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Unfolded protein response pathways regulate Hepatitis C virus replication via modulation of autophagy

Yoshiyasu Shinohara [a,b,1,2], Kento Imajo [a,1], Masato Yoneda [a,1], Wataru Tomeno [a,1], Yuji Ogawa [a,1], Hiroyuki Kirikoshi [a,1], Kengo Funakoshi [b,2], Masanori Ikeda [c,b,2,3], Nobuyuki Kato [c,b,2,3], Atsushi Nakajima [a,1], Satoru Saito [a,*]

[a] Gastroenterology Division, Yokohama City University School of Medicine, 3-9, Fukuura, Kanazawa-ku, Yokohama City 236-0004, Japan
[b] Department of Neuroanatomy, Yokohama City University Graduate School of Medicine, 3-9, Fukuura, Kanazawa-ku, Yokohama City 236-0004, Japan
[c] Department of Tumor Virology, Okayama University Graduate School of Medicine, 2-5-1, Shikata-cho, Okayama 700-8558, Japan

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ABSTRACT

Background: Hepatitis C virus (HCV) induces endoplasmic reticulum (ER) stress which, in turn, activates the unfolding protein response (UPR). UPR activates three distinct signalling pathways. Additionally, UPR induces autophagy (UPR-autophagy pathways). On the other hand, it has become clear that some positive-single-strand RNA viruses utilize autophagy. Some groups have used the siRNA silencing approach to show that autophagy is required for HCV RNA replication. However, the mechanism of induction of the UPR-autophagy pathways remain unclear in the cells with HCV.

Background: Method and results: we used a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) in hepatoma cells (HuH-7-derived OR6 cells). As control, we used OR6c cells from which the HCV genome had been removed by treatment with interferon-α. The UPR-autophagy pathways were activated to a greater degree in the OR6 cells as compared to the OR6c cells. Rapamycin, mTOR-independent autophagy inducer, activated HCV replication in the OR6 cells. On the other hand, HCV replication in the cells was inhibited by 3-methyladenine (3-MA), which is an inhibitor of autophagy. Salubrinal (*Eukaryotic Initiation Factor 2*(eIF2)-alpha phosphatase inhibitor), 3-ethoxy-5, 6-dibromosalicylaldehyde (X-box binding protein-1 (XBP-1) splicing inhibitor) and sp600125 (c-Jun N-terminal kinases (JNK) inhibitor) inhibited HCV replication and autophagy. Additionally, HCV replication and autophagy were inhibited more strongly by combination of these inhibitors.

Conclusion: Our results suggest that UPR-autophagy pathways exert an influence on HCV replication. Therefore, control these pathways may serve as a novel therapeutic strategy against replication of HCV.

1. Introduction

Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [1]. With over 170 million people chronically infected with HCV worldwide, this disease has emerged as a serious global health problem. HCV is an enveloped flavivirus with a 9.6-kb positive single-strand RNA genome [2]. Development of a HCV RNA replicon capable of replication in the human hepatoma cell line Huh7 has been a significant advance [3,4].

HCV replication occurs in a ribonucleoprotein replication complex associated with an endoplasmic reticulum (ER)-derived membranous web [5]. Some groups have shown that HCV protein disrupts normal ER functions and induces ER stress [6,7]. Mammalian cells trigger a response called the unfolded protein response (UPR) to cope with abnormal ER functions and ER stress [8–10]. UPR activates three distinct signalling pathways, namely, the activating transcription factor 6 (ATF-6) pathway, the inositol-requiring enzyme 1 (IRE1) pathway and the double-stranded RNA-activated protein kinase-like ER kinase (PERK) pathway [11–13]. Some groups have reported the existence of some relationships among the three kinds of UPR-autophagy pathways [14,15]. Autophagy is a major intracellular pathway involved in the degradation and recycling of long-lived proteins and cytoplasmic organelles, and plays an essential role in the maintenance of homeostasis in response to starvation and other cellular stresses [16]. On the other hand, autophagy also plays important roles in a variety of other cellular processes, including restriction of intracellular pathogen multiplication. Recently, the significance of autophagy in the establishment of virus infection has become clear; in particular, some positive single-strand RNA viruses

* Corresponding author. Fax: +81 45 784 3546.
E-mail address: ssai1423@yokohama-cu.ac (S. Saito).
[1] Fax: +81 45 784 3546.
[2] Fax: +81 45 782 7251.
[3] Fax: +81 86 235 7392.

