Activation of transcription factor Nrf2 by hepatitis C virus induces the cell-survival pathway

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Oxidative stress has been implicated in various human diseases, including the pathogenesis of hepatitis C virus (HCV). Previous studies have shown the induction of oxidative stress in cultured cells expressing HCV genes. The transcription factor Nrf2 is known to be activated in response to oxidative stress, but the mechanism of its activation is not clearly understood. In this study, we first determined the induction of Nrf2 and then investigated the mechanism of Nrf2 activation in human hepatoma cells infected with HCV (JFH-1). Our results showed the induction and nuclear translocation of Nrf2 in a time-dependent manner. The HCV-mediated activation of Nrf2 was abrogated in the presence of an antioxidant, PDTC (pyrrolidine dithiocarbamate), and a Ca^{2+} chelator, BAPTA-AM [1,2-bis(aminophenoxy)ethane N,N,N,N-tetraacetic acid tetra(acetoxymethyl) ester], which suggests a role for both reactive oxygen species and Ca^{2+} signalling in the Nrf2-activation process. By using inhibitors of cellular kinases, we showed further that HCV-mediated phosphorylation/activation of Nrf2 is mediated by the mitogen-activated protein (MAP) kinases p38 MAPK and janus kinase. We also observed enhanced phosphorylation of Akt and its downstream substrate Bad in HCV-infected cells. Furthermore, by using a small interfering RNA approach, our results suggest a potential role for HCV-mediated Nrf2 activation in the survival of HCV-infected cells, a condition favourable for liver oncogenesis. Taken together, these results provide an insight into the mechanisms by which HCV induces intracellular events relevant to chronic HCV infection.

INTRODUCTION

Chronic liver disease due to hepatitis C virus (HCV) infection is a major global health problem; more than 170 million individuals worldwide are infected with HCV (Di Bisceglie, 1997). HCV infection leads to chronic hepatitis in up to 60–80% of infected adults and progresses to liver fibrosis, cirrhosis and eventually hepatocellular carcinoma (Di Bisceglie, 1997). HCV is an enveloped, single-stranded, positive-sense RNA virus with a genome approximately 9.6 kb in length, which encodes a single polyprotein of about 3000 aa (Bartenschlager & Lohmann, 2000). This polyprotein is cleaved post-translationally by a combination of host-cell signal peptidases and viral proteinases into structural (core, E1 and E2) and non-structural (NS2–NS5B) proteins (Bartenschlager & Lohmann, 2000). Studies of the molecular mechanisms of HCV replication and pathogenesis have been hampered by the lack of an efficient cell-culture system and a suitable small-animal model. Recently, a robust and productive HCV (genotype 2a) infection system that allows the production of infectious virus in cell culture has been demonstrated (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005).

HCV replicates within the ribonucleoprotein complexes in the modified membranous structures extending from the endoplasmic reticulum (ER) membrane (Dubuisson et al., 2002; Shi et al., 2003; Waris et al., 2004). Previously, we have shown that HCV gene expression in the ER membrane induces oxidative stress through Ca^{2+} signalling in the ER (Gong et al., 2001; Tardif et al., 2002). Several HCV proteins, including core, NS3 and NS5A, and the HCV subgenomic replicon have been shown to induce oxidative stress in human hepatoma cells (Bureau et al., 2001; Gong et al., 2001; Okuda et al., 2002; Waris et al., 2005; Machida et al., 2006; Kasprzak & Adamek, 2008).

Several studies have shown the upregulation of transcription factor NF-E2-related factor 2 (Nrf2) via an uncharacterized mechanism in cells undergoing oxidative stress (Motohashi & Yamamoto, 2004). Nrf2, together with p45 NF-E2, Nrf1 and Nrf3, belongs to the CNC (cap ‘n’ collar) family of basic leucine zipper (b-ZIP) transcription factors (Motohashi et al., 2004). Nrf2 exerts its role in host protection against oxidative injury via binding to the antioxidant-response element (ARE) to activate transcription of antioxidant/defence and cytoprotective genes (Rushmore et al., 1991; Motohashi & Yamamoto, 2004;...
Motohashi et al., 2004). Once in the nucleus, Nrf2 forms heterodimers with other b-ZIP proteins, such as Maf, c-Jun and ATF4 (Jeyapaul & Jaiswal, 2000; He et al., 2001). Kelch-like ECH-associated protein 1 (Keap1) is a cytoplasmic, actin-bound protein that represses Nrf2 activity (Kobayashi et al., 2002; Itoh et al., 2003). Under normal conditions, Keap1 sequesters Nrf2 in the cytoplasm and prevents its nuclear translocation (Itoh et al., 2003). Several studies have demonstrated the role of protein kinase pathways in transducing Nrf2-mediated gene expression in the nucleus. Among the cellular kinases, ERK1/2, p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase/Akt (PI3k-Akt) and protein kinase C (PKC) have been shown to phosphorylate and activate Nrf2 (Yu et al., 2000; Zipper & Mulcahy, 2000; Kang et al., 2001; Huang et al., 2002). Previous studies have shown that PERK-dependent phosphorylation of Nrf2 triggers dissociation of Nrf2–Keap1 complexes, which is critical for cell survival (Cullinan et al., 2003; Cullinan & Diehl, 2004).

