Antiviral Research 96 (2012) 260–268

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Interference with ERK and STAT signaling pathways and inhibition of hepatitis C virus replication by ribavirin

Lan-Juan Zhao [a,1], Wen Wang [a,1], Yuan Liu [a,b], Hao Ren [a], Zhong-Tian Qi [a,*]

[a] Department of Microbiology, Shanghai Key Laboratory of Medical Biodefense, Second Military Medical University, Shanghai 200433, China
[b] Department of Clinical Laboratory, Military General Hospital of Chengdu, Sichuan 610083, China

ARTICLE INFO

Article history:
Received 11 June 2012
Revised 4 September 2012
Accepted 4 September 2012
Available online 15 September 2012

Keywords:
Ribavirin
Hepatitis C virus
ERK
STAT

ABSTRACT

Ribavirin in combination with interferon (IFN)-α is the approved treatment for hepatitis C virus (HCV) infection. Interference of ribavirin with signaling events is involved in its biological activities. However, little is known of signaling pathways induced by ribavirin following HCV infection. In human hepatoma cells, effects of ribavirin on ERK and signal transducers and activators of transcription (STAT) pathways, HCV replication, and antiviral gene expression were evaluated before and after cell culture-derived HCV infection. Ribavirin reduced phosphorylation of Raf, MEK, ERK, Tyk2, and STAT1, but selectively increased STAT3 phosphorylation. IFN-α synergistically regulated ERK and STAT3 phosphorylation with ribavirin, and up-regulated expression and phosphorylation of STAT1. Ribavirin dose-dependently decreased HCV RNA replication and HCV protein expression, with slight induction of IFN regulatory factor 9 and IFN-stimulated gene 15. Ribavirin and IFN-α exerted a synergetic inhibitory effect on HCV. ERK and STAT pathways were down-regulated by ribavirin following HCV infection. These results suggest that ribavirin may mediate anti-HCV activity through interference with ERK and STAT pathways.

1. Introduction

Ribavirin, a guanosine analogue, possesses antiviral activity against numerous RNA and DNA viruses (Feld and Hoofnagle, 2005). Hepatitis C virus (HCV), a positive single-strand RNA virus, is a major pathogen of human liver disorders, including chronic hepatitis C, cirrhosis, and hepatocellular carcinoma (Moradpour et al., 2007). Ribavirin for HCV infection only leads to improvements in patient serum aminotransferase levels but not in HCV clearance (Hoofnagle et al., 1996), while ribavirin combined with interferon (IFN)-α exhibits marked improvements in the treatment response (Fried et al., 2002; McHutchison and Poynard, 1999). The combination of ribavirin with pegylated IFN-α is approved to be the standard treatment for HCV infection. Recently, novel treatment options are focused on new HCV specific inhibitors against NS3/4A and NS5B polymerases (Avolio and Summa, 2010). Multiple mechanisms underlying ribavirin's anti-HCV activity have been proposed, including direct inhibition of HCV replication, inosine monophosphate dehydrogenase inhibition, mutagenesis and error catastrophe, and immunomodulation (Feld and Hoofnagle, 2005). However, how ribavirin influences cellular signaling events following HCV infection and whether ribavirin exerts HCV inhibition through interference with signaling pathways have remained elusive.

Ribavirin is shown to interfere with some signaling pathways and such interference correlates with its immunomodulatory, antitumor, and even antiviral activities. As a novel immunomodulatory mechanism, down-regulation of interleukin-15 receptors signaling by ribavirin is responsible for suppression of natural killer cell function (Ogbomo et al., 2010). Ribavirin impairs eukaryotic translation initiation factor eIF4E dependent Akt signaling and thereby inhibits apoptotic rescue (Tan et al., 2008). Ribavirin inhibits leukemic cell proliferation through the modulation of key molecular and metabolic pathways (Kökény et al., 2009), and decreases hepatic stellate cell proliferation by decreasing p-c-Jun levels (Khan et al., 2008). Activation of mammalian target of rapamycin and p53 by ribavirin is a cellular mechanism involving the enhancement of IFN signaling (Su et al., 2009). In mumps virus-infected cells, suppression of interferon-stimulated genes (ISGs) expression is restored with ribavirin treatment through the IFN pathway (Fujii et al., 1999). In respiratory syncytial virus-infected cells, ribavirin potentiates IFN-stimulated response element signaling to enhance the expression of ISGs (Zhang et al., 2003). In patients with chronic hepatitis C, treatment with pegylated IFN and ribavirin leads to activation of the signaling molecules involved in immune cell regulation and IFN signaling (Younossi et al., 2011). Therefore, signaling events induced by ribavirin may play an active role in its biological activities.

* Corresponding author. Address: Department of Microbiology, Second Military Medical University, 800 Xiang-Yin Road, Shanghai 200433, China. Tel./fax: +86 2181870988.
E-mail address: qizt@smmu.edu.cn (Z.-T. Qi).
[1] These authors contributed equally to this work.

0166-3542/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.antiviral.2012.09.002

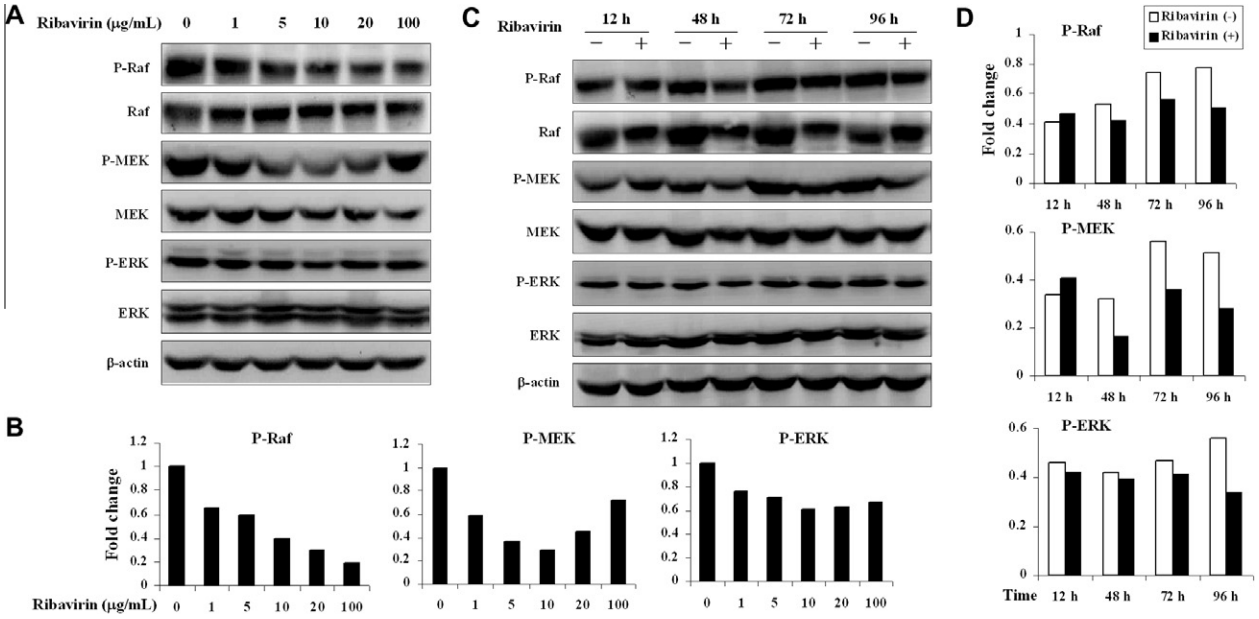

Fig. 1. Huh7.5.1 was cultured with ribavirin at the indicated doses for the varying time periods. (A) Cells were treated for 24 h with the increasing doses of ribavirin, and total and phosphorylated (P-) Raf, MEK, and ERK kinases were analyzed by Western blotting. β-actin is shown as a loading control. Results are representative of at least three experiments. (B) The signals for phosphorylated Raf, MEK, and ERK from the shown experiment above were quantified using GeneTools software. Data are expressed as fold change of the levels of phosphorylated kinases over the levels of untreated control after being normalized to the levels of β-actin. (C) Cells were treated with (+) and without (−) 20 μg/mL ribavirin for the varying lengths of time, and Raf, MEK, and ERK were analyzed by Western blotting. Results are representative of three experiments. Levels of phosphorylated Raf, MEK, and ERK were quantified. Data are expressed as fold change of the levels of phosphorylated kinases after being normalized to the levels of β-actin (D).

