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Curcumin inhibits hepatitis C virus replication via suppressing the Akt-SREBP-1 pathway

KyeongJin Kim [a], Kook Hwan Kim [a], Hye Young Kim [a], Hyun Kook Cho [a], Naoya Sakamoto [b], JaeHun Cheong [a,*]

[a] Department of Molecular Biology, College of Natural Sciences, Pusan National University, Busan 609-735, Republic of Korea
[b] Department of Gastroenterology and Hepatology, Tokyo Medical and Dental University, Tokyo 113-8519, Japan

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ABSTRACT

A polyphenolic compound from the curry spice turmeric, curcumin, is known to show anti-viral activity against the influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus. However, it remains to be determined whether curcumin can inhibit the replication of hepatitis C virus (HCV). In this study, we showed that curcumin decreases HCV gene expression via suppression of the Akt-SREBP-1 activation, not by NF-κB pathway. The combination of curcumin and IFNα exerted profound inhibitory effects on HCV replication. Collectively, our results indicate that curcumin can suppress HCV replication in vitro and may be potentially useful as novel anti-HCV reagents.

1. Introduction

The hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an enveloped virus with a single-stranded 9.6 kb RNA genome. HCV infection is characterized by a high rate of progression to fibrosis and chronic hepatitis, resulting in cirrhosis, and ultimately in hepatocellular carcinoma [1]. The best anti-viral therapy presently known involves the combination of pegylated interferon (IFN) alpha and ribavirin, but almost half of all patients manifest no response to exogenous IFNα [2]. Therefore, the development of novel drugs for the safer and more efficient treatment of HCV is urgently required.

Many bioactive polyphenolic compounds have been shown to perform candidate agent functions in chemoprevention and in cancer chemotherapy [3]. Among this class, curcumin (diferuloylmethane) is one of the most widely studied compounds. Curcumin is the major component of the curry spice tumeric (*Curcuma longa* Linn) and can affect the metabolism of cells and organisms in a number of ways, including anti-inflammatory, anti-oxidant, and anti-proliferative properties via the modulation of multiple cellular mechanisms [4,5]. Furthermore, some recent reports have shown that these compounds show anti-viral activity against the influenza virus, adenovirus, coxsackievirus, and the human immunodeficiency virus [6–9]. Also, curcumin has been shown to suppress transcription activation by the host protein AP-1, leading to diminished HTLV-1 and HPV-mediated cellular transformation [10]. However, it remains to be determined whether curcumin can inhibit the gene expression of HCV.

On the basis of our previous knowledge of the regulation of HCV replication and the biological properties of curcumin, we evaluated the effects of curcumin on the intracellular replication of the HCV genome in vitro, using an HCV replicon system. We showed that curcumin at concentrations that do not affect cell viability reduced HCV RNA replication in vitro to a significant degree. Curcumin inhibited a lipogenic transcription factor, sterol regulatory element binding protein-1 (SREBP-1)-induced HCV replication via the PI3K/Akt pathway. Finally, the combination of curcumin and IFNα showed cooperative inhibitory effects on HCV RNA replication. Our results indicate that curcumin may potentially prove useful as a treatment for HCV infection.

2. Materials and methods

2.1. Plasmid constructs

pEMCV/IRES-Rluc was utilized as a control for the analysis of translation efficiency mediated by an encephalomyocarditis virus internal ribosome entry site (EMCV-IRES) which mediates the translation of the HCV non-structure gene of replicon constructs, Huh7/Rep-Feo [11]. pCIneo-Rluc-IRES-Fluc was constructed in

* Corresponding author. Fax: +82 51 513 9258.
E-mail addresses: molecule85@pusan.ac.kr, jhcheong2@lycos.co.kr (J. Cheong).

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order to evaluate HCV internal ribosome entry site (IRES)-mediated translation efficiency [12]. The plasmid expressed a bicistronic RNA, in which Rluc was translated in a cap-dependent manner and Fluc was translated via HCV IRES-mediated initiation.

