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Commentary

Endoplasmic reticulum (ER) stress: hepatitis C virus induces an ER-nucleus signal transduction pathway and activates NF-κB and STAT-3

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Abstract

Human hepatitis C virus (HCV) is the leading cause of chronic hepatitis, which often results in liver cirrhosis and hepatocellular carcinoma. The HCV RNA genome codes for at least ten proteins. The HCV non-structural protein 5A (NS5A) has generated considerable interest due to its effect on interferon sensitivity via binding and inactivating the cellular protein kinase, PKR. It has been shown that NS5A engages in the endoplasmic reticulum (ER)-nucleus signal transduction pathway. The expression of NS5A in the ER induces an ER stress ultimately leading to the activation of STAT-3 and NF-κB. This pathway is sensitive to inhibitors of Ca^{2+} uptake in the mitochondria (ruthenium red), Ca^{2+} chelators (TMB-8, EGTA-AM), and antioxidants (PDTC, NAC, Mn-SOD). The inhibitory effect of protein tyrosine kinase (PTK) inhibitors indicates the involvement of PTK in NF-κB activation by NS5A. This implicates an alternate pathway of NF-κB activation by NS5A. The actions of NS5A have also been studied in the context of an HCV subgenomic replicon inducing a similar intracellular event. Thus, activation of NF-κB leads to the induction of cellular genes, which are largely antiapoptotic in function. These studies suggest a potential function of NS5A in inducing chronic liver disease and hepatocellular carcinoma associated with HCV infection.

Keywords: HCV; NS5A; ER stress; EOR; STAT-3; NF-κB

1. Introduction

HCV, a major health problem worldwide [1,2], is the causative agent of acute and chronic hepatitis [1,3], which can, in turn, lead to liver cirrhosis and heptocellular carcinoma [4–6]. HCV is also one of the primary causes of liver transplants in the United States and other countries [7].

Upon viral infection, HCV replicates from the cytoplasmic side of the ER membrane and forces cells to produce large amounts of viral proteins that must be processed through the ER. This causes ER stress, which can lead to cell death. To survive, cells adapt by activating the ER to nucleus signaling pathways. HCV-infected cells can also alter the typical course of these signaling pathways to prolong their survival in hepatocytes.

2. HCV and the HCV NS5A

HCV has been classified in a separate genera of the virus family (hepacivirus, *Flaviviridae* [8,9]) because of its unique genomic organization, biochemical properties, and molecular features. The viral genome is composed of a 9.6-kb positive-sense single-stranded RNA containing a 5′-nontranslated region (NTR), a single open reading frame, and a 3′-NTR (Fig. 1A). The 5′-NTR contains an IRES, which regulates the translation of viral polyprotein. The 5′-NTR is also the most highly conserved region among HCV isolates. The open reading frame of the HCV genome encodes an ∼3000 amino acid polyprotein precursor that is co- and post-translationally cleaved by viral proteases and host cell signal peptidases. This results in at least three structural proteins (core, E1, and E2) and

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Abbreviations: EOR, endoplasmic reticulum overload response; ER, endoplasmic reticulum; HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosome entry site; NAC, *N*-acetyl L-cysteine; NF-κB, nuclear factor-κB; NS5A, non-structural protein 5A; PDTC, pyrrolidine dithiocarbamate; PTK, protein tyrosine kinase; SOD, superoxide dismutase; and UPR, unfolded protein response.