Mitogen-activated protein kinases (MAPKs), composed of ERK1/2, p38MAPK, SAPK/JNK, and ERK5 subfamilies, have become important therapeutic targets for control of a wide range of human diseases (Johnson and Lapadat, 2002). Signal transducers and activators of transcription (STAT) family has seven members, including STATs 1, 2, 3, 4, 5a, 5b, and 6, and the members have been focused because of their distinct functions in IFN signaling (Schindler and Levy, 2007). Although regulation of the MAPK and

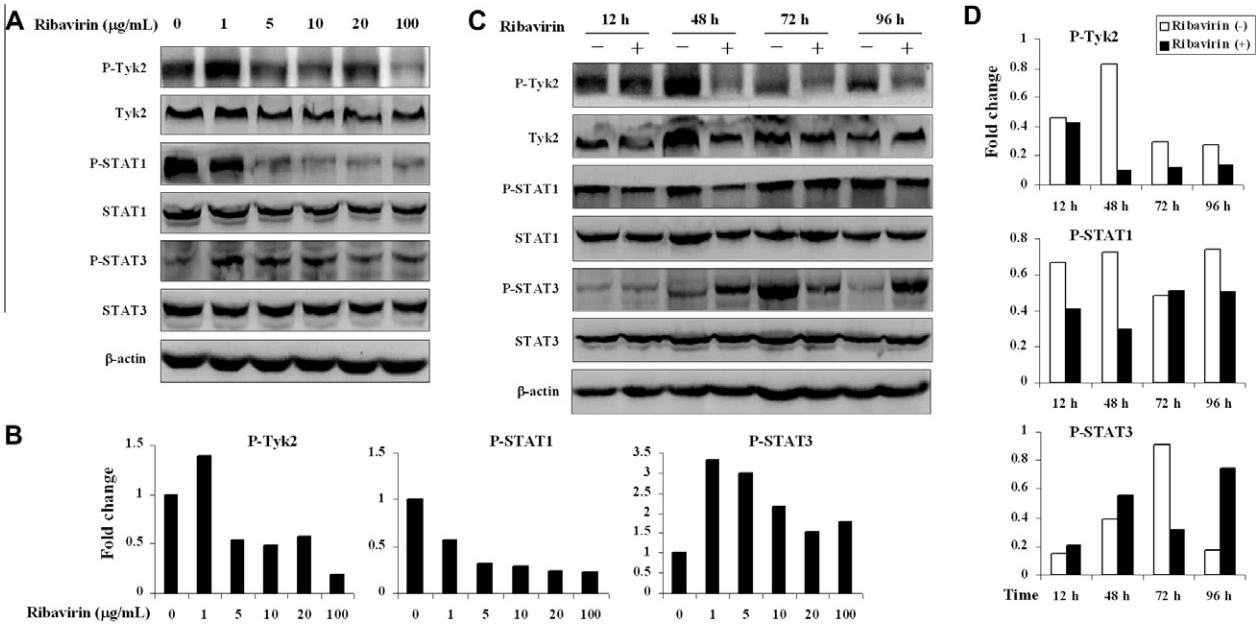

Fig. 2. Huh7.5.1 was cultured with ribavirin at the indicated doses for the varying time periods. (A) Cells were treated for 24 h with the indicated doses of ribavirin, and total and phosphorylated (P-) Tyk2, STAT1, and STAT3 were analyzed by Western blotting. β-actin is shown as a loading control. Results are representative of at least three experiments. (B) The signals for phosphorylated Tyk2, STAT1, and STAT3 from the shown experiment above were quantified. Data are expressed as fold change of the levels of phosphorylated Tyk2, STAT1, and STAT3 over the levels of untreated control after being normalized to the levels of β-actin. (C) Cells were treated with (+) and without (−) 20 μg/mL ribavirin for the varying time lengths, and Tyk2, STAT1, and STAT3 were determined by Western blotting. Results are representative of three experiments. Levels of phosphorylated Tyk2, STAT1, and STAT3 were quantified. Data are expressed as fold change of the levels of phosphorylated Tyk2, STAT1, and STAT3 after being normalized to the levels of β-actin (D).

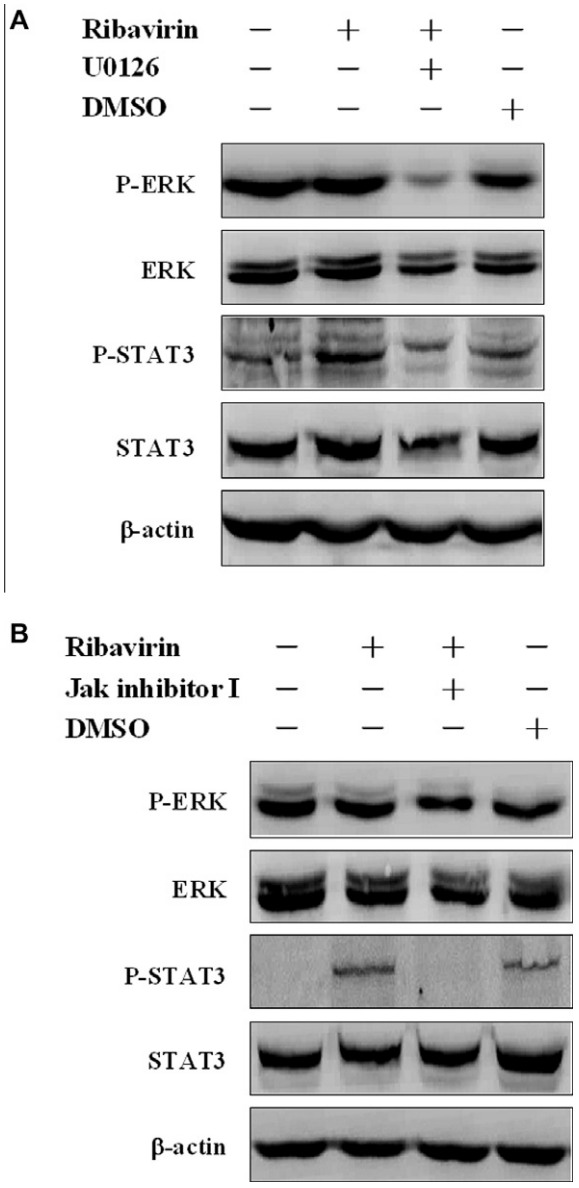

Fig. 3. Huh7.5.1 was pretreated with kinase inhibitors at a concentration of 1 μM for 1 h prior to addition of 20 μg/mL ribavirin for 24 h. Total and phosphorylated (P-) ERK and STAT3 in cells with (+) and without (−) U0126 pretreatment (A) or Jak Inhibitor I pretreatment (B) were analyzed by Western blotting. β-actin is shown as a loading control. Results are representative of four experiments.