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utilize autophagy to generate the cytoplasmic membrane structures that they require for genome replication [17]. HCV has also been shown to induce ER stress and trigger UPR [18–21]. Furthermore, UPR activation has been proposed to be responsible for subsequent induction of autophagy [22]. However, the induction of UPR-autophagy pathways by HCV has not been defined elucidated yet.

In this study, we attempted to examine the influence on HCV replication of the UPR-autophagy pathways, using a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with luciferase as the reporter, which facilitated prompt and precise monitoring of HCV RNA replication in hepatoma cells (HuH-7-derived OR6 cells).

2. Materials and methods

2.1. Reagents and antibodies

3-Ethoxy-5, 6-dibromosalicylaldehyde (3-E-5, 6-D), tunicamycin (TM), rapamycin, 3-methyladenine (3-MA), and interferon (IFN)-alpha were purchased from Sigma (St. Louis, MO, USA). sp600125 and Salubrinal were purchased from Calbiochem (Los Angeles, CA, USA). Anti-Core (CP11) was purchased from Institute of Immunology (Institute of Immunology, Tokyo, Japan), anti-non-structure protein (NS) 4A from abcam (abcam, Cambridge, UK); anti-microtubule-associated protein 1 light chain 3 (LC3) and anti-p62 were purchased from Medical & Biological Laboratories (MBL, Tokyo, Japan), and anti-eIF2-alpha, anti-phospholylated-*Eukaryotic Initiation Factor 2* (eIF2)-alpha, anti-IRE1, anti-c-Jun N-terminal kinases (JNK), anti-phospholylated-JNK, anti-c-Jun, anti-phospholylated-c-Jun and anti-GAPDH from Cell Signaling (Cell Signaling, Inc.).

2.2. Cell cultures

OR6 cells have been described in detail by Ikeda et al. [23]. The OR6 cell line is a clone of ORN/C-5B/KE (Strain O of genotype 1b) RNA replicating Huh-7 cells. OR6 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, penicillin, streptomycin and G418 (300 μg/ml; Calbiochem, Darmstadt, Germany) and passaged twice a week at a 5:1 split ratio. Control cells (OR6c) were established by eliminating genome-length HCV RNA from the OR6 cells by IFN-alpha treatment (500 IU/ml for 2 weeks; Sigma–Aldrich, St. Louis, MO, USA) without G418, as described previously.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated using the RNA Prep mini kit (QIAGEN). Template cDNA synthesis was carried out with 1 μg of total RNA using the High Capacity RNA-to-cDNA kit (Applyed Biosystems, Inc.). One-tenth of the synthesized cDNA was subjected to PCR with the following primer pairs: XBP-1, 5′-TTACGAGAGAAAACT-CATGGC-3′ and 5′-GGGTCCAAGTTGTCCAGAATGC-3′; HCV, 5′-AGA-GCCATAGTGGTCTGCGG-3′ and 5′-CAAGCACCCTATCAGGCAGTA-3′; and GAPDH, 5′-GACTCATGACCACAGTCCATGC-3′ and 5′-GAG-GAGACCACCTGGTGCTCAG-3′.

2.4. Western blot analysis

For Western blot analysis, 4−4.5 × 10^4 cells harboring OR6 cells were plated on six-well plates and cultured for 24 h, and treated with each of the reagents for 72 h. Samples were subjected to native gel and 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis [24]. The proteins were transferred to a polyvinylidene difluoride membrane (Millipore) and reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce) and detected using an LAS-3000 image analyzer system (Fujifilm).

2.5. Fluorescent microscopy

Cells were cultured on glass slides and then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 30 min. After two washes with PBS, the cells were permeabilized at room temperature for 20 min with PBS containing 0.25% saponin, and then blocked with PBS containing 0.2% gelatin (gelatin-PBS) for 60 min at room temperature. The cells were incubated with gelatin-PBS containing appropriate antibodies at 37 °C for 60 min and washed three times with PBS containing 1% Tween 20 (PBST). The resulting cells were incubated with gelatin-PBS containing the corresponding fluorescent-conjugated secondary antibodies at 37 °C for 60 min and then washed three times with PBST. The stained cells were covered with VECTASHIELD® Mounting Medium containing DAPI (4′, 6-diamidino-2-phenylindole) (Vector Laboratories Inc.) and observed under a FluoView FV1000 laser scanning confocal microscope (Olympus).