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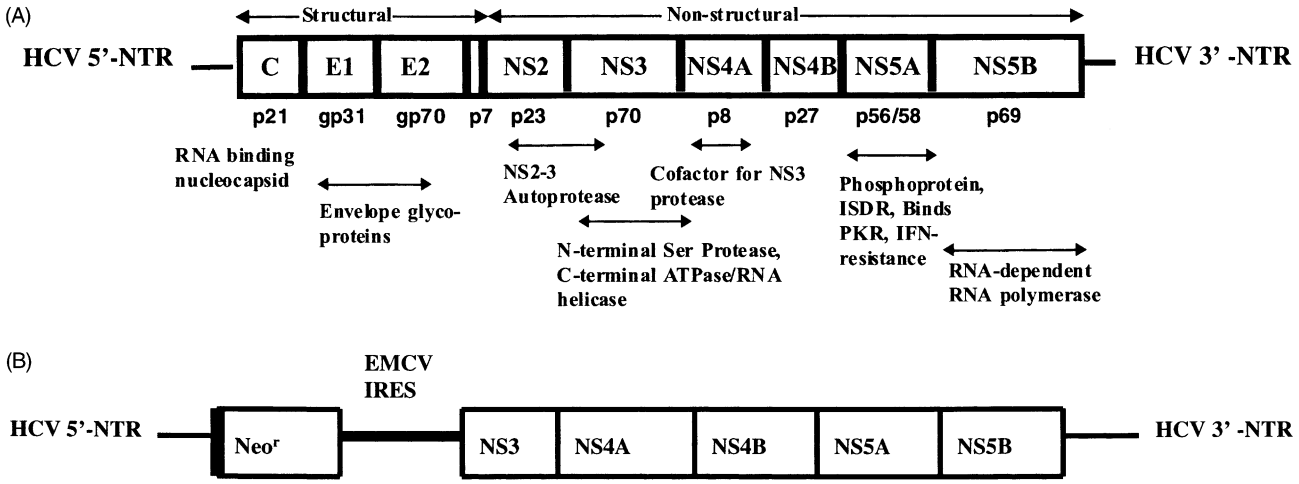

Fig. 1. (A) Schematic of the complete HCV genome. The 5′- and 3′-NTRs flank the open reading frame (ORF) with the structural proteins located in the NH_2-terminal portion of the polyprotein, and the remainder encodes the non-structural proteins (NS2 to NS5B). (B) Physical map of the subgenomic HCV replicon. The HCV 5′-NTR fused to a small portion of the capsid coding region (solid box), the *neo* gene, EMCV IRES (solid line), and HCV NS3 to NS5B coding region (open box), followed by the 3′-NTR structure [20].

six non-structural proteins (NS2-NS5B). The 3′-NTR can be divided into three distinct regions: a 40 base variable sequence, a variable length poly-UC rich tract, and a highly conserved 98 nucleotide structural domain termed 'X' [10–12].

HCV replicates through a negative strand intermediate [13]. The HCV RNA-dependent RNA polymerase, NS5B, presumably initiates transcription from both positive and negative strands of the HCV RNA. Transcriptional initiation is directed from both the 5′- and 3′-NTRs in coordination with viral proteins and host proteins. Several host proteins have been found to bind to the 5′- and 3′-NTR including polypyrimidine tract-binding protein (PTB), La autoantigen, hnRNPC, and NS3 [14–17].

Several attempts to produce a cell culture system that supports HCV replication have failed [18,19]. However, the recent development of HCV subgenomic replicons permits high level expression and replication of HCV RNA in cells. Lohmann *et al.* [20] recently developed selectable HCV subgenomic replicons in a human hepatoma cell line, Huh-7, which supported RNA replication. These replicons are bicistronic constructs composed of the HCV-IRES (nucleotides 1–377 of the 5′-NTR), the neomycin phosphotransferase (*neo*) gene, the encephalomyocarditis IRES, which mediates the translation of HCV non-structural proteins NS3 through NS5, and the 3′-NTR (Fig. 1B).

All of the HCV non-structural proteins make up a ribonucleoprotein replication complex associated with the ER membrane [21]. The HCV NS5A protein is expressed in the context of all other HCV proteins from this membrane-associated replication complex. Although a number of studies have been dedicated to determine the functions of HCV non-structural proteins, the biochemical functions of NS5A are largely unknown [22,23].

NS5A has been the subject of numerous studies, since it was found to interact with the IFN-induced double-stranded RNA-activated protein kinase PKR [24]. Upon induction by IFN, PKR inhibits the initiation of protein synthesis by phosphorylating the translation initiation factor eIF2α [25]. However, NS5A reduces the antiviral activity of IFN by interfering with the enzymatic activities of PKR, allowing translation to continue in the presence of IFN.