2.2. Cell cultures and treatments

Huh7 cells expressing the HCV replicon (Huh7/Rep-Feo) were maintained in DMEM supplemented with 10% FBS containing 500 μg/ml of G418 (Calbiochem). Huh7/Rep-Feo and Huh7 cells were plated at 70–80% confluence and treated with various concentrations of curcumin or vehicle controls and incubated for 24 h at 37 °C. The concentration ranges of 5–15 mM curcumin (obtained from Sigma) were tested. Control vehicle treatment (DMSO) was equivalent to the highest concentrations in the dose range experiments for each of the tested drugs.

2.3. HCV replicons

An HCV subgenomic replicon plasmid, pRep-Feo (Fig. 1A), was derived from pRep-Neo (originally referred to as pHCVIbneo-delS) [13]. Replicon RNA was synthesized in vitro using T7-RNA polymerase (Promega, Madison, WI) and transfected into the Huh7 cells via electroporation. After culturing in the presence of G418, the cell lines stably expressing the replicons were established and designated Huh7/Rep-Feo.

2.4. Transient transfection and luciferase reporter assay

Plasmid transfection was conducted using PolyFect (QIAGEN) in accordance with the manufacturer's instructions. The pcDNA3.1 empty vector was added to the transfections in order to achieve the same total amount of plasmid DNA per transfection. The cells were lysed in cell culture lysis buffer (Promega). The luciferase activity was evaluated using an analytical luminescence luminometer in accordance with the manufacturer's instructions.

2.5. MTT assay

For cell viability assay, Huh7/Rep-Feo cells were seeded in a 24-well tissue culture plate and incubated for 24 h. Cells were treated with curcumin or Bay11-7082. After 24 h, MTT (3-(4,5-dimethylthiozol-2-ly)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/ml) was added to each well. After incubation for 2 h at 37 °C, formazan crystals in viable cells were soluble in 200 μl of DMSO. The soluble formazan product was spectrophotometrically quantified using an ELISA leader at 570 nm.

2.6. siRNA design and siRNA transfection

RNA oligonucleotides were synthesized by Bioneer (Daejeon, Republic of Korea). The sequences of siRNA targeting human p65 were sense, 5′-GAU UGA GGA GAA ACG UAA A-3′ and antisense, 5′-UUU ACG UUU CUC CUC AAU C-3′. The sequence of siRNA targeting human SREBP-1 were sense, 5′-UGA GUG GCG GAA CCA UCU U-3′ and antisense, 5′-AAG AUG GUU CCG CCA CUC A-3′. The scramble control siRNA sequences were sense, 5′-CCU ACG CCA CCA AUU UCG U-3′ and antisense, 5′-ACG AAA UUG GUG GCG UAG G-3′. The cells were transfected with siRNA using HiPerFect (QIAGEN) according to the instructions of the manufacturer.

2.7. RT-PCR analysis

Total RNA from the curcumin-treated Huh7/Feo cells was prepared using TRIzol reagent (Invitrogen) by following the manufacturer's instructions. cDNA was used as a template for real-time PCR using gene-specific primers: NS3, 5′-TCG TGG CAA CAG ACG CTC TAA TGA-3′ (forward) and 5′-AGA ACT CCA G AT GGT CCT GGC AAA-3′ (reverse); NS5A, 5′-TAG CAG TGC TCA CTT CCA TGC TCA-3′ (forward) and 5′-AGG ATC TCC GCC GCA ATG GAT ATT-3′ (reverse); β-actin, 5′-GAC TAC CTC ATG AAG ATC-3′ (forward), 5′-GAT CCA CAT CTG CTG GAA-3′ (reverse).