STAT pathways by IFN is associated with the anti-HCV effect of IFN, few studies have looked at signaling pathways induced by ribavirin following HCV infection and interference of IFN-α with ribavirin-induced pathways, which might be involved in the anti-HCV effect of ribavirin in combination with IFN-α. With the development of cell culture-derived HCV (HCVcc), it became possible to investigate signaling events induced by ribavirin following HCVcc infection in vitro.

We wondered how ribavirin influenced cellular signal transduction and whether such influence correlated with the anti-HCV activity. In human hepatoma Huh7.5.1 cells, we examined effects of ribavirin on signal transduction of ERK and STAT pathways, HCV replication, and antiviral gene expression. Our results showed that both ERK and STAT pathways were down-regulated by ribavirin following HCV infection. Ribavirin exhibited inhibition of HCV replication, and synergistic inhibition was observed when in combination with IFN-α. Additionally, levels of interferon regulatory factor 9 (IRF9) and ISG15 mRNA were slightly increased by ribavirin.

2. Materials and methods

2.1. Reagents and antibodies

Ribavirin and Jak Inhibitor I were from Merck (Calbiochem, Darmstadt, Germany). Recombinant human IFN-α 2a was obtained from PBL Interferon Source (Piscataway, NJ, USA). MEK inhibitor U0126 was purchased from Cell Signaling Technology (Beverly, MA, USA). Bovine serum albumin (BSA) and dimethylsulfoxide (DMSO) were obtained from Sigma (St Louis, MO, USA). Trizol reagent was from Invitrogen (Carlsbad, CA, USA). CellTiter 96®AQueous One Solution Cell Proliferation Assay and M-MLV reverse transcriptase were from Promega (Madison, WI, USA). SYBR Green PCR kit was from Qiagen (Valencia, CA, USA).

Rabbit polyclonal antibodies against Tyk2, STAT1, STAT3, c-Raf, MEK, ERK, phospho-Tyk2 (Tyr1054/1055), phospho-STAT1 (Tyr701), phospho-STAT3 (Tyr705), phospho-c-Raf (Ser338), phospho-MEK (Ser217/221), phospho-ERK (Thr202/Tyr204), and β-actin were purchased from Cell Signaling Technology. HCV NS3 mouse monoclonal antibody (mAb) was a product of Abcam (Cambridge, UK). HCV E2 mouse mAb was generously provided by Michael Houghton (Chiron Corporation, Emeryville, CA, USA). Horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG was from Invitrogen (Camarillo, CA, USA) and Bio-Rad (Hercules, CA, USA).

2.2. Cell and virus

Human hepatoma Huh7.5.1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. All products used for cell culture were from HyClone (Logan, UT, USA). Unless otherwise indicated, cells were cultured in the above medium. J6/JFH1 HCVcc (HCV genotype 2a) was produced by transfection of FL-J6/JFH1 (kind gift from Charles M. Rice, The Rockefeller University, NY, USA) into Huh7.5.1 cells (Lindenbach et al., 2005). HCVcc stocks, 10^5 focus-forming units/mL, were generated by collection of culture medium from Huh7.5.1 cells infected with HCVcc as described (Tong et al., 2011; Zhong et al., 2005).

2.3. Ribavirin treatment

Ribavirin was dissolved in phosphate-buffered saline (PBS) at a concentration of 10 mg/mL. To assess dose-effect of ribavirin treatment, cells seeded in 6-well plates the day before were washed twice with PBS and maintained in the medium containing increasing amounts of ribavirin (0, 1, 5, 10, 20, and 100 μg/mL) for 24 h at 37 °C. To assess time course of ribavirin treatment, cells were maintained in the media with and without 20 μg/mL ribavirin for 12, 48, 72, and 96 h, respectively. After the above treatment, cells were harvested and lysed for Western blot analysis.

2.4. IFN-α treatment

IFN-α (5.4×10^7 U/mL) was diluted in 0.1% BSA for appropriate dilutions. Cells seeded in 6-well plates the day before were washed twice with PBS and maintained in the medium containing 100 U/mL IFN-α or in combination with ribavirin (20 and 100 μg/mL) for 48 and 72 h, respectively. Cell lysates were prepared for Western blotting.

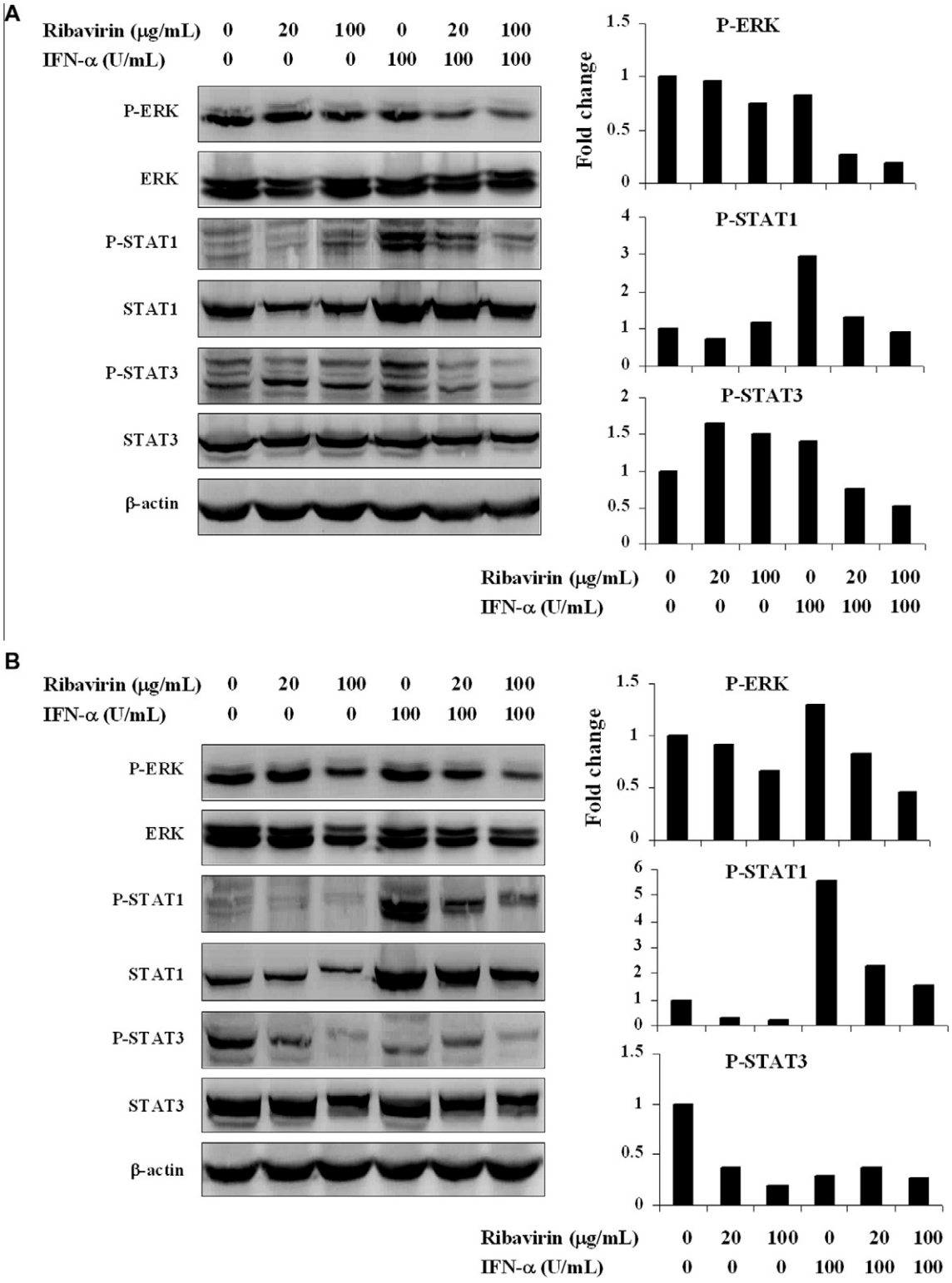

Fig. 4. Huh7.5.1 was treated for 48 or 72 h with 100 U/mL IFN-α in the presence or absence of 20 or 100 μg/mL ribavirin. Total and phosphorylated (P-) ERK, STAT1, and STAT3 were analyzed by Western blotting on the extracts from cells treated with IFN-α or/and ribavirin for 48 h (A) and for 72 h (B), respectively. β-actin is shown as a loading control. Levels of phosphorylated ERK, STAT1, and STAT3 were quantified. Data are expressed as fold change of the levels of phosphorylated ERK, STAT1, and STAT3 over the levels of untreated control after being normalized to the levels of β-actin. Results are representative of three experiments.