2.6. Renilla luciferase (RL) assay

To monitor the effects of the reagents, the RL assay was performed as described previously [25]. Briefly, the cells were plated onto 24-well plates (2 × 10^4 cells per well) in triplicate and cultured with the medium in the absence of G418 for 24 h. The cells were then treated with each reagent at various concentrations for 72 h. After treatment, the cells were subjected to a luciferase assay using the RL assay system (Promega, Madison, WI). After these assays, the 50% effective concentration (EC50) of each reagent for HCV replication was determined.

2.7. WST-1 cell proliferation assay

The cells were plated onto 96-well plates (1 × 10^3 cells per well) in triplicate and then treated with various concentrations of the reagents for 72 h. After the treatment, the cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. Based on these assay results, the 50% cytotoxic concentration (CC50) of the reagents was estimated. The selective index (SI) values of the reagents were also estimated by dividing the CC50 values by the EC50 values.

2.8. Statistical analysis

The luciferase activities were statistically compared between the various treatment groups using Student's *t*-test. *P* values of <0.05 were considered to signify statistically significant differences. The means ± standard deviations were determined from at least three independent experiments.

3. Results

3.1. HCV induced activation of UPR and autophagy

To determine whether autophagy was induced in the OR6 cells, we investigated the phosphoethanolamine (PE) conjugation of LC3 in the OR6 cells showing autonomous replication of HCV RNA. The amount of LC3-II was significantly increased in the OR6 cells as compared to the OR6c cells (Fig. 1A). Autophagy is involved in the response to ER stress, which is represented by UPR; in this case, double-membrane vesicles are formed but do not fuse with the

lysosomes. Accumulation of p62 has been reported to be caused by lysosomal fusion inhibition [14,15]. Also, in our experiment, significant accumulation of p62 was observed in the OR6 cells (Fig. 1A). Fig. 1B shows an increase in the amount of phosphorylated eIF2-alpha (Ser51) [9] in the OR6 cells as compared to the OR6c cells, suggesting that the PERK pathway was activated by HCV. Fig. 1C indicates the increase in the amount of spliced XBP1 in the OR6 cells as compared to the OR6c cells, suggesting that the ATF-6 and IRE1 pathways were activated too [10,26]. Fig. 1D shows an increase in the amount of IRE1 in the OR6 cells as compared to the OR6c cells. Moreover, increases in the amounts of phosphorylated JNK (Thr183/Tyr185) and phosphorylated c-Jun (Ser63) were also observed in the OR6 cells as compared to the OR6c cells (Fig. 1D), suggesting that the IRE1 pathway was activated as well [8].

On the other hand, treatment of Huh7 cells with tunicamycin, which triggers UPR by inhibiting glycosylation, resulted in activation of the ATF-6, PERK and IRE1 pathways. These results confirm that the delay in the appearance of UPR was not due to an inherent defect in the Huh7 cells (Fig. S1B, S1C and S1D). Tunicamycin treatment also induced LC3-II and p62 accumulation (Fig. S1A). These data confirm that the HCV replicon cells showed activation of both autophagy and UPR (Fig. 1).

3.2. Autophagy regulates HCV replication in the HCV replicon cells

We investigated whether activation of autophagy had any influence on the HCV replication. Rapamycin, a mTOR-independent autophagy inducer [27], enhanced HCV replication and formation of autophagosomes in the OR6 cells, whereas 3-MA, an inhibitor of autophagy [27], suppressed HCV replication and formation of autophagosomes in the OR6 cells (Fig. 2A and B). These data suggest that autophagy seems to somehow facilitate HCV replication.

3.3. UPR inhibitors can suppress HCV replication and autophagy

Salubrinal is a selective inhibitor of the phosphatase complexes that dephosphorylate eIF2-alpha in PERK pathway [28]. 3-E-5, 6-D selectively inhibits XBP-1 splicing [29]. sp600125 down-regulates

