly higher than those seen in genome-length replicon cells (Fig. 1b, Lane 3). Both replicon cells and JFH1-infected cells express comparable levels of NS3. Comparison to recombinant core (amino acids 1–179) standards demonstrated that core in JFH1 and replicon cells is similar in size to core 1–179 (data not shown). This suggests that core protein was properly processed in genome-length replicon cells.

Core protein has multiple cellular localizations and when expressed as a single protein has been shown to associate with mitochondria [20] as well as other sites. The mitochondrial localization of core protein in HCV-infected cells and replicons was assessed by mitochondrial isolation and susceptibility to proteinase K digestion. As shown in Fig. 2, digestion of mitochondria with 50 μ g/mL of proteinase K eliminated most of the outer membrane protein, Tom20, without removing the intermembrane space protein, cytochrome *c*, the inner membrane protein, complex III core 2 subunit or the matrix protein, mtHSP70. All proteins were digested by proteinase K when mitochondrial membranes were disrupted by Triton X-100. In both JFH1-infected cells and genome-length replicons, mitochondrially associated core protein was accessible at the outer membrane. This was similar to the situation for TOM20. This indicates either a direct outer membrane association of core protein or localization of core with an extramitochondrial membrane fraction, such as the MAM fraction of the ER [36], that remains closely associated with the mitochondria. This is identical to the situation in cells expressing core protein alone or in transgenic mice expressing the HCV structural proteins [24,25].

Mitochondrial effects in HCV infected cells

Core protein expression has been shown to cause ER stress and have specific mitochondrial effects such as inhibiting electron transport, increasing superoxide production and sensitizing cells to secondary forms of stress such as alcohol and exogenous peroxides [25,26]. To determine whether this phenomenon occurred in the context of viral infection, we examined hydrogen peroxide production and mitochondrial function in JFH1-infected cells. Figure 3a demonstrates that JFH1 infection results in a progressive increase in hydrogen peroxide release into the medium that peaks after 7 days of infection. We next examined whether there was increased sensitivity to tBOOH, associated with the increase in reactive oxygen species (ROS) production in infected cells. tBOOH is an exogenous lipid peroxide and has been shown to deplete the mitochondrial reduced glutathione (GSH) pool and induce MPT [37–39]. Infected or uninfected cells were treated with 200 μ M tBOOH overnight and assessed for membrane permeability by flow cytometry with the membrane impermeant compound 7AAD. Figure 3b shows that JFH1-infected cells undergo cell death when exposed to tBOOH. Under similar conditions, uninfected cells are resistant to this treatment. The cell death process was associated with an increased sensitivity to tBOOH-induced mitochondrial depolarization as measured by JC-1 fluorescence and flow cytometry (Fig. 3c). In each of these cases, the behaviour of JFH1-infected cells was similar to that reported for cells expressing core protein alone and was different than uninfected or cells without expression of core.

Mitochondrial effects in subgenomic and genome-length replicons

In order to define the viral proteins responsible for the greater sensitivity of HCV-infected cells to oxidant-induced apoptosis, we next examined subgenomic and genome length replicon cells. Figure 4 demonstrates that there was no cytotoxicity in either of the replicon cells without exogenous oxidative treatment. However, as shown in Fig. 4a, when cells were exposed to 100 μ M of tBOOH overnight, there was a significant increase in cytotoxicity in genome-length when compared to subgenomic replicon cells ($30 \pm 6\%$ vs $12 \pm 4\%$, $P = 0.034$). Similar to the case in JFH1-infected cells, genome-length replicon cells underwent an increase in oxidant-induced mitochondrial depolarization (Fig. 4b). To determine whether the difference in sensitivity between the two replicon cells could be an effect mediated by the mitochondria, mitochondrial membrane potential ($\Delta\psi_m$) was evaluated by JC-1 flow cytometry. As shown in Fig. 4b, in the absence of exogenous oxidant, minimal mitochondrial depolarization was observed in both genome-length and subgenomic replicon cells. After overnight incubation with tBOOH, the number of cells with depolarized mitochondria was nearly 1.8-fold greater in genome-length compared to subgenomic cells ($48 \pm 7\%$ vs $27 \pm 5\%$, $P = 0.01$), demonstrating that genome-length cells are more sensitive to oxidative stress than subgenomic cells.