2.5. Kinase inhibitor treatment

For 10 mM stock, Jak Inhibitor I and U0126 were resuspended in DMSO. Cells seeded in 6-well plates the day before were washed twice with PBS and pretreated with DMSO, U0126, and Jak Inhibitor I at a final concentration of 1 μM for 1 h at 37 °C. After washing with PBS, cells were cultured with 20 μg/mL ribavirin for 24 h. Cell lysates were prepared for measurement of ERK and STAT3.

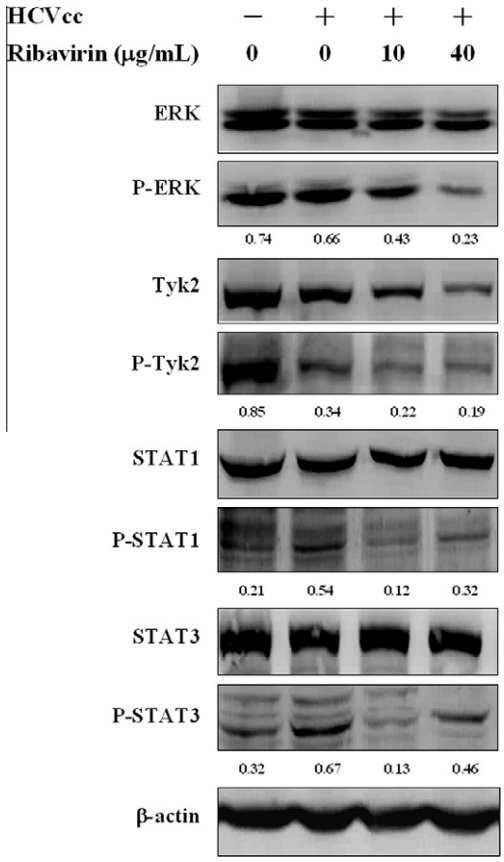

Fig. 5. Huh7.5.1 infected with HCVcc was treated with ribavirin at the indicated doses for 96 h. Levels of ERK, Tyk2, STAT1, and STAT3 were analyzed by Western blotting on the extracts from HCVcc-infected (+) and uninfected (−) cells. β-actin is shown as a loading control. Changes in the levels of phosphorylated (P-) ERK, Tyk2, STAT1, and STAT3 after being normalized to the levels of β-actin are shown below each blot. Results are representative of at least three experiments.

2.6. HCVcc infection

Cells were seeded in 12-well plates or 60-mm dishes 24 h before HCVcc infection. Cells were washed twice with PBS and HCVcc stock was added to 12-well plates (60 μL/well) or 60-mm dishes (300 μL/dish) for 2 h at 37 °C. After three washes with PBS to remove unbound virus, the fresh medium containing ribavirin (0, 1, 5, 10, 20, 40, 80, and 100 μg/mL) or in combination with IFN-α (5, 10, and 20 U/mL) were added and allowed to culture for 96 h. Cellular RNA and cell lysates were prepared for reverse transcription real-time PCR and Western blot analyses.

2.7. Cell viability assay

Cells seeded in 96-well plates (2×10^4 cells/well) were treated with increasing amounts of ribavirin (0, 1, 10, 20, 40, 80, and 100 μg/mL) for 96 h. Cell viability was determined using the CellTiter 96®AQueous One Solution reagent according to the manufacture's instructions. The absorbance at 490 nm was read on a Synergy 2 Multi-Mode Microplate Reader (BioTek, Winooski, VT). The background absorbance in the wells with medium only was subtracted.

2.8. Real-time PCR

Total cellular RNA was extracted with Trizol reagent according to the manufacturer's instructions. Reverse transcription was performed with random hexamers and M-MLV reverse transcriptase. Levels of HCV, IRF9, ISG15, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were measured by semi-quantitative reverse transcription real-time PCR with QuantiTect SYBR Green PCR kit on a Rotor-Gene 3000 real-time thermal cycler (Corbett, Sydney, Australia). Primers used were as follows: HCV, 5′-CTTCACGCAGAAAGCGTCTA-3′(forward) and 5′-CAAGCACCCTATCAGGCAGT-3′(reverse); IRF9, 5′-CCACCGAAGTTCCAGGTAACAC-3′ (forward) and 5′-AGTCTGCTCCAGCAAGTATCGG-3′ (reverse); ISG15, 5′-CTCTGAGCATCCTGGTGAGGAA-3′ (forward) and 5′-AAGGTCAGCCAGAACAGGTCGT-3′ (reverse); GAPDH 5′-TGGGCTACACTGAGCACCAG-3′ (forward) and 5′-AAGTGGTCGTTGAGGGCAAT-3′ (reverse). Results were normalized to endogenous reference GAPDH and analyzed using Rotor-Gene 6.1.81 software. The data were expressed as relative percentage (for HCV) or increase (for antiviral genes) over the corresponding mRNA levels in untreated control.

2.9. Western blot

Cells were lysed with Blue Loading Buffer Pack (Cell Signaling Technology) following the manufacturer's protocol. Proteins in cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidence difluoride membranes using Mini-Protein II system (Bio-Rad), and blocked for 2 h with 5% nonfat milk. The membranes were incubated overnight at 4 °C with primary antibodies, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:1000) or goat anti-mouse IgG (1:2000) for 1 h at room temperature. The primary antibodies used were antibodies against total or phosphorylated kinases (1:1000) including Tyk2, STAT1, STAT3, c-Raf, MEK, and ERK, HCV NS3 mAb (1:1000), and HCV E2 mAb (1:500). The immune complex was detected with chemiluminescence reagents (Pierce; Rockford, IL, USA) on a GeneGnome HR image capture (Cambridge, UK) using GeneTools software.

2.10. Statistical analysis

Statistical analysis was performed using Student's *t* test. *P*-values lower than 0.05 were considered statistically significant.