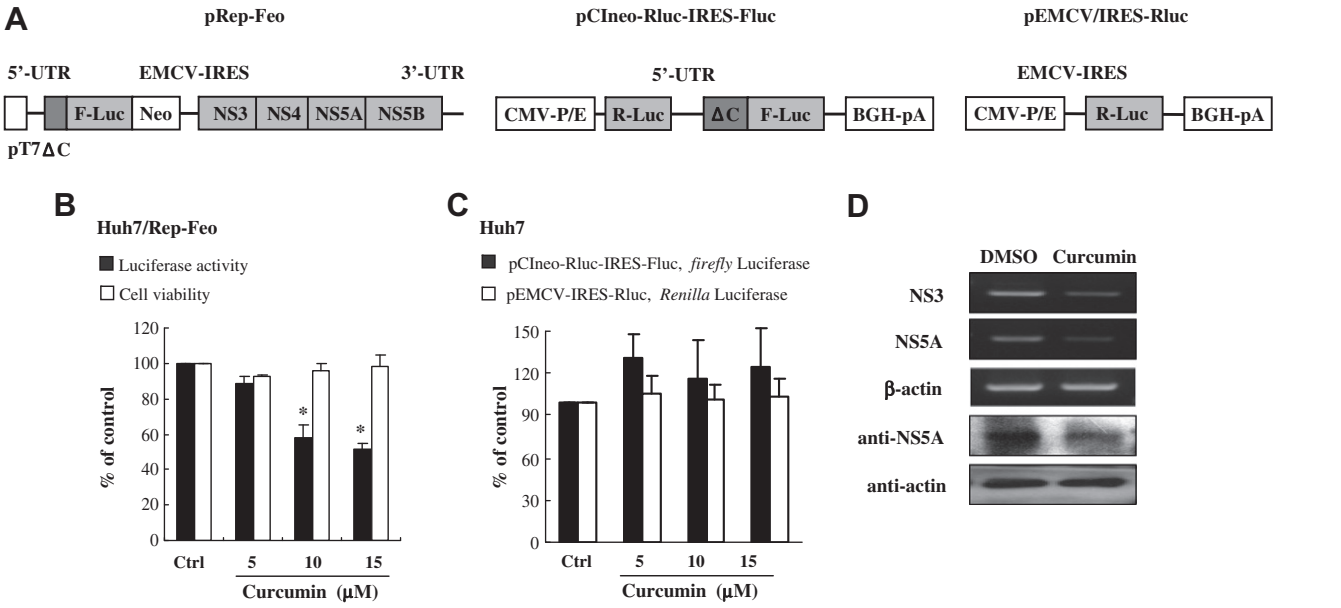

Fig. 1. Curcumin suppresses HCV RNA replication. (A) Structures of the hepatitis C virus (HCV) replicon, pEMCV/IRES-Rluc, and pCIneo-Rluc-IRES-Fluc plasmids. (B) The effects of curcumin on Huh7/Rep-Feo cells on luciferase activity and MTT assays. For the luciferase assay, the Huh7/Rep-Feo cells were cultured in the presence of the indicated curcumin concentrations, and the luciferase activity was determined at 24 h of treatment. The values are expressed as the means ± S.D. for at least three independent experiments. *$P < 0.05$ compared with control. (C) The luciferase activity effects on HCV IRES-mediated translation and EMCV/IRES-mediated translation by curcumin. The data are expressed as the means ± S.D. ($n = 3$). (D) The inhibitory effect of HCV RNA and protein level by curcumin. Huh7/Rep-Feo cells were cultured for 24 h in the presence of 15 μM curcumin. Total RNA was extracted from the cells, and the levels of NS3 and NS5A mRNA were determined by RT-PCR and β-actin expression is shown as an mRNA-loading control. Total protein extracts were blotted with anti-NS5A antibody and actin expression is shown as a protein-loading control. Three independent experiments were reproduced.

2.8. Nuclear/cytosolic fractions analysis

Cells were lysed in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM DTT, 0.5% NP-40, 1 mM PMSF, protease inhibitors) and incubated for 10 min on ice. The supernatants (cytosolic lysates) were collected by centrifugation (3300×*g*) at 4 °C for 5 min. The nuclear pellets were then washed with ice-cold PBS to avoid contamination of cytosolic proteins and lysed in buffer B (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 25% glycerol, 420 mM NaCl, 0.2 mM EDTA, 1 mM DTT, 0.5% NP-40, 1 mM PMSF, protease inhibitors). After incubation on ice for 25 min, the supernatants (nuclear lysates) were collected by centrifugation (13 000×*g*) at 4 °C for 5 min.

2.9. Statistical analysis

Statistical analyses were carried out by unpaired or paired *t* test as appropriate. All data are reported as means ± S.D. *P* value of <0.05 was considered significant.

