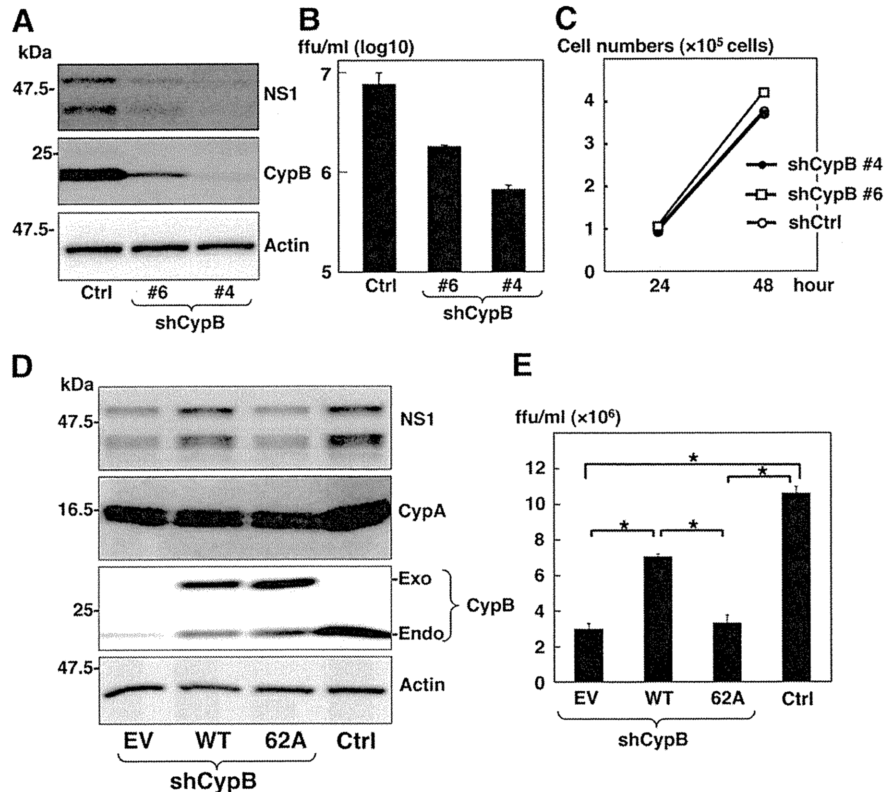


Fig. 4. PPIase activity of CypB is crucial for the propagation of JEV. Huh7 cell lines expressing shRNA targeted to CypB or the control were infected with JEV at an MOI of 0.1 for 1 h and cultured in 10% FBS DMEM for 48 h. The expressions of NS1, CypB, and actin were detected by immunoblotting (A). The propagation of JEV was determined by focus-forming assay (B). Growth kinetics of the stable CypB-knockdown cell lines were determined by the method of trypan blue dye exclusion (C). The stably knocked-down cell lines were transfected with the siRNA-resistant FLAG-tagged wild- or Ala^{62}-CypB, or empty vector and cultured for 1 week in the presence of 1 μg/ml puromycin. The remaining cells were infected with JEV at an MOI of 1. The expressions of NS1, CypA, endogenous and exogenous CypBs, and actin were detected by immunoblotting (D). Virus production in the culture supernatant at 36 h post-infection was determined by a focus-forming assay (E). The results are representative of three independent assays, with the error bars indicating the standard deviations. Asterisks indicate significant differences (*$P<0.01$).

through the interaction with the NS5 polymerase (Qing et al., 2009), CypB was colocalized and specifically co-immunoprecipitated with JEV NS4A. CypA is abundantly expressed in the cytoplasm of mammalian cells (Galigniana et al., 2004) and NS5 is predominantly detected on the cytoplasmic side of the ER (Zhang et al., 1992). Thus, it is conceivable that an interaction between CypA and NS5 occurs on the cytoplasmic side of the ER. On the other hand, CypB is localized in the ER lumen and targeted to the secretory pathway via its ER signal sequence (Price et al., 1994, 1991). NS4A is predicted to be a three-transmembrane protein with its C-terminal end localized in the ER lumen (Miller et al., 2007). Therefore, it is plausible that CypB interacts with NS4A within the ER lumen and confers proper folding to form the RNA replication complex of JEV. Expression of DENV NS4A alone has been shown to induce rearrangement of the cytoplasmic membrane to form the convoluted membrane required for viral replication (Roosendaal et al., 2006). It might be feasible to speculate that JEV NS4A undergoes conformational change through the interaction with CypB and induces formation of the convoluted membrane in the ER essential for genome replication of JEV. It was reported that HCV NS5A from CsA resistant mutant exhibits an enhanced interaction with CypB and NS5B facilitates a stronger binding of the mutant NS5A to endogenous CypB than wild-type in cell culture (Fernandes et al., 2010). Study of the molecular mechanism underlying the CsA resistant of JEV may shed light on the complex interaction among Cyps and viral proteins.

In conclusion, we have demonstrated that CsA suppresses the propagation of JEV by inhibiting the interaction between CypB and NS4A, which is required for viral RNA replication. Further studies are needed to elucidate the precise molecular mechanism underlying the involvement of cellular Cyps in the efficient propagation of JEV. Three inhibitors of the PPIase activity of Cyps, DEBIO-025, SCY635, and NIM811, are currently under clinical trial for the treatment of hepatitis C patients (Puyang et al., 2010). The PPIase inhibitor may be an attractive therapeutic target for the treatment of patients infected with not only HCV but also other flaviviruses.

Materials and methods

Plasmids

The human CypB gene was amplified from the total cDNA of Huh7 by PCR using *LA taq* (Takara Bio Inc., Shiga, Japan) and cloned into pcDNA3.1 and pCAGPM (Mori et al., 2007). The plasmids encoding the NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 of the JEV AT31 strain were generated by PCR and cloned into pCAGPM. The pSilencer-CypB, carrying an shRNA targeted to CypB under the control of the U6 promoter, was constructed by cloning of the oligonucleotide pair 5′-GATCCGGTGGAGAGCACCAAGACATTCAAGAGATGTCTTGGTGCTCTC-CACCTTTTTTGGAAA-3′-5′-AGCTTTTCCAAAAAAGGTGGAGAGCACCAA-GACATCTCTTGAATGTCTTGGTGCTCTCCACCG-3′ between the *Bam*HI and *Hin*dIII sites of pSilencer 2.1-U6 hygro (Ambion, Austin, TX). A plasmid coding a mutant CypB resistant to shRNA was prepared by insertion of four silent mutations (the nucleotides at positions 543, 549, 555, and 561 were changed from G to A, G to A, C to G, and A to C, respectively) into CypB cDNA by the method of splicing by overlap extension (Ho et al., 1989). The pSilencer negative-control plasmid (Ambion) has no homology to any human gene. The pJErep plasmid was kindly provided by Dr. Konishi (Kobe University, Kobe, Japan). A puromycin-resistant gene under the internal ribosomal entry site (IRES) of encephalomyocarditis virus was inserted into pJErep and designated as pJErepIRESpuro.

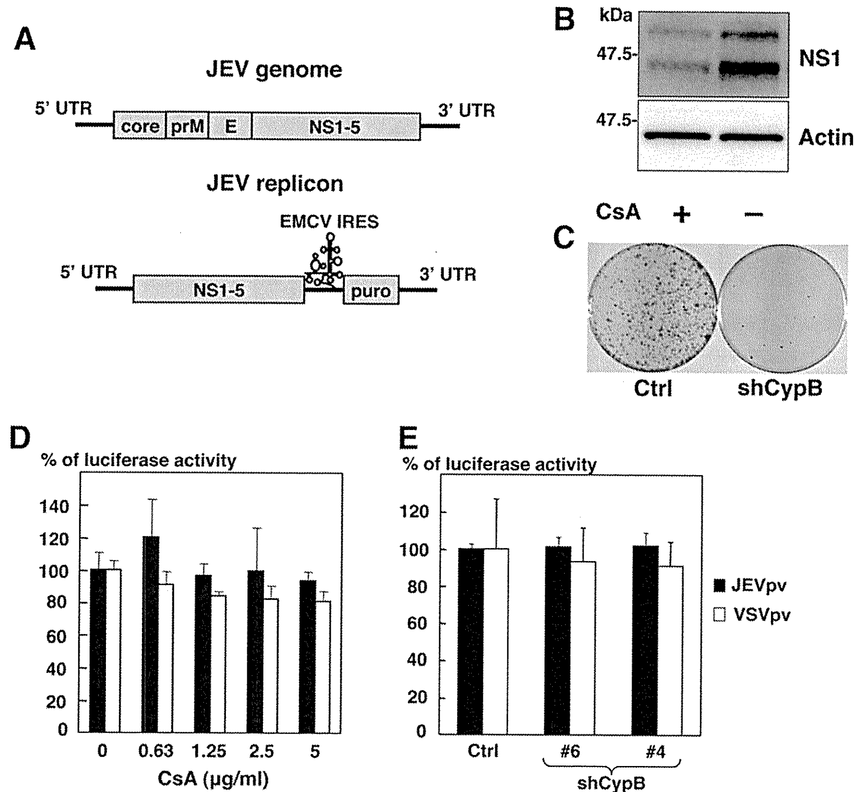


Fig. 5. CypB participates in the replication but not in the entry of JEV. (A) Schematic representations of the JEV genome and its subgenomic replicon. (B) JEV replicon cells were treated with CsA (1 μg/ml) for 6 days, and the expressions of NS1 and actin were detected by immunoblotting. (C) The stable CypB-knockdown and control cell lines were electroporated with the JEV replicon RNA and cultured for 3 weeks in the presence of 1 μg/ml of puromycin. The remaining cells were fixed with 4% paraformaldehyde and stained with crystal violet. (D) Huh7 cells treated with the indicated concentrations of CsA for 1 h were infected with the pseudotype viruses, JEVpv and VSVpv, and luciferase activities were determined at 24 h post-infection. (E) The stable CypB-knockdown and control cell lines were incubated with the pseudotype viruses, and the luciferase activities were determined. The results shown are representative of three independent assays, with error bars indicating standard deviations.

Cells and viruses

All cell lines were cultured at 37 °C under the condition of a humidified atmosphere and 5% CO_2. The human embryonic kidney cell line, 293T, African green monkey kidney cell line, Vero, hepatocellular carcinoma cell line, Huh7, mouse neural cell line, N18, and baby hamster kidney cell line, BHK, were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, non-essential amino acid (Sigma), and 10% fetal bovine serum (FBS). The mosquito C6/36 cell line (*Aedes albopictus*) was cultured at 27 °C and maintained in modified Eagle's medium (MEM) (Sigma). Huh7 cells were transfected with pSilencer-CypB or control plasmid and drug-resistant clones were selected by treatment with hygromycin B (Wako, Tokyo, Japan) at a final concentration of 50 μg/ml. Huh7 cells were electroporated with *in vitro*-transcribed RNA from pJErepIRESpuro and drug-resistant clones were selected by treatment with puromycin (InvivoGen, San Diego, CA) at a final concentration of 1 μg/ml. Wild-type JEV strain AT31 was used as described previously (Tani et al., 2010). The wild-type JEV was amplified on C6/36 cells and stored at −80 °C. Pseudotype VSVs bearing JEV PrM and E proteins (JEVpv) and VSVG (VSVpv) were produced in 293T cells transfected with pCAG105E and pCAGVSVG, respectively, as described previously (Tani et al., 2010). The infectivities of JEV and the pseudotype VSVs were assessed by both a focus-forming assay and luciferase activity as described previously (Tani et al., 2010). Cell viability was determined by using CellTiter-Glo (Promega Corporation, Madison, WI) according to the manufacturer's protocol.

