Hepatitis C Virus proteins Core and NS5A are highly sensitive to oxidative stress-induced degradation after eIF2a/ATF4 pathway activation

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Abstract

HCV infection is accompanied by increased oxidative stress as a consequence of viral replication, production of viral proteins, and inflammatory signals from immune cells. We investigated the role of stress pathways and autophagy-related proteins in the resistance of HCV protein expressing-cells to oxidative stress-induced damage. Huh7 cells stably expressing HCV Core, NS3/4A or NS5A proteins were treated with menadione. Production of reactive oxygen species and activation of caspase 3 were quantified. The activation of the eIF2a/ATF4 pathway and changes in the steady state levels of the autophagy-related proteins LC3 and p62 were determined by qPCR and Western blotting. Huh7 cells expressing Core or NS5A demonstrated reduced oxidative stress and apoptosis. In addition, phosphorylation of eIF2a and increased ATF4/CHOP expression were observed with subsequent HCV Core and NS5A protein degradation.

Our results suggest that autophagy plays an important role in the degradation of HCV proteins leading to decreased oxidative stress and apoptosis and providing hepatocytes with a survival advantage.
Introduction

Hepatitis C virus (HCV) is a member of the *Flaviviridae* family and was identified in 1989 as the infectious agent of non-A, non-B hepatitis. Currently, HCV is the leading cause of end stage liver disease as a result of cirrhosis and/or hepatocellular carcinoma (HCC). An estimated 71 million people are chronically infected and approximately 400,000 associated deaths occur each year worldwide (1,2). Although safe, tolerable and curative therapies for HCV infection have emerged in recent years, the prevention, clinical management and access to treatment remain important determinants in the control of HCV infection. Despite the recent therapeutic advances, HCV pathophysiology is still not entirely elucidated justifying continued research in this field (3).

HCV-infected hepatocytes are exposed to several stressors that may affect their function and viability. These stressors include viral replication and viral protein production within hepatocytes, as well as the inflammatory response of the host. It is known that HCV infection leads to increased oxidative stress in the liver and in particular in the hepatocytes (4). Since HCV replication and protein expression are also closely linked to the endoplasmic reticulum (ER), both ER stress and oxidative stress may contribute to the progression of chronic HCV-related liver disease (5–9).

HCV contains a positive sense single-stranded RNA (ssRNA+) genome that encodes for a polyprotein of approximately 3,100 amino acids, depending on the genotype, that is cleaved co- and post-translationally by cellular and viral proteases to produce 10 viral proteins with various structural and biochemical functions (Core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) (10). The role of HCV proteins in the generation of oxidative stress and ER stress has been demonstrated and Core and the non-structural proteins NS3/4A and NS5A are the most potent inducers (11–13).

In mammalian cells, different signaling pathways have evolved to mediate the cellular stress response. One of the most conserved regulatory events activated in response to stress is the phosphorylation of the a subunit of eukaryotic translation Initiation Factor 2 (eIF2a) at serine 51 and subsequent ATF4 (Activation Transcription Factor 4) activation (14). It has been demonstrated that activation of the eIF2a/ATF4 pathway directs an autophagy gene transcriptional program to overcome the cellular stress. Furthermore, the transcription factors ATF4 and CCAAT/Enhancer-Binding Protein
Homologous Protein (CHOP) are involved in the transcriptional activation of other autophagy genes, including p62/SQSTM1 (Sequestosome 1) [hereafter referred to as p62] (15).

Autophagy can also be induced via activation of the Unfolded Protein Response (UPR) through phosphorylation of the ER stress sensors: Protein Kinase R (PKR)-like endoplasmic reticulum kinase (PERK) and Inositol-Requiring protein 1 (IRE1, or Endoplasmic reticulum to nucleus signaling 1, human homologue). Both sensors can induce and activate Beclin-1, as well as the expression of autophagy-related genes (ATGs), ATG5 and ATG12 (16,17). Additionally, PERK phosphorylation is known to couple distinct upstream stress signals to eIF2a/ATF4 pathway activation and further autophagy gene expression (15). In Huh7 hepatoma cells, infection with HCV leads to induction of the UPR and subsequently to an inhibition of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian Target of the Rapamycin (mTOR) signaling pathway, resulting in induction of autophagy (16). HCV-induced autophagy can also be mediated via an UPR-independent mechanism, since silencing of the UPR-sensor IRE1 in HCV-infected Huh7.5 cells did not affect HCV replication or the induction of autophagy (18).

In a previous study, we established a model of double injury in Huh7 cells, as well as in primary rat hepatocytes, to investigate the adaptive responses activated under HCV protein expression and external cellular oxidative stress. HCV Core or NS3/4A were transiently expressed and subsequently treated with menadione, a superoxide anion donor. We observed that in this double injury condition, hepatocytes adapt to oxidative stress via a reduction in reactive oxygen species (ROS) production as well as reduction of oxidative stress induced-apoptosis. In addition, we observed an increased degradation of the HCV Core protein together with the autophagy adaptor protein p62 in hepatocytes resistant to oxidative stress (Rios-Ocampo et al., submitted).

