Review

The cellular stress response in hepatitis C virus infection: A balancing act to promote viral persistence and host cell survival

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ABSTRACT

Oxidative- and endoplasmic reticulum (ER)-stress are common events during hepatitis C virus (HCV) infection and both regulate cell survival and determine clinical outcome. In response to intrinsic and extrinsic cellular stress, different adaptive mechanisms have evolved in hepatocytes to restore cellular homeostasis like the anti-oxidant response, the unfolded protein response (UPR) and the integrated stress response (ISR). In this review, we focus on the cellular stress response in the context of acute and chronic HCV infection. The mechanisms of induction and modulation of oxidative- and ER-stress are reviewed and analyzed from both perspectives: viral persistence and cell survival. Besides, we delve into the activation of the eIF2α/ATF4 pathway and selective autophagy induction; pathways involved in the elimination of harmful viral proteins after oxidative stress induction. For this, the negative role of autophagy upon HCV infection or negative regulation of viral replication is analyzed. Finally, we hypothesize that the cellular stress response in hepatocytes plays a major role for HCV control thus acting as an important host-factor for virus clearance during the early stages of HCV infection.

1. Introduction

Mammalian cells are continuously exposed to internal and external stimuli. The adverse effects of these stimuli are defined as cellular stress and the ability to respond rapidly to these insults is essential for cell survival (Spriggs et al., 2010). The molecular pathways to handle cellular stress are controlled by both transcriptional and non-transcriptional regulators that can sense changes in the cellular environment and transmit the information to elicit adaptive responses (Haigis and Yankner, 2010). In the cellular response to endoplasmic reticulum (ER) stress and oxidative stress these molecular pathways are of major importance. Alterations in protein homeostasis at the ER can trigger the activation of signal transduction pathways defined as the Unfolded Protein Response (UPR) to restore protein homeostasis through the enhancement of the folding capacity of the ER or the Integrated Stress Response (ISR) which leads to global decrease in translation (Walter and Ron, 2011; Sano and Reed, 2013; Harding et al., 2003). Additionally, cells are able to respond to deleterious and toxic products like reactive oxygen species (ROS) during oxidative stress through the expression and/or activation of endogenous antioxidant molecules and enzymes. In this context, the Nuclear factor [erythroid-derived 2]-like/

Kelch-like ECH-associated protein 1 (Nrf2/Keap1) pathway plays an important role in ROS detoxification and restauration of cellular homeostasis (Espinosa-Diez et al., 2015).

During viral infections, virus replication and synthesis of viral proteins can also impose cellular stress and contribute to the imbalance of cellular homeostasis. Viral infection can be considered as an additional stimulus for intrinsic cellular stress and increase the risk of cell death. As the case of hepatitis C virus (HCV) infection, hepatocytes correspond to its principal target cells, however, they have evolved special mechanisms to avoid cell death induction from cellular stress (Feldman et al., 2010). Likewise, the establishment of a chronic infection during hepatitis C requires that cell death of hepatocytes is avoided. Thus, the cellular stress response can determine both cell survival and viral persistence and respond in different ways to stress, depending on the inducers of damage (Chen et al., 2010) (Fig. 1).

Several studies have demonstrated that HCV induces and modulates different signaling pathways related to oxidative stress, ER-stress, autophagy and apoptosis (Tardif et al., 2005; Paracha et al., 2013). In this review, we summarize the knowledge about the mechanisms of induction and modulation of cellular stress in the context of HCV infection (Fig. 1). In addition, the adaptive response to oxidative stress and

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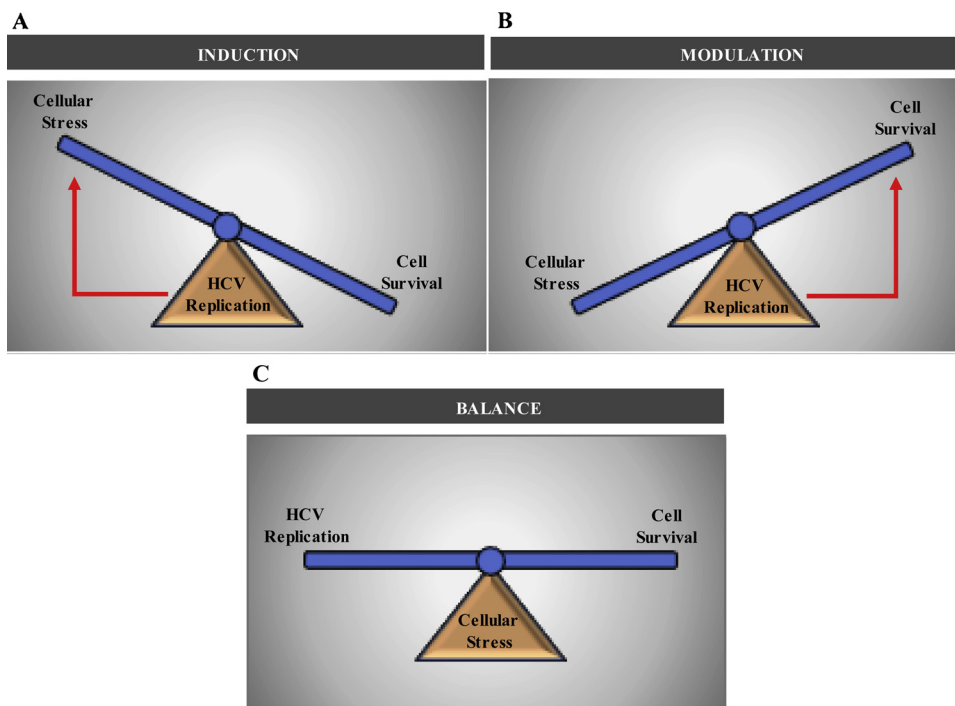 *E-mail address:* wilson.rios@udea.edu.co (W.A. Ríos-Ocampo).