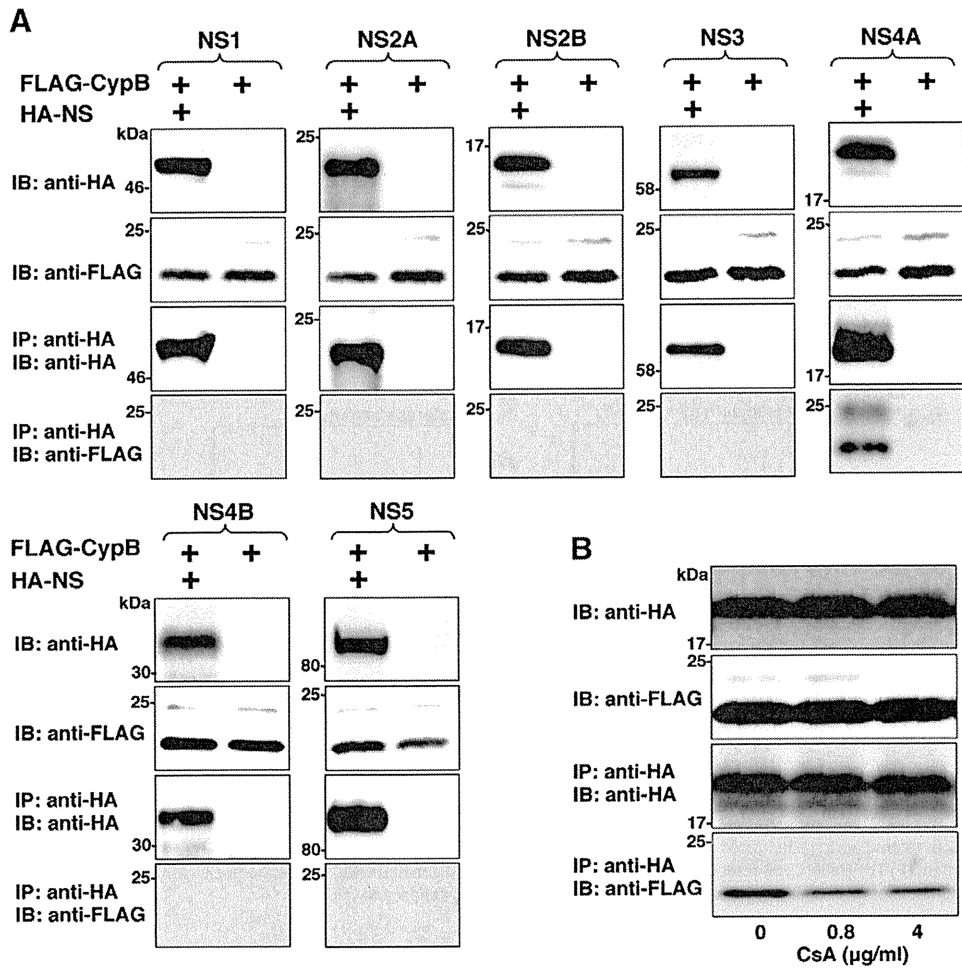
Reagents and antibodies

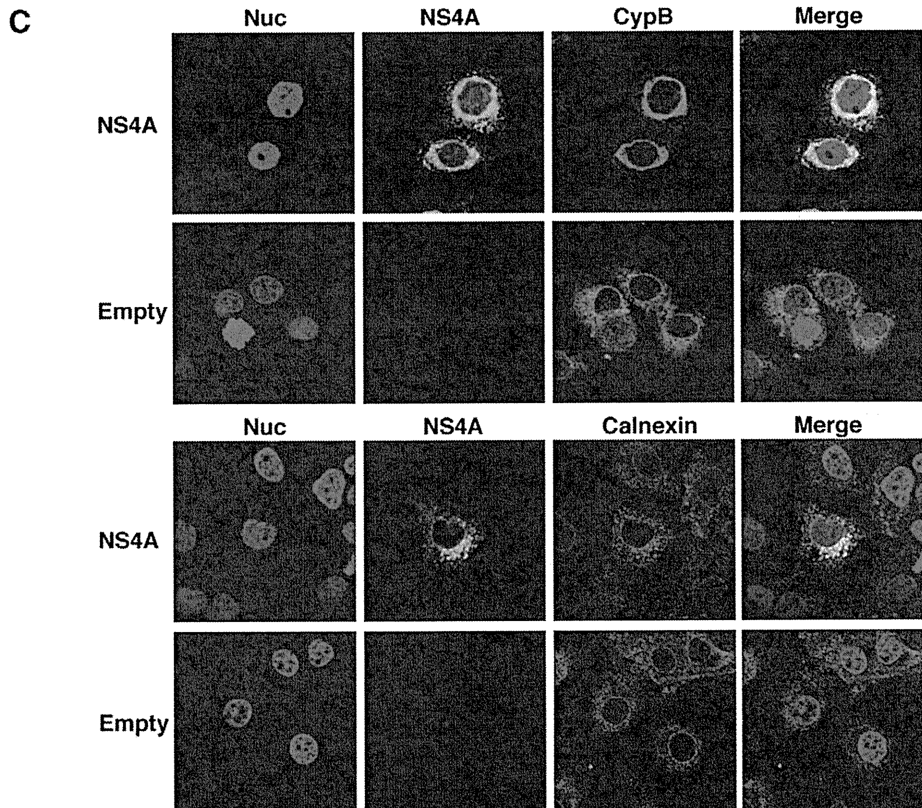
CsA and FK506 were purchased from Sigma, and CsD and CsH from Eton Bioscience Inc. (San Diego, CA). Mouse monoclonal antibodies to tags of HA and FLAG and β-actin were previously described (Taguwa et al., 2009). Rabbit polyclonal antibodies to CypA and CypB were purchased from Upstate Cell Signaling (Lake Placid, NY) and Affinity BioReagents (Golden, CO), respectively. Rabbit polyclonal antibody to calnexin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to JEV NS1 protein (34A1) was kindly provided by Dr. Yasui.

Transfection, immunoblotting, and immunoprecipitation

Transfection and immunoprecipitation were carried out as described previously (Taguwa et al., 2009). Immunoprecipitates boiled in loading buffer were subjected to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene

Fig. 6. NS4A protein recruits CypB to the replication complex in the JEV-infected cells. (A) FLAG-tagged CypB was co-expressed with HA-tagged NS1, NS2A, NS2B, NS3, NS4A, NS4B, or NS5 in 293T cells and immunoprecipitated with anti-HA antibody. The immunoprecipitates were subjected to immunoblotting by using either anti-FLAG or anti-HA antibody. (B) FLAG-tagged CypB was co-expressed with HA-tagged NS4A in 293T cells. The cell lysates obtained after lysis with the buffer containing CsA were immunoprecipitated with anti-FLAG antibody. The immunoprecipitates were subjected to immunoblotting by using either anti-FLAG or anti-HA antibody. (C) Huh7 cells transfected with an expression plasmid encoding HA-tagged NS4A or empty vector were fixed at 48 h post-transfection, permeabilized, and stained with the appropriate antibodies to HA (green), calnexin (red), and CypB (red). Cell nuclei were stained with DAPI (blue). Intracellular localization of CypB and NS4A was examined by confocal microscopy.


A
NS1
NS2A
NS2B
NS3
NS4A
FLAG-CypB
HA-NS
kDa
IB: anti-HA
IB: anti-FLAG
IP: anti-HA
IB: anti-HA
IP: anti-HA
IB: anti-FLAG
NS4B
NS5
B
0
0.8
4
CsA (μg/ml)


C
Nuc
NS4A
CypB
Merge
NS4A
Empty
Nuc
NS4A
Calnexin
Merge
NS4A
Empty

difluoride membranes (Millipore, Bedford, MA) and were reacted with the appropriate antibodies. The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Gene silencing by siRNA

The siRNAs against CypA and CypB were 5′-AAGCATACGGGTCCTGG-CATC-3′ and 5′-AAGGTGGAGAGCACCAAGACA-3′, respectively (QIAGEN, Tokyo, Japan). FlexTube siRNAs against CypC and the negative control were purchased from QIAGEN. The cells were grown on 6-well plates and transfected with 35 nM siRNA by using Dharmafect (Dharmacon, Buckinghamshire, UK) according to the manufacturer's protocol. The transfected cells were incubated in DMEM supplemented with 10% FBS.

Quantitative RT-PCR

RNA was determined by the method described previously (Taguwa et al., 2009). The total RNA was prepared from cells by using an RNeasy mini kit (QIAGEN). First-strand cDNA was synthesized using an RNA LA PCR™ *in vitro* cloning kit (Takara Bio Inc.) and random primers. Each cDNA was determined by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, San Diego, CA) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems, Tokyo, Japan).

In vitro transcription and RNA transfection

Plasmid pJErepIRESpuro linearized at the *Swa* I site was transcribed *in vitro* using an mMESSAGE mMACHINE (Ambion) according to the manufacturer's protocol. The *in vitro*-transcribed RNA was introduced into Huh7 cells at 5 million cells/0.5 ml by electroporation at 270 V and 960 μF using Gene Pulser™ (Bio-rad, Hercules, CA).

Colony formation assay

Colony formation was determined as previously described (Taguwa et al., 2009). Briefly, *in vitro*-transcribed RNA was electroporated into Huh7 cells and plated on DMEM containing 10% FBS and non-essential amino acids. The medium was replaced with fresh DMEM containing 10% FBS, non-essential amino acids, and 1 μg/ml puromycin at 24 h post-transfection. The remaining colonies were fixed with 4% paraformaldehyde (PFA) and stained with crystal violet at 3 weeks after electroporation.

Indirect immunofluorescence assay

Cells cultured on glass slides were fixed with 4% PFA in phosphate buffered saline (PBS) at room temperature for 30 min. After washing three times with PBS, the cells were permeabilized for 20 min at room temperature with PBS containing 0.25% saponin and blocked with phosphate buffer containing 2% BSA for 1 h at room temperature. The cells were incubated with blocking buffer containing mouse anti-HA or rabbit anti-CypB at room temperature for 1 h, then washed three times with PBS and incubated with blocking buffer containing AF488-conjugated anti-mouse IgG and AF594-conjugated anti-rabbit IgG at room temperature for 1 h. Cell nuclei were stained blue with DAPI. Finally, the cells were washed three times with PBS and observed a FluoView FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan).

Statistical analysis

Results are expressed as the means ± standard deviation. The significance of differences between the means was determined by Student's *t*-test.