In the present study, autophagy-related proteins and changes in their steady state protein levels were investigated and related to protection of HCV-infected cells against oxidative stress and ER stress. We observed that selective degradation of HCV Core and NS5A proteins via activation of the eIF2a/ATF4 pathway plays an important role in the adaptive response of hepatocytes to stress.
Material and methods

Cell lines and culture

Huh7 cells were maintained in Dulbecco’s Modified Eagle Medium (1X) + GlutaMAX™-I (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and 1% penicillin-streptomycin (Gibco) at 37°C and 5% CO₂. The stable cell lines Huh7_puro [3 µg/ml puromycin] (containing the empty vector), Huh7_Core_[Jc1]_bla [10 µg/ml blasticidin], Huh7_NS3/4A_[Con1] [0.75 µg/ml G418] and Huh7_NS5A_[JFH1]_puro [3 µg/ml puromycin] were generated and kindly provided by Prof. Dr. Ralf Bartenschlager from the University of Heidelberg, Germany (19).

Reagents and treatments

Cells (3.0x10⁵) were grown until 80% confluence in 6-well plates 24 hours (h) prior to treatment. Cells were treated for 6 h with 50 µmol/L menadione (Sigma) or 5 mmol/L hydrogen peroxide (H₂O₂) (Sigma) to induce oxidative stress. Subsequently, cells were harvested and viability was determined by trypan blue exclusion staining. For the kinetic assays cells were collected after menadione treatment every hour for 6 h. In control experiments, 5 mmol/L N-Acetyl-L-Cysteine (NAC, Sigma) was added 30 minutes prior to menadione treatment. In some experiments 50 mmol/L chloroquine (CQ) diphosphate salt ( Sigma) was used to inhibit autophagic flux and 100 nmol/L bafilomycin A1 (Sigma) or a mixture of 20 mmol/L ammoniumchloride (NH₄Cl) / 100 mmol/L leupeptin / 100 mmol/L pepstatin was used to inhibit the lysosomal degradation pathway. For proteasome inhibition 10 mmol/L MG132 (Sigma) was added 3 h prior to menadione treatment. Experiments were conducted in duplicate and the results are expressed as the average of three independent experiments.

Tissue samples

Liver tissue samples from patients submitted for liver transplantation were obtained from the tissue bank of the Gastrohepatology Group, University of Antioquia, Colombia. The samples were selected according to etiology and used for RNA isolation and qPCR analysis. The liver tissues were from patients with cirrhosis associated to HCV infection (6 patient samples), HCV-associated hepatocellular carcinoma (HCC) (7 patient samples), hepatitis B virus (HBV)-associated cirrhosis
(4 patient samples) and non-viral liver disease (4 patient samples). The demographic and clinical characteristics of the samples are presented in Table 1. For total RNA isolation 100 mg of tissue was processed using TriZOL reagent (Invitrogen) following the manufacturer’s instructions. Total RNA (2.5 μg) was used for reverse transcription (RT). Complementary DNA (cDNA) was diluted 20 X in nuclease free water and stored at -20°C until use.

**Cell culture, RNA isolation and qPCR**

After treatment, Huh7 cells stably expressing the empty vector, Core, NS3/4A and NS5A were harvested on ice and washed three times with ice-cold 1X Hank’s Balanced Salt Solution (HBSS) with Ca²⁺ and Mg²⁺ (Gibco). TRI reagent (Sigma) was added to the cells for total RNA isolation according to the manufacturer’s instructions. Total RNA (2.5 μg) was used for RT in 1X RT buffer (500 mmol/L Tris-HCl -pH 8.3--; 500 mmol/L KCl; 30 mmol/L MgCl₂; and 50 mmol/L DTT), 1 mmol/L deoxynucleotides triphosphate (dNTPs, Sigma), 10 ng/μL random nanomers (Sigma), 0.6 U/μL RNaseOUT™ (Invitrogen) and 4 U/μL reverse transcriptase M-MLV (Invitrogen) in a final volume of 50 μL. cDNA was diluted 20 X in nuclease free water and qPCR was carried out in a StepOnePlus™ (96-well) PCR System (Applied Biosystems) using TaqMan probes; the sequences of the probes and primers are described in Supplementary Table 1. For qPCR, 2X reaction buffer (dNTPs, HotGoldStar DNA polymerase, 5 mmol/L MgCl₂) (Eurogentech, the Netherlands), 5 μmol/L fluorogenic probe and 50 μmol/L of primers sense and antisense (Invitrogen) were used. mRNA levels were normalized to 18S gene expression and compared between groups (20). The experiments were performed in duplicate and presented as the average of three independent experiments.
Table 1. Liver biopsy samples: characteristics of patients with liver diseases.

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*Sample code at the tissue bank of the Gastrohepatology Group, University of Antioquia.
F=Feminine/M=Masculine/- =No viral liver disease

Determination of cellular oxidative stress

Total cytoplasmic ROS was quantified using the fluorogenic probe CellROX® Deep Red (Invitrogen) following the manufacturer’s instructions. After induction of oxidative stress, 5 µmol/L of CellROX reagent was added to the cells and cells were subsequently incubated at 37°C and 5 % CO₂ for 30 minutes. Media was removed and cells were washed three times with 1X HBSS with Ca²⁺, Mg²⁺ (Gibco) and subsequently harvested using 1X trypsin (Gibco) and analyzed by flow cytometry using a BD FACSVerse system and a 635nm laser. Three independent experiments were carried out and the results are expressed as an average.