3. Results

3.1. Influence of ribavirin on ERK pathway

We previously demonstrated that the up-regulation of MAPK pathways by HCV envelope protein E2 via the relevant cellular receptors was implicated in the viral pathogenesis (Chen et al., 2010; Zhao et al., 2005, 2006, 2007). To investigate influence of ribavirin on ERK pathway, Huh7.5.1 cells were cultured for various time periods with ribavirin at doses ranging from 1 to 100 μg/mL. Cell lysates were prepared for kinase measurement by Western blot analysis. After treatment for 24 h, ribavirin inhibited phosphorylation of Raf, MEK, and ERK kinases when compared with the untreated control (Fig. 1A). Levels of total Raf, MEK, and ERK were also determined. The changes in the levels of phosphorylated kinases were clearly presented in Fig. 1B. The Raf phosphorylation was inhibited by ribavirin in a dose-dependent manner, and the MEK phosphorylation was potently inhibited at a dose of 10 μg/mL. In comparison with Raf and MEK, ERK phosphorylation was slightly inhibited by ribavirin. To determine duration of Raf–MEK–ERK signaling, the kinases were further examined at the various time points after treatment with 20 μg/mL ribavirin. In the presence of ribavirin, levels of the kinase phosphorylation were reduced. As shown in Fig. 1C and D, the phosphorylation of Raf, MEK, and ERK was suppressed after ribavirin treatment for 48, 72, or

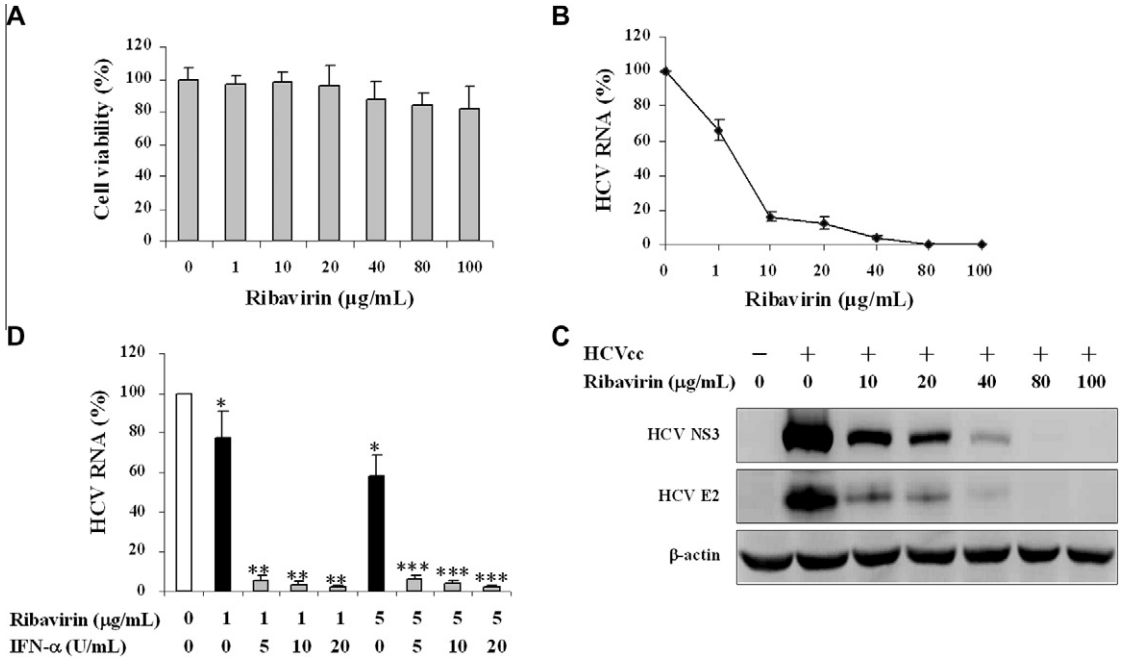

Fig. 6. Huh7.5.1 infected with HCVcc was treated with varying doses of ribavirin or in combination with IFN-α at the indicated doses for 96 h. (A) Huh7.5.1 was treated with the indicated doses of ribavirin for 96 h, and then subjected to cell viability assay. Columns represent the mean of triplicate of one representative experiment; error bars indicate the standard deviation. (B) Real-time RT-PCR was performed on the extracted RNA from HCVcc-infected cells with ribavirin treatment, using GAPDH for normalization. HCV RNA levels were shown as relative percentages of the untreated control. For each sample, real-time PCR analysis was performed three times. Symbols represent the mean of triplicate of one representative experiment; error bars indicate the standard deviation. (C) Western blot analysis of HCV NS3 and E2 protein expression was carried out on the extracts from HCVcc-infected (+) and uninfected (−) cells. β-actin is shown as a loading control. One representative experiment out of two is shown. (D) HCV RNA levels were determined on the extracted RNA from HCVcc-infected cells with ribavirin or in combination with IFN-α treatment. For each sample, real-time PCR analysis was performed three times. Columns represent the mean of triplicate of one representative experiment; error bars indicate the standard deviation; $^{*}P < 0.05$ relative to the untreated control; $^{**}P < 0.05$ relative to 1 μg/mL ribavirin; $^{***}P < 0.05$ relative to 5 μg/mL ribavirin.

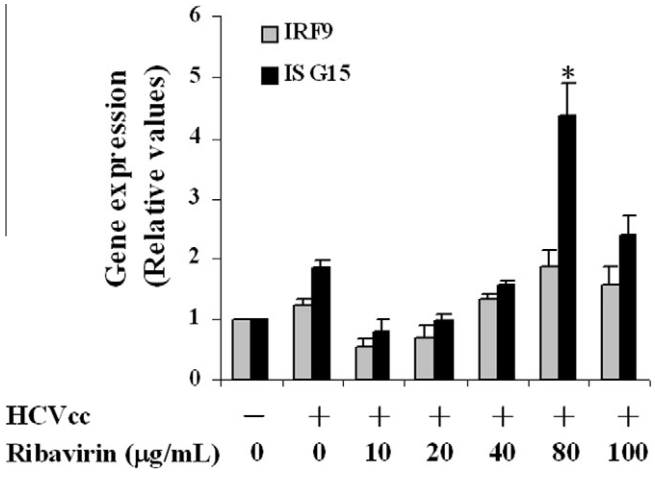

Fig. 7. Huh7.5.1 infected with HCVcc was treated with the varying doses of ribavirin for 96 h. Real-time RT-PCR analysis of IRF9 and ISG15 mRNA levels after ribavirin treatment in cells with (+) and without (−) HCVcc infection. The levels of IRF9 and ISG15 mRNA were expressed as fold increase over the corresponding mRNA levels in uninfected and untreated control. For each sample, real-time PCR analysis was performed three times. Columns represent the mean of triplicate of one representative experiment; error bars indicate the standard deviation; $^{*}P < 0.05$ relative to the untreated control.

96 h. These results indicate inhibition of Raf, MEK, and ERK phosphorylation by ribavirin.

3.2. Influence of ribavirin on STAT pathway

Next, we studied influence of ribavirin on STAT pathway. Both upstream Tyk2 and downstream STAT1 and STAT3 were analyzed by Western blotting on the lysates from Huh7.5.1 cells treated with the increasing doses of ribavirin for the various time periods. Fig. 2A and B showed that treatment with the ribavirin for 24 h resulted in reduced expression and phosphorylation of Tyk2 at doses ranging from 5 to 100 μg/mL. STAT1 phosphorylation was dose-dependently inhibited by ribavirin, while STAT3 phosphorylation was increased. Time course of the kinase cascades showed that levels of Tyk2 and STAT1 phosphorylation were reduced after 20 μg/mL ribavirin treatment for 12, 48, 72, or 96 h (Fig. 2C and D). In contrast, STAT3 phosphorylation was increased after the treatment for 12, 48, or 96 h, but markedly reduced at the 72 h. Thus, ribavirin inhibits phosphorylation of Tyk2 and STAT1 but selectively enhances STAT3 phosphorylation.

3.3. Effects of U0126 and Jak Inhibitor I on ERK and STAT3

MEK inhibitor U0126 is able to inhibit ERK activation. Jak Inhibitor I displays potent inhibitory activity against Jak, an upstream kinase of STAT pathway. To address specificity of the ERK and STAT pathways induced by ribavirin, Huh7.5.1 cells were pretreated for 1 h with U0126 or Jak Inhibitor I prior to addition of 20 μg/mL ribavirin for 24 h. Levels of ERK and STAT3 phosphorylation were analyzed in the presence or absence of the kinase inhibitors. As shown in Fig. 3A, 1 μM U0126 pretreatment reduced phosphorylation of ERK and STAT3 as compared with ribavirin alone treatment. However, 1 μM Jak Inhibitor I pretreatment only abolished STAT3 phosphorylation induced by ribavirin without affecting ERK phosphorylation (Fig. 3B). In addition, levels of phosphorylated ERK and STAT3 in the cells treated with DMSO were presented to exclude any inhibitory effects of DMSO on the kinases. The inhibition of ERK and STAT3 phosphorylation by the inhibitors indicates specific regulation of the pathways by ribavirin.