In the present study, we first determined the induction of Nrf2 and then investigated the mechanism of Nrf2 activation in HCV-infected cells. Our results demonstrate that the activation of Nrf2 in HCV-infected cells is mediated by Ca\(^{2+}\) signalling in the ER and by elevation of reactive oxygen species (ROS) in the mitochondria. Further, we demonstrate that p38 MAPK and janus kinase (JNK) are involved in the phosphorylation and subsequent nuclear translocation of Nrf2 in HCV-infected cells. By using Nrf2 small interfering RNA (siRNA), our results demonstrate further that HCV-mediated Nrf2 activation contributes to the survival of HCV-infected cells.

### RESULTS

#### HCV infection induces Nrf2

To examine whether HCV infection induces Nrf2, we incubated Huh-7 cells with HCV at an m.o.i. of 1 as described previously (Wakita et al., 2005; Zhong et al., 2005; Waris et al., 2007). At 48 h post-infection (p.i.), HCV RNA replication was analysed by quantitative RT-PCR. The results showed a significant increase (approx. 600-fold) in HCV RNA levels in Huh-7 cells infected with HCV (Fig. 1a, compare bars 1 and 2). In order to demonstrate that Nrf2 is induced in HCV-infected cells, mock-infected and HCV-infected cells were collected at various time points; cellular lysates were prepared and subjected to immunoblot analysis using an anti-Nrf2 antibody. The results revealed the induction of Nrf2 at day 2 p.i. (1.5-fold), continuing up to day 6 p.i. (2.7-fold) (Fig. 1b, lanes 5–7), compared with mock-infected Huh-7 lysates (lane 1). These results demonstrate that HCV gene expression is required for the induction of Nrf2, but not the early steps of the HCV life cycle. At days 2 and 4, we observed approximately 35–40 and 75–80% HCV core-positive cells by immunofluorescence (Fig. 1d). As a positive control, Huh-7 cells were incubated with tert-butyldihydroquinone (tBHQ), a known inducer of Nrf2 (Nguyen et al., 2003). The results showed an approximately 15-fold induction of Nrf2 in Huh-7 cells incubated with tBHQ (Fig. 1b, lane 9). To determine whether the induction of Nrf2 was due to an increase in Nrf2 transcription, total cellular RNA was extracted from mock-infected and HCV-infected cells, and the level of Nrf2 mRNA was quantified by real-time RT-PCR. The results showed a statistically significant increase (P<0.01; Student’s t-test) in Nrf2 mRNA expression in HCV-infected cells at days 2 (3-fold) and 4 (4-fold) compared with mock-infected cells (Fig. 1c). Taken together, these results indicate clearly that HCV infection induces the transcriptional stimulation, as well as synthesis, of Nrf2 protein.

#### HCV infection triggers the nuclear translocation of Nrf2

Because HCV induced Nrf2 in a time-dependent manner, we examined the colocalization of Keap1 and Nrf2 in mock-infected and HCV-infected cells by immunofluorescence. The results showed that the induction and nuclear staining of Nrf2 began at day 2, and a robust increase in nuclear staining was observed at day 4 in HCV-infected cells (Fig. 2b, c), compared with a very low amount of Nrf2 staining in the cytoplasm in mock-infected cells (Fig. 2a). To demonstrate the expression of HCV antigen in Huh-7 cells infected with HCV, we co-stained the infected cells for HCV core protein. The results showed the perinuclear staining of HCV core in HCV-infected cells (Fig. 2b, c, f and g).