Caspase 3 activity determination

After treatment, cells were scraped on ice and lysed by three cycles of freezing (liquid nitrogen) and thawing (37°C) in lysis buffer (25 mmol/L HEPES, 150 mmol/L KAc, 2 mmol/L EDTA, 0.1% NP-40) supplemented with protease and phosphatase inhibitors (10 mmol/L NaF, 50 mmol/L PMSF, 1 µg/µL of α-protenin/pepsatin/leupeptin and 1
mmol/L DTT) followed by centrifugation for 10 minutes at 12,000 rpm. For the caspase 3 activity assay (21), 30 μg of protein was mixed with the synthetic fluorogenic caspase 3 substrate, Ac-DEVD-AMC and the release of fluorogenic AMC was quantified in a spectrofluorometer at an excitation wavelength of 380 nm and emission wavelength of 430 nm. The arbitrary units of fluorescence (AUF) from three independent experiments were used to depict the results.

**Transfection of siRNA**

For silencing of p62, 4x10^4 cells were seeded in 12-well plates pre-treated with 1.5 mL Lipofectamine 3000 (Invitrogen) and 50 mmol/L esiRNA human p62/SQSTM 1 (esiRNA1) (Sigma, Cat. #EHU027651) or scrambled siRNA (esiRNA Egfp Cat. #EHUEGFP-20UG, Sigma) as a control. The Lipofectamine 3000 and the esiRNAs were prepared in 75 mL OPTI-MEM™ I (1X) reduced serum medium (Gibco) following the manufacturer’s instructions then wells were cover with the mixture (reverse transfection). After cells were added, media was completed to 750 mL final volume per well. 12 h post-transfection, media were replaced and a second transfection round was performed using the same amounts of Lipofectamine 3000 and esiRNA for 12 h. Subsequently, cells were treated with 50 mmol/L menadione for 6 h. After treatment cells were scraped and lysed by freezing and thawing cycles in lysis buffer containing protease and phosphatase inhibitors as described above, followed by centrifugation for 10 minutes at 12,000 rpm. Supernatant was collected and stored at -20°C until use. Three independent experiments were performed and the results are expressed as means +/- S.D.

**Western Blotting**

Cell lysates (20 mg) were resolved on Mini-PROTEAN® TGX Stain-Free™ Precast Gels (BioRad) and semi-dry blotting transfer was performed using Trans-Blot Turbo Midi Nitrocellulose Membrane with Trans-Blot Turbo System (BioRad). To confirm the electrophoretic transfer, Ponceau S 0.1% w/v (Sigma) staining was used. The monoclonal antibodies human anti-HCV Core B12-F8 (kindly provided by prof. Dr. Mondelli, University of Pavia, Italy) (22), mouse anti-HCV NS3/4A (8 G-2) (Abcam) and mouse anti-HCV NS5A (9E10) (kindly provided by prof. Dr. Charles M. Rice), were used at a dilution of 1:1000 and mouse anti-Glyceraldehyde 3-phosphate
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dehydrogenase (GAPDH) (Calbiochem) at a dilution of 1:10000. ER stress markers were also determined using the polyclonal rabbit antibodies anti-peIF2a (Cell Signaling), anti-eIF2a (total) (Cell Signaling), anti-ATF4 (Cell Signaling) and anti-Glucose-Regulated Protein of 78kDa (GRP78) (Cell Signaling) at 1:1000 dilution. Polyclonal rabbit anti-Microtubule Associated Protein 1 Light Chain 3 Beta (LC3B) (Cell Signaling) and anti-p62 (Cell Signaling) were also used at 1:1000 dilution. For detection of ubiquitinated proteins mouse anti-a-Ubiquitin (1/1000) (Hycult, Biotech) was used. Secondary horseradish peroxidase (HRP)-bound antibodies were used. The blots were analyzed by chemiluminescence in a ChemiDoc XRS system (Bio-Rad). Protein band intensities were quantified by ImageLab software (BioRad).

Statistical analysis

All experiments were performed at least three times and the mean ± standard deviation (s.d.) is depicted. The Graphpad Prism 5 software (GraphPad Software) was used and comparisons were evaluated by unpaired, two-tailed t-test. For the group analysis two tails Anova and Bonferroni post-test were performed. A p value of <0.05 was considered statistically significant.