3. Results

3.1. Curcumin suppresses HCV RNA replication

In order to evaluate the effects of curcumin on the intracellular replication of the HCV genome, Huh7/Rep-Feo cells were treated with various concentrations of curcumin. The luciferase activity of the Huh7/Rep-Feo cells demonstrated that the replication of the HCV replicon was suppressed by curcumin. The MTT assay showed no effects on cell viability at various concentrations of these compounds (Fig. 1B). Moreover, the efficiency of EMCV-IRES-mediated translation was not affected by curcumin (Fig. 1C). These data indicate that the inhibitory effects on HCV replication exerted by curcumin are not attributable to cytotoxicity or to an artificial effect on the EMCV-IRES, which directly translates the HCV non-structure protein of the replicon. We then attempted to determine whether these effects of curcumin on the replication of the HCV replicon are involved in HCV IRES-dependent translation. We determined that curcumin gives no effects on the activity of *Firefly* luciferase in Huh7 cells that were transiently transfected with the pCIneo-Rluc-IRES-Fluc reporter (Fig. 1C). Taken together, these results indicate that curcumin suppresses HCV replication in vitro and that these effects are not involved in cell viability, EMCV-IRES-mediated translation, or HCV IRES-dependent translation.

In an effort to confirm these inhibitory effects on the HCV replicon by curcumin, we attempted to determine whether curcumin affects the HCV RNA and protein level. HCV replicon RNA level was detected by RT-PCR by using primers specific to NS3 and NS5A and protein level was assayed by Western blots with the anti-NS5A antibody. As shown in Fig. 1D, curcumin-treated cells expressed lower levels of the HCV replicon RNA as compared with the control vehicle-treated Huh7/Rep-Feo cells. Also, we showed that curcumin decreases the protein level of HCV NS5A, translated from the