Because core protein has specifically been shown to increase mitochondrial Ca^{2+} uptake, we examined the effect of intracellular Ca^{2+} chelation on mitochondrial depolarization and cell killing. Cells were incubated with BAPTA-AM, an intracellular Ca^{2+} chelator and then exposed to tBOOH overnight. As demonstrated in Fig. 4a, chelation of Ca^{2+} completely prevented oxidant-induced cell death in both genome-length and subgenomic replicon cells. The role of Ca^{2+} was further confirmed by demonstrating that chelation of Ca^{2+} also completely inhibited oxidant-induced depolarization in both replicons (Fig. 4b). Both the Ca^{2+} -dependence and the core-induced sensitization to tBOOH were nearly identical to that reported previously [25] in Huh7 cells with or without inducible expression of core protein (Fig. 4b inset).

Role of core protein in Ca^{2+} induced mitochondrial permeability transition

Because Ca^{2+} is involved in oxidant-induced mitochondrial depolarization, we next examined the role of core protein in direct Ca^{2+} -induced MPT. Replicon bearing cells were first pre-loaded with a mitochondrially localized cationic fluorophore, Rhodamine 123 (Rh123). Rh123 accumulates in mitochondria as a direct function of the membrane potential and is released

upon mitochondrial depolarization. Following Rh123 loading, the cells were treated with an inhibitor of the endoplasmic reticulum Ca^{2+} pump, thapsigargin, which induces release of intracellular Ca^{2+} stores and results in MPT and mitochondrial depolarization [40]. In the absence of thapsigargin, both subgenomic and genome-length replicon cells displayed similarly strong Rh123 fluorescence in mitochondria (Figs 5a,e). After exposing the subgenomic cells to thapsigargin, there was only a slight reduction in Rh123 intensity after 8 min of treatment (Figs 5b–d). On the contrary, in genome-length cells, Rh123 intensity decreased dramatically over time (Figs 5f–h). Rh123 intensity was quantified (Fig. 5i) and the markedly increased susceptibility to thapsigargin-induced MPT in genome-length cells was consistently observed in a series of experiments (Fig. 5j). While Rh123 intensity in genome-length cells was reduced to about 60% 8 min after thapsigargin treatment, subgenomic replicon cells still maintained their Rh123 fluorescence ($94 \pm 2\%$ of control, $n = 3$, $P < 0.01$).

We next tested whether core protein itself accounted for differences in mitochondrial MPT. To do so, we isolated mitochondria from subgenomic and genome-length replicon cells and used a proteinase K accessibility assay with digestion of cytochrome *c* as our endpoint for MPT. Mitochondria were isolated from replicon cells and incubated with various concentrations of Ca^{2+} . The amount of cytochrome *c* remaining in the intermembrane space was then evaluated after digestion with proteinase K. As shown in Fig. 6, the amount of proteinase K resistant cytochrome *c* decreased with increasing concentrations of Ca^{2+} . Furthermore, the Ca^{2+} concentration-dependent release of cytochrome *c* from the intermembrane space was different between subgenomic and genome-length mitochondria. Cytochrome *c* release from subgenomic mitochondria required a higher concentration of Ca^{2+} than that required in genome-length mitochondria (200 vs 50 μM). However, incubation of subgenomic mitochondria with exogenous core protein (20 ng/mg mitochondrial protein) generated a Ca^{2+} sensitivity profile of cytochrome *c* release similar to that of genome-length mitochondria. These results suggest that HCV core protein can directly change mitochondrial response to Ca^{2+} in cells actively replicating HCV RNA.

DISCUSSION

Cell culture infection with the JFH1 strain of HCV has now clearly shown that the virus is cytotoxic and has the capacity to induce cell death via a mitochondrial based mechanism [4, 5]. The present study examined the role of core protein in this system and specifically examined whether the direct effects of core on mitochondria, observed in simple protein expression systems [7,14], are present during viral infection and account for some of the observed effects.