Results

ROS generation is attenuated in Huh7 cells expressing NS3/4A and NS5A

We first investigated the generation of ROS in Huh7 cells, stably expressing either Core, NS3/4A or NS5A (Supplementary Figure S1) with and without menadione treatment as second external stressor. As expected, Huh7 cells transfected with empty vector showed an increase in ROS production after menadione treatment and this effect was suppressed by NAC pre-treatment (Figure 1A). A similar response was observed for Huh7 cells expressing Core (Figure 1B). Remarkably, the ROS generation in response to menadione in Huh7 cells stably expressing NS3/4A (Figure 1C) or NS5A (Figure 1D) was much less prominent compared to empty vector and Core expressing cells, suggesting that after external oxidative stress the HCV proteins NS3/4A and NS5A attenuate menadione-induced ROS generation and therefore cellular oxidative stress. Importantly, basal levels of ROS production were similar in all 4 cell lines.
Figure 1. ROS generation is attenuated in Huh7 cells expressing NS3/4A and NS5A. Total ROS production was determined in stably transfected Huh7 cells expressing the empty vector (A), Core (B), NS3/4A (C) and NS5A (D). Cells were plated in 6-well plates and 24 h post-seeding treated with menadione (50 mmol/L) for 6 h. In some experiments, cells were pre-treated with 5 mmol/L NAC 30 min prior to addition of menadione. ROS production was determined using flow cytometry and the relative main signal intensity is depicted. The graphs show means ± SD of three independent experiments. t test was performed to compare the means and the asterisks represent the p value **<0.0093 and *<0.0127. n.s = non-significant, (p values > 0.05 are considered not statistically significant).

Huh7 cells expressing HCV Core and NS5A are protected against apoptosis induced by oxidative stress

We next investigated the effect of expression of HCV proteins on susceptibility to oxidative stress-induced toxicity. As shown in Figure 2A menadione treatment alone induced caspase 3 activation in Huh7 cells expressing the empty vector and this effect could be completely reversed by the antioxidant NAC (Figure 2A). Huh7 cells expressing Core or NS5A (Figure 2B and Figure 2D), but not NS3/4A (Figure 2C), were completely protected against menadione-induced caspase 3 activation (Figure 2B and Figure 2D). Interestingly, NAC treatment abolished the protective effect observed in Huh7 cells expressing HCV Core or NS5A on menadione-induced apoptosis (Figure 2B and Figure 2D). These results suggest that after external oxidative stress, hepatocytes expressing Core or NS5A viral proteins activate an adaptive response to attenuate caspase 3 activation and thereby the apoptotic effect of oxidative stress.
eIF2α/ATF4 pathway is involved in the adaptive response against external oxidative stress-induced damage

To elucidate the mechanism behind the protective effect of Core and NS5A against menadione-induced apoptosis, first, the transcriptional regulation of genes encoding antioxidant enzymes (copper zinc superoxide dismutase [CuZnSOD, SOD1] and catalase [CAT]) were determined. No regulation of antioxidants genes was observed (Supplementary Figure S2), suggesting that other mechanisms are involved in the adaptive resistance to oxidative stress.

An important pathway in the adaptation to cellular oxidative stress is the phosphorylation of eIF2α and subsequent induction of ATF4 and CHOP expression (14). The eIF2α/ATF4 pathway has been associated with the induction of autophagy after cellular stress as an essential mechanism of survival (15). Therefore, we investigated the eIF2α/ATF4 pathway in our model. Menadione robustly induced phosphorylation on serine 51 of eIF2α of Huh7 cells expressing the empty vector, Core, NS3/4A or NS5A, which was completely abolished by antioxidant treatment with NAC (Figure 3A). Remarkably, mRNA levels of ATF4 were not significantly increased after menadione treatment in any of the 4 Huh7 cell lines (Supplementary Figure S3). However, at the protein level, increased ATF4 protein expression was observed after menadione treatment in Huh7 cells expressing the empty vector and all the HCV viral proteins and this increase was reversed by the antioxidant NAC (Figure 3B). The expression of CHOP (DDIT3) is required for the transcription of a set of autophagy genes after activation of the eIF2α/ATF4 pathway. DDIT3 mRNA levels were significantly increased after menadione treatment in cells expressing Core and NS5A even after treatment with NAC (Figure 3C). The expression of the ER stress marker GRP78 (HSPA5) was also analyzed to determine whether the eIF2α/ATF4 pathway was activated as a consequence of ER stress. We did not observe any regulation of HSPA5 mRNA or GRP78 protein levels in any of the cell lines and by any of the treatments (Figure 3A and Figure 3D). This result suggests that the eIF2α/ATF4 pathway was not activated in response to ER stress, but as an adaptive response to attenuate external oxidative stress.
Figure 2. Huh7 cells expressing HCV Core and NS5A are protected against apoptosis induced by oxidative stress. Apoptosis was determined by measuring caspase 3 activity in Huh7 cells expressing (A) empty vector, (B) Core, (C) NS3/4A and (D) NS5A after menadione treatment (50 mmol/L). In some experiments, cells were pre-treated with 5 mmol/L NAC 30 min prior to addition of menadione. Cells were plated in 6-well plates and 24 h post-seeding treated with menadione for 6 h. Caspase 3 activity was determined as described in Materials and Methods and represented as fold change in arbitrary units of fluorescence (AUF). The graphs depict means ± SD of three independent experiments. t test was performed to compare the means and the asterisks represent the p value ***<0.0009, **<0.002 and *<0.02. n.s = non-significant, (p values > 0.05 are considered not statistically significant).

Expression of ATF4 and DDIT3 (CHOP) is induced in HCV-related cirrhosis

To confirm our in vitro data in clinical samples, we determined the mRNA expression of transcription factors ATF4 and DDIT3 in liver biopsies from patients with end-stage liver diseases (Table 1). Both ATF4 and DDIT3 mRNA expression were significantly increased in samples of patients with HCV-related cirrhosis, where HCV viral load is fluctuating compared to tumor tissue of patients with HCV-associated HCC where HCV viral load decrease (23) (Figure 4A and Figure 4B). The increased expression of ATF4 and DDIT3 was specific for HCV-related cirrhosis since their expression was
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not induced in liver samples of patients with non-HCV end-stage liver disease as HBV associated cirrhosis (Figure 4A and Figure 4B).