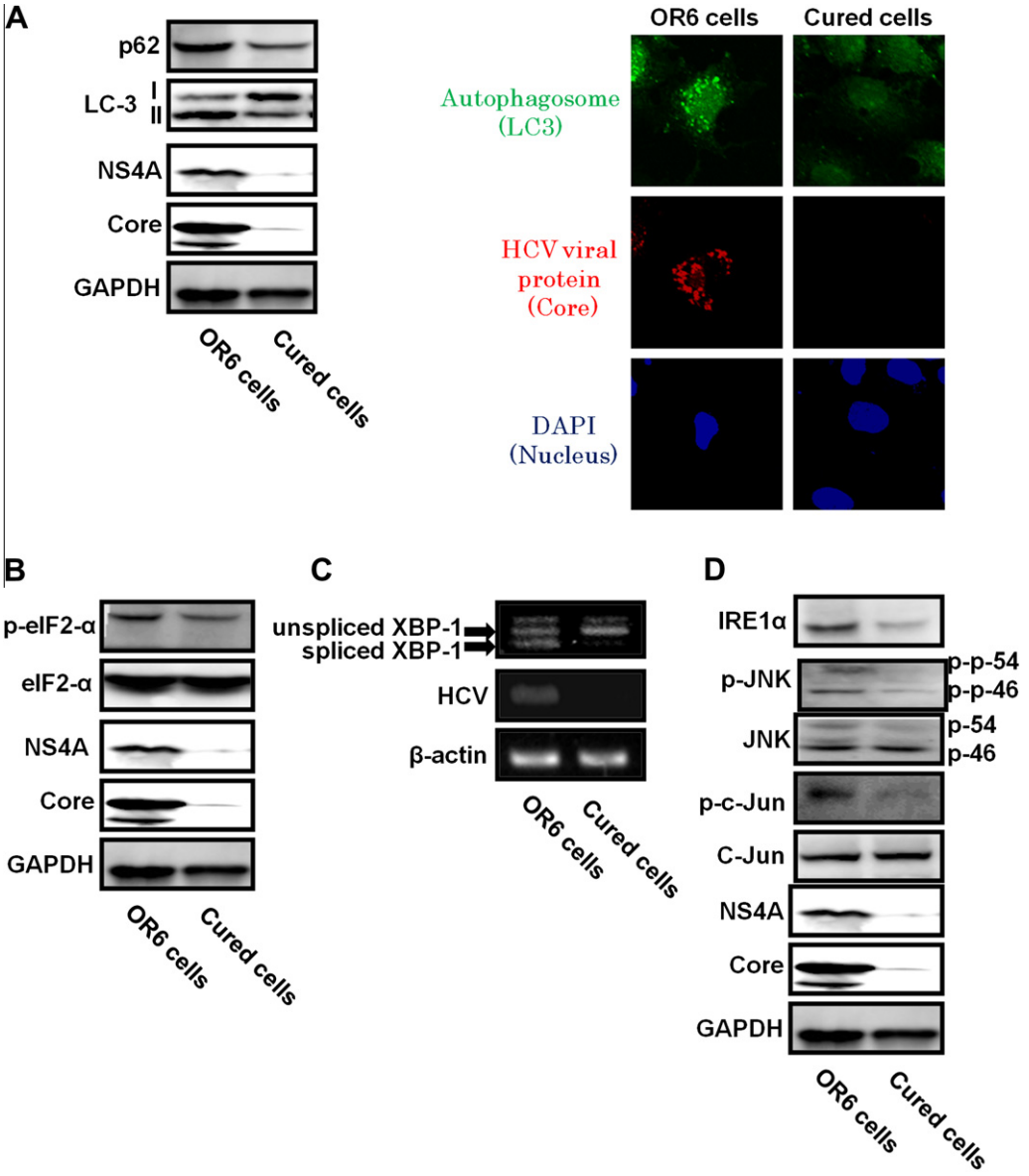

Fig. 1. Induction of UPR-autophagy pathways in HCV replicon cells. (A) Left panel: Western blot analysis for autophagy in OR6 cells and OR6c cells. Right panel: fluorescence microscopy. Green: LC3 staining of the autophagosomes; blue: DAPI staining of the nuclei; red: staining of the HCV core protein. (B) Western blot analysis for PERK pathway in OR6 cells and OR6c cells. (C) RT-PCR for XBP-1 in OR6 cells and OR6c cells. (D) Western blot analysis for IRE1 pathway in OR6 cells and OR6c cells.