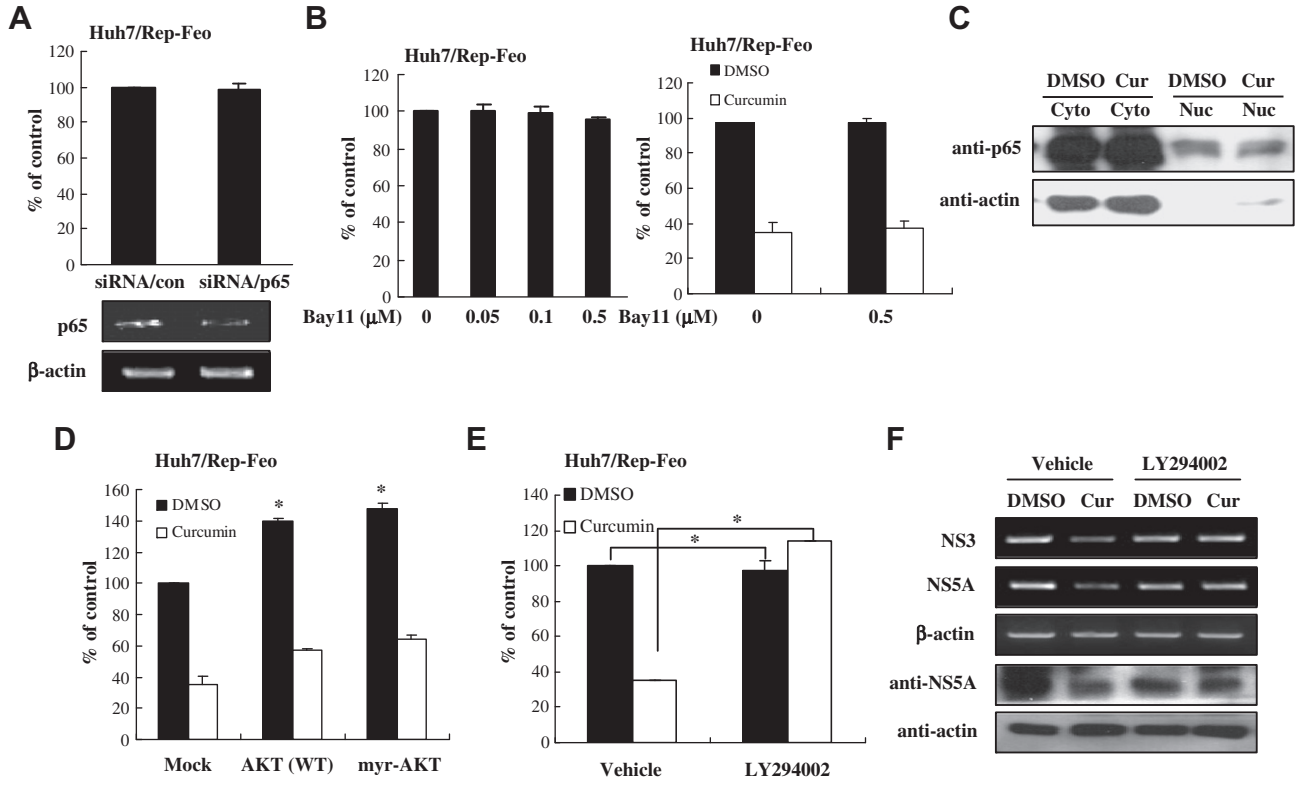

Fig. 2. Curcumin suppressed HCV replication via the PI3 K/Akt pathway. (A) The effects of p65 siRNA on HCV replication. Huh7/Rep-Feo cells were seeded in 6-well culture plates and transfected with scramble control siRNA and p65 siRNA and luciferase activities are measured ($n = 3$). (B) The effects of Bay11-7082 on HCV replication. Huh7/Rep-Feo cells were seeded in 24-well culture plates and treated with the Bay11-7082. The effects of curcumin on Huh7/Rep-Feo cells were identified by MTT assays (left) and luciferase activity (right) ($n = 3$). (C) The effects of curcumin on the nuclear localization of p65 protein. Huh7/Rep-Feo cells were seeded in 6-well culture plates and treated with curcumin (15 μM). The cyto or nuc indicates the cytosolic or nuclear extracts. (D) The effect of Akt on HCV RNA replication. Huh7/Rep-Feo cells were transfected with the plasmid for Akt (WT) or myr-Akt and cells were treated for 24 h with 15 μM curcumin or vehicle (DMSO). Luciferase activity was measured and the values are expressed as the means ± S.D. for at least three independent experiments. (E and F) The effect of LY249002 on curcumin-suppressed HCV RNA replication. Huh7/Rep-Feo cells were pretreated with 25 μM LY294002 for 2 h, and then incubated further in the absence or presence of 15 μM curcumin for 24 h. Luciferase activities were measured ($n = 3$) (E). NS3 or NS5A mRNA levels were detected using RT-PCR and protein levels of NS5A were detected by Western blotting ($n = 2$) (F).

HCV replicon (Fig. 1D). These data correlate well with the luciferase activity of Huh7/Rep-Feo cells.

3.2. Curcumin inhibits HCV replication via the PI3K/Ak pathway, not NF-κB pathway

In an effort to gain insight into the molecular mechanism by which curcumin suppresses HCV replication, we then analyzed the signaling pathways involved in curcumin-inhibited HCV replication. Previously, it was suggested that the activation of the NF-κB pathway is involved in the increase of HCV replication [14,15]. To evaluate the involvement of NF-κB pathway on the curcumin-mediated inhibition of HCV replication, p65 (of NF-κB) siRNA and Bay11-7082 (a specific inhibitor of NF-κB pathway) were exploited. However, in contrast to our expectations, p65 siRNA and Bay11-7082 had no effect on HCV replication (Fig. 2A and B). Furthermore, curcumin also had no effect on the nuclear localization of p65 (Fig. 2C). These results show that PDTC and curcumin exert a synergic-inhibitory effect on HCV replication via NF-κB-independent pathway.

We next explored other potential pathways for HCV replication by curcumin. Several signaling pathways and transcription factors including AP-1, mitogen-activated protein kinases (MAPKs), and cell cycle machinery have been suggested as the targets of curcumin. We examined the effect of several pathways on HCV replication using treatments of specific kinase inhibitors or transfection of plasmid for specific kinases, including ERK, JNK, p38, Akt, and PKA. Among many signaling pathways, transfection of plasmid for wild type (WT)-Akt or constitutive active (myr)-Akt significantly augmented the luciferase activities of HCV replicon (Fig. 2D). Also, LY294002, a specific inhibitor of the PI3K/Akt pathway, inhibited curcumin-suppressed activity of HCV replicon (Fig. 2E). To confirm these effects on the HCV replicon induced by curcumin, we examined the HCV RNA and protein levels. As shown in Fig. 2F, LY294002-treated cells restrained the RNA and protein levels of HCV replicon inhibited by curcumin. Taken together, these results suggest that curcumin suppresses HCV replicon expression via the PI3K/Akt pathway.