![Figure 3. eIF2a/ATF4 pathway is involved in the adaptive response in the double injury model. Huh7 cells, expressing empty vector, Core, NS3/4A and NS5A HCV proteins were treated with and without 50 µmol/L menadione. In some experiments, cells were pre-treated with 5 mmol/L NAC 30 min prior to addition of menadione. Protein levels of total- and phosphorylated-eIF2a, GRP78 and GAPDH (A) and ATF4 (B) were determined by Western blotting as described in Materials and Methods. mRNA levels of DDIT3 (C) and HSPA5 (D) were evaluated by qPCR. The relative expression was normalized based on the expression of 18S. t test was performed to compare the means and the asterisks represent the p value ** < 0.007 and * < 0.03. n.s = non-significant, (p values > 0.05 are considered not statistically significant).

**HCV Core and NS5A are preferentially degraded after menadione treatment: involvement of autophagy adaptor proteins**

Activation of the eIF2a/ATF4 pathway in response to stress can induce autophagy as a survival mechanism (15). Therefore, we investigated changes in the steady state protein levels of LC3-II and the autophagy adaptor protein p62 and determined whether these changes correlated with increased resistance to external oxidative stress. In cells expressing the empty vector, or any of the HCV proteins, the steady
state levels of LC3-II diminished significantly after menadione treatment and were recovered by the antioxidant NAC. A similar pattern was observed with the expression of p62 (Figure 5A-5D). Thus, after menadione treatment, both p62 and LC3-II were virtually absent in all Huh7 cell lines.

Figure 4. Expression of **ATF4** and **DDIT3** (CHOP) is induced in HCV-related cirrhosis. Total mRNA was isolated from liver tissues obtained from patients with chronic liver diseases and the expression levels of **ATF4** (A) and **DDIT3** mRNA (B) were determined by qPCR and compared. Patient characteristics are in Table 1. The relative expression of the genes was normalized based on the expression of 18S mRNA. t test was performed to compare the means and the asterisks represent the p value as follow ***<0.0006 and *<0.04. n.s = non-significant, (p values > 0.05 are considered not statistically significant).

Menadione treatment also induced the loss of HCV Core and NS5A in the Huh7 cells. This degradation was reversed by the antioxidant NAC (Figure 5B and 5D). This result was not observed for NS3/4A (Figure 5C) which was apparently upregulated, suggesting that HCV Core and NS5A degradation is a selective process. The degradation of Core and NS5A viral proteins occurred simultaneously with a decrease in the levels of p62 and LC3-II, which may account for their degradation, suggesting that autophagy adaptor proteins may play an important role in the selective elimination of Core and NS5A after external oxidative stress induction.

To confirm that oxidative stress affects the protein levels of HCV Core and NS5A, we used H$_2$O$_2$ as an alternative source of external oxidative stress and indeed we obtained similar results: a pronounced loss of Core which was reversed by the anti-oxidant NAC (Supplementary Figure 4A). NS3/4A levels did not change in the presence of H$_2$O$_2$ (Supplementary Figure 4B). In contrast, NS5A protein level diminished after
Hepatitis C Virus proteins Core and NS5A are highly sensitive to oxidative stress-induced degradation after eIF2a/ATF4 pathway activation. H$_2$O$_2$ treatment, which indicates increased susceptibility of NS5A to degradation after external oxidative stress. This effect was not reversed by NAC treatment (Supplementary Figure 4C). These data suggest that HCV proteins Core and NS5A are highly sensitive to oxidative stress-induced degradation.

![Figure 5. HCV Core and NS5A are preferentially degraded after menadione treatment.](image)

LC3-I/II and p62 autophagy markers were detected by Western blotting in Huh7 cells stably expressing the empty vector (A), Core (B) NS3/4A (C) and NS5A (D) HCV proteins. Non-treated cells (-/-) were used as control. Oxidative stress was induced by 50 mmol/L menadione (+/-) and in some experiments cells were treated with 5 mmol/L NAC (+/+). Protein band intensities were quantified using ImageLab software (BioRad). The relative protein expression was calculated based on the expression of GAPDH and compared to the expression of the control cells. The graphs depict means ± SD of three independent experiments. t test was performed to compare the means and the asterisks represent the p value ***<0.0007, **<0.0072 and *<0.04. (p values > 0.05 are considered not statistically significant).
The ubiquitin-proteasome pathway is not involved in HCV Core and NS5A degradation

The ubiquitin-proteasome pathway selectively degrades a wide range of protein substrates employing a ubiquitin conjugation cascade. We investigated whether the proteasome pathway is involved in HCV Core and NS5A degradation after external oxidative stress. Huh7 cells were treated with the proteasome inhibitor MG132 and accumulation of ubiquitinated proteins was observed after 2 hours (Figure 6A). As already shown in Figure 5, menadione treatment reduced protein levels of Core and NS5A, but not NS3/4A (Figures 6B-6D). Pre-treatment of Huh7 cells expressing Core or NS5A with MG132 did not prevent the degradation of Core and NS5A in Huh7 cells exposed to menadione (Figure 6B and 6C). Huh7 cells expressing HCV NS3/4A were used as a control and MG132 treatment did not affect protein levels of NS3/4A (Figure 6D). These results suggest that the proteasome pathway is not involved in the degradation of HCV Core and NS5A proteins.