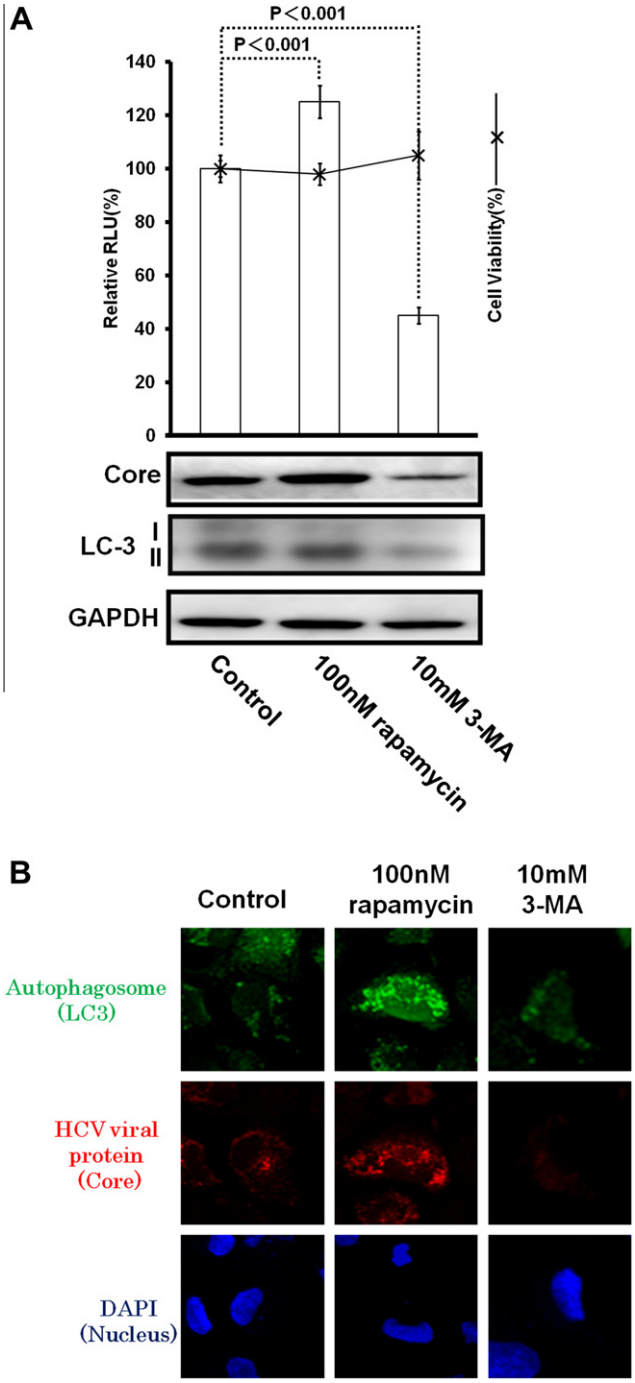

Fig. 2. Regulation of HCV replication by autophagy. The efficacy of HCV replication in replicon cells using OR6 assay systems. (A) OR6 cells were treated with each reagent for 72 h, following which the RL assay (bar graph) and WST-1 assay (line graph) were performed. The relative values (%) calculated at each point, with the level in the non-treated cells set at 100%, are presented here. Western blot analysis for the HCV core protein and LC3 was also performed (lower panels). (B) fluorescence microscopy. Green: LC3 staining of the autophagosomes; blue: DAPI staining of the nuclei; red: staining of the HCV core protein.

the expression of IRE1 and dephosphorylates JNK [30]. To further investigate whether inhibition of the UPR-autophagy pathway might affect the rate of HCV replication, we determined the EC50, CC50 and SI values of Salubrinal (eIF2-alpha inhibitor), 3-E-5, 6-D (X-box binding protein-1 (XBP-1) inhibitor) and sp600125 (JNK inhibitor) using the OR6 assay system. The results for the EC50, CC50 and SI values were as follows: (Salubrinal: EC50; 6.25 μM, CC50 > 100 μM, SI > 16 (Fig. 3A), 3-E-5, 6-D: EC50; 2 μM, CC50; 20 μM, SI; 10 (Fig. 3B) and sp600125: EC50; 1 μM, CC50; 12.5 μM, SI; 12.5 (Fig. 3C). Meanwhile, the percentage of viable cells was determined by the WST-1 assay. Cytotoxic effects were observed at >25 μM of the Salubrinal (Fig. 3A), >6.25 μM of the 3-E-5, 6-D (Fig. 3B), and >3.13 μM of the sp600125 (Fig. 3C). On the other hand, no cytotoxic effects were observed in the presence of <12.5 μM of the Salubrinal (Fig. 3A), <3.13 μM of the 3-E-5, 6-D (Fig. 3B), and <1.56 μM of the sp600125 (Fig. 3C). In addition, autophagosome formation in the OR6 cells was decreased in the presence of EC50 concentrations of the Salubrinal, 3-E-5, 6-D and sp600125 (Fig. 3D).