3.3. SREBP-1 increases the HCV replicon expression via the PI3K/Akt pathway

To identify the downstream target of the PI3K/Akt pathway on HCV RNA replication, we tested several transcription factors regulated by the PI3K/Akt pathway. Among them, two SREBP-1 isoforms, SREBP-1a and SREBP-1c, induced the luciferase activity of the Huh7/Rep-Feo cells. Especially, SREBP-1a showed a profound effect on HCV replication (Fig. 3A). Previously, it has been suggested that the activation of Akt was able to increase the SREBP-1 gene expression [16]. Also, in an effort to determine whether SREBP-1 performs a function in HCV RNA replication, we attempted to knockdown SREBP-1 expression using siRNA for SREBP-1. As shown in Fig. 3B, siRNA/SREBP-1-transfected cells decreased the luciferase activity of the Huh7/Rep-Feo cells.

In an effort to confirm these effects on the HCV replicon exerted by SREBP-1, we attempted to determine whether SREBP-1 affects the HCV RNA and protein level. As shown in Fig. 3C, SREBP-1a-transfected cells enhanced the levels of the HCV replicon RNA as

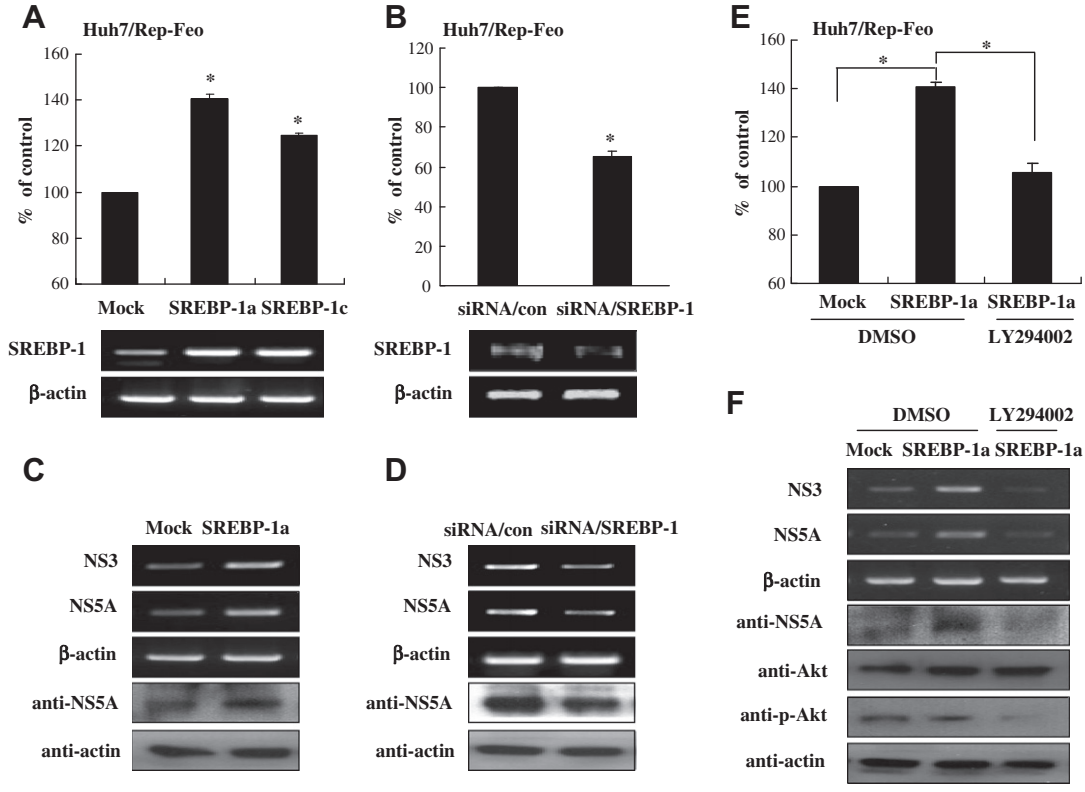

Fig. 3. SREBP-1 increases the HCV replicon expression via the PI3 K/Akt pathway. (A and B) The effects of SREBP-1 on HCV replication. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a or SREBP-1c and luciferase activities were measured ($n = 3$) (A). Huh7/Rep-Feo cells were transfected with scramble control siRNA and SREBP-1 siRNA and luciferase activities were measured ($n = 2$) (B). *$P < 0.05$ compared with control. (C and D) The effects of SREBP-1 on HCV RNA and protein levels. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a (C) or siRNA for SREBP-1 (D). NS3 or NS5A mRNA levels were detected using RT-PCR and protein levels of NS5A were detected by Western blotting ($n = 2$). (E and F) The effect of LY294002 on SREBP-1-induced HCV replication. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a and then incubated further in the absence or presence of 25 μM LY294002 for 24 h. Luciferase activities were measured ($n = 3$) (E) and NS3 or NS5A mRNA and NS5A protein levels were determined using RT-PCR or Western blotting ($n = 2$) (F).

compared with the control transfected-Huh7/Rep-Feo cells. Also, we showed that the knockdown of SREBP-1 expression decreases the RNA and protein level of HCV replicon (Fig. 3D). Furthermore, SREBP-1a-transfected cells did not induce the HCV RNA replication in the presence of LY294002 (Fig. 3E and F). Taken together, SREBP-1 can increase the HCV replication via the PI3 K/Akt pathway.