Menadione-induced NS5A degradation involves the autophagy-related proteins LC3 and p62

Autophagic flux was blocked using chloroquine (CQ) to determine whether lysosomal degradation of HCV proteins occurs. The experiments were performed using Huh7 cells expressing NS5A since in these cells we observed a complete degradation of the viral protein after menadione treatment and strong recovery after NAC treatment (Figure 5D). As shown in Figure 7A, treatment of Huh7 cells expressing NS5A with only CQ showed accumulation of LC3-II and p62 which indicates inhibition of the late steps of autophagy (Figure 7A). Consistent with previous results, menadione treatment reduced the levels of LC3-II, p62 and NS5A. When autophagic flux was blocked in cells exposed to menadione (+/+), no recovery of NS5A or p62 protein expression was observed suggesting that at least p62 is involved in NS5A degradation and supporting the hypothesis that p62 plays a role in the elimination of NS5A (Figure 7A). We also explored the effect of other inhibitors of lysosomal degradation, Bafilomycin A1 and a mix of protease inhibitors (NH₄Cl/Leupeptin/Pepstatin). After cells were treated with Bafilomycin A1 and exposed to menadione (+/+), NS5A protein levels were partially restored. Though full restoration of NS5A protein levels was not observed, the recovery of the NS5A protein was statistically significant as
Hepatitis C Virus proteins Core and NS5A are highly sensitive to oxidative stress-induced degradation after eIF2a/ATF4 pathway activation demonstrated by densitometry analysis (Figure 7B). Similar results were observed using NH₄Cl/Leupeptin/Pepstatin as inhibitor of the lysosomal pathway (Figure 7C). The above results suggest that although lysosomal degradation inhibition partially restored NS5A protein, other mechanism must be involved in its elimination and even the transcriptional regulation of NS5A and p62 should be analyzed.

Figure 6. Ubiquitin-proteasome pathway is not involved in HCV Core and NS5A degradation. Huh7 cells were plated in 6-well plates and treated with 10 mmol/L MG132 for 3 h. After treatment, Western blotting was performed to detect ubiquitinated proteins, GAPDH was used as loading control (A). Huh7 cells expressing HCV Core, NS3/4A, and NS5A were treated with menadione (50 mol/L) (+/−). In some experiments 10 mol/L MG132 was added for 3 h followed by addition of menadione (+/+/−). The expression of HCV Core (B), NS5A (C) and NS3/4A (D) was determined by Western blotting. GAPDH was used as a loading control. The relative protein expression was calculated based on the expression of GAPDH and compared to the expression of the control cells using ImageLab software (BioRad). The graphs depict means ± SD of three independent experiments. t test was performed to compare the means and the asterisks represent the p value **˂0.0039 and *˂0.0163. (p values 0.05 are considered not statistically significant).
Figure 7. Menadione-induced NS5A degradation involves LC3-II and p62 autophagy markers. Huh7 cells expressing HCV NS5A were exposed or not to 50 mol/L menadione. Autophagic flux (A) was blocked using 50 mmol/L chloroquine (CQ; +/-) and the lysosomal pathway (B, C) was blocked using bafilomycin A1 (B) or NH4Cl/Leupeptin/Pepstatin (C). The expression of NS5A, LC3-I/II, p62 and GAPDH were determined by Western blotting. The graphs depict means ± SD of three independent experiments. t test was performed to compare the means and the asterisks represent the p value ***<0.0007, **<0.0072 and *<0.04. (p values > 0.05 are considered not statistically significant.

A role for the autophagic adaptor protein p62 in the degradation of Core and NS5A

To further analyze the role of p62 in HCV Core and NS5A protein degradation, we performed a time course of the degradation of HCV Core, NS5A and p62 in Huh7 cells (Figure 8A and B). Protein levels of HCV Core and NS5A remained relatively stable during the first 2 h of menadione treatment, after which their levels rapidly dropped in the following hour(s) (Figure 8A and B).
Hepatitis C Virus proteins Core and NS5A are highly sensitive to oxidative stress-induced degradation after eIF2a/ATF4 pathway activation

Figure 8. Role for the autophagic adaptor protein p62 in the degradation of Core and NS5A. Huh7 cells expressing Core and NS5A were treated or not with 50 mmol/L menadione. Expression of Core (A), NS5A (B), p62 and GAPDH proteins were evaluated by Western blotting at 1 h intervals for 6 h as described in Materials and Methods. For p62 silencing, Huh7 cell expressing HCV Core (C) and NS5A (D) were transfected twice with esiRNAs. Cells were plated and reverse transfected with esiRNA p62 and random esiRNAs as described in Materials and Methods. Transfected cells were treated with menadione (50 mmol/L) and the expression Core, NS5A and p62 protein were detected by Western blot. The experiments were repeated three times. The relative HCV Core and NS5A expression was determined by densitometry and t test was performed to compare the means and the asterisks represent the p value as follow ** ˂ 0.001 and * ˂ 0.04. n.s = non-significant, (p values > 0.05 are considered not statistically significant).