NS5A may also contribute to viral replication and HCV-mediated pathogenesis by influencing gene expression. Although NS5A has not been detected in the nucleus of cells, an N-terminal deletion of the protein has been shown to activate transcription [26]. Interestingly, NS5A is anchored to the ER membrane by an amphipathic N-terminal helix, and removing this anchoring sequence alters the localization of the protein from the ER to the nucleus [27]. NS5A can also affect gene expression by physically associating with transcription factors. NS5A binds to the tumor suppressor p53, inhibiting its transcriptional up-regulation of the cyclin-dependent kinase inhibitor *p21/waf1* gene [28] and preventing p53-dependent G_1 arrest. NS5A may also regulate cell growth through its interaction with the novel cellular transcription factor SCRAP [29].

3. HCV NS5A and ER stress

The ER serves several critical cellular functions. The organelle is the production site for all parts of cell membranes, proteins, lipids, and sterols. Proteins are also folded, with the aid of chaperones, and glycosylated in the ER lumen before their transport to other organelles, secretion, or expression on the cell surface. Because these processes are required for cell survival, any disruption in

the ER functions can have dire consequences for the cell. The ER is sensitive to a variety of cellular stresses including disturbances in calcium homeostasis and inhibition of protein glycosylation [30]. These stresses prevent protein folding in the ER and result in the accumulation of misfolded proteins in the organelle [31]. Overloading the ER with correctly folded proteins can also induce ER stress. Each stress elicits a unique response activating ER-nucleus signal transduction pathways. ER stress responses can include transcriptional induction, translational attenuation, and protein degradation. HCV induces ER stress in hepatocytes and triggers two functionally distinct ER stress response pathways: the EOR and the UPR.

The EOR is stimulated by HCV NS5A expressed alone or in the context of an HCV replicon associated with the ER [32]. NS5A activates the EOR after disturbing intracellular calcium levels, leading to the activation of NF-κB and STAT-3. Numerous viral proteins have been shown to induce an ER overload–ER stress response, including influenza hemagglutinin [33], hepatitis B virus MHBS^t [34], adenovirus E3/19K [35], and hepatitis C virus NS5A protein [32].

A limited number of viral proteins induce the UPR in reaction to ER stress. The hepatitis B virus large surface protein [36] and the HCV E2 envelope protein [37] both activate the UPR. The HCV E2 protein is a structural component of the viral envelope and is probably not essential for viral replication [20]. Recently, cells expressing HCV replicons were found to activate the UPR in the absence of the HCV structural protein E2 [38]. Cells expressing HCV replicons activate the ER stress transducer, ATF6, by proteolytically cleaving a transcriptionally active N-terminal domain of ATF6 (pATF6α(N)) from the ER membrane [39]. The N-terminal domain of ATF6 is translocated to the nucleus where it activates the transcription of selective genes with a conserved ER stress response element (ERSE) [39,40].

When HCV NS5A was expressed alone, the protein did not induce the UPR [38]. Since NS5A can induce the EOR when it is expressed alone, UPR activation by HCV replicons and HCV non-structural proteins is distinct from NS5A induction of the EOR. This does not exclude the ability of NS5A to stimulate the UPR when it is expressed with all the non-structural proteins in the context of the HCV replicon.

Another intracellular event characteristic of the UPR includes translational attenuation. However, cells expressing HCV replicons were found to have lower levels of eIF2α phosphorylation [38], indicating elevated translation initiation. This is likely the result of NS5A inhibition of PKR activity [24]. Furthermore, cap-dependent and HCV IRES- and GRP78 IRES-mediated cap-independent translation was enhanced in cells expressing HCV replicons [38]. These results illustrate how HCV alters the typical course of the UPR to prolong its survival in hepatocytes.

4. Activation of transcription factors

A result of NS5A-induced ER stress is the efflux of calcium from the ER. The calcium released from the ER is taken up by the mitochondria, where it alters the transmembrane potential and induces oxidative stress exhibited by the elevation of reactive oxygen species (ROS) [31,41,42]. Elevated mitochondrial calcium ($[Ca^{2+}]_m$) levels directly affect mitochondrial ROS production, leading to the activation of transcription factors, STAT-3 and NF-κB, in an ER overload–ER stress response [32]. The downstream events from the ER to the activation of STAT-3 or NF-κB are illustrated in Fig. 2.