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Oxidative Stress Induces Anti-Hepatitis C Virus Status via the Activation of Extracellular Signal-Regulated Kinase

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Recently, we reported that β-carotene, vitamin D_2, and linoleic acid inhibited hepatitis C virus (HCV) RNA replication in hepatoma cells. Interestingly, in the course of the study, we found that the antioxidant vitamin E negated the anti-HCV activities of these nutrients. These results suggest that the oxidative stress caused by the three nutrients is involved in their anti-HCV activities. However, the molecular mechanism by which oxidative stress induces anti-HCV status remains unknown. Oxidative stress is also known to activate extracellular signal-regulated kinase (ERK). Therefore, we hypothesized that oxidative stress induces anti-HCV status via the mitogen activated protein kinase (MAPK)/ERK kinase (MEK)–ERK1/2 signaling pathway. In this study, we found that the MEK1/2-specific inhibitor U0126 abolished the anti-HCV activities of the three nutrients in a dose-dependent manner. Moreover, U0126 significantly attenuated the anti-HCV activities of polyunsaturated fatty acids, interferon-γ, and cyclosporine A, but not statins. We further demonstrated that, with the exception of the statins, all of these anti-HCV nutrients and reagents actually induced activation of the MEK–ERK1/2 signaling pathway, which was inhibited or reduced by treatment not only with U0126 but also with vitamin E. We also demonstrated that phosphorylation of ERK1/2 by cyclosporine A was attenuated with *N*-acetylcysteine treatment and led to the negation of inhibition of HCV RNA replication. We propose that a cellular process that follows ERK1/2 phosphorylation and is specific to oxidative stimulation might lead to down-regulation of HCV RNA replication. *Conclusion:* Our results demonstrate the involvement of the MEK–ERK1/2 signaling pathway in the anti-HCV status induced by oxidative stress in a broad range of anti-HCV reagents. This intracellular modulation is expected to be a therapeutic target for the suppression of HCV RNA replication. (HEPATOLOGY 2009;50:678-688.)

Hepatitis C virus (HCV), which belongs to the family Flaviviridae, is a single-stranded positive-sense RNA virus of approximately 9.6 kb.[1,2] Persistent infection with HCV causes chronic hepatitis, which often leads to liver cirrhosis and hepatocellular carcinoma.[3] Therefore, HCV infection is a major health problem worldwide. Interferon (IFN)-based therapies, including the combination of pegylated IFN with ribavirin, are the current standard strategies for chronic hepatitis, but their sustained virological response rates are unsatisfactory.[4,5] There is thus an urgent need for novel partners with IFN or more effective reagents that may improve the sustained virological response rate.

Following the development in 1999 of a cell culture system to support efficient HCV RNA replication,[6] numerous studies have identified reagents that inhibit HCV RNA replication and enhance the effect of IFN treatment.[7-9] Some of these reagents are already available for clinical use. Previously, we also developed a genome-length HCV RNA (strain O of genotype 1b) replication system (OR6) with Renilla luciferase (RL) as a reporter in hepatoma cell lines.[10] Using this OR6 assay system, we found that mizoribine,[11] as an immunosuppressant, and

Abbreviations: AA, arachidonic acid; BC, β-carotene; CsA, cyclosporine A; CyPA, cyclophilin A; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor: EPA, eicosapentaenoic acid; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; FLV, fluvastatin; HCV, hepatitis C virus; IFN, interferon; LA, linoleic acid: MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; NS5A, nonstructural 5A; PTV, pitavastatin; PUFA, polyunsaturated fatty acid; RL, renilla luciferase; ROS, reactive oxygen species; VD2, vitamin D_2; VE, vitamin E.

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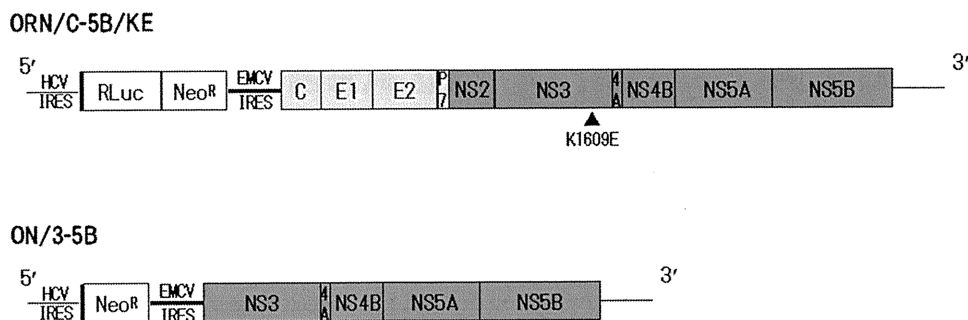

Fig. 1. Schematic gene organization of the genome-length and subgenomic HCV RNA used in this study. ORN/C-5B/KE encoding the RL gene was replicated in OR6 cells and ON/3-5B in sO cells. RL in OR6 cells was expressed as a fusion protein with neomycin phosphotransferase (Neo^R). The arrowhead indicates the position of K1609E, an adaptive mutation.

fluvastatin (FLV) and pitavastatin (PTV),[9,12] as the reagents for hypercholesterolemia, suppressed genome-length HCV RNA replication. Furthermore, in a recent study[13] in which we comprehensively analyzed the activities of ordinary nutrients on HCV RNA replication, three nutrients, β-carotene (BC), vitamin D_2 (VD2), and linoleic acid (LA), were found to suppress HCV RNA replication and enhance the antiviral activity of IFN-α or cyclosporine A (CsA) in an additive or a synergistic manner. Because the anti-HCV activities of these three nutrients, as well as CsA, were canceled by treatment with antioxidants such as vitamin E (VE) or selenium, we suggested that oxidative stress might be involved in the anti-HCV activities of these three nutrients and CsA. However, the detailed molecular mechanism via which the oxidative effects of these three nutrients and CsA suppress HCV RNA replication has not been explored.

The production of reactive oxygen species (ROS) plays a pivotal role in various cellular processes, including cell proliferation, differentiation, and apoptosis.[14] Whereas high-level production of ROS resulting from external stimuli is recognized as an important component of the pathogenesis of inflammatory and cancerous diseases, endogenously produced ROS at low concentrations are shown to function as signaling mediators of cellular responses.[15,16] Emerging evidence indicates that these ROS-triggered responses are mediated primarily via cellular signaling cascades, including a signaling pathway of extracellular signal-regulated kinase (ERK)1/2, namely p44/42 mitogen-activated protein kinase (MAPK), which belongs to the MAPK family.[17,18]

Several studies have revealed that certain viral proteins initiate activation of the MAPK/ERK kinase (MEK)–ERK1/2 signaling pathway, which may facilitate the viral replication and infectivity in the infected cells.[19,20] The HCV core protein[21] and the envelope protein[22] have also been reported to up-regulate this signaling pathway. However, another study reported that the HCV nonstructural 5A (NS5A) protein suppressed activating protein-1 activation by inhibiting the phosphorylation of ERK1/2 in replicon cells.[23] Moreover, recent studies using an inhibitor specific to the MEK–ERK1/2 signaling pathway reported that the direct anti-HCV activities of IFN-γ[24] and acetylsalicylic acid[25] are mediated in part through the induction of this cascade.

We demonstrate that the activation of MEK–ERK1/2 signaling plays a significant role in the anti-HCV activity caused by oxidative stress in a broad range of anti-HCV reagents.

Materials and Methods

Reagents and Antibodies. Dimethyl sulfoxide (DMSO), BC, VD2, VE, LA, arachidonic acid (AA), eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and IFN-γ were purchased from Sigma Aldrich (St. Louis, MO), and CsA, FLV, U0126, PD98059, SB203580, and c-Jun N-terminal kinase inhibitor II were obtained from Calbiochem (San Diego, CA). Epidermal growth factor (EGF) was purchased from Toyobo (Osaka, Japan). PTV was purchased from Kowa Company, Ltd. (Tokyo, Japan). Anti-HCV core antibody (CP11) was purchased from the Institute of Immunology (Tokyo, Japan), and anti-HCV NS5A antibody was the generous gift of Dr. A. Takamizawa (Research Foundation for Microbial Diseases, Osaka University). Antibodies specific to ERK1/2 (p44/42 MAPK), MEK1/2, and phosphorylated (S217/221) MEK1/2 were purchased from Cell Signaling Technology (Beverly, MA), and anti-phosphorylated (T202/Y204) ERK1/2 antibody was obtained from BD Biosciences (San Jose, CA). Anti–β-actin antibody was purchased from Sigma Aldrich.

Cell Cultures. The cell lines OR6 and sO were cloned from ORN/C-5B/KE RNA and subgenomic replicon RNA (ON/3-5B)–replicating cells, respectively (Fig. 1). These cells were derived from the hepatoma cell line HuH-7, cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), peni-

cillin, streptomycin, and 300 μg/mL of G418 (Geneticin; Invitrogen, Carlsbad, CA), and passaged twice a week at a 5:1 split ratio. ORN/C-5B/KE and ON/3-5B were derived from HCV-O (strain O of genotype 1b).[10]

OR6 Reporter Assay. For the RL assay, 1.0-1.5 $\times 10^4$ OR6 cells were plated onto 24-well plates in triplicate and precultured for 24 hours. The cells were pretreated with DMSO or a specific inhibitor for 1 hour and then were treated with each anti-HCV nutrient or compound in either the absence (DMSO) or presence of a specific inhibitor for 72 hours. After the treatment, the cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol.

Western Blot Analysis. For analysis of the effect of a specific inhibitor on the anti-HCV activity, 6.0-6.5 $\times 10^4$ OR6 cells were plated onto 6-well plates and precultured for 24 hours. The pretreatment with DMSO or a specific inhibitor for 1 hour and subsequent treatment for 72 hours was performed in the same manner as for the OR6 reporter assay. For analysis of the activities of each anti-HCV nutrient or reagent on the MEK–ERK1/2 signaling pathway, 1.0 $\times 10^5$ OR6 or sO cells were plated onto 6-well plates and precultured in 10% FBS-containing medium for 24 hours. After the preculture, the culture medium was changed to FBS-free medium and the cells were cultured for 48 hours prior to treatment with each nutrient or reagent. When the effect of a specific inhibitor or VE on ERK1/2 phosphorylation was analyzed, the cells were pretreated with the specific inhibitor or VE for 1 hour prior to each treatment. Preparation of the cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were then performed as described.[26]

Measurement of ROS. OR6 cells in 24-well plates were left untreated or were treated with hydrogen peroxide (1 mM), LA (200 μM), and CsA (15 μg/mL) for 30 minutes and then incubated with dihydrodichlorocarboxyfluorescein diacetate (Invitrogen) (5 μM) for 15 minutes. Fluorescence was measured with a FLUOROSKAN ASCENT fluorescence plate reader (Thermo Fisher Scientific, Waltham, MA) at an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Cell Growth Assay. To examine the activity of EGF on OR6 cell growth, 6.0-6.5 $\times 10^4$ OR6 cells were plated onto 6-well plates in triplicate and were pre-cultured for 24 hours. The cells were treated with or without EGF for 72 hours, and the number of viable cells was counted after trypan blue dye treatment as described.[11]

Statistical Analysis. Statistical comparison of the luciferase activities between the various treatment groups was performed using the Student t test. P values of less than 0.05 were considered statistically significant.