Remarkably, a virtually identical response of p62 to menadione was observed (Figure 8A and B). Since degradation of p62 parallels the elimination of HCV Core and NS5A, and since p62 functions as an adaptor protein, we further investigated the relation between p62 and the viral proteins by selective p62 silencing. Expression of p62 was suppressed in Huh7 cells using siRNA technology and Huh7 cells transfected with a random control siRNA served as controls (Figure 8C and D). As expected, degradation of Core, NS5A and p62 was observed after exposure to menadione, (Figure 8C and D). When p62 was silenced prior to menadione treatment of Huh7 cells, the expression of
Core and NS5A were significantly recovered (Figure 8C and D). These results confirm the role of p62 as an adaptor protein for selective degradation of HCV Core and NS5A after external oxidative stress.

**Discussion**

The goal of the present study was to investigate the adaptive response of hepatocytes to multiple stressors resulting from HCV infection. To investigate this adaptive response, we used external oxidative stress. We found that Huh7 cells expressing HCV Core and NS5A resist the deleterious effects from additional oxidative stress through selective degradation of these viral proteins and autophagy adaptor proteins such as p62 and LC3. The degradation occurs in response to activation of the eIF2a/ATF4 pathway and suggest elimination of harmful viral proteins (Figure 9).

Infection of cells by viruses constitutes an important stress to the host cells. Cells must support viral replication and synthesis and shedding of newly synthesized virions. In addition, in most viral infections, cells are also exposed to immune and/or inflammatory response to the viral infection. Therefore, cells have to adapt to multiple stressors in order to survive and thus also sustain the viral replication cycle. HCV infection of hepatocytes is an example in which cells are subjected to multiple stressors: viral protein synthesis may lead to ER stress and HCV infection is also accompanied by oxidative stress (4,24). In a previous study, we observed increased resistance to menadione-induced oxidative stress using Huh7 cells and primary rat hepatocytes transiently expressing HCV Core or NS3/4A proteins. Both mitochondrial ROS production and menadione-induced apoptosis were significantly decreased together with reduced levels of the ER stress markers GRP78 and sXBP1 in Huh7 cells expressing HCV Core and NS3/4A proteins. This increased resistance was accompanied by increased degradation of HCV Core protein in these cells, suggesting that selective degradation of one stressor may be in part responsible for this increased resistance (Rios-Ocampo et al., submitted). In the present study, using stably-transfected Huh7 cells, we confirm increased resistance to oxidative stress and the selective degradation of HCV proteins Core and NS5A, as well as the activation of the stress pathway eIF2a/ATF4.
Figure 9. Graphical abstract: HCV proteins are susceptible to degradation after oxidative stress induction. In Huh7 cells expressing HCV viral proteins and under oxidative stress conditions, we observed that HCV Core or/and NS5A proteins were susceptible to degradation after induction of external oxidative stress. The phosphorylation of eIF2α was followed by increased expression of ATF4 and CHOP. Autophagy-related proteins (LC3-II/p62) are involved in the degradation of HCV proteins.

The increased resistance to oxidative stress, demonstrated by reduced apoptosis, observed in Huh7 cells expressing the HCV proteins Core and NS5A is probably not caused by altered levels of anti-oxidant enzymes. Gene expression of prominent anti-oxidant genes like SOD1 and CAT were not changed by any of the treatments, although we cannot rule out a regulation at the level of antioxidant enzyme activity.

Autophagy has been described as an important adaptive survival mechanism to cope with cellular stress, e.g. ER stress and oxidative stress (25). The initial step in activation of the autophagic program is activation of the eIF2α/ATF4 pathway (15,26). In our model, we were able to demonstrate activation of the eIF2α/ATF4 pathway and downstream events like increased ATF4 and CHOP (DDIT3) expression. Increased levels of autophagy markers have also been observed in liver biopsies of patients with HCV infection and in cell culture models of HCV infection (27,28). In our study, we demonstrate increased expression of the transcription factors ATF4 and CHOP...
DDIT3), key elements of the eIF2a/ATF4 pathway, in liver biopsies from patients with HCV-related cirrhosis, but not in liver tumor tissue of patients with HCV-related HCC or liver tissue of non-HCV-related cirrhosis. These clinical data support our experimental data and also highlight the specificity of the observed changes for HCV during its replication at early stages of the infection and during cirrhosis but not for late stages of the infection as HCC because HCV replication considered low.

Sir et al. demonstrated that HCV infection induces the accumulation of autophagosomes in cells without increasing autophagic protein degradation, whereas accumulation of autophagosomes and protein degradation were increased in Huh7 cells under starvation, the ‘gold standard’ for induction of autophagy (29). We did observe increased viral protein degradation in HCV Core and NS5A expressing Huh7 cells after exposure to external oxidative stress. However, this degradation was independent of the ubiquitin-proteasomal degradation pathway and was also not prevented by blocking autophagic flux with chloroquine. Blocking the lysosomal pathway partially restored HCV protein levels, however additional experiments are required to demonstrate the involvement of selective autophagic pathway and role of autophagy adaptor markers (30).