3.4. Interference of IFN-α with ribavirin-induced ERK and STAT pathways

As the combination of IFN-α and ribavirin is the standard treatment for HCV infection, we thus wondered whether IFN-α interfered with ribavirin-induced signaling pathways. For this purpose, Huh7.5.1 cells were treated for 48 h with 20 or 100 μg/mL ribavirin alone or in combination with 100 U/mL IFN-α. Fig. 4A showed that levels of ERK phosphorylation were reduced following the treatment of IFN-α combined with ribavirin, compared with the untreated control as well as the treatment of ribavirin or IFN-α alone. In contrast to the inhibition of STAT1 phosphorylation by ribavirin (Fig. 2), IFN-α enhanced not only STAT1 phosphorylation but also STAT1 expression. The enhancement of STAT1 was attenuated due to the addition of ribavirin. Ribavirin enhanced STAT3 phosphorylation, and the combination of IFN-α and ribavirin reduced the phosphorylation (Fig. 4A). Moreover, levels of the kinases were further examined in cells treated with the ribavirin or in combination with the IFN-α for 72 h. As shown in Fig. 4B, the IFN-α combined with ribavirin reduced ERK phosphorylation. The enhancement of expression and phosphorylation of STAT1 by IFN-α was attenuated by ribavirin. Consistent with the data presented in Fig. 2C, STAT3 phosphorylation was reduced following the ribavirin treatment for 72 h. IFN-α exhibited inhibitory effect on STAT3 phosphorylation, and synergetic inhibitory effect on the kinase was observed when combined with ribavirin (Fig. 4B).

3.5. Down-regulation of ERK and STAT by ribavirin following HCV infection

We wondered whether different patterns of ERK and STAT pathways induced by ribavirin occurred following HCVcc infection. Huh7.5.1 cells infected with HCVcc were treated with ribavirin for 96 h. It seemed that HCVcc infection increased phosphorylation of STAT1 and STAT3 but reduced phosphorylation of ERK and Tyk2. Fig. 5 showed that ERK phosphorylation was obviously inhibited by ribavirin. Levels of phosphorylated and total Tyk2 were sharply reduced after ribavirin treatment. Also, phosphorylation of STAT1 and STAT3 was inhibited by ribavirin at the doses between 10 and 40 μg/mL. In contrast to the enhancement of STAT3 phosphorylation after the ribavirin treatment for 96 h (Fig. 2C), STAT3 phosphorylation was reduced in HCVcc-infected cells treated with ribavirin. ERK and STAT pathways are down-regulated by ribavirin following HCVcc infection.

3.6. Inhibition of HCV replication by ribavirin

Ribavirin is the approved drug for HCV infection. Here in vitro antiviral potency of ribavirin was evaluated in HCVcc-infected cells. A recent study showed that 100 μg/mL ribavirin displayed no observable toxicity in Huh7.5.1 cells (Thomas et al., 2011). In the present study, Huh7.5.1 cells were treated with the increasing doses of ribavirin for 96 h. Influence of ribavirin on cell viability of Huh7.5.1 was then evaluated. Fig. 6A showed that ribavirin did not exert significant cytotoxic effect at doses up to 100 μg/mL. To explore effect of ribavirin on HCV replication, Huh7.5.1 cells infected with HCVcc were treated with ribavirin for 96 h. Total RNA was extracted and reverse transcribed, and cDNA was amplified using primers specific for HCV by real-time PCR assay using GAPDH for normalization. As shown in Fig. 6B, ribavirin treatment led to a dose-dependent decrease in HCV RNA levels, and ribavirin at 1 μg/mL significantly decreased the RNA levels as compared with the untreated control ($P < 0.05$). At higher dose up to 80 μg/mL, ribavirin had minimal additional effect on HCV RNA levels. Moreover, similar results were observed at HCV protein expression level. HCV nonstructural protein NS3 and structural protein E2 were expressed at a high level in HCVcc-infected cells, and such expression was inhibited by ribavirin in a dose-dependent manner (Fig. 6C). Eighty microgram per milliliter ribavirin treatment eliminated the NS3 and E2 expression, which was consistent with the significant decreased HCV RNA levels upon 80 μg/mL ribavirin treatment (Fig. 6B). Additionally, whether the antiviral potency of ribavirin could be enhanced by IFN-α at a low dose was further examined. HCV RNA levels were determined after treatment with ribavirin in the presence or absence of IFN-α for 96 h. Fig. 6D showed that ribavirin at 1 and 5 μg/mL significantly decreased HCV RNA levels ($P < 0.05$), and that treatment with 5, 10, or 20 U/mL IFN-α in combination with the ribavirin resulted in greater inhibition of the virus replication than that of ribavirin at the dose of 1 and 5 μg/mL ($P < 0.05$), suggesting that ribavirin and IFN-α exert a synergetic inhibitory effect on HCV replication.

3.7. Induction of antiviral genes by ribavirin

We performed the dose-response experiment to reveal profile of antiviral gene expression induced by ribavirin. Huh7.5.1 cells were infected with HCVcc and then treated for 96 h with ribavirin at the indicated doses. Real-time PCR assay was carried out to determine mRNA levels of IRF9 and ISG15. Fig. 7 showed that mRNA levels of IRF9 and ISG15 were slightly increased after ribavirin treatment at doses ranging from 40 to 100 μg/mL, peaking at 80 μg/mL, and declining at 100 μg/mL. Treatment with 80 μg/mL ribavirin significantly increased ISG15 level when compared with the untreated control ($P < 0.05$). The up-regulation of IRF9 and ISG15 expression by 80 μg/mL ribavirin was consistent with the inhibition of HCV RNA level and the elimination of NS3 and E2 expression by ribavirin at the same dose (Fig. 6B and C).

4. Discussion

The precise mechanism by which ribavirin induces cellular signaling events involved in antiviral activity remain to be elucidated. In the present study, the kinase cascades of ERK and STAT signaling induced by ribavirin were analyzed in human hepatoma cells with and without HCVcc infection. The interference of IFN-α with ribavirin-induced ERK and STAT pathways was also evaluated. Moreover, effects of ribavirin on HCV replication and antiviral gene induction were examined.

For its ability to support HCVcc replication, Huh7.5.1 was selected to be a cell model for evaluation of signaling events. First, we addressed the influence of ribavirin on Raf, MEK, and ERK kinases of ERK pathway in Huh7.5.1 without HCVcc infection. We found that ribavirin treatment led to the reduced phosphorylation of Raf, MEK, and ERK (Fig. 1). Supporting our data concerning the inhibition of ERK phosphorylation by ribavirin, previous studies showed that 100 μM ribavirin treatment for 2 or 4 days decreased ERK activation, and 20 μg/mL ribavirin also inhibited ERK phosphorylation (Ogbomo et al., 2010; Vallée et al., 2000). We provided evidence for the first time that the Raf–MEK–ERK signaling cascades other than ERK alone are inhibited by ribavirin and that the regulation correlates with the dose and the time length of treatment, which was confirmed by the dose-response and time-course experiments with ribavirin. The impairment of signaling events by ribavirin accounts for its activities (Borden and Culjkovic-Kraljacic, 2010; Khan et al., 2008; Li et al., 1999; Ogbomo et al., 2010; Simone et al., 2010). Our results suggest the down-regulation of ERK pathway by ribavirin may be responsible for its antiviral activity by impairing the viral survival.