Results

Effects of MEK1/2-Specific Inhibitors on the Anti-HCV Activities of BC, VD2, and LA in OR6 Cells. Our recent study suggested the involvement of oxidative stress in the suppressive mechanism of three anti-HCV nutrients: BC, VD2, and LA.[13] Because there have been reports of negative regulation of HCV RNA replication via the MEK–ERK1/2 signaling pathway,[24,25] which is one of the oxidative stress-induced cellular signaling pathways, we hypothesized that the suppression of HCV RNA replication by these three nutrients might be mediated via this cascade (Supporting Fig. 1). To test this hypothesis, we first used an OR6 assay system to examine the effects of U0126 and PD98059, inhibitors specific to MEK1/2, on the three anti-HCV nutrients at 60% inhibitory concentration. As shown in Fig. 2A, treatment with either 5 μM of U0126 or 10 μM of PD98059 slightly enhanced HCV RNA replication in comparison with the control. However, U0126 attenuated the anti-HCV activities of the three nutrients more clearly than PD98059 (Fig. 2A,B). U0126 prevented the anti-HCV activities of the three nutrients in a significant and dose-dependent manner and exerted complete inhibition against the anti-HCV activities of BC and LA (Fig. 2C,D), while the inhibitory effect of PD98059 was more mild (Fig. 2E,F). As shown in Fig. 2G, we also found that U0126 treatment restored the expressions of HCV proteins, core, and NS5A in a dose-dependent manner. We further demonstrated that knockdown of MEK1 or MEK2 by small interfering RNA negated the anti-HCV activity of LA (Supporting Fig. 2A-C). These inhibitions by U0126 against the anti-HCV activities of the three nutrients were not due to the enhancement of encephalomyocarditis virus/internal ribosomal entry site–driven RL activity, because this activity was not increased by U0126 (data not shown). Moreover, treatment with neither SB203580 (an inhibitor specific to p38 MAPK) nor c-Jun N-terminal kinase inhibitor, both of which belong to the same cascade family as MEK–ERK1/2, significantly affected the anti-HCV activities of the three nutrients (data not shown). These results imply that the activation of the MEK–ERK1/2 signaling pathway might be required for the suppression of genome-length HCV RNA replication by the three nutrients in cell culture.

Effect of U0126 on the Suppressive Effects of Polyunsaturated Fatty Acids and Anti-HCV Reagents in OR6 Cells. Previous studies using a cell culture system have shown that polyunsaturated fatty acids (PUFAs), including LA, act as anti-HCV nutrients.[27,28] A recent study reported that lipid peroxidation of PUFAs was correlated with their anti-HCV activities, which were pre-

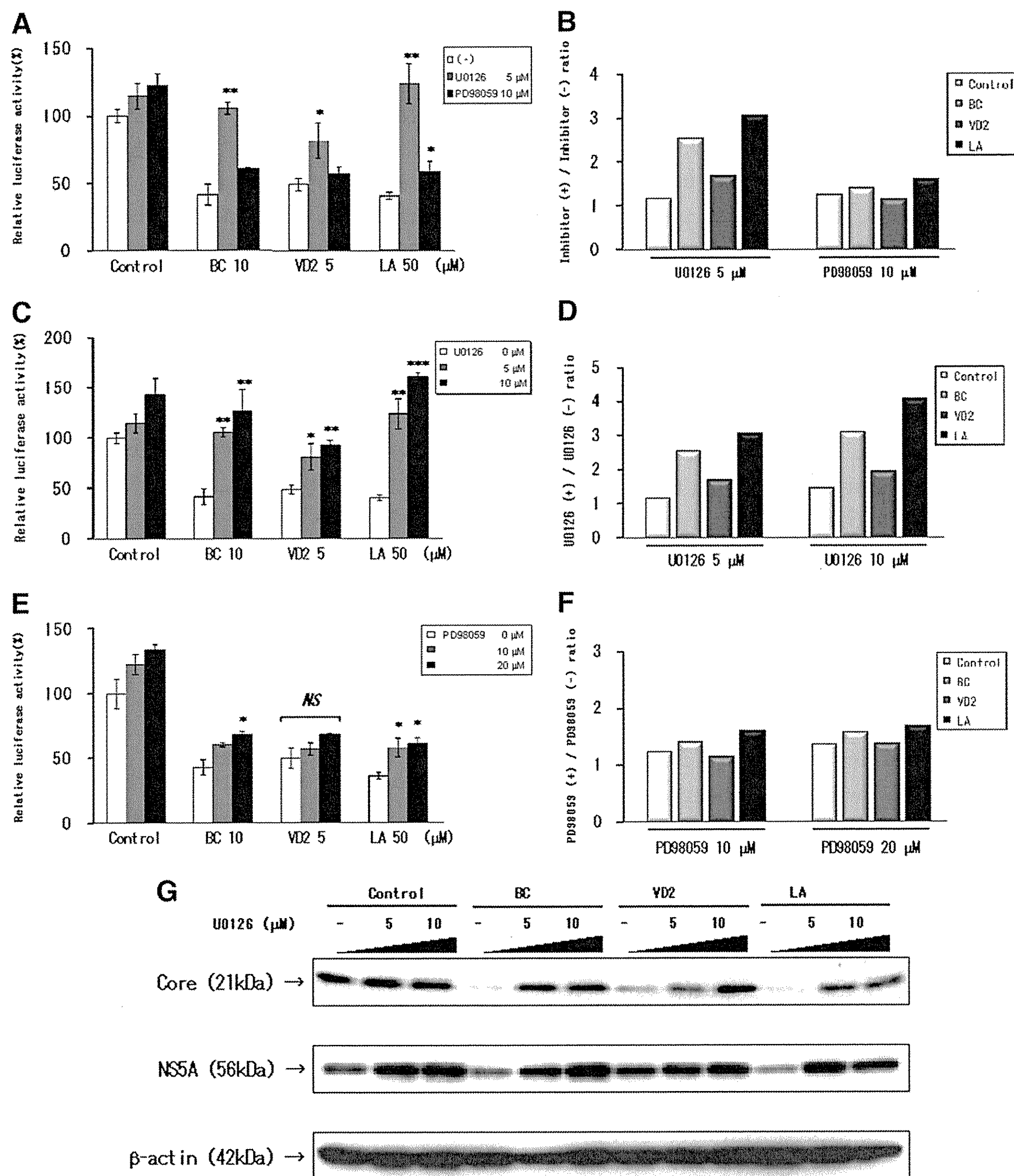

Fig. 2. U0126 strongly inhibited the anti-HCV activities of the anti-HCV nutrients BC, VD2, and LA in OR6 cells. (A,B) Effects of MEK-specific inhibitors on the three nutrients at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO, 5 μM U0126, or 10 μM PD98059 for 1 hour. The cells were then treated with control medium, 10 μM BC, 5 μM VD2, or 50 μM LA in either the absence (DMSO) or presence of each specific inhibitor for 72 hours. After treatment, RL assay was performed as described in Materials and Methods. Shown here is the relative luciferase activity (%) calculated when the RL activity of the control was assigned as 100%. Data are expressed as the mean $\pm$ standard deviation of triplicate samples from at least three independent experiments. Asterisks indicate significant difference from treatment with DMSO (*$P < 0.05$; *$P < 0.01$) (A). The ratio of the RL activity in the presence of the MEK-specific inhibitor to the RL activity in the absence of the inhibitor was then calculated (B). (C-F) OR6 reporter assays of the dose effects of MEK1/2-specific inhibitors on the three nutrients. OR6 cells were pretreated with DMSO, U0126 (C), or PD98059 (E) at the indicated concentrations for 1 hour. Treatment of the cells with control medium or each of the three nutrients in either the absence (DMSO) or presence of each specific inhibitor and the RL assay of harvested OR6 cell samples were performed as described in panels A and B. Asterisks indicate significant difference from treatment with DMSO (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; NS, not significant). Next, we calculated the ratio of RL activity in the presence of the MEK-specific inhibitor, U0126 (D), or PD98059 (F), to the RL activity in the absence of the inhibitor. (G) Western blot analysis of the dose effects of U0126 on three nutrients. OR6 cells were pretreated and then treated as in panel C. The production of HCV core and NS5A in the cells was analyzed by way of immunoblotting using antibodies specific to HCV core (top row) and NS5A (middle row). β-actin was used as a control for the amount of protein loaded per lane (bottom row).

vented by treatment with VE.[29] This result coincides with our previous observations on the effects of LA.[13] We proposed that the MEK–ERK1/2 signaling pathway might be involved in the anti-HCV activity of PUFAs, including LA, because lipid peroxidation is known to be a ROS-triggered cellular modification.[16] As expected, treatment with U0126 attenuated the anti-HCV activities of four representative PUFAs in a significant and dose-dependent manner (Fig. 3A,B).

Moreover, because the anti-HCV activities of BC, VD2, LA, and CsA, but not FLV, were found to be negated by VE,[13] we were also interested in the potent role of the MEK–ERK1/2 signaling pathway in the anti-HCV mechanism of CsA. Furthermore, the previous study using a subgenomic replicon system had already shown the partial involvement of this cascade in the antiviral activity of IFN-γ.[24] Therefore, we examined the effects of U0126 on various anti-HCV reagents: IFN-γ, CsA, and statins (FLV and PTV). We confirmed that also in genome-length HCV RNA replication cells, U0126 significantly inhibited the anti-HCV activity of IFN-γ (Fig. 3C,D). Interestingly, consistent with the effects of treatment with VE,[13] the anti-HCV activity of CsA was completely abrogated by U0126 in a significant and dose-dependent manner, whereas statins were unaffected (Fig. 3C,D).

U0126 restored the reduced expression of HCV proteins by PUFAs, IFN-γ, and CsA in a dose-dependent manner, whereas statins were unaffected (Fig. 3E,F). These results were supported by additional real-time reverse-transcription polymerase chain reaction and immunofluorescence analyses (Supporting Fig. 3A-C). We also observed that knockdown of MEK1 or MEK2 by small interfering RNA did not affect the anti-HCV activity of PTV (Supporting Fig. 2A-C). Collectively, these findings suggest that the MEK–ERK1/2 signaling pathway may play a critical role in the negative regulation of HCV RNA replication by the anti-HCV nutrients BC and VD2, PUFAs, and the anti-HCV reagents IFN-γ and CsA, but not statins.

Activation of the MEK–ERK1/2 Signaling Pathway by Anti-HCV Nutrients and Reagents. To further ensure the involvement of the MEK–ERK1/2 signaling pathway in the suppressive mechanisms of anti-HCV nutrients and reagents, we next examined whether these nutrients and reagents could actually initiate the activation of this signaling pathway. After treating the HCV RNA replicating cells with each of the nutrients and reagents, we performed immunoblotting specific to the phosphorylation of ERK1/2 and MEK1/2. In the same way as EGF, a potent activator of these kinases, the three anti-HCV nutrients (BC, VD2, and LA) enhanced the phosphorylation of ERK1/2 and MEK1/2 in both genome-length and subgenomic HCV RNA replication cells (Fig. 4A,B). IFN-γ, CsA, and all of the PUFAs also up-regulated this cascade in OR6 cells (Fig. 4C,D). The increase in phosphorylation of ERK1/2 was not observed after either statin treatment (Fig. 4D). The activation of MEK–ERK1/2 by the three anti-HCV nutrients was apparent until 1 hour after their application and subsequently attenuated, although EGF exhibited persistent enhancement of MEK–ERK1/2 phosphorylation (Fig. 4E). Because the experiments regarding ERK1/2 phosphorylation were performed in FBS-free conditions, we checked the anti-HCV activity of PTV, CsA, and LA in FBS-free medium. The results revealed that these anti-HCV reagents and nutrients also inhibited HCV RNA replication in FBS-free conditions (Supporting Fig. 4). Taken together, these findings indicate that the anti-HCV nutrients and reagents activated the MEK–ERK1/2 signaling pathway in HCV RNA replicating cells, providing further confirmation that this signaling cascade might be involved in their anti-HCV activities.

MEK1/2-Specific Inhibitors Attenuated the Increased Phosphorylation of ERK1/2 by Anti-HCV Nutrients/Reagents and EGF. We next tested whether MEK1/2-specific inhibitors could prevent not only the suppression of HCV RNA replication but also the activation of ERK1/2 by the anti-HCV nutrients BC, VD2, and PUFAs and the anti-HCV reagents IFN-γ and CsA. Consistent with the inhibitory effects on their anti-HCV activities, U0126 more markedly abrogated the increase in ERK1/2 phosphorylation by anti-HCV nutrients, reagents, and EGF than did PD98059 (Fig. 5A,B). As shown in Fig. 5C, the enhanced ERK1/2 phosphorylation by the three nutrients and EGF was reduced by U0126 in a dose-dependent manner.