ATF6 activation in cells expressing HCV replicons is required for initiation of the UPR. ATF6 induces the transcription of ER chaperone genes, such as *GRP78*, and the *XBP1* gene in an ERSE-dependent manner [43]. XBP1 mRNA is spliced by the ER stress transducer, IRE1, and the XBP1 transcription factor expressed from the spliced mRNA effectively activates the mammalian UPR through ERSE sequences, similar to ATF6 [43,44]. XBP1 has been shown to be essential for hepatocyte growth and differentiation [45]. Activation of the transcription factors ATF6 and XBP1 in the UPR may contribute to liver pathogenesis associated with the HCV infection.

5. NF-κB and STAT-3

The EOR activates a well-characterized transcription factor, NF-κB, which controls cell survival by the activation of pro-survival genes [46]. In a variety of cell types, NF-κB stays latent in the cytoplasm complexed with IκB, its inhibitory subunit [47,48]. Activation of NF-κB occurs via Ser^{32} and Ser^{36} phosphorylation of IκB for ubiquitination and subsequent degradation by 26S proteasome [47,49,50]. The evidence to support the transactivation of NF-κB by NS5A is based on the use of inhibitors of calcium uptake in the mitochondria (ruthenium red, Ru 360), calcium chelators (TMB-8, EGTA-AM) which are known to adversely affect calcium signaling, and antioxidants (PDTC, NAC, Mn-SOD) [32]. The antioxidants may act by directly scavenging radicals, chelating metal ions, or sustaining the activity of antioxidant enzymes.

An alternative mechanism for the activation of NF-κB involves the phosphorylation at Tyr^{42}, and PEST sequences of IκB under oxidative stress [51,52]. Tyrosine phosphorylation of IκBα has been observed during ischemia/reperfusion of the liver [53], suggesting a functional role in this pathway. Livolsi *et al.* [51] have shown that tyrosine kinases act at several levels to dissociate IκBα–NF-κB complexes. We observed that HCV NS5A and HCV replicon expression similarly activated NF-κB via tyrosine phosphorylation (unpublished results). The use of Ser and Tyr mutants in our study dissected the IκBα pathway. MG132, a proteasome inhibitor, did not affect the ability of NS5A to