To demonstrate the effect of HCV-induced Ca\(^{2+}\) signalling and elevation of ROS on Nrf2 nuclear translocation, we incubated mock-infected and HCV-infected cells at day 4 with a Ca\(^{2+}\) chelator [1,2-bis(aminophenoxy)ethane N,N,N,N-tetraacetic acid tetra(acetoxymethyl) ester; BAPTA-AM] or an antioxidant (pyrrolidine dithiocarbamate; PDTC). We observed that the nuclear staining of Nrf2 in HCV-infected cells was inhibited in the separate presence of Ca\(^{2+}\) chelator as well as antioxidant (Fig. 2f, g). However, these inhibitors did not show any effect on Nrf2 in mock-infected cells (Fig. 2d, e). The cytoplasmic induction of Nrf2 in HCV-infected cells was unaffected by inhibitor treatments (Fig. 2f, g). These results demonstrate that HCV-induced Ca\(^{2+}\) signalling and elevation of ROS induce the activity of Nrf2 and its translocation into the nucleus.

#### HCV infection induces phosphorylation of Nrf2

Our previous studies have shown that HCV-induced ROS and Ca\(^{2+}\) signalling activate cellular kinases (Waris & Siddiqui, 2005; Waris et al., 2005). To investigate the role of ROS-induced cellular kinases in stimulating Nrf2 phosphorylation, Huh-7 cells were infected with HCV (m.o.i. of 1). At day 4 p.i. (approx. 75% infection), cells were incubated with antioxidant (PDTC), Ca\(^{2+}\) chelator...
(BAPTA-AM), JNK inhibitor (SP600125), p38 MAPK inhibitor (SB203580), PI3K inhibitor (LY294002) or PKC inhibitor (Go6976). To determine the specificity of JNK and p38 MAP kinases in inducing Nrf2 phosphorylation, Huh-7 cells were transfected with siRNAs against p38 MAPK, JNK and green fluorescent protein (GFP; negative control), followed by HCV infection. Cellular lysates were immunoprecipitated with anti-Nrf2 antibody and Western blotting was performed with an anti-phosphoserine antibody. Increased phosphorylation of Nrf2 was observed in HCV-infected cells at day 4 (Fig. 3a–c, lane 2) but, in the presence of the above inhibitors or siRNAs, Nrf2 phosphorylation was inhibited to varying degrees (Fig. 3a, lanes 3–6; Fig. 3c, lanes 4 and 5), whereas treatment of HCV-infected cells with LY294002 or Go6976 did not affect Nrf2 phosphorylation (Fig. 3b, lanes 3 and 4). Treatment of HCV-infected cells with these inhibitors or transfection with Nrf2 siRNA did not affect the expression levels of Nrf2 protein (Fig. 3a–c). We observed respectively 61 and 66% reduction in p38 MAPK and JNK protein levels in the presence of the corresponding siRNAs (Fig. 3c, lanes 4 and 5). In order to demonstrate the phosphorylation of MAP kinases in HCV-infected cells, cellular lysates from mock-infected and HCV-infected cells were immunoblotted with anti-phospho-p38 MAPK and phospho-JNK antibodies. The results showed the phosphorylation of p38 MAPK and JNK in HCV-infected cells (Fig. 3d, lane 2) compared with mock-infected cells (lane 1). Collectively, these results demonstrate that HCV infection stimulates the phosphorylation of Nrf2 via oxidative stress, Ca$^{2+}$ signalling and activation of MAP kinases.

**HCV-induced Nrf2 activates the ARE–luciferase reporter**

To validate Nrf2 induction by HCV infection further, cell-based luciferase reporter assays were performed. Mock-infected and HCV-infected cells at day 4 (approx. 75% infection) were transfected with ARE-Luc and ARE-mut-Luc. At 30 h post-transfection (p.t.), cellular lysates were
prepared and assayed for luciferase activity. The results displayed increased activity of ARE-Luc in HCV-infected cells, whereas the ARE-mut-Luc containing mutated Nrf2-binding sites was not induced in HCV-infected cells (Fig. 4a, b). In order to demonstrate that ARE-Luc activity in HCV-infected cells is mediated by Nrf2, Huh-7 cells were transfected with Nrf2 siRNA or GFP siRNA. At 24 h p.t., cells were infected with HCV (m.o.i. of 1) for 48 h, followed by another transfection with ARE-Luc plasmid. The results displayed reduced ARE-Luc activity in HCV-infected cells transfected with Nrf2 siRNA compared with those transfected with GFP siRNA (Fig. 4a). These results indicate clearly that HCV-induced Nrf2 is involved in the activation of ARE-Luc.