VE Attenuated the Increased Phosphorylation of ERK1/2 by Anti-HCV Nutrients/Reagents and EGF. Because the suppression of HCV RNA replication by BC, VD2, LA, and CsA were completely negated by the treatment with VE in our recent study,[13] we investigated whether VE could also inhibit ERK1/2 activation by anti-HCV nutrients and reagents. As expected, VE also attenuated the enhanced phosphorylation of ERK1/2 by not only anti-HCV nutrients and CsA but also IFN-γ and EGF (Fig. 6A,B). We also demonstrated that phosphorylation of ERK1/2 by CsA was attenuated with *N*-acetylcysteine treatment and led to the negation of inhibition of HCV RNA replication (Supporting Fig. 5A-C). The anti-HCV nutrients and reagents, whose activities were negated by U0126, were also inhibited by VE. In contrast, the anti-HCV activities of statins were not negated by U0126 or VE. We also demonstrated that LA and CsA induce ROS (Fig.

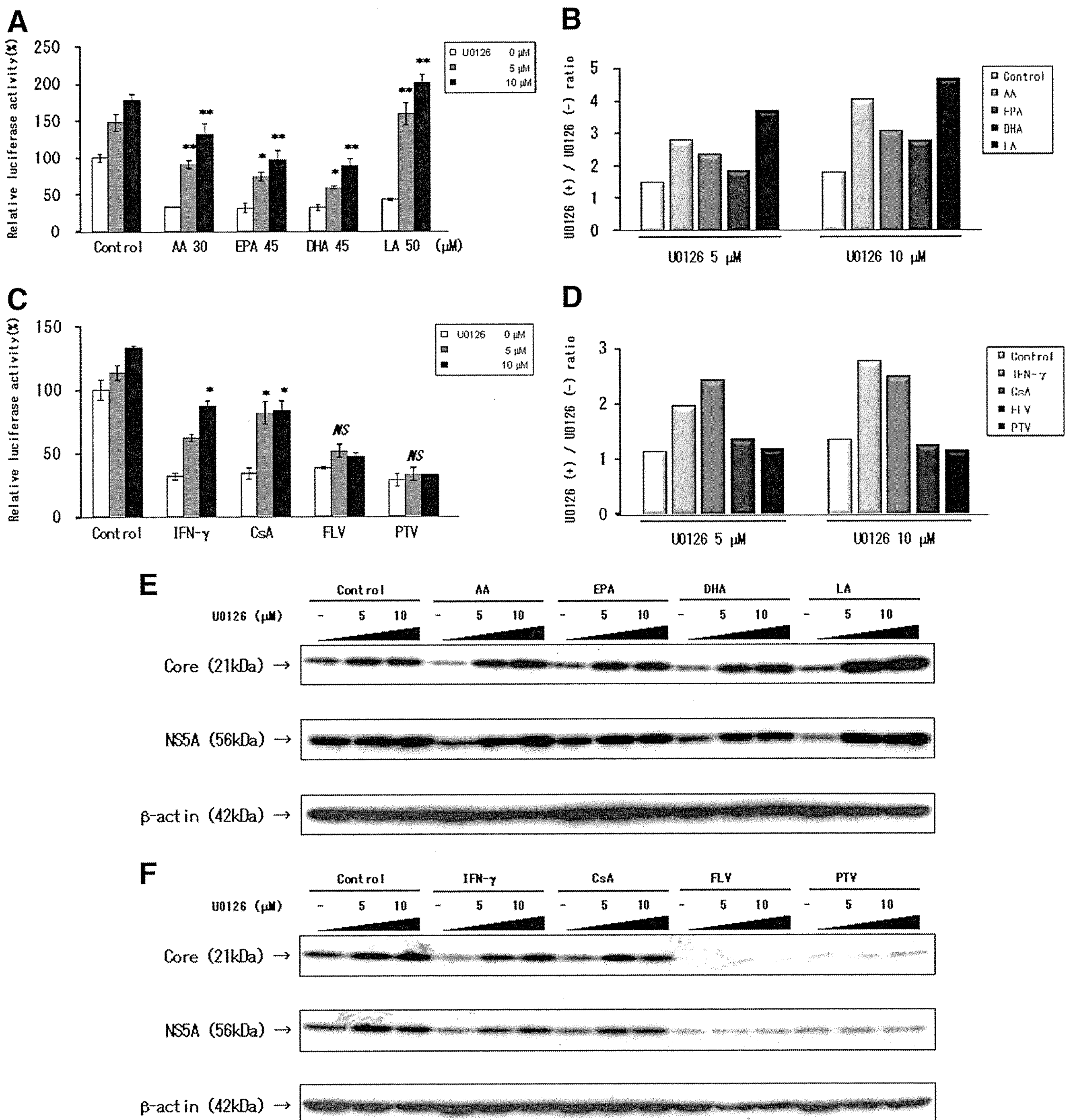

Fig. 3. U0126 dose-dependently attenuated the anti-HCV activities of PUFAs, IFN-γ, and CsA, but not the statins. (A-D) OR6 reporter assays of the dose effects of U0126 on the PUFAs and anti-HCV reagents at the 60% inhibitory concentration. OR6 cells were pretreated with DMSO or U0126 as in Fig. 2C and then treated with control medium, 30 μM AA, 45 μM EPA, 45 μM DHA, or 50 μM LA (A) and control medium, 0.4 IU/mL IFN-γ, 0.2 μg/mL CsA, 3 μM FLV, or 1 μM PTV (C), respectively, in either the absence (DMSO) or presence of U0126 for 72 hours. After the treatment, the RL assay of harvested OR6 cell samples was performed as described in Fig. 2A and 2B. Asterisks indicate significant difference from treatment with DMSO (*$P < 0.05$; **$P < 0.01$; NS, not significant). The ratio of the RL activity in the presence of U0126 to the RL activity in the absence of U0126 was then calculated (B, D). (E, F) Western blot analysis of the dose effects of U0126 on the PUFAs and anti-HCV reagents. The production of HCV core (top row) and NS5A (middle row) in the cells treated as in panel A (E) and panel C (F) was analyzed as described in Fig. 2G. β-actin was used as a control for the amount of protein loaded per lane (bottom row).

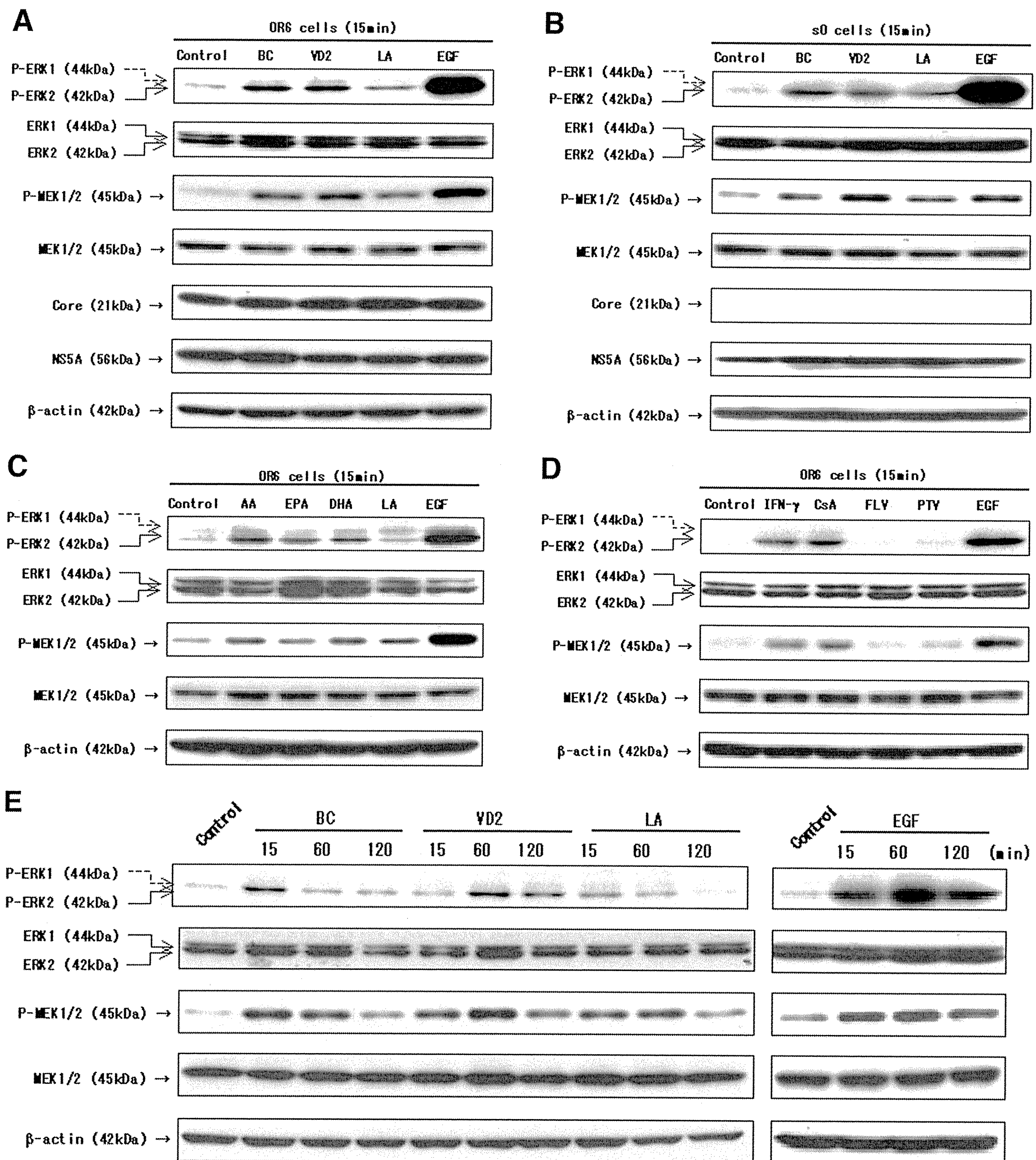

Fig. 4. U0126 attenuated the MEK-ERK1/2 signaling pathway activated by anti-HCV nutrients and reagents. (A, B) Three anti-HCV nutrients—BC, VD2, and LA—increased the phosphorylation of MEK-ERK1/2 in both full-length and subgenomic HCV RNA replication cells. OR6 cells (A) or sO cells (B) were maintained in FBS-free medium for 48 hours and then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF for 15 minutes. After treatment, cell lysates underwent western blot analysis using antibodies specific to phosphorylated ERK1/2, ERK1/2, phosphorylated MEK1/2, and MEK1/2. The appropriate expression of HCV core and NS5A was determined by way of immunoblotting with their respective antibodies. (C, D) IFN-γ, CsA, and the PUFAs, but not the statins, increased the phosphorylation of MEK-ERK1/2 in OR6 cells. OR6 cells were precultured as described in panels A and B, then treated with control medium, 100 μM AA, EPA, DHA, or LA, or 50 ng/mL EGF (C) and control medium, 2 IU/mL IFN-γ, 2 μg/mL CsA, 5 μM of FLV or PTV, or 50 ng/mL EGF (D), respectively, for 15 minutes. (E) Time-course western blot analysis of the increase of MEK-ERK1/2 phosphorylation by the three anti-HCV nutrients and EGF. Samples for analysis were harvested prior to treatment with the control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF (0 time point) and at 15, 60, and 120 minutes posttreatment. After all of the treatments (C-E), cell lysates were subjected to western blot analysis of the activation of the MEK-ERK1/2 signaling pathway as described in panels A and B. β-actin was used as a control for the amount of protein loaded per lane in all analyses.