Apart from ERK, STAT pathway was subsequently investigated in response to the ribavirin treatment. Our results showed the

inhibition of Tyk2 and STAT1 phosphorylation and the enhancement of STAT3 phosphorylation by ribavirin (Fig. 2). The inhibition of STAT3 phosphorylation by Jak Inhibitor I confirmed the specific regulation of the pathway by ribavirin (Fig. 3). In interleukin-15-activated natural killer cells, the phosphorylation of STAT1 and STAT3 was sharply inhibited by 20 μg/mL ribavirin (Ogbomo et al., 2010). The effects of ribavirin on signal molecules may be due to the cell types, the strength, or the duration of signaling. Moreover, we extended the analysis to examine the interference of IFN-α with ribavirin-induced ERK and STAT pathways. In contrast to ribavirin, IFN-α strongly enhanced not only STAT1 phosphorylation but also STAT1 expression (Fig. 4), indicating different effects of the antiviral agents on signal molecules. IFN-α and ribavirin synergistically regulated ERK and STAT3, which may be involved in the mechanisms underlying the clinical efficacy of IFN-α in combination with ribavirin. Similarly, Stevenson et al. reported that IFN-α-induced STAT3 phosphorylation was increased in hepatocytes treated with ribavirin and IFN-α (Stevenson et al., 2011). Interestingly, we found that the enhancement of STAT3 phosphorylation by ribavirin occurred at the indicated time points but not 72 h, which need to be further verified.

Although ribavirin in combination with pegylated IFN-α displays strong clinical efficacy against chronic hepatitis C, the precise role of ribavirin is still being defined (Reddy et al., 2009). The cell culture system for HCV is an effective tool for evaluation of antiviral drugs on the complete HCV life cycle. Using genotype 2a HCV produced in cell culture, we observed that ribavirin itself could inhibit HCV replication, as evidenced that levels of HCV RNA and HCV NS3 and E2 proteins were reduced following the ribavirin treatment (Fig. 6B and C). In addition, ribavirin and IFN-α exerted the synergetic inhibitory effect on HCV replication (Fig. 6D). In supporting our data, the IFN half-inhibition concentration was significantly lower for HCV after exposure to 50 μM ribavirin for 1 month than for the virus in the absence of ribavirin by the use of HCVcc system (Brochot et al., 2007). As for the ribavirin treatment, the doses used were determined based on the previous studies (Ogbomo et al., 2010; Stevenson et al., 2011; Thomas et al., 2011; Vallée et al., 2000). Moreover, the levels of ribavirin in the serum of patients with hepatitis C are reported to be 1–4 μg/mL (Maynard et al., 2008; Toyoda et al., 2008). Also, studies showed that the concentration of ribavirin in erythrocytes reached over 100 times greater than that in plasma (Baiocchi et al., 2010; Homma et al., 2004). Indeed, we observed the dose-dependent inhibition of HCV RNA replication and HCV protein expression by ribavirin. Studies showed that ribavirin induced IRF7, IRF9, and ISG15 in patients with hepatitis C and in HCV cell culture models (Taylor et al., 2007; Thomas et al., 2011). While Taylor et al. reported that treatment with ribavirin had very little effect on the gene expression, whereas treatment with pegylated IFN-α had a dramatic effect in peripheral blood cells (Taylor et al., 2004). We found that ribavirin treatment slightly increased IRF9 and ISG15 mRNA levels in HCVcc-infected cells and the obvious up-regulation of IRF9 and ISG15 expression was consistent with the inhibition of HCVcc replication by 80 μg/mL of ribavirin (Fig. 7), implying that ribavirin exerts anti-HCV activity through induction of the antiviral genes.

Aberrant regulation of ERK signaling cascades occurs during virus infection and has an important implication for viral pathogenicity. IFN-α -induced activation of STAT pathway is essential for the induction of an antiviral state. Ribavirin is shown to exert antiviral activity by promoting the IFN signaling (Feld et al., 2010; Su et al., 2009; Zhang et al., 2003). A recent study showed that the antiviral activity of ribavirin is mediated through the inhibition of inosine monophosphate dehydrogenase (Mori et al., 2011). Our data showed that the ERK and STAT pathways were down-regulated by ribavirin following HCVcc infection (Fig. 5). Further experiments performed with kinase inhibitors or siRNAs targeting ERK or STAT would be necessary to clarify the association between ERK and STAT pathways and ribavirin-mediated anti-HCV effect. Taken together, ribavirin inhibits HCV RNA replication and HCV protein expression. The down-regulation of ERK and STAT signaling pathways by ribavirin might be involved in the molecular mechanisms underlying anti-HCV effect of ribavirin.

Acknowledgments

This work was supported by the Natural Science Foundation of China Grants (30771928 and 81171564) and the Shanghai Leading Academic Discipline Project (B901).

References

Avolio, S., Summa, V., 2010. Advances in the development of macrocyclic inhibitors of hepatitis C virus NS3-4A protease. Curr. Top. Med. Chem. 10, 1403–1422.
Baiocchi, L., De Leonardis, F., Delle Monache, M., Nosotti, L., Conti, R.L., Lenci, I., Carbone, M., Di Paolo, D., Cucchiarelli, S., Angelico, M., 2010. Plasma/erythrocyte ribavirin ×100 ratio as an indicator of sustained virological response in HCV genotype 1 patients with early virological response. Antivir. Ther. 15, 633–639.
Borden, K.L., Culjkovic-Kraljacic, B., 2010. Ribavirin as an anti-cancer therapy: acute myeloid leukemia and beyond? Leuk. Lymphoma 51, 1805–1815.
Brochot, E., Duverlie, G., Castelain, S., Morel, V., Wychowski, C., Dubuisson, J., François, C., 2007. Effect of ribavirin on the hepatitis C virus (JFH-1) and its correlation with interferon sensitivity. Antivir. Ther. 12, 805–813.
Chen, Q.L., Zhu, S.Y., Bian, Z.Q., Zhao, L.J., Cao, J., Pan, W., Qi, Z.T., 2010. Activation of p38 MAPK pathway by hepatitis C virus E2 in cells transiently expressing DC-SIGN. Cell Biochem. Biophys. 56, 49–58.
Feld, J.J., Hoofnagle, J.H., 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature 436, 967–972.
Feld, J.J., Lutchman, G.A., Heller, T., Hara, K., Pfeiffer, J.K., Leff, R.D., Meek, C., Rivera, M., Ko, M., Koh, C., Rotman, Y., Ghany, M.G., Haynes-Williams, V., Neumann, A.U., Liang, T.J., Hoofnagle, J.H., 2010. Ribavirin improves early responses to peginterferon through improved interferon signaling. Gastroenterology 139, 154–162.
Fried, M.W., Shiffman, M.L., Reddy, K.R., Smith, C., Marinos, G., Gonçales Jr., F.L., Häussinger, D., Diago, M., Carosi, G., Dhumeaux, D., Craxi, A., Lin, A., Hoffman, J., Yu, J., 2002. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N. Engl. J. Med. 347, 975–982.
Fujii, N., Yokosawa, N., Shirakawa, S., 1999. Suppression of interferon response gene expression in cells persistently infected with mumps virus, and restoration from its suppression by treatment with ribavirin. Virus Res. 65, 175–185.
Homma, M., Matsuzaki, Y., Inoue, Y., Shibata, M., Mitamura, K., Tanaka, N., Kohda, Y., 2004. Marked elevation of erythrocyte ribavirin levels in interferon and ribavirin-induced anemia. Clin. Gastroenterol. Hepatol. 2, 337–339.
Hoofnagle, J.H., Lau, D., Conjeevaram, H., Kleiner, D., Di Bisceglie, A.M., 1996. Prolonged therapy of chronic hepatitis C with ribavirin. J. Viral. Hepat. 3, 247–252.
Johnson, G.L., Lapadat, R., 2002. Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 298, 1911–1912.
Khan, F., Peltekian, K.M., Peterson, T.C., 2008. Effect of interferon-alpha, ribavirin, pentoxifylline, and interleukin-18 antibody on hepatitis C sera-stimulated hepatic stellate cell proliferation. J. Interferon Cytokine Res. 28, 643–651.
Kökény, S., Papp, J., Weber, G., Vaszkó, T., Carmona-Saez, P., Oláh, E., 2009. Ribavirin acts via multiple pathways in inhibition of leukemic cell proliferation. Anticancer Res. 29, 1971–1980.
Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309, 623–626.
Li, W., Shen, F., Weber, G., 1999. Ribavirin and quercetin synergistically downregulate signal transduction and are cytotoxic in human ovarian carcinoma cells. Oncol. Res. 11, 243–247.
Maynard, M., Pradat, P., Gagnieu, M.C., Souvignet, C., Trepo, C., 2008. Prediction of sustained virological response by ribavirin plasma concentration at week 4 of therapy in hepatitis C virus genotype 1 patients. Antivir. Ther. 13, 607–611.
McHutchison, J.G., Poynard, T., 1999. Combination therapy with interferon plus ribavirin for the initial treatment of chronic hepatitis C. Semin. Liver Dis. 19 (Suppl. 1), 57–65.
Moradpour, D., Penin, F., Rice, C.M., 2007. Replication of hepatitis C virus. Nature 5, 453–463.
Mori, K., Ikeda, M., Ariumi, Y., Dansako, H., Wakita, T., Kato, N., 2011. Mechanism of action of ribavirin in a novel hepatitis C virus replication cell system. Virus Res. 157, 61–70.
Ogbomo, H., Michaelis, M., Altenbrandt, B., Doerr, H.W., Cinatl Jr., J., 2010. A novel immunomodulatory mechanism of ribavirin in suppressing natural killer cell function. Biochem. Pharmacol. 79, 188–197.
Reddy, K.R., Nelson, D.R., Zeuzem, S., 2009. Ribavirin: current role in the optimal clinical management of chronic hepatitis C. J. Hepatol. 50, 402–411.