Selective autophagy, also known as microautophagy, in which lysosomes invaginate and directly sequester cytosolic components, has been suggested to play an important role in the elimination of harmful proteins. In this pathway, LC3-II and p62 play an important role as adaptor proteins (31). In our study, we confirm that selective HCV Core and NS5A degradation can be induced in response to oxidative stress. The identification of autophagy receptor proteins such as p62, which also binds to ubiquitinated proteins, has provided a molecular link between the ubiquitination pathway and autophagy. p62 is a scaffold protein that has been implicated in processes like signal transduction, cell proliferation, cell survival, cell death and oxidative stress response (32) and also plays an important role as receptor for selective autophagy (33). In our study, the HCV Core and NS5A degradation occurred simultaneously with a decrease of p62 protein level, suggesting the involvement of p62 as receptor protein for degradation of viral proteins after oxidative stress induction. Supporting this hypothesis is our observation that silencing of p62 allows the recovery of the expression of HCV Core and NS5A after external oxidative stress. Since we observed a partial restauration of NS5A expression after p62 silencing (Figure 8D), other mechanism must also be considered. It is also
important to demonstrate a direct interaction between HCV Core or NS5A with p62 by immunoprecipitation assay. Although we have performed some experiments with promising results, we need to optimize the immunoprecipitation conditions (Data not show).

Wang et al., suggested that macroautophagy and chaperone-mediated autophagy (CMA) are required for hepatocyte resistance to oxidative stress, because inhibition of macroautophagy sensitized cells to apoptotic and necrotic cell death induced by menadione (34). Our results indicate a more prominent role for autophagy adaptor proteins like p62. The reason for this difference may be due to differences in the model systems used, but are in accordance with the suggestion of Czaja et al., who propose that two types of autophagy are better that one to face the effects of oxidative stress in hepatocytes and that adaptor proteins play an important role in this effect (25).

In summary, our study demonstrates that hepatocytes can adapt to multiple stressors, like HCV protein expression and external oxidative stress. We conclude that activation of the eIF2/ATF4 pathway and subsequent selective degradation, involving LC3-II and p62 contributes to the resistance of hepatocytes to oxidative stress by selective removal of one of the stressors (HCV proteins). This mechanism suggests an important role for autophagy in viral replication and persistence of viral infection and may provide new leads for clinically applicable therapeutic interventions. In addition, it might be suggested to re-investigate in more detail the value of anti-oxidants in HCV patients, since anti-oxidants may abolish the adaptive response of hepatocytes against hepatocytes and prevent the degradation of viral proteins in hepatocytes.
Acknowledgments

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Supplementary figures and tables

Supplementary Figure 1. HCV Core-, NS3/4A- and NS5A-protein expression. The expression of HCV Core, NS3/4A and NS5A proteins was determined by Western blotting in stably transfected Huh7 cells. The expression of GAPDH was used as a loading control.

Supplementary Figure 2. Transcriptional regulation of antioxidant enzyme genes is not involved in the protection against menadione-induced apoptosis. The mRNA levels of antioxidant enzymes SOD1 (A) and CAT (B) were quantified using qPCR in Huh7 cells expressing the empty vector, HCV Core and NS5A. Cells were treated with menadione (50 mmol/L) to induce oxidative stress and in some experiments NAC (5 mmol/L) was used as antioxidant. The relative mRNA expression was normalized relative to 18S. The graphs depict means ± s.d. of three independent experiments. t test was performed to compare the means (p values > 0.05 are considered not statistically significant).
Supplementary Figure 3. Degradation of HCV Core and NS5A is independent of menadione treatment. \( \text{H}_2\text{O}_2 \) (5 mmol/L) was used as an alternative inducer of oxidative stress to confirm the results obtained using menadione. Huh7 cells expressing HCV Core, NS3/4A and NS5A were treated with \( \text{H}_2\text{O}_2 \) for 6 h. In some experiments cells were treated with 5 mmol/L NAC 30 min prior to \( \text{H}_2\text{O}_2 \) treatment. Protein band intensities were quantified using ImageLab software (BioRad). The relative protein expression was calculated based on the expression of GAPDH and compared to the expression of the control cells.

Supplementary Figure 4. \( \text{ATF4} \) mRNA expression. \( \text{ATF4} \) mRNA expression in Huh7 cells treated with menadione was quantified using qPCR in Huh7 cells expressing the empty vector, HCV Core, NS3/4A and NS5A. Cells were treated with 50 mol/L menadione and/or 5 mmol/L NAC. The relative expression was normalized relative to 18S. The graphs depict means ± s.d. of three independent experiments. \( t \) test was performed to compare the means (\( p \) values 0.05 are considered not statistically significant).
Hepatitis C Virus proteins Core and NS5A are highly sensitive to oxidative stress-induced degradation after eIF2a/ATF4 pathway activation.

### Supplementary Table 1. Primers and probes for qPCR.

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F=Forward/R=Reverse/P=Probe/Pr=Primer
References


Hepatitis C Virus proteins Core and NS5A are highly sensitive to oxidative stress-induced degradation after eIF2α/ATF4 pathway activation.


Chapter 4