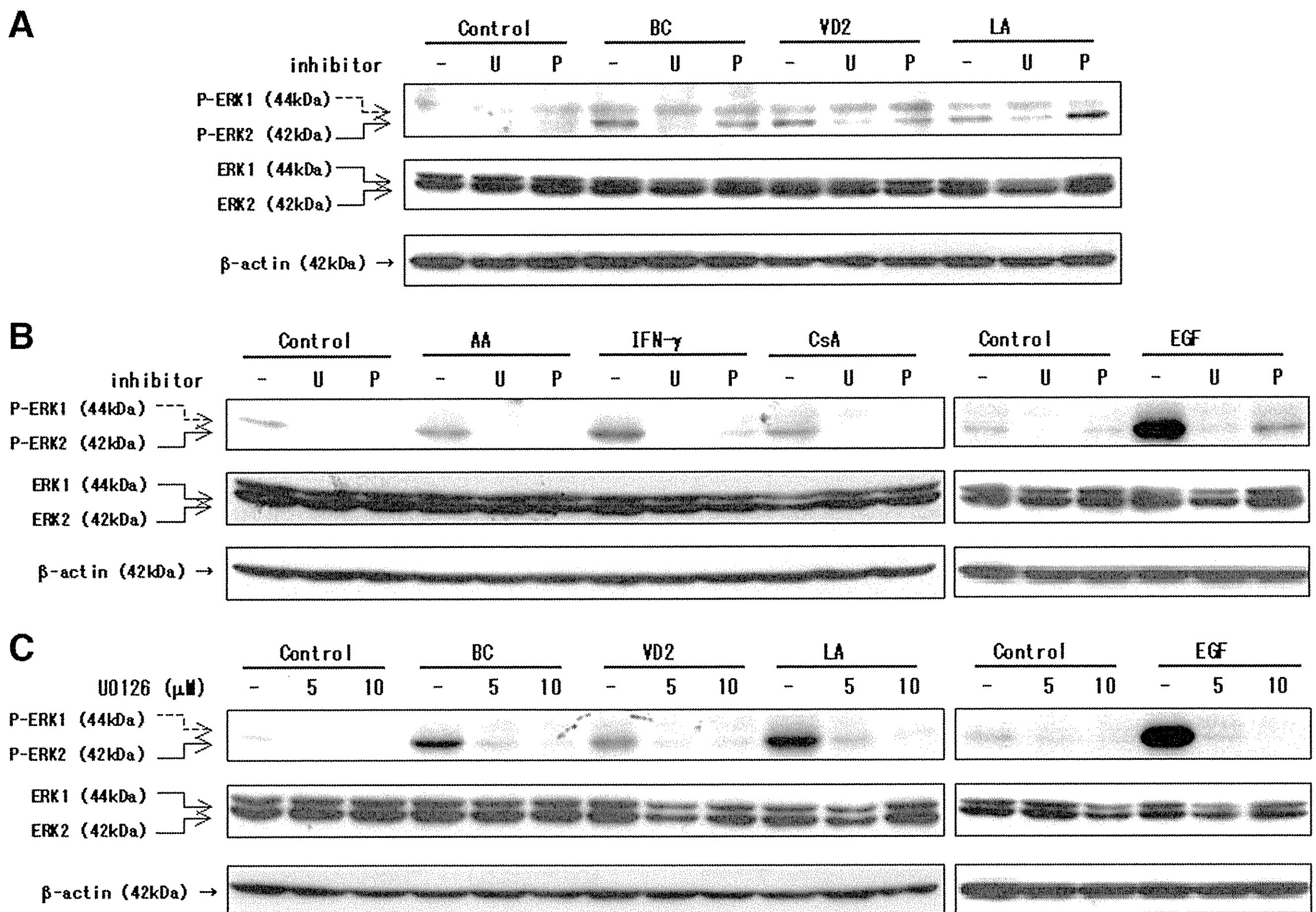

Fig. 5. U0126 strongly abolished ERK1/2 phosphorylation by the anti-HCV nutrients, anti-HCV reagents, and EGF. (A,B) Effects of the MEK1/2-specific inhibitors on ERK1/2 phosphorylation by anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with DMSO (−), 10 μM U0126: (U), or 20 μ M PD98059: (P) for 1 hour. Subsequently, the cells were treated with control medium, 20 μM BC, 10 μM VD2, or 100 μM LA (A) and control medium, 100 μM AA, 2 IU/mL IFN-γ, 2 μg/mL CsA, or 50 ng/mL EGF (B), respectively, in either the absence (DMSO) (−) or presence of U0126 (U) or PD98059 (P) for 15 minutes. (C) Dose effects of U0126 on ERK1/2 phosphorylation by the three anti-HCV nutrients and EGF. OR6 cells were precultured as described in Figs. 4A and 4B, then pretreated with DMSO (−) or 5 or 10 μM U0126 for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF in either the absence (−) or presence of U0126 for 15 minutes. After all treatments (A-C), cell lysates were subjected to western blot analysis using antibodies specific to phosphorylated ERK1/2 (top row) and ERK1/2 (middle row). β-actin was used as a control for the amount of protein loaded per lane (bottom row).

7). Collectively, these results suggest that these nutrients and reagents induce ROS as an oxidant in HCV RNA replicating cells, leading to activation of the MEK–ERK1/2 signaling pathway and suppression of HCV RNA replication.

The Effects of EGF on HCV RNA Replication were Different than Those of the Anti-HCV Nutrients/Reagents. Because the study by Huang et al.[24] showed that EGF time-dependently suppressed the expressions of HCV nonstructural proteins in subgenomic replicon-harboring cells, we wondered whether EGF could suppress genome-length HCV RNA replication. EGF inhibited HCV RNA replication by approximately 25% at a concentration of 100 ng/mL. This anti-HCV activity was weaker than that of the anti-HCV nutrients and reagents tested in this study. However, as shown in the cell growth assay, EGF promoted OR6 cell proliferation in a dose-dependent manner (Supporting Fig. 6). These cell growth effects of EGF may have caused us to underestimate the actual anti-HCV activity of EGF. The other reagents and nutrients did not affect cell proliferation compared with EGF (Supporting Fig. 7).

Discussion

The previous studies using the MEK1/2-specific inhibitor and subgenomic replicon system showed that induction of the MEK–ERK1/2 signaling pathway might be required for the suppression of HCV RNA replication by some reagents.[24,25] In agreement with the study by Huang

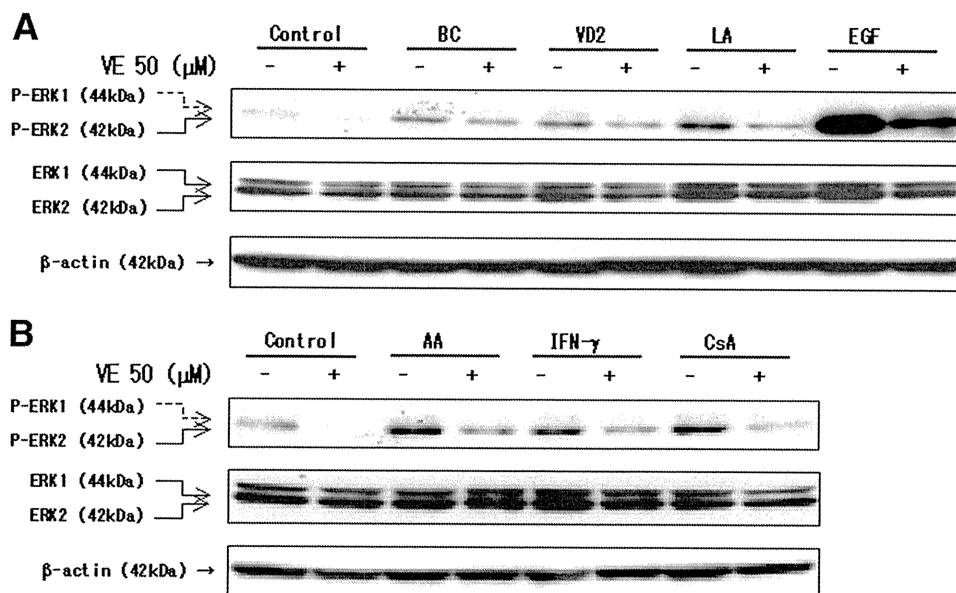

Fig. 6. VE attenuated ERK1/2 phosphorylation by the anti-HCV nutrients and reagents. OR6 cells were precultured as described in Figs. 4A and B, and then pretreated with ethanol (−) or 50 μM VE (+) for 1 hour. The cells were then treated with control medium, 20 μM BC, 10 μM VD2, 100 μM LA, or 50 ng/mL EGF (A) and control medium, 100 μM AA, 2 IU/mL IFN-γ, and 2 μg/mL CsA (B), respectively, in either the absence (ethanol) (−) or presence (+) of 50 μM VE for 15 minutes. After the treatment, cell lysates underwent western blot analysis as described in Fig. 5.

et al.,[24] we also confirmed that U0126 inhibited the anti-HCV activity of IFN-γ in OR6 cells stably replicating genome-length HCV RNA. Although they did not identify the direct activation of the MEK–ERK1/2 signaling pathway by IFN-γ, we demonstrated that IFN-γ could stimulate this cascade in HCV RNA replication cells. Moreover, this stimulation was not only inhibited by U0126 but also by antioxidant VE. This result indicates the involvement of oxidative stress in the anti-HCV activity of IFN-γ as well as the MEK–ERK1/2 signaling pathway. IFNs induce the transcription of IFN-stimulated genes through the JAK–STAT pathway, but the induction of IFN-stimulated genes by IFN-γ has been far more complex than that by IFN type I.[30] A study using a

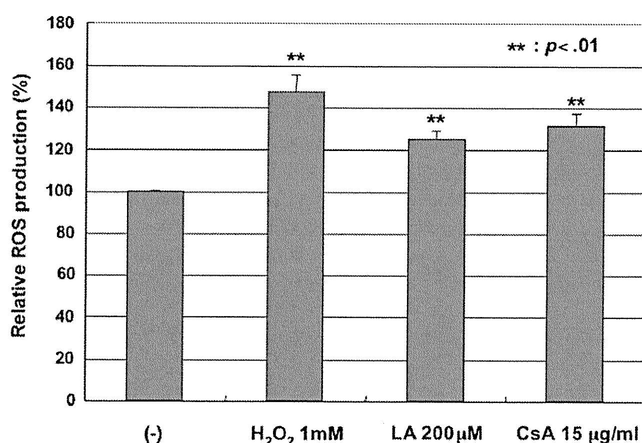

Fig. 7. ROS production by H_2O_2, LA, and CsA. OR6 cells were untreated or treated with H_2O_2 (1 mM), LA (200 μM), and CsA (15 μg/mL) and then incubated with dihydrodichlorocarboxyfluorescein diacetate. Fluorescence was measured with a fluorescence plate reader. **$P < 0.01$ versus untreated cells.

macrophage cell line revealed that IFN-γ activated ERK1/2, followed by the expression of IFN-γ–stimulated genes downstream of the JAK–STAT signaling pathway.[31] Another study reported that the defensive activity of IFN-γ against hepatitis B virus in hepatoblastoma cells was mediated through the induction of oxidative stress.[32] Furthermore, ROS itself has been reported to suppress HCV RNA replication in human hepatoma cells.[33] These reports support our proposal regarding anti-HCV activity of oxidative stress that the generation of intracellular ROS inhibits HCV RNA replication through activation of the MEK–ERK1/2 signaling pathway. Waris and Siddiqui[34] reported that calcium-dependent ROS generation induced cyclooxygenase-2 and prostaglandin E(2) via the activation of nuclear factor kappa B, leading to the suppression of HCV RNA replication. Choi et al.[35] also demonstrated that elevated calcium suppressed HCV RNA replication. The activation of nuclear factor kappa B by ROS was mediated through the MEK–ERK1/2 signaling pathway. Therefore, we suggest that the oxidative reagents and nutrients in this study also may induce anti-HCV status by calcium-dependent ROS generation.