Next, to determine the role of HCV-induced Ca\textsuperscript{2+} signalling, induction of ROS and activation of cellular kinases in ARE-Luc activity, HCV-infected cells were transfected with ARE-Luc followed by incubation with antioxidant (PDTC), Ca\textsuperscript{2+} chelator (BAPTA-AM), p38 MAPK inhibitor (SB203580), JNK inhibitor (SP600125), PI3k inhibitor (LY294002) or PKC inhibitor (Go6976). Cellular lysates were subjected to dual-luciferase assays. The results showed reduced ARE-Luc activity in the separate presence of antioxidant, Ca\textsuperscript{2+} chelator and MAPK inhibitors, whereas PI3k and PKC inhibitors did not affect ARE-Luc activity in HCV-infected cells (Fig. 4b). These results demonstrate that Nrf2 transactivation in HCV-infected cells is indeed mediated through Ca\textsuperscript{2+} signalling, elevation of ROS and activation of MAP kinases.

**HCV infection activates Nrf2 target genes**

To determine whether HCV-infected cells can induce Nrf2 target genes such as haem oxygenase 1 (HO-1), NAD(P)H:quinone reductase (NQO1) and glutamylcysteine synthetase heavy subunit (γGCSH), total cellular

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**Fig. 2.** Nuclear translocation of Nrf2 in HCV-infected cells. Mock-infected and HCV-infected cells at days 2 and 4, and mock-infected and HCV-infected cells incubated with the antioxidant PDTC (100 μM for 4 h) or calcium chelator BAPTA-AM (50 μM for 2 h) were grown in eight-well chamber slides. The cells were fixed, permeabilized and stained with antibodies against Nrf2, Keap1 and HCV core. DAPI was used as a nuclear stain. Bar, 10 μm.
RNA was extracted from mock-infected and HCV-infected cells at day 4 (approx. 75% infection). The mRNA levels of HO-1, NQO1 and γGCSH were quantified by real-time RT-PCR. The results showed that HCV infection enhanced the transcript levels of these Nrf2 target genes by approximately 3.5- to 4-fold at day 4 (Fig. 5a). In order to demonstrate that the induction of Nrf2 target genes is mediated by ROS and subsequent activation of Nrf2, HCV-infected cells at day 4 were incubated with the antioxidant PDTC or transfected with Nrf2 siRNA prior to HCV infection. The results displayed downregulation of Nrf2 target genes in HCV-infected cells incubated with antioxidant or transfected with Nrf2 siRNA, whereas GFP siRNA did not show any effect on the Nrf2 target genes (Fig. 5a). To determine the effect of Nrf2 and GFP siRNAs on the expression levels of Nrf2 mRNAs, total mRNA was extracted and subjected to quantitative RT-PCR. We observed an approximately 68% reduction in the expression of Nrf2 mRNA in cells transfected with Nrf2 siRNA, compared with approximately 7% in GFP siRNA-transfected cells (Fig. 5b). These results suggest that HCV-induced ROS and activation of Nrf2 are indeed responsible for the stimulation of the HO-1, NQO1 and γGCSH genes.

Nrf2 induces the cell-survival pathway in HCV-infected cells

Previously, we have demonstrated that the HCV subgenomic replicon activates cell-survival proteins such as Akt, GSK-3β, Bad and Bcl-XL via oxidative stress (Waris & Siddiqui, 2005; Waris et al., 2005), and Nrf2 has been shown to induce cell survival through the activation of PERK (Cullinan & Diehl, 2004). It was therefore of particular interest to investigate whether HCV-mediated Nrf2 activation can induce the activation of cell-survival proteins. To determine whether Nrf2 is necessary for HCV-mediated phosphorylation of Akt and its downstream substrates, Bad, we used siRNA against Nrf2 and GFP (negative control). Huh-7 cells were transfected with Nrf2 or GFP siRNAs at 24 h p.t., cells were infected with HCV (m.o.i. of 1). At 48 h p.i., cellular lysates were prepared and subjected to immunoblot analysis using antibodies against Akt, p-Akt, Bad and p-Bad. The results showed the phosphorylation of Akt and Bad in HCV-infected cells, which was reduced in HCV-infected cells transfected with Nrf2 siRNA (Fig. 6a). Transfection of HCV-infected cells with GFP siRNA did not affect the phosphorylation of
these proteins (Fig. 6a). In order to determine the effect of Nrf2 and GFP siRNAs on the expression of Nrf2 protein, we harvested the cells at 72 h post-siRNA transfection and subjected cell lysates to Western blot analysis. We observed 71% reduction in the expression of Nrf2 protein in HCV-infected cells transfected with Nrf2 siRNA (Fig. 6a). Next, we examined the effect of Nrf2 and GFP siRNA on cells via a cell-death assay. Mock-infected and HCV-infected cells at day 4 were transfected with ARE-Luc and ARE-mut-Luc reporter plasmids. At 30 h p.t., cells were serum-starved for 4 h and incubated with the antioxidant PDTC (100 μM for 4 h), Ca²⁺ chelator BAPTA-AM (50 μM for 2 h), p38 MAPK inhibitor SB203580 (10 μM for 12 h), JNK inhibitor SP600125 (30 μM for 12 h), PI3k inhibitor LY294002 (50 μM for 12 h) or PKC inhibitor Go6976 (10 μM for 2 h); cellular lysates were assayed for luciferase activity. Data represent means ± SD of three independent experiments performed in duplicate.