A

INDUCTION

Cellular Stress

Cell Survival

HCV Replication

B

MODULATION

Cell Survival

Cellular Stress

HCV Replication

C

BALANCE

HCV Replication

Cell Survival

Cellular Stress

Fig. 1. The cellular stress response during HCV infection and the balance between cell survival and viral persistence. During HCV infection, cellular stress is increased together with the risk of cell death (**A**). However, for persistence, HCV has evolved different mechanisms to modulate the cellular stress response and suppress death stimuli (**B**). Finally, the cellular stress response determines both cell survival and viral persistence (**C**).

ER-stress as a positive or negative factor in HCV replication is discussed. We will also discuss the different types of autophagy -macroautophagy, microautophagy and chaperone-mediated autophagy (CMA)- as adaptive mechanisms to attenuate oxidative stress and ER-stress after HCV infection and the role of autophagic pathways in viral propagation and persistence.

2. The cellular stress response to oxidative stress in HCV infection

2.1. Oxidative stress is induced during HCV infection

HCV was identified in 1989 as the infectious agent that caused non-A, non-B post-transfusion hepatitis (Choo et al., 1989). According to reports from the World Health Organization (WHO) an estimated 3% of the human population is infected by HCV. Approximately 71 million individuals have a chronic infection and annually almost 400.000 patients die from HCV worldwide, making this viral entity one of the major causes of morbidity and mortality worldwide (World Health Organization, 2017). HCV contains a 9.6-kb positive single-stranded RNA genome with a single open reading frame encoding a polyprotein precursor of about 3000 amino acids (aa) that is co- and post-translationally processed by cellular and viral proteases into the mature structural proteins, Core, E1, E2, p7 and the non-structural (NS) proteins, NS2, NS3, NS4 A, NS4B, NS5A and NS5B (Moradpour et al., 2007). As was mentioned before, hepatocytes in the liver are the predominant targets for HCV and the infection is associated with alterations in the redox state of the host cells, either by the oxidative stress generated by the immune response to eliminate the virus or by viral proteins acting as pro-oxidant molecules in different signaling pathways (Ivanov et al., 2011).

The occurrence of oxidative stress during HCV infection has been extensively demonstrated in liver tissue of chronic HCV-infected patients and in in vitro models using HCV-infected cells or cells expressing individual viral proteins (De Maria et al., 1996; Ivanov et al., 2013). A method referred to as radical-probe electron paramagnetic resonance (EPR) was developed to measure ROS in human hepatic tissue. A significant increase in the production of ROS was observed in liver biopsies from patients with chronic HCV infection compared to liver biopsies from patients with non-viral liver disease or healthy controls

(Valgimigli et al., 2002). Other, more indirect, approaches included the measurement of antioxidants molecules, the levels and activity of antioxidant enzymes and the products of ROS-modified macromolecules, e.g. DNA and protein oxidation (Ivanov et al., 2013).

The increased generation of ROS by HCV has been associated with its pathogenic role during development of chronic liver disease. From the 10 viral proteins, HCV Core protein is the strongest inducer of ROS, followed by the non-structural proteins NS3 and its cofactor NS4 A and NS5A (Paracha et al., 2013; Weinman, 2002). Ivanov et al, using transfected Huh7 cells with several plasmids expressing the full-length HCV Core protein or truncated forms, depicted several mechanisms by which HCV Core can induce oxidative stress. Several enzymes involved in ROS production were induced by HCV Core. In particular, the N-terminal region of the viral protein induced the expression of nicotinamide adenine dinucleotide phosphate oxidase 1 (NOX1), NOX4 and cyclo-oxygenase 2 (COX2). In addition, the expression of cytochrome P450-2E1 and Endoplasmic Reticulum Oxidoreductase 1 A (ERO1 A) were increased upon the expression of the truncated form (aa 37–191) of HCV Core. This study not only demonstrated that HCV Core caused the induction of ROS-producing enzymes but also that different regions of HCV Core are responsible for this induction (Ivanov et al., 2015). Meanwhile, other studies have shown a differential contribution of HCV viral proteins to oxidative stress. Garcia-Mediavilla et al explored the effect of Core and NS5A HCV protein expression on the production of ROS and other reactive molecules like nitric oxide (NO) in Huh7 cells. They observed that both proteins increased the production of superoxide anions to a similar extent, whereas production of hydrogen peroxide (H_2O_2), NO and peroxynitrite was predominantly mediated through NS5A expression, suggesting a differential contribution of these proteins to the production of free radicals (García-Mediavilla et al., 2005). These previous studies indicate that HCV has the capability to induce oxidative stress through the expression of individual viral proteins. In addition, some viral proteins like HCV Core uses multiple mechanisms to trigger ROS production.

HCV can also generate ROS indirectly, e.g. via activation of the host immune response against infection (Kayesh et al., 2017), thus immune-mediated cytotoxicity has been suggested as a key factor in the pathogenesis of HCV-related liver damage and oxidative stress (Vescovo et al., 2016). E.g. human monocytes from healthy blood donors were

incubated with Core, NS3, NS4 A and NS5A HCV recombinant proteins and ROS production was measured. Surprisingly, only NS3 triggered ROS production through the activation of the stress-activated protein kinase, p38 and phosphorylation of the p47^{PHOX} factor resulting in activation of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation (Bureau et al., 2001). In addition to ROS, NO production can have detrimental effects as well. The production of NO occurs after overexpression of inducible nitric oxide synthase (iNOS) by immune cells during the pro-inflammatory response to infection. Interestingly, the expression of iNOS has been described in patients with HCV infection and correlated with the content of viral HCV RNA in the liver (Mihm et al., 1997).