Schindler, C., Levy, D.E., 2007. Decker T. JAK-STAT signaling: from interferons to cytokines. J. Biol. Chem. 282, 20059–20063.

Simone, O., Tortorella, C., Zaccaro, B., Napoli, N., Antonaci, S., 2010. Impairment of TLR7-dependent signaling in dendritic cells from chronic hepatitis C virus (HCV)-infected non-responders to interferon/ribavirin therapy. J. Clin. Immunol. 30, 556–565.

Stevenson, N.J., Murphy, A.G., Bourke, N.M., Keogh, C.A., Hegarty, J.E., O'Farrelly, C., 2011. Ribavirin enhances IFN-α signalling and MxA expression: a novel immune modulation mechanism during treatment of HCV. PLoS One 6, e27866.

Su, W.C., Liu, W.L., Cheng, C.W., Chou, Y.B., Hung, K.H., Huang, W.H., Wu, C.L., Li, Y.T., Shiau, A.L., Lai, M.Y., 2009. Ribavirin enhances interferon signaling via stimulation of mTOR and p53 activities. FEBS Lett. 583, 2793–2798.

Tan, K., Culjkovic, B., Amri, A., Borden, K.L., 2008. Ribavirin targets eIF4E dependent Akt survival signaling. Biochem. Biophys. Res. Commun. 375, 341–345.

Taylor, M.W., Grosse, W.M., Schaley, J.E., Sanda, C., Wu, X., Chien, S.C., Smith, F., Wu, T.G., Stephens, M., Ferris, M.W., McClintick, J.N., Jerome, R.E., Edenberg, H.J., 2004. Global effect of PEG-IFN-alpha and ribavirin on gene expression in PBMC in vitro. J. Interferon Cytokine Res. 24, 107–118.

Taylor, M.W., Tsukahara, T., Brodsky, L., Schaley, J., Sanda, C., Stephens, M.J., McClintick, J.N., Edenberg, H.J., Li, L., Tavis, J.E., Howell, C., Belle, S.H., 2007. Changes in gene expression during pegylated interferon and ribavirin therapy of chronic hepatitis C virus distinguish responders from nonresponders to antiviral therapy. J. Virol. 81, 3391–3401.

Thomas, E., Feld, J.J., Li, Q., Hu, Z., Fried, M.W., Liang, T.J., 2011. Ribavirin potentiates interferon action by augmenting interferon-stimulated gene induction in hepatitis C virus cell culture models. Hepatology 53, 32–41.

Tong, Y., Zhu, Y., Xia, X., Liu, Y., Feng, Y., Hua, X., Chen, Z., Ding, H., Gao, L., Wang, Y., Feitelson, M.A., Zhao, P., Qi, Z.T., 2011. Tupaia CD81, SR-BI, Claudin-1, and Occludin support hepatitis C virus infection. J. Virol. 85, 2793–2802.

Toyoda, H., Kumada, T., Kiriyama, S., Sone, Y., Tanikawa, M., Hisanaga, Y., Kanamori, A., Atsumi, H., Nakano, S., Arakawa, T., Honda, T., Hayashi, K., Katano, Y., Goto, H., 2008. Correlation of serum ribavirin concentration with pretreatmentrenal function estimates in patients with chronic hepatitis C receiving combination antiviral therapy with peginterferon and ribavirin. J. Viral. Hepat. 15, 651–658.

Vallée, S., Fouchier, F., Braguer, D., Marvaldi, J., Champion, S., 2000. Ribavirin-induced resistance to heat shock, inhibition of the Ras-Raf-1 pathway and arrest in G(1). Eur. J. Pharmacol. 404, 49–62.

Younossi, Z.M., Limongi, D., Stepanova, M., Pierobon, M., Afendy, A., Mehta, R., Baranova, A., Liotta, L., Petricoin, E., 2011. Protein pathway activation associated with sustained virologic response in patients with chronic hepatitis C treated with pegylated interferon (PEG-IFN) and ribavirin (RBV). J. Proteome Res. 10, 774–779.

Zhang, Y., Jamaluddin, M., Wang, S., Tian, B., Garofalo, R.P., Casola, A., Brasier, A.R., 2003. Ribavirin treatment up-regulates antiviral gene expression via the interferon-stimulated response element in respiratory syncytial virus-infected epithelial cells. J. Virol. 77, 5933–5947.

Zhao, L.J., Wang, L., Ren, H., Cao, J., Li, L., Ke, J.S., Qi, Z.T., 2005. Hepatitis C virus E2 protein promotes human hepatoma cell proliferation through the MAPK/ERK signaling pathway via cellular receptors. Exp. Cell Res. 305, 23–32.

Zhao, L.J., Zhang, X.L., Zhao, P., Cao, J., Cao, M.M., Zhu, S.Y., Liu, H.Q., Qi, Z.T., 2006. Up-regulation of ERK and p38 MAPK signaling pathways by hepatitis C virus E2 envelope protein in human T lymphoma cell line. J. Leukoc. Biol. 80, 424–432.

Zhao, L.J., Zhao, P., Chen, Q.L., Ren, H., Pan, W., Qi, Z.T., 2007. Mitogen-activated protein kinase signaling pathways triggered by the hepatitis C virus envelope protein E2: implications for the prevention of infection. Cell Prolif. 40, 508–521.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. USA 102, 9294–9299.