The results demonstrate that core protein directly associates with the mitochondrial outer membrane both in infection and in replicons. This is similar to the situation in transfected cells. HCV infection increased cellular ROS production and sensitized the cells to a process of ROS and Ca^{2+} induced mitochondrial depolarization and cell death. The role of core protein in this process was specifically demonstrated by the results with replicon cells. Replicon cells containing core were more sensitive to both oxidant and Ca^{2+} -induced MPT, while subgenomic replicon cells without core did not demonstrate this mitochondrial sensitivity. Isolated mitochondria from genome-length replicon cells retained the hypersensitive phenotype while subgenomic mitochondria did not. Moreover, *in vitro* incubation of subgenomic mitochondria with core reproduced the increased sensitization seen when core is present. These observations are consistent with previous data obtained from Huh-7 cells over-expressing core protein and transgenic mouse liver showing that expression of the core protein depolarized mitochondria and increased ROS production [24–26,30]. It is noteworthy that mitochondrial effects were seen with core protein derived from both genotype 1a (replicon cells) and genotype 2a (JFH1 infection). This indicates that the results are not genotype specific.

The results show that Ca^{2+} is required to mediate oxidant-induced mitochondrial depolarization because intracellular Ca^{2+} chelation completely prevented tBOOH-induced cell death and mitochondrial depolarization. The mechanisms of the observed effects could be because of an increased Ca^{2+} release from ER, a greater mitochondrial Ca^{2+} uptake or an increased susceptibility to Ca^{2+} . Several studies have shown that the mitochondria-associated core protein is either directly or indirectly associated with the outer membrane [20,36] and we and others have shown a direct effect of core to increase mitochondrial Ca^{2+} entry, increase basal mitochondrial Ca^{2+} , and most importantly dramatically increase the transfer of Ca^{2+} from ER to mitochondria upon ER Ca^{2+} release [23,30]. It appears that the direct effect of core on the mitochondrial Ca^{2+} uptake step is the most likely explanation for our results given the fact that *in vitro* incubation of subgenomic mitochondria with core can replicate Ca^{2+} and ROS sensitivity of MPT. Our studies here confirmed the specific association of core protein with the mitochondrial outer membrane in the HCV infected cells and genome-length replicons. We thus believe that the direct effects of core on the mitochondrial Ca^{2+} uptake step is the most likely explanation.

An alternative explanation is that core protein primarily affects ER Ca^{2+} release and mitochondria are innocent bystanders. This is less likely because the difference in sensitivity is retained in mitochondria isolated from the respective replicons and can be reproduced in isolated mitochondria incubated with recombinant core protein. Although ER fragments are present in our mitochondrial preparations, this ER would lack the ability to concentrate Ca^{2+} in the absence of ATP. Furthermore, if the difference between genome-length and subgenomic mitochondria is primarily because of ER Ca^{2+} release, isolated mitochondria from both sources would have been expected to have identical responsiveness once extra-mitochondrial Ca^{2+} is controlled.

Other researchers have also observed mitochondrial effects of HCV proteins, replicons and viral infection. As mentioned, expression of core protein in mice, or cells has uniformly produced an increase in ROS production and some degree of mitochondrial dysfunction. Expression of either core or NS5A can result in ER stress and this may secondarily alter mitochondrial function via changes in Ca^{2+} homeostasis [22,41–43]. JFH1 infection has been observed in several studies to induce apoptosis. This appears to be a type II apoptosis process which is associated with Bax-induced mitochondrial depolarization, cytochrome *c* release and apoptosome and caspase activation [4,5]. It has also been associated with enhanced sensitivity to TRAIL [44]. In the present study, we have observed a marked sensitization to a Ca^{2+} /ROS induced cell death pathway that is present whenever core is expressed in the context of replicating virus, replicating RNAs only, or even simple protein transfection. While ER stress is present in some models, it does not appear to be necessary for the mitochondrial sensitization as we found the ER stress levels are similar in both genome-length and subgenomic replicon cells (data not shown).

In conclusion, our study shows that the mitochondrial effects produced by HCV core protein are present when core is present in the context of viral replication. Core protein renders the cells and isolated mitochondria more susceptible to undergo oxidant-induced and Ca^{2+} -induced MPT. This study further supports the likelihood that mitochondrial effects induced by HCV core protein contribute to the pathogenesis of chronic Hepatitis C and enhance liver injury in alcoholic and inflammatory liver diseases.