2.2. HCV can modulate the response against oxidative stress

The cellular response to oxidative stress involves, among others, the expression of low-molecular weight antioxidants and phase II detoxifying enzymes for the elimination of ROS and free radicals. The Nrf2/ARE (Nuclear factor erythroid 2 related factor 2/Antioxidant Response Elements) pathway is the major determinant for the expression of γ-glutamylcysteine synthetase (GCL), Glutathione peroxidase (GPX), Glutathione S-transferase (GST), Heme oxygenase-1 (HO-1), N-acetyl-transferase (NAT), NADPH quinine oxidoreductase 1 (NQO-1), Peroxiredoxin (PRX), Thioredoxin reductase (TrxR) and many other genes (Itoh et al., 1997). During HCV replication in cell culture, the activation of the Nrf2/ARE pathway protects the cells from oxidative stress-induced apoptosis, suggesting that HCV, besides its pro-oxidative role, also modulates the cellular response to increase cell survival (Burdette et al., 2010). Indeed, the expression of HCV proteins Core, E1, E2, NS4B and NS5A in Huh-7 cells resulted in the activation of the Nrf2/ARE pathway. Furthermore, in Huh-7 cells expressing Core and NS5A HCV proteins, the activation of the above mentioned antioxidant response was dependent on protein kinase C (PKC), casein kinase 2 (CK2) and phosphoinositide-3-kinase (PI3K) activation. Therefore, it was suggested that in the early stages of viral infection and viral protein expression, the activation of the Nrf2/ARE pathway plays an important role to control the harmful effects of HCV-induced oxidative stress (Ivanov et al., 2011). On the other hand, HCV-dependent inhibition of the Nrf2/ARE pathway and its regulated genes has also been demonstrated (Carvajal-yepes et al., 2011).

3. Endoplasmic reticulum stress and HCV infection

3.1. ER stress and the unfolded protein response

HCV protein synthesis takes place in the cytoplasm in a membrane network generated from the ER. Therefore, infection is strongly dependent on cellular ER function (Moradpour et al., 2007). The sequence of events by which HCV modifies the ER structure and its functions to establish a viral factory are not fully understood; however, it has been shown that several non-structural viral proteins such as NS4B and NS5A cause rearrangements in the ER membrane structure to facilitate viral genome replication, biosynthesis of envelope proteins and assembly of viral particles (Biswas et al., 2016; David et al., 2015). The consequences of this are ER-stress and the activation of the UPR pathway.

The UPR is the cellular adaptive response to restore ER homeostasis using several mechanisms. It constitutes a transcriptional and translational program activated to: i) promote protein folding capacity via the synthesis of chaperone molecules to reduce protein load at the ER, ii) to inhibit protein synthesis, iii) to activate the ER-associated degradation (ERAD) pathway to eliminate unfolded proteins and iv) to expand the ER membrane (Walter and Ron, 2011). There are three molecular sensors located at the ER membrane: i) inositol requiring enzyme 1 (IRE1), ii) double-stranded RNA-activated protein kinase (PKR)–like ER kinase (PERK) and iii) activating transcription factor 6 (ATF6). These sensors operate in parallel and use unique signal transduction

pathways. IRE1 is an ER-transmembrane factor with a dual function as kinase and endoribonuclease. Its activation occurs after direct binding of unfolded proteins and consecutive autophosphorylation and oligomerization (Sidrauski and Walter, 1997). IRE1 cleaves the mRNA encoding the X-box binding protein 1 (XBP1), a UPR-specific transcription factor for the synthesis of chaperones and ERAD proteins. PERK is a kinase that is activated by dimerization and autophosphorylation upon sensing ER-stress. It specifically phosphorylates the alpha-subunit of the eukaryotic translation initiation factor 2 (eIF2α) causing inhibition of protein synthesis. PERK enhances the translation of the transcription factor ATF4 and subsequently C/EBP homologous protein (CHOP) and growth arrest and DNA damage-inducible 34 (GADD34) (Walter and Ron, 2011). ATF6 works as a transcription factor that is initially synthetized as an ER-resident transmembrane protein. Upon accumulation of unfolded proteins, ATF6 is translocated to the Golgi apparatus and processed by the resident site-1 (SP1) and site-2 (SP2) proteases. It then moves to the nucleus and regulates the expression of UPR-genes such as the chaperone immunoglobulin heavy-chain binding protein (BiP) also known as glucose-regulated protein 78 kDa (GRP78), protein disulfide isomerase and glucose-regulated protein 94 (GRP94) (Schindler and Schekman, 2009).

3.2. HCV replication can modulate the response against ER-stress

Several mechanism(s) of HCV replication induced ER-stress and modulation of the UPR pathway have been described in all steps of the virus life cycle. These mechanisms include formation of the replication complex on the ER, virus assembly and maturation of the envelope glycoproteins (Chan, 2014). The expression of HCV Core, NS2, NS4 A, and NS5A/5B in Huh7 cells has been associated with increased levels of the UPR marker Binding Immunoglobulin Protein (BiP) also known as Glucose-Regulated Protein, 78 kDa (Grp78) and CHOP. ER stress induced by HCV Core protein expression was associated with lipid droplet formation during virus assembly (Mclauchlan et al., 2002), while expression of NS2 during virus assembly was associated with UPR activation (Lindenbach and Rice, 2014). Similar to NS2, NS4B is important in membranous web formation and it was also suggested that NS4B can induce ATF6 cleavage and XBP1 splicing (Tardif et al., 2002, 2004). HCV-infected hepatocytes from chimeric SCID/Alb/uPA mice infected with strain H77 genotype 1a also demonstrated increased levels of the UPR marker BiP/Grp78 (Joyce et al., 2009; Malhi and Kaufman, 2011).