In the course of our study of the anti-HCV activities of these three nutrients, we found that treatment with U0126 more strongly inhibited their anti-HCV activities than treatment with PD98059. U0126 has been shown to possess approximately 100-fold-higher MEK1/2-specific inhibitory activity than PD98059.[36] This different potential between the two inhibitors was considered to cause a gap in their effects on anti-HCV activities. We further found that, much like EGF, all three nutrients enhanced the phosphorylation of ERK1/2 and MEK1/2, which was reduced by treatment with U0126 or VE. In addition, the

present study was the first to observe that BC, which has been shown to produce ROS,[37] activates the MEK–ERK1/2 signaling pathway, an action that VD2[38] and LA[39] have already been shown to exhibit in leukemia cell and dendritic cell lines, respectively. Furthermore, we found the involvement of the MEK–ERK1/2 signaling pathway in the anti-HCV mechanism of the three nutrients as well as various PUFAs, which were reported to be mediated through lipid peroxidation.[29] These results suggest that the anti-HCV nutrients BC, VD2, and PUFAs, including LA, as well as IFN-γ may suppress HCV RNA replication via activation of the MEK–ERK1/2 signaling pathway in response to ROS production.

We also investigated the involvement of the MEK–ERK1/2 signaling pathway in the suppressive mechanism of anti-HCV reagents other than IFN-γ. In our previous study, the anti-HCV activity of CsA, but not FLV, was prevented by VE.[13] Consequently, these results implied that CsA, but not statins, could be potent activators of the MEK–ERK1/2 signaling pathway as oxidants, leading to down-regulation of HCV RNA replication. CsA has been demonstrated to bind to cyclophilins and suppress HCV RNA replication by abolishing their interaction with NS5B polymerase.[40] This CsA binding to cyclophilins, especially cyclophilin A (CyPA), has been shown to result in the generation of ROS through inhibition of the peptidylprolyl-cis-trans-isomerase–like activity of CyPA.[41] Moreover, CyPA was reported to be secreted in response to oxidative stress,[42] and to bind to a cell surface receptor, CD147, followed by ERK1/2 activation.[43] These reports and our results suggest that CsA, acting as an oxidant, may trigger activation of the MEK–ERK1/2 signaling pathway, both directly by producing ROS by way of interaction with CyPA in the early phase, and indirectly by secreting CyPA in the late phase. Both activations could lead to an inhibition of HCV RNA replication. Thus, CyPA may play a critical role as an intermediator in the oxidative anti-HCV activity of CsA. In the latest study, CyPA was identified as the most essential cellular cofactor of HCV RNA replication among cyclophilins.[44] Further studies will be needed to clarify whether CyPA is required for the oxidative suppressive mechanism of anti-HCV nutrients/reagents other than CsA.

Although we expected that strong activation of the MEK–ERK1/2 signaling pathway would suppress HCV RNA replication, EGF exhibited only slight anti-HCV activity in OR6 cells. The promotion of cell growth by EGF might prevent its primary inhibitory effect on HCV RNA replication. A portion of the ERK1/2 phosphorylation by EGF was also reduced by treatment with VE (Fig. 6A), suggesting that EGF might stimulate the MEK–ERK1/2 signaling pathway, in part, as an oxidant, and that this oxidative activity of EGF could exhibit its slight anti-HCV activity.

In this study, using MEK1/2 specific inhibitors, we revealed that the MEK–ERK1/2 signaling pathway is involved in the oxidative antiviral mechanism of the anti-HCV nutrients BC, VD2, and PUFAs and the anti-HCV reagents IFN-γ and CsA. Our results suggest that this oxidative induction of the MEK–ERK1/2 signaling pathway could be a novel therapeutic strategy for the eradication of HCV infection. Although oxidants themselves cause liver damage, they may work as anti-HCV factors during therapy in patients with chronic hepatitis C.

In conclusion, this study suggests that the anti-HCV activity of oxidative stress is closely linked to the activation of the MEK–ERK1/2 signaling pathway.

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ORIGINAL ARTICLE

Double-stranded RNA-induced interferon-beta and inflammatory cytokine production modulated by hepatitis C virus serine proteases derived from patients with hepatic diseases

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Abstract We previously demonstrated that hepatitis C virus (HCV) serine protease NS3-4A was unable to cleave TRIF (adaptor protein of Toll-like receptor 3), resulting in a lack of suppression of the TRIF-mediated pathway, whereas NS3-4A cleaved Cardif (adaptor protein of retinoic acid-inducible gene I or melanoma differentiation-associated gene-5), resulting in an interruption of the Cardif-mediated pathway in non-neoplastic human hepatocyte PH5CH8 cells. To elucidate these observations, we examined the cleavage potential of NS3-4A for TRIF in PH5CH8 cells, genome-length HCV RNA-replicating O cells, and HCV-infected cells, and we demonstrated that NS3-4A lacked the ability to cleave endogenous TRIF, regardless of HCV strains derived from patients with different stages of hepatic disease. Furthermore, we demonstrated that inflammatory cytokine production by NF-κB activation via the TRIF-mediated pathway also remained unsuppressed by NS3-4A. These results suggest that the inhibitory effects of NS3-4A on antiviral signaling pathways are limited to the Cardif-mediated pathway in human hepatocytes.

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Introduction

Hepatitis C virus (HCV) infection causes a number of liver diseases such as acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [5, 24, 32, 33]. The progression of liver disease from chronic hepatitis to hepatocellular carcinoma by persistent HCV infection is a serious health problem [37]. In order to elucidate the relationship between the mechanism of persistent HCV infection and liver disease progression, it will be necessary to examine the virus life cycle and develop more effective anti-HCV reagents based on these observations. HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae* [20, 36]. The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues, which is cleaved co- and post-translationally into at least ten proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and a virally encoded serine protease located in the amino-terminal domain of NS3. The serine protease activity of NS3 requires NS4A, a protein that consists of 54 aa residues, to form a stable complex with NS3 [11, 12, 19].

During infection by RNA viruses such as HCV, double-stranded RNA (dsRNA) is produced by viral RNA replication in virus-infected cells, and dsRNA is in turn recognized by Toll-like receptor (TLR) 3, which is expressed on the cell surface or in endosome vesicles [3, 13]. Additionally, dsRNA is recognized by retinoic-acid-inducible gene I (RIG-I) and/or melanoma differentiation-associated gene 5 (MDA5), which are both localized in the cytoplasm [18, 40, 41]. The stimulation of TLR3 by extracellular dsRNA leads to the activation of two signaling pathways that bifurcate at TRIF [17, 34], i.e., interferon

(IFN)-β production is induced via activation of the TRIF/TRAF3/TBK1/IRF-3 pathway, and inflammatory cytokines such as IL-6 or IL-8 are produced via activation of the TRIF/TRAF6/TAK1/NF-κB pathway (see Fig. 5a). On the other hand, the stimulation of RIG-I or MDA5 by intracellular dsRNA may induce both IFN-β and inflammatory cytokine production by similar signaling pathways that bifurcate at Cardif (i.e., the Cardif/TRAF3/TBK1/IRF-3 pathway and the Cardif/TRAF6/TAK1/NF-κB pathway) [39]. IFN-β and the inflammatory cytokines are upregulated to induce an antiviral state in virus-infected cells, and then these production levels return to a steady state in virus-eliminated cells.

Several groups, including ours, have previously reported that the HCV serine protease NS3-4A inhibited intracellular dsRNA-induced IFN-β production via the cleavage of Cardif [6, 29, 30]. The findings of these reports have indicated that Cardif is a key molecule for establishing persistent HCV infection. On the other hand, we also previously demonstrated that NS3-4A (1B-1 and O strains of genotype 1b) was not able to inhibit extracellular dsRNA-induced IFN-β production due to a lack of ability to cleave TRIF [6]; however, in another previous report, it was demonstrated that NS3-4A (N strain of genotype 1b) was able to inhibit IFN-β production via the cleavage of TRIF [27]. These latter results, taken together, suggest that among HCV strains, NS3-4A possesses a range of ability to cleave TRIF. In the present study, NS3-4As derived from patients with different stages of liver disease were used to compare the potential of NS3-4As to inhibit IFN-β production and NF-κB activation via intracellular or extracellular dsRNA.

Materials and methods

Cell culture

Non-neoplastic human hepatocyte PH5CH8 cells susceptible to HCV infection and supportive of HCV replication were cultured as reported previously [16]. Genome-length HCV RNA-replicating O cells [14], their cured Oc cells [14] and other HuH-7-derived RSc cells [25] were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carsbad, CA, USA) supplemented with 10% fetal bovine serum.

Construction of expression vectors

Retroviral vectors pCX4bsr and pCX4pur [1], which contain the resistance gene for blasticidin and puromycin, respectively, were used to construct various expression vectors. pCX4pur/myc-TRIF(P367D), (P367E), (P367D/S368C), or (P367E/S368D) mutants were constructed using PCR mutagenesis with primers containing base alterations. pCX4pur/myc-TRIF [6] was used as the template for PCR mutagenesis. The NS3-4A expression vectors used in this study were constructed using oligonucleotides (Supplementary Table S1 in Electronic Supplementary Material) as described below. RNA was extracted using an ISOGEN extraction kit (Nippon Gene, Toyama, Japan) and serum from 13 HCV-infected patients: three healthy carriers (1B-3, 1B-4, and 1B-5 strains [15]), a patient with acute hepatitis (AH1 strain [21]), and seven patients with chronic hepatitis (CH1, CH3, CH4, CH5, CH6, CH7, and CH8 strains). In addition, serum was obtained from two patients with hepatocellular carcinoma (HCC1 and HCC2 strains [2]). Informed consent was obtained from each patient before the study. The DNA fragments, including the NS3-4A region, were amplified by RT-nested PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) and oligonucleotides for cDNA synthesis, first-round PCR, and second-round PCR (Supplementary Table S1). The obtained DNA fragments were subcloned into the *Xba*I site of pBR322MC [22], and the nucleotide sequences of the NS3-4A regions were determined. The oligonucleotides for the construction of the NS3-4A expression vector were designed from the nucleotide sequences of the NS3-4A regions (Supplementary Table S1). The DNA fragments encoding NS3-4A were amplified by PCR using KOD-plus DNA polymerase and the specifically designed oligonucleotides, and the amplified fragments were cloned into the *Eco*RI and *Not*I sites of pCX4bsr. The nucleotide sequences of the constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Molecular evolutionary analysis

Molecular evolutionary trees were constructed from the aa sequences of the NS3-4A regions using the UPGMA method and the program GENETYX-MAC (Software Development, Tokyo, Japan).

JFH-1 infection experiments

The infection of RSc cells with JFH-1 was performed as described previously [25]. Briefly, 1.0×10^5 RSc cells were seeded onto 6-well plates 24 h before infection. Then, an inoculum of JFH-1 was added to the cells at a multiplicity of infection of 0.1. After 96 h of JFH-1 infection, cell lysates were prepared as described below.