3.4. Combined treatment with the UPR inhibitors resulted in efficient suppression of HCV replication and autophagy

We found these inhibitors resulted in efficient suppression of HCV replication and autophagy. We then attempted to clarify which of the three UPR-autophagy pathways might be most closely involved in the activation of autophagy by investigating the effects of combined use of the Salubrinal, 3-E-5, 6-D and sp600125 on the rate of HCV replication using the OR6 assay system. Salubrinal inhibited phasphorylated eIF2-alpha, LC3-II and HCV core protein, but not inhibited spliced XBP1 and phasphorylated JNK. 3-E-5, 6-D and sp600125 inhibited phasphorylated JNK, spliced XBP1, LC3-II and HCV core protein, but not inhibited phasphorylated eIF2-alpha. Combined treatment with three inhibitors of Salubrinal, 3-E-5, 6-D and sp600125 strongly enhanced the inhibition of both HCV replication and autophagy. These results indicated that combined use of the inhibitors was more effective than use of any of the inhibitors alone in suppressing HCV replication and autophagy (Fig. 4). In addition, the concentrations of each of the inhibitors used in combination did not affect the cell viability rate (Fig. 4). Thus, in regard to the efficacy of inhibition for HCV replication and autophagy, combined use of 3-E-5, 6-D and sp600125 appears to be the most efficient as compared to use of other combinations.

4. Discussion

4.1. In this study, we showed, using a replicon system, activation by HCV of the three main signaling pathways from UPR to autophagy (Fig. 1)

In regard to the UPR-ATF6-XBP1 pathway, Tardif KD et al. reported, from a study using subgenomic replicons, that HCV suppresses the IRE1-XBP1 pathway to stimulate the synthesis of its viral proteins [31]. The differences from our results could be attributable to the fact that we used full-genome replicon or different HCV strain for our experiments. In addition, some other groups have shown that HCV induces ER stress and UPR activation [18–21].

On the other hand, several groups have reported that autophagy and/or autophagy genes are likely to play both antiviral and proviral roles in the life cycles and pathogenesis of many different virus families. Especially, replication and spread of some positive-strand RNA viruses are regulated by their interactions with the host autophagy machinery [17]. Components of the autophagic machinery seem to be subverted to promote replication of RNA viruses, such as in the case of infections with the coronavirus [mouse hepatitis virus (MHV)], poliovirus, or rhinoviruses 2 and 14, by generation of a membrane scaffold for RNA replication [32,33]. We then attempted to determine if inhibitors of UPR signaling might control HCV replication and autophagy by inhibiting the signaling pathways from URP to autophagy (Figs. 2 and 3). Our results showed that Salubrinal (eIF2-alpha phosphatase inhibitor), 3-E-5, 6-D