However, the occurrence of ER-stress in patients with HCV infection is less well described. ER-stress and UPR were investigated in liver biopsies from individuals with chronic HCV infection without treatment and liver biopsies from adults with normal liver histology. The HCV group was further subdivided according to fibrosis classification (mild and advanced). Electron microscopic analysis revealed a more dilated and disorganized ER structure in HCV cases compared to the controls. Moreover, the activation of the three ER-stress sensors, ATF-6, IRE1, and PERK was demonstrated in advanced chronic hepatitis C, suggesting increased ER-stress in HCV-related fibrosis (Asselah et al., 2010). In another study, ER-stress markers were investigated in tissue samples from patients with HCV-associated HCC. In these samples, UPR markers like sXBP1, BiP, and ATF6 were increased (Shuda et al., 2003). However, contradictory results have also been published: Mcperson et al, concluded that the UPR does not play a prominent role in development of liver injury, since no significant variation in the mRNA levels of UPR-genes such as GRP94, processing of XBP1 or expression of ERAD proteins was observed in liver biopsies of 124 patients with a chronic HCV infection (Mcpherson et al., 2011).

In vitro studies repeatedly demonstrated the activation of the three ER-stress sensors after HCV infection. Huh7.5.1 cells infected with HCV showed an acute ER-stress response including phosphorylation of IRE1 and eIF2α, XBP1 splicing, ATF6 cleavage and increased expression of ER-stress markers GADD34, ATF4, and CHOP (Merquiol et al., 2011). The above results were confirmed using Huh7.5 cells infected with
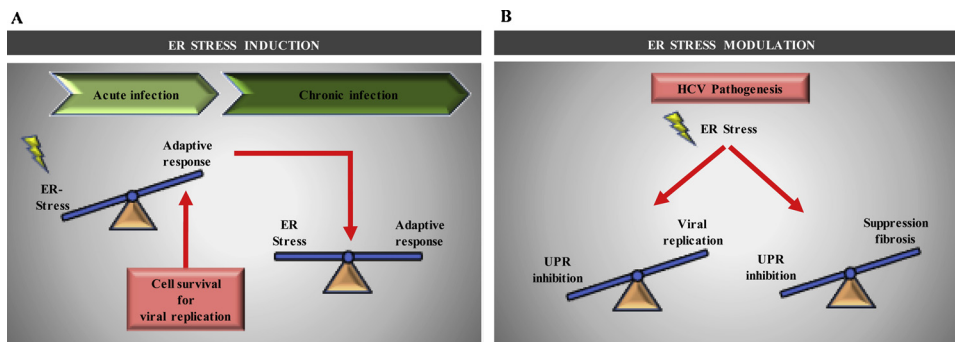
Fig. 2. ER-Stress induction and modulation in response to HCV infection. In acute and chronic HCV infection, the induction of ER-stress has been demonstrated. To overcome ER-stress, an adaptive response is initiated during acute infection to ensure cell survival and allow viral replication. During chronic infection both ER-stress and the adaptive responses to stress are balanced (**A**). ER-stress modulation via inhibition of one or more of the UPR pathways during chronic infection is related to HCV-pathogenesis and may affect viral replication, cell death and fibrogenesis (**B**).

HCV: an acute ER-stress response, peaking at 6–9 days post-infection was observed followed by attenuation of the ER-stress response 15–22 days post-infection (Ke and Chen, 2011). These results are compatible with a model in which HCV infection induces an early and strong ER-stress response, followed by a cellular adaptive response that attenuates the ER-stress and allows cell survival and sustained viral replication (Fig. 2).

Inhibition of (parts of) the UPR by HCV plays an important role in HCV replication and pathogenesis. The IRE1/XBP1 pathway was repressed in Huh7 cells expressing a subgenomic HCV replicon resulting in increased internal ribosome entry site (IRES)-mediated HCV protein translation (Tardif et al., 2004). On the other hand, HCV ER-stress induction is also linked to viral pathogenicity: Chusri et al demonstrated that inhibition of the IRE1/XBP1 pathway decreases the expression of the fibrogenic cytokine transforming growth factor-β1 (TGF-β1). This suggests that inhibition of HCV-induced ER-stress and UPR activation may attenuate fibrogenesis (Chusri et al., 2016). HCV-induced pathogenicity linked to the ER-stress response has also been proposed for insulin resistance: peroxisome proliferator-activated receptor gamma coactivator 1 α (PGC-1α) is an inducible transcription factor that controls cellular energy metabolism and has been linked to insulin resistance (Finck and Kelly, 2006; Yoon et al., 2001). Yao et al, observed that HCV infection induced-ER-stress increases PGC-1α levels, facilitating HCV replication via phosphorylation of cyclic AMP (cAMP)-responsive element-binding protein (CREB). In contrast, the pharmacological inhibition of HCV-induced ER-stress impaired PGC-1α expression and decreased CREB phosphorylation (Yao et al., 2014).