Immunoprecipitation and Western blot analysis

The preparation of cell lysates from PH5CH8 cells stably expressing NS3-4A and two mutants (S1165A and W1528A) [6] was performed as described previously [31].

Cell lysates were subjected to immunoprecipitation using anti-TRIF antibody (Exalpha Biologicals, Maynard, MA, USA) or anti-Cardif antibody (Bethyl Laboratories, Montgomery, TX, USA). Bound proteins were collected from cell lysates using Protein G Sepharose (Amersham) and were subjected to immunoblot analysis. Anti-NS3 antibody (polyclonal R212; a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), and anti-NS4A antibody (C14II3-3; also a generous gift from Dr. Kohara) were used to detect NS3 and NS4A proteins. Anti-myc antibody (PL14; Medical and Biological Laboratories, Nagoya, Japan), anti-EGFP antibody (JL-8; Clontech), and anti-β-actin antibody were used in this study as primary antibodies. Immunocomplexes were detected using a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA, USA).

Luciferase reporter assay

For the dual luciferase assay, we used a firefly luciferase reporter vector, pIFN-β (−125)-Luc [4], containing the IFN-β gene promoter region (−125 to +19) and pNF-κB-Luc (Stratagene). The reporter assay was carried out as described previously [8]. Briefly, a total of 0.3×10^5 cells were seeded onto 24-well plates 24 h before transfection. Then, PH5CH8 cells were transfected with 0.1 μg pIFN-β (−125)-Luc, 0.2 μg NS3-4A expression pCX4bsr vectors (NS3-4A series), and 0.2 ng pRL-CMV (Promega, Madison, WI, USA), used as an internal control reporter, for the measurement of IFN-β promoter activity. For the measurement of NF-κB promoter activity, PH5CH8 cells were transfected with 0.01 μg pNF-κB-Luc, 0.2 μg NS3-4A expression pCX4bsr vectors (NS3-4A series), and 0.02 ng pRL-CMV. The cells were cultured for 48 h, and then a dual luciferase assay was performed according to the manufacturer's protocol (Promega). In some cases, the cells were cultured for 42 h, and then poly(I-C) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was added to the medium for 6 h at 50 μg/ml (M-pIC) before the reporter assay. Three independent triplicate transfection experiments were conducted in order to verify the reproducibility of the results. Relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

RNA interference and real-time LightCycler PCR

siRNA duplexes targeting the coding regions of human TLR3 [31], TRIF (Dharmacon; catalog no. M-012833-00), and luciferase GL2 (Dharmacon), used as a control, were chemically synthesized. Using PH5CH8 cells with drastically decreased TLR3 or TRIF mRNA levels [6], NF-κB promoter activity was measured as described above, and dsRNA-induced inflammatory cytokine production levels were examined by using a primer set for IL-6 or IL-8 [38]. Total cellular RNA extraction and real-time LightCycler PCR were performed as described previously [6, 7].

Results

NS3-4A lacks the ability to cleave endogenous TRIF

We recently reported that NS3-4A serine protease (1B-1 strain of genotype 1b) was unable to cleave TRIF expressed in human PH5CH8 hepatocyte cells [6]. To account for this lack of cleavage ability, we examined the ability of NS3-4A to cleave TRIF mutants converted to a sequence similar to the consensus sequence required for cleavage ability by NS3-4A (Fig. 1a). The results obtained with

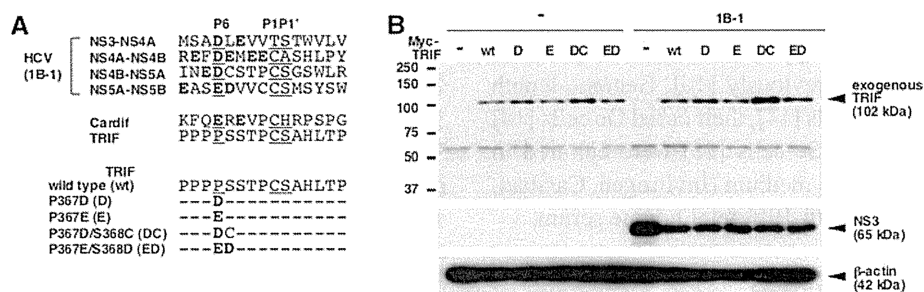

Fig. 1 NS3-4A does not cleave exogenous TRIF or its mutants at the P6 position. **a** The alignment of amino acid sequences surrounding the site cleaved *in trans* or *in cis* by NS3-4A. The consensus sequences required for cleavage by NS3-4A are underlined (P6, P1, and P1′ positions). The amino acids with a negative charge are indicated in boldface type. **b** TRIF mutants with a negative charge at the P6 position also remain uncleaved by NS3-4A. Wild-type TRIF and TRIF mutants (P367D, P367E, P367D/S368C, and P367E/S368D) are indicated as wt, D, E, DC, and ED, respectively. PH5CH8 cells stably expressing NS3-4A (1B-1) were transfected with the pCX4pur vector (as a control, –) or myc-TRIF expression vectors (wild-type strain or mutants). Production of myc-TRIF and NS3 in the cells was analyzed by immunoblot analysis using anti-myc and anti-NS3 antibody, respectively. PH5CH8 cells infected with retrovirus pCX4bsr were used as a control (–). β-actin was used as a control for the amount of protein loaded per lane

PH5CH8 cells revealed that NS3-4A (1B-1 strain) was still unable to cleave the TRIF mutants possessing D or E at the P6 position, even though an acidic aa (D or E) is known to be important for cleavage by NS3-4A [23] (Fig. 1b). Although we demonstrated that exogenously expressed Cardif, but not TRIF, was cleaved by NS3-4A (1B-1 or O strain) [6], no studies had determined whether endogenous Cardif or TRIF can be cleaved by NS3-4A. To clarify these issues, we selected anti-Cardif and anti-TRIF antibodies, and we immunoprecipitated lysates from PH5CH8 cells [in which NS3-4A (1B-1 or O strain) was overexpressed] and lysates from genome-length HCV RNA-replicating O cells [14]. We then performed immunoblot analyses using anti-Cardif, anti-TRIF, or NS3 antibodies. The results revealed that endogenous TRIF was also not cleaved by the NS3-4A expressed in PH5CH8 and O cells, whereas endogenous Cardif (75 kDa) was efficiently cleaved to the expected size (70 kDa) in PH5CH8 and O cells (Fig. 2a). On the other hand, we observed that NS3 interacted with TRIF, but not with Cardif, in both PH5CH8 and O cells (Fig. 2a), as had also been observed previously by another group [10]. In addition, we demonstrated that endogenous Cardif was cleaved by the NS3-4A/W1528A mutant, which lacks RNA helicase activity, but not by the NS3-4A/S1165A mutant, which lacks the serine protease activity (Fig. 2b). Furthermore, we examined whether or not endogenous Cardif and TRIF are cleaved in JFH-1-infected RSc cells. The results revealed that endogenous TRIF was also not cleaved in JFH-1-infected RSc cells, whereas endogenous Cardif was efficiently cleaved in these cells (Fig. 2c). In addition, we also observed that NS3 interacted weakly with TRIF, but not with Cardif, in these cells (Fig. 2c). We therefore concluded that endogenous TRIF is not cleaved by NS3-4A derived from at least the 1B-1 (genotype 1b), O (genotype 1b) or JFH-1 (genotype 2a) strain.

None of the NS3-4As derived from patients with different hepatic disease diagnoses prevented extracellular dsRNA-induced IFN-β transcription via the TRIF-mediated pathway

Although we demonstrated that NS3-4As derived from healthy carriers (1B-1 and O) was unable to suppress IFN-β production induced by the TRIF-mediated pathway [6], there is still no evidence that NS3-4As derived from patients with various hepatic disease diagnoses carry out such suppression. To obtain more evidence, we first amplified NS3-4A-encoding regions by RT-PCR using sera derived from five HCV-positive healthy carriers (including strains 1B-1 and O), one patient with acute hepatitis, seven patients with chronic hepatitis, and two patients with hepatocellular carcinoma; using these samples, we next constructed 15 types of NS3-4A expression vector. Although we observed that all of the NS3-4As expressed in PH5CH8 cells were processed into NS3 and NS4A by an intramolecular reaction, there were some size differences (60–65 kDa) of NS3 and NS4A (Fig. 3a). These size differences may be related to the aa sequence variation, as described below. Sequence analysis of these NS3-4A-encoding regions revealed that the aa sequences involved

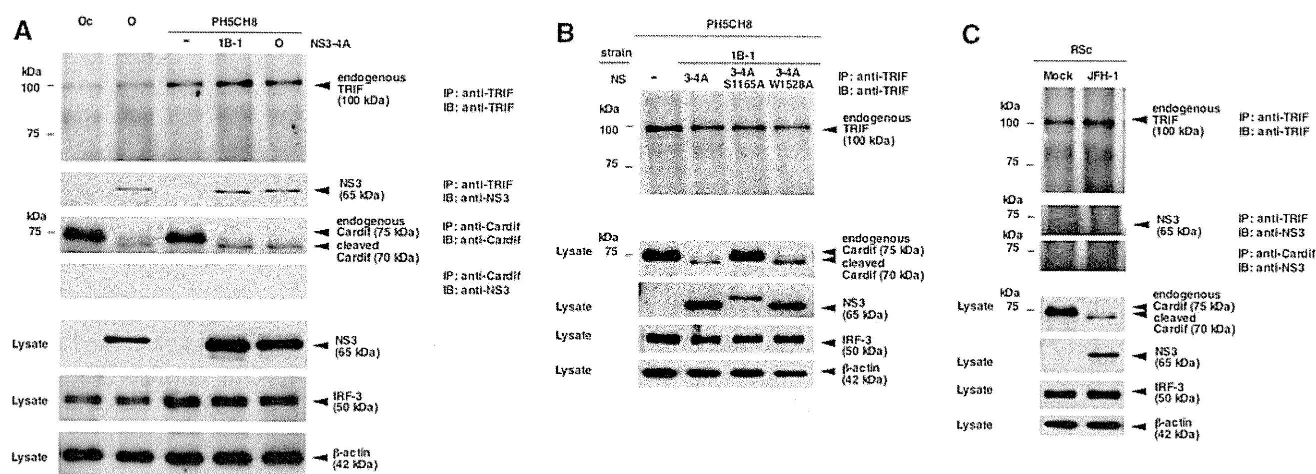

Fig. 2 NS3-4A lack the ability to cleave endogenous TRIF, but not Cardif. **a** Endogenous Cardif, but not TRIF is cleaved by NS3-4As from 1B-1 and O strains. Cell lysates were prepared and subjected to immunoprecipitation using anti-TRIF or anti-Cardif antibody. Bound proteins were collected from cell lysates using Protein G Sepharose and were subjected to immunoblot analysis using anti-TRIF, anti-Cardif, or anti-NS3 antibody. NS3, IRF3, and β-actin in the cell lysates were detected by anti-NS3, anti-IRF3, and anti-β-actin antibody, respectively. **b** Endogenous Cardif is cleaved by the serine protease activity of NS3-4A. The cell lysates were prepared and subjected to immunoprecipitation and followed by immunoblot analysis as described in **a**. **c** Endogenous Cardif, but not TRIF, is cleaved in JFH-1-infected RSc cells. The cell lysates were prepared and subjected to immunoprecipitation followed by immunoblot analysis as described in **a**