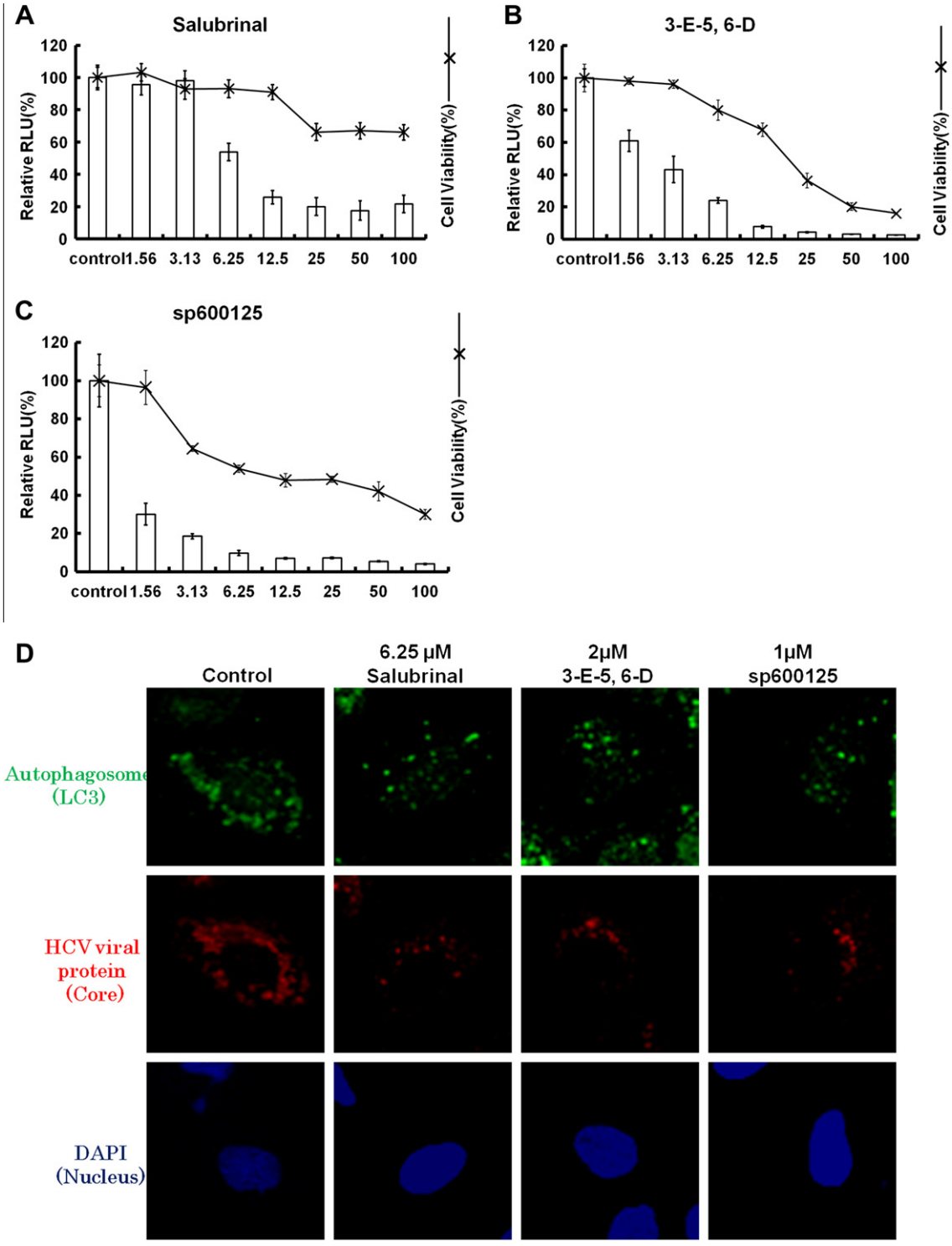

Fig. 3. Regulation of HCV replication by inhibitors of UPR. The effects of Salubrinal (eIF2-alpha phosphatase inhibitor), 3-E-5, 6-D (XBP-1 splicing inhibitor) and sp600125 (JNK inhibitor) on HCV replication in the OR6 assay system were examined. OR6 cells were treated with Salubrinal (A), 3-E-5, 6-D (B) or sp600125 (C) inhibitor for 72 h, following which the RL assay (bar graph) and WST-1 assay (line graph) were performed. The relative values (%) calculated at each point, with the level in the non-treated cells set at 100%, are presented here. (D) fluorescence microscopy. Green: LC3 staining of the autophagosomes; blue: DAPI staining of the nuclei; red: staining of the HCV core protein.

(XBP-1 inhibitor) and sp600125 (JNK inhibitor) could regulate HCV replication and autophagy in the OR6 cells (Fig. 3). Next, we attempted to determine combinated inhibitiors which UPR-autophagy pathways might have the greatest influence in the regulation of HCV replication. When the concentrations of the inhibitors used were under the EC50, the cell viability of the HCV replicons was almost 100%. In particular, combined treatment with the 3-E-5, 6-D (XBP-1 splicing inhibitor) and sp600125 (JNK inhibitor) was the most efficient at reducing HCV replication (Fig. 4). Some interesting findings have been reported previously. eIF2-alpha phosphatase inhibits the assembly of the 80S ribosome and inhibits protein synthesis [34]. On the other hand, IRE1 and ATF6 promote transcription of the UPR target genes. IRE1 and ATF6 process XBP1 mRNA to generate mature XBP1 mRNA. Spliced XBP1 binds directly