4. Oxidative stress and ER-stress converge into an integrated stress response during HCV infection

The previous sections convincingly demonstrate that HCV is a master in the manipulation of the cellular stress responses and that HCV infection triggers adaptive mechanisms to overcome cellular stress. The Integrated Stress Response (ISR) refers to the activation of multiple stress responses in eukaryotic cells (induced by multiple stressors) to restore cellular homeostasis (Pakos-zebrucka et al., 2016). The central event of the ISR is the phosphorylation of serine 51 of the alpha subunit of eIF2α (Fig. 3). eIF2a phosphorylation causes a reduction in total protein synthesis and allows the translation of selected genes such as ATF4 and CHOP (Walter and Ron, 2011). If the ISR fails to restore homeostasis, e.g. when the cellular stress is too severe, additional pathways are activated that lead to cell death via activation of caspase-3, caspase-7 and caspase-8 (Saelens et al., 2001). Depending on the environmental or physiological stress, there are four protein kinases able to phosphorylate eIF2α. These are: i) PERK; ii) double-stranded RNA-dependent protein kinase (PKR); iii) heme-regulated eIF2α kinase and iv) general control nonderepressible 2 (GCN2) kinase. These kinases are activated after the accumulation of unfolded proteins at the ER, viral infections, oxidative stress or nutrient deprivation, respectively (Fig. 3) (Wek et al., 2006). ATF4 is the best characterized effector molecule of the ISR. It is a basic leucine zipper (bZIP) transcription

factor involved in the regulation of metabolic and redox-related processes. ATF4-mediated transcription leads to the induction of additional bZIP transcriptional regulators, such as Activation Transcription Factor 3 (ATF3) and CHOP via binding to C/EBP-ATF response elements. Due to the presence of a leucine zipper domain, ATF4 can interact with other proteins forming homodimers and heterodimers and this interaction can influence the outcome of the ISR (Pakos-zebrucka et al., 2016). Ohoka et al, demonstrated that ATF4/ATF3 interactions enhance cellular homeostasis whereas ATF4/CHOP interaction promotes cell death or autophagy after ER-stress (Ohoka et al., 2005; Talloczy et al., 2002).

The ISR is primarily a homeostasis-restoring program in response to stress and correlates with the activation of other pro-survival signals like autophagy. Thus, the phosphorylation of eIF2a appears to be crucial for stress-induced autophagy (Fig. 3). Several studies have implied a role for ATF4 and CHOP in the induction of autophagy (B'Chir et al., 2013; Rzymski et al., 2010). These transcription factors can bind either individually or as a complex to specific *cis* promotor elements leading to the activation of genes involved in autophagy (B'Chir et al., 2013). Since our studies also investigated the role of autophagy markers in HCV infection we will briefly introduce some general concepts of cellular stress induced-autophagy in the next section.

5. Autophagy is a rescue signal activated after cellular stress

Several groups have shown that autophagy can act as a cell survival mechanism under stress conditions through activation of the eIF2α/ATF4 pathway (B'Chir et al., 2013; Kroemer et al., 2010). B´chir et al, using mouse embryonic fibroblasts treated with tunicamycin, an agent to induce ER-stress, demonstrated that the eIF2α/ATF4 pathway directs an autophagy gene transcriptional program involved in adaptation to stress (B'Chir et al., 2013). After ER-stress induction the activation of PERK leads to eIF2α phosphorylation and subsequent ATF4 translation. A set of ATF4/CHOP target genes implicated in the formation, elongation and function of autophagosomes was identified: the expression of *Atg16l1, Map1lc3b, Atg12, Atg3* and *Gabarapl2* were ATF4-dependent. The interaction between ATF4 and CHOP controlled the expression of *p62/Sqstm1* and *Atg7*, while *Atg10* and *Atg5* were CHOP-dependent (B'Chir et al., 2013). A similar study using electron microscopic analysis of neuroblastoma SK-N-SH cells exposed to ER stressors demonstrated an increased autophagosome formation during ER-stress. Activation of the IRE1 signaling pathway was required for autophagy induction which protected the cells from ER-stress- induced apoptosis, since cells treated with the autophagy inhibitor 3-methyladenine (3-MA) underwent cell death (Ogata et al., 2006).

Autophagy constitutes a major protective mechanism in response to cellular stress. In its simplest form, as in yeast, autophagy represents the adaptation to starvation. However, autophagy has multiple roles leading to both adaptive and harmful outcomes (Puri and Chandra, 2014). Three types of autophagy have been described: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). These three autophagic pathways all contribute to lysosomal
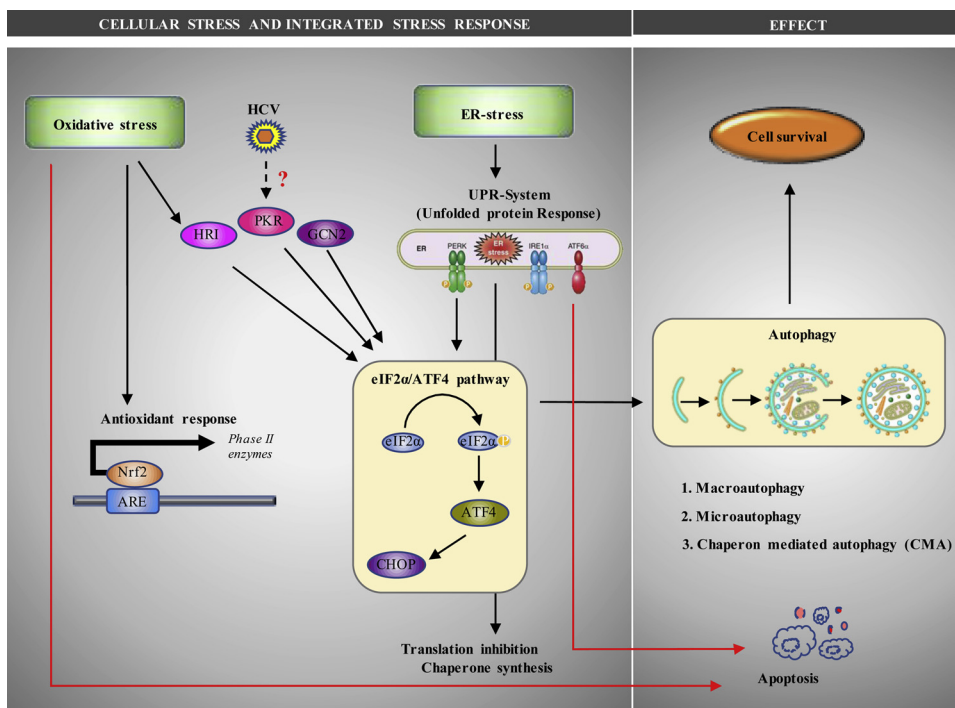
Fig. 3. Cellular stress and the integrated stress response. Oxidative stress and ER-stress induce adaptive responses such as the antioxidant response and UPR response, to overcome stress stimuli. Sustained stress stimuli can induce cell death (red arrows). The induction of adaptive mechanisms to stress is called the ISR. The central event of the ISR is the activation of the eIF2α/ATF4 signaling pathway which has been associated with induction of autophagy and cell survival. Viral infections like hepatitis C as well as oxidative stress can activate the eIF2α/ATF4 pathway.