Fig. 4. (a) HCV activates the ARE–luciferase reporter through Nrf2. Huh-7 cells were transfected with Nrf2 or GFP siRNAs. At 24 h p.t., cells were infected with HCV (m.o.i. of 1). At 24 h p.i., cells were transfected with ARE-Luc reporters for another 24 h. Cellular lysates were prepared and subjected to dual-luciferase assay. (b) Mock-infected and HCV-infected cells at day 4 were transfected with ARE-Luc and ARE-mut-Luc reporter plasmids. At 30 h p.t., cells were serum-starved for 4 h and incubated with the antioxidant PDTC (100 μM for 4 h), Ca²⁺ chelator BAPTA-AM (50 μM for 2 h), p38 MAPK inhibitor SB203580 (10 μM for 12 h), JNK inhibitor SP600125 (30 μM for 12 h), PI3k inhibitor LY294002 (50 μM for 12 h) or PKC inhibitor Go6976 (10 μM for 2 h); cellular lysates were assayed for luciferase activity. Data represent means ± SD of three independent experiments performed in duplicate.

Fig. 5. HCV infection induces the expression of ARE-containing Nrf2 target genes. (a) Huh-7 cells were transfected with Nrf2 and GFP siRNAs. At 24 h p.t., cells were infected with HCV (m.o.i. of 1) for 48 h. In the case of antioxidant treatment, HCV-infected cells at day 4 were serum-starved for 4–5 h followed by treatment with PDTC (100 μM for 4 h) (filled bars). Total cellular RNA was extracted with TRizol and cDNA was prepared from mock-infected (empty bars) and HCV-infected (light-grey bars) cells at day 4 and from those silenced with Nrf2 siRNA (dark-grey bars) or GFP siRNA (hatched bars) or treated with the antioxidant PDTC (100 μM for 4 h) (filled bars). Equal amounts of cDNA were subjected to quantitative RT-PCR using primers specific for HO-1, NQO1, γGCSH and SYBR green probe. The experiment was performed in duplicate. GAPDH mRNA was used as an internal control. (b) Cellular mRNA from mock-infected and HCV-infected cells and from those transfected with GFP or Nrf2 siRNAs was extracted at 72 h and subjected to quantitative RT-PCR using primers specific for Nrf2 and SYBR green probe.
day 4 (approx. 75% infection) were transfected with Nrf2 and GFP siRNAs and subjected to propidium iodide (PI) staining followed by fluorescence-activated cell sorting (FACS) analysis. The results showed increased PI staining of HCV-infected cells silenced with Nrf2 siRNA compared with GFP siRNA (Fig. 6b). Taken together, these results demonstrate the role of HCV-induced/activated Nrf2 in cell survival, which is mediated by the induction of ROS.