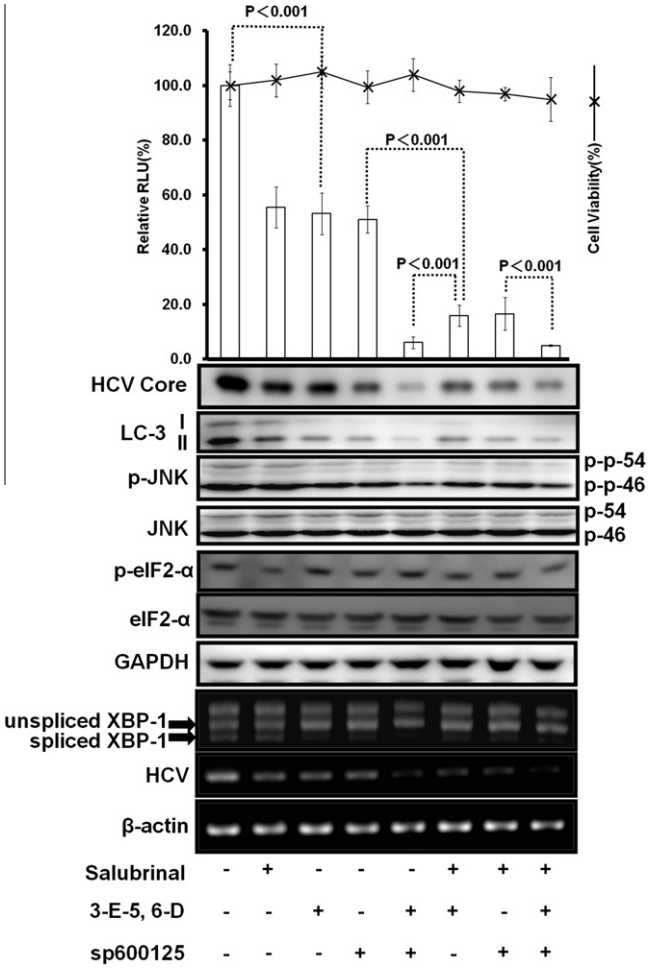

Fig. 4. Combined treatment with inhibitors of UPR signaling enhanced the inhibition of HCV replication. The effects of combined treatment with Salubrinal (eIF2-alpha phosphatase inhibitor), 3-E-5, 6-D (XBP-1 splicing inhibitor) and sp600125 (JNK inhibitor) on HCV replication in the OR6 assay system were examined. OR6 cells were treated with the reagents for 72 h, following which the RL assay (bar graph) and WST-1 assay (line graph) were performed. The relative values (%) calculated at each point, with the level in the non-treated cells set at 100%, are presented here. Western blot analysis of the treated cells for the HCV core protein and LC3 was also performed (lower panels).

to the ER stress response element and unfolded protein response elements and activates transcription of the molecular chaperones of the ER. JNK activation, mediated by IRE1, is required for autophagosome formation [35]. The PERK pathway is independent of UPR, however, the IRE1 and ATF6 pathways play complementary roles in UPR signaling [36–38]. These findings indicate that inhibition of UPR-autophagy pathways may have significant influence in the suppression of HCV replication, and 3-E-5, 6-D and sp600125 might inhibit strongly HCV replication and autophagy.

In conclusion, HCV stimulates the three signaling pathways from UPR to autophagy. Salubrinal (eIF2-alpha phosphatase inhibitor), 3-E-5, 6-D (XBP-1 inhibitor) and sp600125 (JNK inhibitor) acted as a potent anti-autophagy agent and limit both inhibition of autophagy and HCV replication. We lead to the notion that HCV may hijack the UPR-autophagy pathway for HCV replication (Fig. 5). Our study demonstrates control of the UPR-autophagy pathway machinery as a possible antiviral drug target.

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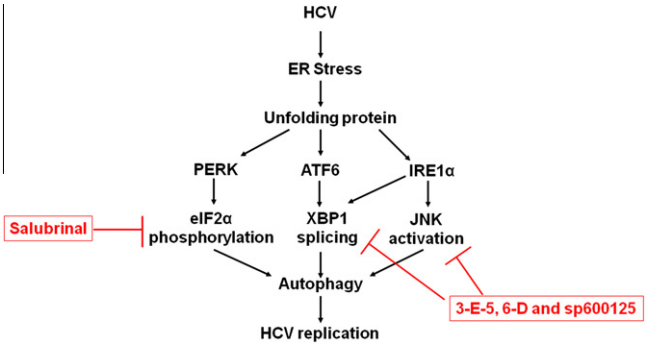

Fig. 5. Diagram showing the schema of UPR-autophagy pathways in the HCV replicon cells.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.01.103.

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