degradation but differ in their regulation, type of cargo and the mechanisms that determine the targeting of the cargo to the lysosomal compartment for degradation. In macroautophagy, isolated membranes, referred to as phagophores have the capability to engulf intracellular components including a portion of the cytoplasm, soluble proteins, aggregated proteins, organelles, macromolecular complexes, and foreign bodies resulting in the formation of cellular structures surrounded by a double membrane called autophagosome. The autophagosomes fuse with the lysosomes to form the autolysosome leading to the degradation of engulfed material (Puri and Chandra, 2014; Klionsky et al., 2016).

In microautophagy, the lysosomal membrane is invaginated and differentiated into a structure termed "autophagic tube" to enclose portions of the cytosol. Microautophagy is characterized by several sequential stages. The process starts with invagination of the lysosomal membrane and formation of the autophagic tube. In this stage, specific lipids and lipid-modifying proteins together with dynamin-related GTPases play an important role in membrane fission and fusion events as well as with the expansion and separation of the vesicle from the lysosomal membrane. After separation, the vesicle can move into the lysosomal lumen. Subsequently, hydrolases from the family of autophagy-related proteins (Atg), like Atg15p, degrade the vesicle and its content is recycled by the action of proteins like Atg22p (Life et al., 2012).

Chaperone-mediated autophagy (CMA) is a selective lysosomal pathway for the degradation of cytosolic proteins. In contrast to macro- and microautophagy, it acts as a recycling system that mediates the breakdown of specific proteins and/or as a quality control mechanism to remove damaged or incorrectly synthesized proteins. It may also serve as a cellular defense mechanism for the elimination of harmful proteins from e.g. pathogens (Orenstein and Cuervo, 2010). The selectivity of CMA is determined by the amino acid motif KFERQ present in all substrate proteins in CMA. The exact order of amino acids in this motif is not essential, but rather the charge of the residues in this sequence (Dice, 1990). The CMA targeting motif always contains a glutamine (Q), one acidic aspartic acid (D) or glutamic acid (E) amino acid, a basic lysine (K) or arginine (R) amino acid, a hydrophobic amino acid and a basic or hydrophobic (not negatively charged) amino acid

(Bejarano and Cuervo, 2010). The KFERQ-like CMA targeting motif is recognized in the cytosol by a group of chaperones and co-chaperones. The heat shock cognate protein of 70 kDa (hsc70) transports the substrate to the lysosomal membrane and facilitates substrate unfolding for protein translocation across to the lysosomal membrane. The translocation involves a luminal form of hsc70 (lysosomal (lys)-hsc70) and a set of co-chaperones like heat shock cognate protein of 90 kDa (hsc90), heat shock cognate protein of 40 kDa (hsc40), Bcl-2 associated athanogene 1 (Bag-1), hsc70-hsc90 organizing protein (Hop) and hsc70-interacting protein (Hip) (Orenstein and Cuervo, 2010; Agarraberes and Dice, 2001). Once at the lysosomal surface, the uptake of the substrate is mediated by CMA receptors, including the lysosome-associated membrane protein type 2 A (LAMP-2 A) (Orenstein and Cuervo, 2010).

6. Autophagy is essential for HCV replication and viral persistence

All three types of autophagy are observed in HCV infected cells in liver biopsies and in several cell culture models. Using various techniques, including electron microscopy and immunoblotting for microtubule-associated protein 1 A/1B-light chain 3 (LC3), an increased number of autophagic vesicles in liver tissue of chronic HCV patients was observed compared to liver biopsies from patients with a different etiology of liver disease like HBV infection, nonalcoholic steatohepatitis and alcoholic liver disease (Rautou et al., 2011). In addition, increased levels of autophagy parameters like Beclin-1, activation of the mammalian target of rapamycin (mTOR) signaling pathway and conversion of the cytosolic form of LC3 (LC3-I) to the phosphatidylethanolamine-conjugated form LC3-II (which is recruited to autophagosomal membranes) were observed after HCV infection (Ke and Chen, 2011; Shrivastava et al., 2012; Dreux et al., 2009). HCV-induced autophagy is essential for the persistence of infection since knockdown of autophagy-related factors or the chemical inhibition of autophagy abrogates HCV replication (Ke and Chen, 2011; Dreux et al., 2009; Sir et al., 2008, 2012). It is now well established that HCV induces autophagy to support its own replication. However, the molecular mechanisms by which HCV induces the biogenesis of autophagosomes and how its RNA replication complex is assembled on autophagosomes are largely unknown, in particular the mechanism(s) by which preautophagosomal