**DISCUSSION**

It is well-established that activation of Nrf2 regulates the basal and inducible expression of cytoprotective, detoxifying and antioxidant genes (Motohashi & Yamamoto, 2004). However, the role of Nrf2 in the context of virus infection and cancer development has not been studied in detail. Recently, it has been shown that upregulation of Nrf2 in lung cancer cells stimulated growth of lung cancer-derived cell lines (Padmanabhan et al., 2006; Ohta et al., 2008). Previous studies have demonstrated that Nrf2 in Moloney murine leukemia virus ts1-infected astrocytes was activated in response to thiol depletion and oxidative stress (Qiang et al., 2004). Oxidative and electrophilic stresses are the major stimuli that provoke the induction of Nrf2 target genes (Ohta et al., 2008). Under homeostatic conditions, Nrf2 is tethered onto the actin cytoskeleton in the cytoplasm by Keap1 and degraded by proteasomes (Motohashi & Yamamoto, 2004). Thus, the transcription levels of Nrf2 remain low. Upon stimulation with inducers, Nrf2 dissociates from Keap1, translocates into the nucleus and binds to ARE elements (Itoh et al., 2003). However, the molecular mechanisms underlying the activation of Nrf2 are not clearly understood. Previously, two models for the activation of Nrf2 have been proposed: (i) oxidation of redox-sensitive cysteines within Keap1 or (ii) phosphorylation of Nrf2. Our previous studies have shown that HCV gene expression in the ER induces oxidative stress via Ca\(^{2+}\) signalling (Gong et al., 2001).

In the present study, we investigated the induction and molecular mechanism of Nrf2 activation and the cell-survival cascade in response to Ca\(^{2+}\) signalling, oxidative stress and activation of cellular kinases induced by HCV infection. We observed that HCV infection can induce and translocate Nrf2 in a time-dependent manner, and demonstrated further that HCV-induced Nrf2 activation is sensitive to antioxidants and Ca\(^{2+}\) chelators. This is consistent with previous observations that, in response to virus infection, activation of Nrf2 occurs under conditions of oxidative stress (Qiang et al., 2004; Jiang et al., 2006).

Recently, evidence has been accumulating that Nrf2 is a regulatory target of the MAPK, ERK-1/-2, PKC and PI3-k/Akt pathways (Yu et al., 2000; Zipper & Mulcahy, 2000; Kang et al., 2001; Huang et al., 2002). We have shown previously that HCV gene expression induces the activation of cellular kinases such as JAK, Src, MAPK and PI3-k/Akt via oxidative stress and Ca\(^{2+}\) signalling (Waris & Siddiqui, 2005; Waris et al., 2005). In this study, we observed an increase in serine phosphorylation of Nrf2 in HCV-infected cells, which is mediated by HCV-induced Ca\(^{2+}\) signalling, subsequent elevation of ROS levels and activation of JNK and p38 MAP kinases. Whilst it is...
possible that, under certain conditions, ROS could act directly on Nrf2 or Keap1, from our study it seems likely that oxidative stress-mediated serine phosphorylation of Nrf2 in HCV-infected cells triggers a conformational change that disrupts Nrf2–Keap1 complexes. These results suggest a mechanistic model of ARE-mediated cellular antioxidant response involving the MAPK-catalysed phosphorylation of Nrf2 as a specific trigger for its nuclear translocation and the induction of Nrf2 target genes (Fig. 7).

Previous studies have demonstrated that PERK-induced phosphorylation of Nrf2 and subsequent nuclear translocation are critical for cell survival (Cullinan et al., 2003; Cullinan & Diehl, 2004). In this study, we observed the activation of cell-survival proteins such as Akt and Bad through Nrf2 activation in HCV-infected cells, suggesting a role for Nrf2 in the induction of cell survival, a situation favourable for viral oncogenesis. Modulation of this pathway by the virus provides an alternative to the expression of viral oncogenes or the direct inhibition of pro-apoptotic proteins. The molecular mechanisms underlying Nrf2-mediated cell survival are not clearly understood. Based on previous studies and our results, it is likely that Nrf2-dependent induction of phase II detoxifying enzyme expression will contribute to cell survival by HCV infection.

In summary, these studies demonstrate the mechanism of Nrf2 activation, which involves a disturbance of Ca\(^{2+}\) homeostasis in the ER, elevation of ROS and activation of MAP kinases. Activation of Nrf2 induces cell survival, probably through the induction of phase II detoxifying enzymes. Our results provide important clues to the understanding of the mechanisms of chronic liver disease induced by oxidative stress and other intracellular events associated with HCV infection.

**METHODS**

**Expression plasmids.** The ARE-Luc plasmid containing four ARE elements was kindly provided by Dr M. Yamamoto (University of Tsukuba, Japan). The infectious JFH-1 cDNA (HCV genotype 2a) and the replication-defective JFH-1/GND construct were obtained from Dr Takaji Wakita (Tokyo, Japan).