Acknowledgments

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Abbreviations

7-AAD	7-amino-actinomycin D
MPT	mitochondrial permeability transition
PI	propidium iodide
ROS	reactive oxygen species

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Fig. 1.

Expression of HCV proteins in model systems. (a) Schematic depicting the subgenomic and genome-length constructs used to generate the replicon bearing cell lines. The subgenomic replicon bearing cells express the nonstructural viral proteins including NS3 to NS5B, while the genome-length replicon bearing cells express the full range of HCV proteins including core. (b) Western blot showing expression of HCV core protein and NS3 in a previously generated stable cell line (lane 1), subgenomic (lane 2) and genome-length replicon bearing cells (lane 3) and JFH1-infected cells on day 3 postinfection (lane 4). (c) Immunofluorescence for HCV core protein in control and JFH1-infected cells on day 3 postinfection. Images are composites of DAPI stained nuclei (blue) and core (green). Original magnification 200 $\times$. (d) Immunofluorescence for core and NS5A protein in subgenomic and genome-length replicon bearing cells. Original magnification 200 $\times$.

Fig. 2.

Mitochondrial membrane localization of HCV core protein. Isolated mitochondria from genome-length replicon bearing cells and HCV infected cells were digested with 50 $\mu\text{g/mL}$ proteinase K in the presence or absence of 1% Triton-X-100 and subjected to SDS-PAGE. Immunoblots for core protein, and mitochondrial marker proteins (TOM20, outer membrane; cytochrome *c*, intermembrane space; complex III core 2, inner membrane; mitHSP70, matrix) are shown. Results for replicons were performed by identical procedures in separate experiments, and a composite image is presented as indicated. In the case of replicon bearing cells, spaces indicate where lanes were omitted for clarity (i.e. sample order differed from that shown for JFH1 infected cells).

Fig. 3.

Reactive oxygen species (ROS) production, cell death and mitochondrial depolarization in HCV-infected cells. (a) Hydrogen peroxide production was measured with the Amplex Red assay in control and HCV-infected cells following infection with JFH1. (b) Cell death was measured by flow cytometry of JFH1-infected and uninfected cells after incubation with 7-amino-actinomycin D. Cells were treated with tBOOH (200 μ M) overnight prior to measurement. (c) Mitochondrial depolarization was measured by flow cytometry after 12 h incubation of control and JFH1 infected cells (moi 1.0) with increasing concentrations of tBOOH. Cells were then loaded with JC-1 and analysed by flow cytometry as described in Methods. Percent of cells with depolarized mitochondria is presented.

Fig. 4.

Cell death and mitochondrial depolarization in HCV-replicon bearing cells. Subgenomic and genome-length replicon bearing cells were incubated with no treatment, tBOOH, or pre-incubated with the Ca^{2+} chelator BAPTA for 30 min prior to incubation with tBOOH for 16 h. (a) Cell death was measured by propidium iodide assay. (b) Mitochondrial depolarization was measured by JC-1 flow cytometry. $**P < 0.01$, $*P < 0.05$. Inset indicates similar data obtained with Huh7 cells with (On) or without (Off) the inducible expression of HCV core protein.

Fig. 5. Ca^{2+} induced mitochondrial depolarization in replicon bearing cells. Subgenomic (a–d) and genome-length (e–h) replicon bearing cells were loaded with Rh123 and imaged over 8 min as indicated. Cells were treated with thapsigargin at time zero to induce ER Ca^{2+} release. (i) Quantification of total cellular Rh123 fluorescence for a single experiment displayed in the images. (j) Rh123 fluorescence in subgenomic and genome-length replicon bearing cells after 8 min treatment with thapsigargin ($n = 3$ independent experiments, $P < 0.01$).

Fig. 6. Cytochrome *c* release from isolated mitochondria. (a) Mitochondria were isolated from replicon bearing cells and incubated with or without core protein as described in Methods. After further incubation with the indicated concentrations of Ca^{2+} , mitochondria were pelleted, exposed to proteinase K, electrophoresed on SDS-PAGE and immunoblotted for cytochrome *c* and the mitochondrial matrix protein mtHSP90. (b) Densitometry results of three independent experiments as shown in panel a.