structures (phagophores) elongate their membranes and mature towards fully formed autophagosomes. Phagophores were thought to extend their membranes to form the enclosed autophagosomes, but more recent studies indicate that they can also undergo homotypic fusion to generate autophagosomes (Moreau et al., 2011). Wang et all, using Huh-7 cells harboring an HCV subgenomic RNA replicon identified crescent membrane structures that resembled phagophores, which appeared to be able to undergo homotypic fusion to form autophagosomes. This process was dependent on SNARE (Soluble NSF [N-ethylmaleimide-sensitive factor] Attachment Protein Receptor) protein syntaxin 7 (STX7), which is known to play an important role in mediating the fusion of vesicular membranes in cells (Wang et al., 2017).

There is controversy whether HCV can efficiently induce the fusion between autophagosomes and lysosomes. The term "autophagic flux" is used to denote the dynamic process of autophagosome synthesis, delivery of autophagic substrates to the lysosome, and degradation of autophagic substrates inside the lysosome (Mizushima et al., 2010). Therefore, it seems likely that impairment or inhibition of the final stages of autophagy is mandatory for successful HCV replication. UV Radiation Resistance Associated (UVRAG) protein plays an important role in the maturation of autophagosomes. Together with the homotypic fusion and protein-sorting/class C vacuole protein-sorting (HOPS/class C Vps) complex it activates the GTPase Rab7 and facilitates fusion between autophagosomes and lysosomes (Wirth et al., 2013; Liang et al., 2010). The activity of UVRAG is antagonized by the protein Rubicon (RUN domain and cysteine-rich domain containing Beclin 1-interacting protein), which can sequester UVRAG preventing interaction with HOPS/class C Vps complex and activation of Rab7 and hence, preventing the maturation of autophagosomes. In Huh7.5 cells infected with HCV, the expression levels of Rubicon and UVRAG were determined at various time points after infection. Rubicon levels were increased 6–48 hours post-infection. These results suggest an impaired maturation of the autophagosomes in the first 24 h after HCV infection, allowing enhanced viral replication. These results were confirmed by Rubicon siRNA knockdown and transient Rubicon overexpression. Therefore, HCV can regulate the autophagic flux to enhance its own replication through the differential expression of Rubicon and UVRAG (Wang et al., 2015).

To successfully establish a chronic HCV infection, the virus has evolved sophisticated mechanisms to evade the host immune response. E.g. the HCV NS3/4 A protease can cleave mitochondrial antiviral signaling protein (MAVS) to suppress the induction of type I interferons after the recognition of pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) (Li et al., 2005). Another example is the HCV protein NS3/4 A mediated cleavage of Toll–interleukin-1 [IL-1] receptor domain containing adaptor-inducing interferon beta (TRIF), an adaptor molecule involved in Toll-Like receptor 3 (TLR3) signaling (Ferreon et al., 2005). Recently, tumor necrosis factor receptor (TNFR)-associated factor 6 (TRAF6), an important adaptor molecule that mediates the TNFR family and interleukin-1 (IL-1)/TLR signaling cascades, was shown to be degraded via autophagy in Huh7 cells containing a subgenomic HCV replicon resulting in suppression of proinflammatory cytokines and enhancing HCV replication. Interestingly, treatment of these cells with Bafilomycin 1 A, an inhibitor of autophagic protein degradation via lysosome, prevented TRAF6 degradation, suggesting a link between autophagy induction and impairment of the innate immune response by HCV. In these experiments TRAF6 degradation was mediated by the p62/sequestosome 1 (SQSTM1) protein (Chan et al., 2016).

## 7. Autophagy also regulates HCV protein synthesis during cellular stress

As discussed in Section 4, autophagy may facilitate and promote HCV replication and viral persistence. However, autophagy has also been implicated to control and suppress HCV replication and persistence (Chan and Ou, 2017).

In hepatocytes, the different types of autophagy can be observed simultaneously as a result of cellular stress. The function of macroautophagy and CMA in hepatocyte resistance to oxidative stress was examined in a model of oxidative stress induced by the superoxide anion generator menadione. The inhibition of macroautophagy by autophagy-related gene 5 (ATG5) knockdown resulted in a deleterious effect and sensitized cells to death from a nontoxic concentration of menadione. This cell death was mediated by the c-Jun N-terminal kinase (JNK) signaling pathway, cytochrome c release from mitochondria and effector caspases 3 and 7 activation (Wang et al., 2010). A similar effect was observed after inhibition of CMA by knockdown of LAMP-2 A, although in this case no hyperactivation of JNK was detected. Pharmacological inhibition of macroautophagy in cells with genetic knockdown of CMA aggravated menadione-induced cell death. These results suggest that both types of autophagy play important roles in the resistance against oxidative stress and that there is some redundancy between the two types of autophagy (Wang et al., 2010; Czaja, 2011).

Since different types of autophagy can be activated simultaneously during stress conditions in hepatocytes, a similar effect can be expected during HCV infection. Indeed, all three types of autophagy have been described in HCV infection (Dreux et al., 2009; Kurt et al., 2015; Kim et al., 2014) and probably the activation of some pathways, like macroautophagy, could represent a positive effect for HCV replication while other types like CMA or microautophagy can induce the opposite effect and negatively regulate virus production as has been demonstrated for the control of e.g. Herpes Simplex Virus 1 and Sindbis virus which are susceptible to degradation via selective autophagy (Chan and Ou, 2017; Tallóczy et al., 2006; Orvedahl et al., 2010). Therefore, the net result of the simultaneous activation of different autophagic pathways in HCV infection on viral replication and persistence may be difficult to predict. Although the activation of autophagic pathways appear to be protective to the infected cells, the effect of activation of these pathways on actual HCV replication and HCV protein synthesis may vary depending on the different stages of the infection (acute or chronic).