**HCV cell-culture infection system.** The JFH-1 genomic RNA was transcribed and delivered into Huh-7 cells by electroporation or liposome-mediated transfection. For electroporation, cells were suspended in cytomix buffer at 10^7 cells ml\(^{-1}\). JFH-1 RNA (8–10 \(\mu\)g) was mixed with 0.2 ml cells in a 2 mm cuvette; a Bio-Rad Gene Pulser system was used to deliver a single pulse at 0.27 kV, 140 \(\Omega\) and 960 \(\mu\)F and the cells were plated in 100 mm dishes. Liposome-mediated transfection was performed with Lipofectamine 2000 (Invitrogen). Cells were then plated in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and passaged every 2–3 days; the presence of HCV RNA in the cells and corresponding cell-culture supernatants was determined by quantitative RT-PCR (ABI). The expression of HCV core protein was analysed by using Western blot assays. The HCV cell-culture supernatant was collected at appropriate time points and used to infect naive Huh-7 cells at appropriate dilutions (m.o.i. of 1), followed by 5–6 h incubation at 37°C and in 5% CO\(_2\). Virus titre in cell-culture supernatants was expressed as focus forming units (f.f.u.) ml\(^{-1}\), determined by the mean number of HCV core-positive foci detected at the highest dilutions, as described by Zhong et al. (2005).

**Immunoprecipitation and Western blot analysis.** Mock-infected and HCV-infected cells were harvested and cellular lysates were prepared by incubating in radioimmune precipitation (RIPA) buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.5% sodium
deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM sodium formate, 1 mM PMSF, 10 μg aprotinin ml⁻¹, 10 μg leupeptin ml⁻¹) for 30 min on ice. Cellular lysates were immunoprecipitated with anti-Nrf2 antibody (Santa Cruz) for 4 h at 4 °C. The immune complexes were incubated with protein A-Sepharose (Invitrogen) for 1 h at 4 °C, washed three or four times with RIPA buffer and boiled for 5 min in sample buffer (2 × sample buffer: 100 mM Tris-Cl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol, 200 mM 2-mercaptoethanol). The samples were subjected to SDS-PAGE. Gels were electroblotted onto a nitrocellulose membrane (Thermo Scientific) in 25 mM Tris, 192 mM glycine and 20% methanol. Membranes were incubated for 1 h in blocking buffer (20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.3% polyvinyl pyrrolidone, 0.5% Tween 20), probed with phosphoserine monoclonal antibody (Alexis) for 1 h at room temperature (RT) and washed twice for 10 min with blocking buffer, followed by incubation with secondary antibody for 45 min at RT. After an additional washing step with blocking buffer, immunoblots were visualized by using the ECL detection system (Thermo Scientific).

**Immunofluorescence assay.** Huh-7 cells in eight-well chamber slides (Nalge Nunc) were serum-starved overnight and incubated with HCV virions (m.o.i. of 1) at 37 °C. The cells were washed with PBS, fixed with 4% paraformaldehyde for 10 min at RT, permeabilized for 5 min with 0.2% Triton X-100 and blocked for 45 min with 5% bovine serum albumin in PBS. The cells were next incubated with primary antibodies (Nrf2, Keap1 and HCV core) for 1 h at RT, followed by incubation with secondary antibodies (anti-goat–Alexa Fluor 546, anti-rabbit–Alexa Fluor 488 and anti-mouse–Alexa Fluor 633) (Molecular Probes) for 1 h. After washing with PBS, cells were mounted with an anti-fade reagent containing DAPI (4,6-diamidino-2-phenylindole; Invitrogen) and observed under a fluorescence microscope equipped with the Nikon Metamorph digital imaging system.

**RNA interference.** Huh-7 cells were transfected with Nrf2, p38 MAPK, JNK and GFP siRNAs according to the manufacturer’s protocol (Santa Cruz). Each siRNA consisted of pools of three to five target-specific, 19–25 nt siRNAs designed to knock down the target-gene expression. For each transfection, two solutions were prepared. For solution A, 60 pmol siRNA duplex was mixed with 100 μl transfection medium. For solution B, 6 μl transfection reagent was added to 100 μl siRNA transfection medium. Solutions A and B were allowed to incubate separately at RT for 20 min. After 20 min, solutions A and B were combined and then allowed to incubate for another 20 min at RT. The combined solutions were then added to the cells in six-well plates and then incubated for 5 h at 37 °C and in 5% CO₂, and the transfection solution was replaced with complete DMEM growth medium.