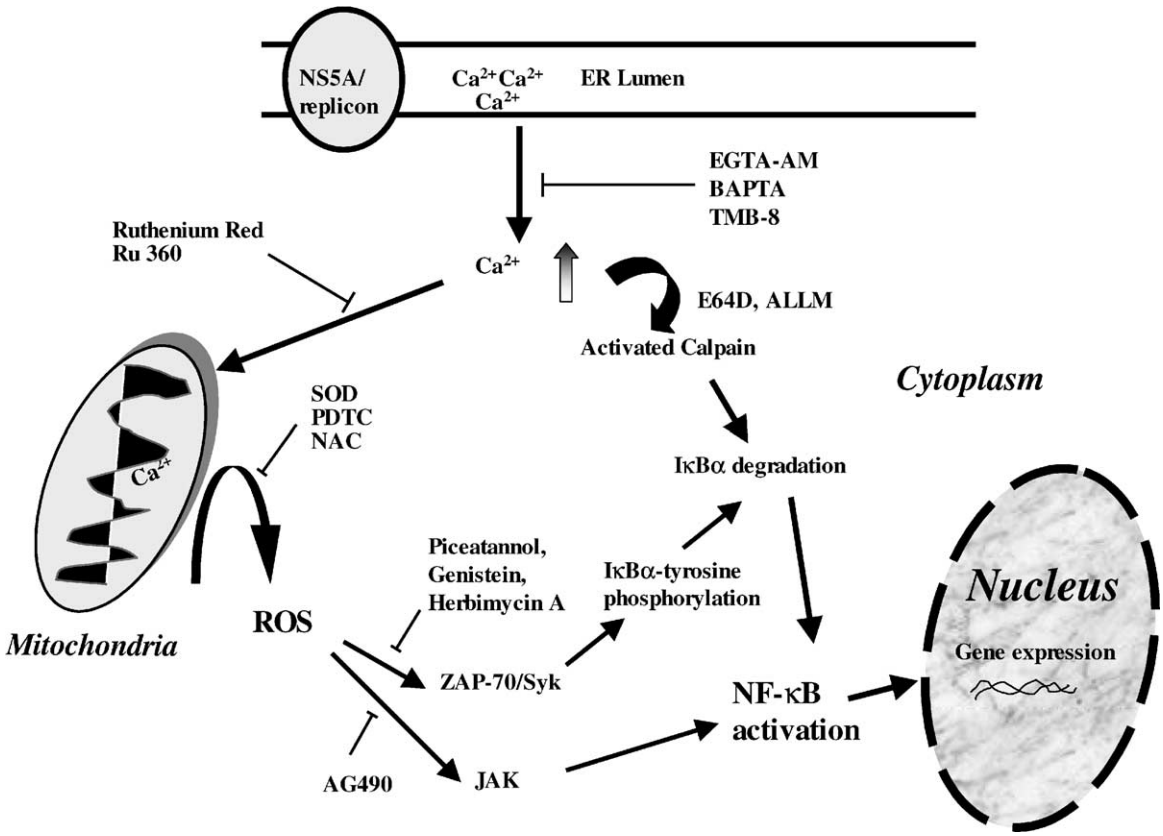

Fig. 2. Model illustrating NS5A-induced activation of NF-κB via Ca^{2+} signaling and oxidative stress. This pathway involves the activation of NF-κB via tyrosine phosphorylation. Expression of HCV non-structural proteins leads to the efflux of Ca^{2+}, which is subsequently taken up by the mitochondria. The elevated $[Ca^{2+}]_m$ induces ROS. By a mechanism not clearly understood, ROS activate STAT-3 and NF-κB via IκBα tyrosine phosphorylation.

transactivate NF-κB, whereas E64D [L-3-*trans*-ethoxycarbonyloxirane-2-carbonyl]-L-leucine (3-methylbutyl) amide] and ALLM (*N*-acetyl-leu-leu-Met-CHO), calpain inhibitors, eliminated the activation of NF-κB (unpublished results).

We also showed that NS5A constitutively activates STAT-3, a transcription factor that is activated by cytokines such as epidermal growth factor or interleukin-6 [54]. Activated STATs form dimers or multimers through their Src-homology domain II, transported into the nucleus, where they bind to the cognate DNA sequences and activate gene expression. Oxidative stress has been shown to trigger STAT-3 tyrosine phosphorylation and nuclear translocation [55], which correlates with the activation of STAT-3 leading to its DNA-binding activity. NS5A does not possess tyrosine kinase activity. There are multiple tyrosine kinase signaling pathways, in addition to the Janus kinase (JAK) family, that can induce STAT signaling [56]. Expression of a constitutive PTK, v-Src, in T-cells has been reported to induce a constitutive activation of NF-κB in the nucleus [57]. Recently, Siebenlist [58] suggested a cross-talk between STATs and NF-κB pathways and identified JAK2 to be essential in this process in certain cell types. The enzyme JAK2 activates by autophosphorylation and then phosphorylates the receptor, which recruits STATs. This cross-talk might involve the well-characterized activation of IκB kinases, as well as the phosphorylation of IκBα on the tyrosine residue(s).

6. Conclusions

HCV, an RNA-containing virus, triggers an ER-nucleus signal transduction pathway. HCV replicon and the HCV protein NS5A-mediated induction of the EOR and UPR ultimately lead to the stimulation of transcription factors, including NF-κB, STAT-3, and ATF6. These activities are essential to the maintenance of chronic hepatitis. Under conditions of ER stress, a signal is generated that activates the transcription factors, which then induce the transcription of genes encoding survival factors. ATF6 and XBP1 induction through the UPR may be essential for hepatocyte growth when cells are under HCV replicon-induced ER stress. A coordinated balance of responses is required to maintain cell survival under conditions of ER stress. These intracellular events suggest a potential function of HCV in inducing chronic liver disease including its progression to hepatocellular carcinoma associated with HCV infection.

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