3.4. Curcumin suppresses the SREBP-1-induced HCV replication

In order to determine whether SREBP-1-increased HCV RNA replication is inhibited by curcumin, Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a in the absence or presence of curcumin treatment. As shown in Fig. 4A, the luciferase activities of Huh7/Rep-Feo cells were inhibited by curcumin in the SREBP-1a transfection. Also, these effects were confirmed on the RNA and protein levels of HCV replicon (Fig. 4B).

3.5. Curcumin and IFNα have synergistic inhibitory effects on HCV replication

Finally, we examined whether curcumin can affect IFNα-based inhibition of HCV. IFNα has been shown to exert inhibitory effects on HCV replication. In order to determine whether curcumin could affect the IFNα-mediated inhibitory effect on HCV replicon, Huh7/Rep-Feo cells were treated with a combination of IFNα and curcumin. As shown in Fig. 5A and B, curcumin significantly augmented the IFNα-mediated inhibition of HCV replication. Thus, these findings demonstrate that cotreatment with curcumin was more effective than treatment with IFNα alone.

4. Discussion

There is abundant evidence indicating that dietary phytochemicals, including epigallocatechin gallate (EGCG), curcumin, resveratrol, and genistein, show anti-viral effects in a variety of virus types [6–9,17,18]. However, it remains to be determined whether dietary phytochemicals have anti-viral activity in cases of Hepatitis C. It was reported that curcumin exhibits anti-oxidant activity by reducing the generation of reactive oxygen species (ROS). Although it has been known that oxidative stress decreases HCV replication [19], a recent study showed that ROS is able to increase the replication of HCV [20]. According to this study, anti-oxidant pyrrolidine dithiocarbamate (PDTC) treatment decreased the expression of HCV RNA in Huh7 cells expressing HCV subgenomic replicons. In the present study, we observed that PDTC reduces the activity of HCV luciferase replicons in Huh7/Rep-Feo cells (data not shown). In addition, curcumin suppressed the HCV replication in cooperation with PDTC. Although curcumin had no effect on the

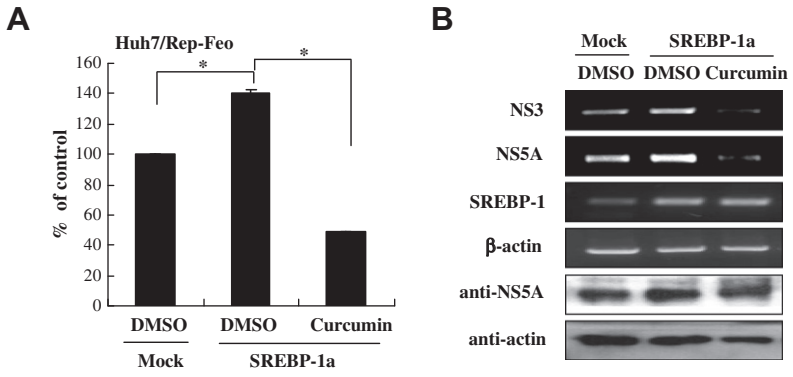

Fig. 4. Curcumin suppresses the SREBP-1-induced HCV replication. (A and B) The effects of SREBP-1 on curcumin-treated HCV replicon. Huh7/Rep-Feo cells were transfected with the plasmid for SREBP-1a and then incubated further with or without 15 μM curcumin for 24 h. Luciferase activities were measured ($n = 3$) (A) and NS3 or NS5A mRNA and NS5A protein levels were determined using RT-PCR or Western blotting ($n = 2$) (B).