**Luciferase assay.** Mock-infected and HCV-infected cells were transfected with 0.1 μg ARE-Luc and ARE-mut-Luc reporter plasmids using Lipofectamine 2000 reagent (Invitrogen). At 30 h p.t., cells were serum-starved overnight followed by treatment with PDTC (100 μM for 6 h), BAPTA-AM (50 μM for 2 h), Sp600125 (30 μM for 12 h), SB203580 (10 μM for 15 h), LY294002 (50 μM for 12 h) or Go6976 (10 μM for 2 h) (all obtained from Calbiochem). Cells were harvested and cellular lysates were analysed for luciferase activity by using a Dual-Luciferase reporter assay kit (Promega). All transfections included a Renilla expression vector to serve as an internal control.

**Cell-survival assay.** Subconfluent Huh-7 cells were transfected with Nrf2 and GFP siRNAs. At 30 h p.t., cells were incubated with HCV cell-culture supernatant (m.o.i. of 1) at 37 °C for 4–5 h. At 48 h post-incubation, cells were incubated further with PI (2 μg ml⁻¹) for 15 min at RT. Analysis of cell death was carried out by using a BD LSR-II flow cytometer (Rosalind Franklin University of Medicine and Science core facility).

**Quantitative real-time RT-PCR.** Total RNA was extracted from mock-infected and HCV-infected cells by using TRIzol (Invitrogen). HCV RNA was quantified by real-time RT-PCR using an ABI PRISM 7500 sequence detector. Amplifications were conducted in duplicate using the following primers and 6-carboxyfluorescein (6FAM)- and tetrachloro-6-carboxyfluorescein (TAMRA)-labelled probes (ABI): HCV Taqman probe, 5′-6FAM-CTGGCGAAAGCGGTAAGTTAC-GCCA-3′, HCV sense primer, CGGGAGAGCCATAGTGCG; HCV antisense primer, AGTACCAAGGCCCCTTCTG. The sequences for the primers and probes were designed by using Primer Express software (ABI). The human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an internal control. Amplification reactions were performed in a 25-μl mix using an RT-PCR core reagents kit (ABI) and the template RNA. Reactions were performed in a 96-well spectrophotofluorometric thermal cycler under the following conditions: 2 min at 50 °C, 30 min at 56 °C, 10 min at 95 °C, 44 cycles of 1 s at 95 °C and 1 min at 60 °C. Fluorescence was monitored during every PCR cycle at the annealing step. At the termination of each PCR run, the data were analysed by the automated system and amplification plots were generated.

Nrf2 and Nrf2 target genes in mock-infected and HCV-infected cells were quantified by real-time RT-PCR using the following primers: Nrf2 sense primer, TACTCCCAAGGTGGCACA; Nrf2 antisense primer, CATCTCAAAAAACCAGAATCTGC; HO-1 sense primer, CACGCAATACCGGCCTACC; HO-1 antisense primer, AAGCGG-GTTCGAGCCCTGCT; NQO1 sense primer, CATCTGGAAAGCTGGTTGA; NQO1 antisense primer, CTAGCTTTGATCTGGTTGCAG; γ-GCSH sense primer, TTTGGAGGAAGCGATGTAC; γ-GCSH antisense primer, GCACATCAAGGTTGTTTGT; GAPDH sense primer, AGACAGGCGATCTCGTGTG; GAPDH antisense primer, CCACAGCTCTGTAGGCGA. Total cellular RNA was extracted by using TRIzol (Invitrogen) and the cDNA was reverse-transcribed from 1 μg total RNA using oligo(dT) primers. Quantitative RT-PCR was carried out by using SYBR green master mix (ABI) and specific primer sets. Amplification reactions were performed under the following conditions: 2 min at 50 °C, 10 min at 95 °C, 40 cycles for 1 s at 95 °C and 34 s at 60 °C. Relative transcript levels were calculated by using the ΔΔCT method as specified by the manufacturer.

**ACKNOWLEDGEMENTS**

This work was supported in part by the ACS Illinois Division, Inc., 09-14 and by the Rosalind Franklin University of Medicine and Science–H. M. Bligh Cancer Research Fund to G. W. We thank T. Wakita (National Institute of Infectious Diseases, Tokyo, Japan) for the generous gift of HCV genotype 2a (IFH-1) infectious cDNA, and Dr Bala Chandran (Rosalind Franklin University of Medicine and Science) for valuable suggestions in preparing the manuscript.

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