Since hepatocytes can activate different types of autophagy to overcome oxidative stress, in previous studies our group has investigated the role of autophagic pathways in cell survival of hepatocytes that overexpress HCV viral proteins and additionally were treated with an inductor of oxidative stress, this two hits inducing oxidative stress model was used to mimic in vitro the in vivo conditions of HCV infection. Huh7 cells expressing the pro-oxidant HCV proteins Core, NS3/4 A or NS5A, were treated with menadione. Surprisingly, Huh7 cells transiently expressing the viral proteins Core and NS3/4 A were more resistant to menadione-induced oxidative stress and cell death. A significant reduction in total and mitochondrial ROS production was observed together with suppression of oxidative-stress induced apoptosis. In addition, in Huh7 cells expressing HCV NS3/4 A and subjected to external oxidative stress (menadione) the expression of ER-stress markers (Grp78, sXBP1) was also reduced (Submitted manuscript). In a subsequent study, using stably transfected Huh7 cells expressing HCV Core, NS3/4 A and NS5A, we observed increased degradation of HCV Core and NS5A proteins after menadione treatment. This menadione-induced degradation was prevented by the antioxidant N-acetyl-L-cysteine (NAC). HCV Core and NS5A degradation also occurred after H_2O_2 treatment, suggesting that the degradation is the consequence of oxidative stress in general and not because of a specific menadione effect. HCV protein degradation correlated with reduced ROS production and oxidative stress-induced apoptosis. Since oxidative stress induced the activation of the eIF2α/ATF4 pathway and expression of CHOP, we hypothesize that selective autophagy is involved in HCV protein degradation (Fig. 4). This hypothesis is further supported by the observation that the autophagic receptor/adaptor protein p62/SQSTM1 is essential for the elimination of the viral proteins Core and NS5A (Manuscript in preparation) (Katsuragi et al., 2015).
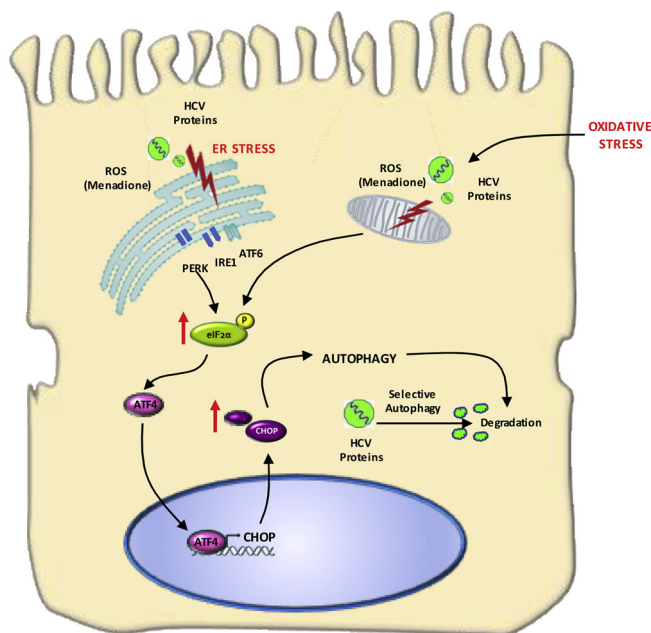
Fig. 4. HCV proteins are selectively degraded in response to oxidative stress. In Huh7 cells expressing HCV viral proteins and subjected to oxidative stress, Core and NS5A HCV proteins as well as the autophagic receptor/adaptor protein p62 are selectively degraded. Involvement of the eIF2α pathway was demonstrated by the increased expression of ATF4 and CHOP.

8. Future perspectives

Since the discovery and identification of HCV in 1989 (Choo et al., 1989), research in this field has grown rapidly with significant advances in the understanding of viral biology and pathophysiology and importantly in the development of successful antiviral treatments. Today, the safety,

tolerability and effectiveness of antiviral treatment has improved to > 90% for all HCV genotypes worldwide (Horner and Naggie, 2015). Therefore, the cure for HCV infection is near and the ambition of the WHO to eliminate viral hepatitis before 2030 is realistic (WHO, 2016). Nonetheless, there are still some unanswered questions and challenges: global HCV eradication, also in less well-developed countries; development of a preventive vaccine; access and cost of the therapy and virus resistance. In addition, there are still (scientific) questions with regard to viral persistence and pathology and host-virus interaction.

The host-HCV interaction is the topic of our research and this review, with special emphasis on the adaptive response of the host cell (hepatocyte) to virus-induced cellular stress, including oxidative stress and ER-stress (Figs. 1 and 4).

We conclude from published studies and our own research that viral protein synthesis activates adaptive responses, including autophagy pathways, that act to limit viral protein load and thereby reduce oxidative stress and cell death (Fig. 4). Exploitation of these pathways to reduce viral replication will be the next goal and might be a valuable addition to antiviral therapy. However, the first challenge will be to translate our in vitro studies to more clinically relevant models and conditions.

Author contributions

Gathering literature information –WARO-; drafting of the manuscript –WARO, MCN-; obtained funding –MCN, HM, TD-; project supervision –HM, TD, MCN-.

Conflict of interest statement

The authors certify that they have NO affiliations with or involvement in any organization or entity with any financial or non-financial interest in the subject matter or materials described in this manuscript.

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