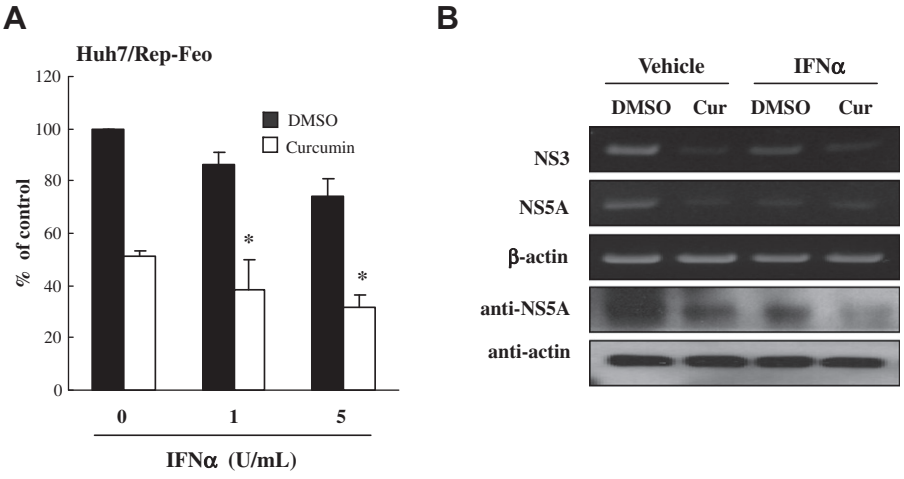

Fig. 5. curcumin and IFNα have synergistic inhibitory effects on HCV replication. (A) Huh7/Rep-Feo cells were cultured in the presence or absence of curcumin (15 μM) and IFNα, (0, 1, 5 U/ml). After 24 h of treatment, the cell lysates were obtained and luciferase activity was measured. The data shown are expressed as the means ± S.D. ($n = 3$). *$P < 0.05$ compared with curcumin alone or IFNα alone. (B) Huh7/Rep-Feo cells were incubated in the presence or absence of curcumin (15 μM) and IFNα (5 U/ml). After 24 h, NS3 or NS5A mRNA levels or NS5A protein levels were detected using RT-PCR or Western blotting ($n = 2$).

gene expression change of anti-oxidant enzymes, such as, Cu/Zn-superoxide dismutase (Cu/Zn-SOD), Mn-superoxide dismutase (Mn-SOD) (data not shown), because curcumin exert the function as anti-oxidant through various pathways, we could not rule out the possibility that curcumin decreases HCV replication as anti-oxidant.

Some reports were demonstrated that despite its beneficial, direct anti-tumor actions, curcumin (and potentially other natural products) may adversely modulate the cellular response to clinically relevant cytokines or cytotoxic activities against a variety of tumor targets [21,22]. Therefore, curcumin experimentally evaluated has been found to be non-toxic or to have effective doses far below its toxic doses in the cancer therapy or blood lipid profile [23,24]. It is necessary to perform experiments to identify the adequate concentrations of curcumin in relation to inhibition of HCV replication in vivo.

Curcumin is considered to be a potentially important chemopreventive agent against a variety of cancers, including liver cancer [25]. Recently, it has been reported that curcumin inhibits the development of human hepatocellular carcinoma [26,27]. In this report, we demonstrated that curcumin inhibits HCV replicon expression via the PI3K/Akt-SREBP-1 pathway. Taken together with previous findings, although hepatocellular carcinoma is the outcome of complicated processes by various genetic factors and environmental factors, our current data suggests the possibility that curcumin may hinder the development of liver cancer via the inhibition of HCV replication in HCV-induced hepatocellular carcinoma.

Acknowledgments

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