Biochemical and Biophysical Research Communications 437 (2013) 127–133

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

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

Valine, the branched-chain amino acid, suppresses hepatitis C virus RNA replication but promotes infectious particle formation

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ARTICLE INFO

Article history:
Received 3 June 2013
Available online 24 June 2013

Keywords:
Hepatitis C virus
Branched-chain amino acid
IRES activity
Infectious virus production
Mammalian target of rapamycin
JAK/STAT pathway

ABSTRACT

Background & aims: Concentrations of the branched-chain amino acid (BCAA) in the serum of patients with liver cirrhosis correlate with their liver function. Oral administration of BCAA can ameliorate hypoalbuminemia and hepatic encephalopathy. In this study, we aim to clarify the role of BCAA in regulating the replication of the hepatitis C virus (HCV).
Methods: HCV sub-genomic replicon cells, genome-length replicon cells, and cells infected with cell culture-infectious HCV (HCVcc) were cultured in media supplemented with various concentrations of BCAA, followed by evaluation of the replicon or HCV abundance.
Results: BCAA was capable of suppressing the HCV replicon in a dose-dependent manner and the effect was independent of the mTOR pathway. Of the three BCAAs, valine was identified as being responsible for suppressing the HCV replicon. Surprisingly, an abundance of HJ3-5(YH/QL), an HCVcc, in Huh7 cells was augmented by BCAA supplementation. In contrast, BCAA suppressed an abundance of HJ3-5(wild), an HCVcc that cannot assemble virus particle in Huh7 cells. Internal ribosome entry site of HCV was shown to be a target of BCAA. Single-cycle virus production assays using Huh7-25 cells, which lacked CD81 expression, revealed that BCAA, especially valine, promoted infectious virus particle formation with minimal effect on virus secretion. Thus, BCAA was found to have two opposing effects on HCV production: suppression of the HCV genome RNA replication and promotion of infectious virus formation.
Conclusions: BCAA accelerates HCV production through promotion of infectious virus formation in infected cells despite its suppressive effect on HCV genome replication.

1. Introduction

Persistent infection of hepatitis C virus (HCV) causes progressive liver disease in humans. Chronic inflammation in the liver leads to the accumulation of fibrosis and an eventual progression to liver cirrhosis. In patients with decompensated liver cirrhosis, a change in plasma amino acid composition is frequently observed. In particular, the ratio of branched-chain amino acid (BCAA) to aromatic amino acid (AAA), known as Fischer's ratio, decreases as the liver function deteriorates [1]. In such cirrhotic patients, hypoalbuminemia occurs, and it has been shown that oral administration of BCAA can ameliorate hypoalbuminemia and hepatic encephalopathy.

Three amino acids valine, leucine, and isoleucine are BCAAs, which are considered to be essential for protein anabolism. In addition to the role of acting as nutrient substrates, recent studies have demonstrated that BCAA also serve as physiologically active substances. BCAA have been shown to have pharmacological effects, such as induction of protein synthesis [2] and glucose metabolism [3]. In rat primary hepatocytes, albumin synthesis is significantly increased by BCAA administration, which is dependent on activation of the mammalian target of rapamycin (mTOR), mainly induced by leucine [4].

HCV replication is controlled by intracellular signaling pathways. In addition to the interferon (IFN)-induced JAK/STAT pathway, which activates interferon-stimulated genes, leading to strong anti-viral activity, activation of ERK [5], PI3 kinase/Akt [6,7], smad [8], PKC [9], and p38 [10], have been shown to be capable of regulating HCV replication. mTOR, one of the downstream molecules of Akt, phosphorylates the two substrates p70 S6 kinase and eukaryotic translation initiation factor 4E binding protein 1

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0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.06.051

(4EBP1). p70 S6 kinase phosphorylates ribosomal S6 protein, resulting in an increase of the protein synthesis complex. Phosphorylated 4EBP1 results in its dissociation from the eukaryotic translation initiation factor 4E (eIF4E), which consequently enables eIF4E to regulate the translation initiation. Thus, together, p70 S6 kinase and 4EBP1 are responsible for the mTOR-dependent regulation of cellular translation. Moreover, both have been demonstrated to be involved in the regulation of HCV replication [6].

The finding that BCAA, per se, can activate signaling pathways suggests that they may affect HCV replication, presumably via the activation of the mTOR pathway. However, to date, no detailed investigation has been reported. Therefore, we attempt to clarify whether BCAA have a role in regulating HCV replication by using the HCV replicon system and cell culture of infectious-HCV (HCVcc). The present study reveals that although BCAA, especially valine, suppresses HCV genome replication, they eventually promote total HCV production by accelerating viral formation.

2. Methods

2.1. Cells

The hepatoma-derived cell line Huh7 and its derivatives, Huh7.5 and Huh7-25 [11], were maintained in DMEM supplemented with 10% FCS. The HCV subgenomic replicon cell line Huh-RepSI [10], and the HCV genome-length replicon cell line 2–3 [12], both harboring the HCV-N strain (genotype 1b), were previously described. The molar ratio of the BCAA mixture was adjusted to Leu:Ile:Val = 2.0:1.0:1.2 according to data from previous studies [13]. For assays to examine the role of BCAA, cells were cultured in BCAA-deficient DMEM with 10% FCS supplemented with BCAA mixtures of various concentrations (0–2 mM).

2.2. Cell culture-infectious HCV

JFH-1 is a cell culture-infectious virus of genotype 2a as previously described [14]. HJ3-5(YH/QL) is a chimeric cell culture-infectious virus with a genome consisting of the core to NS2 sequence of genotype 1a (H77) virus placed within the background of the genotype 2a JFH-1 virus. This virus contained compensatory mutations in E1 (Y361H) and NS3 (Q1251L) [15]. These two mutations rendered the chimeric RNA highly infectious.

2.3. In Vitro transcription and transfection of synthetic RNA

Plasmid DNAs encoding HJ3-5(wild) and HJ3-5(YH/QL), a wild-type chimeric virus and a chimeric virus carrying two mutations, respectively, were linearized by *Xba*I prior to transcription. RNA was synthesized with the T7 RiboMAX Express Large Scale RNA Production System (Promega, Madison, WI, USA) following the

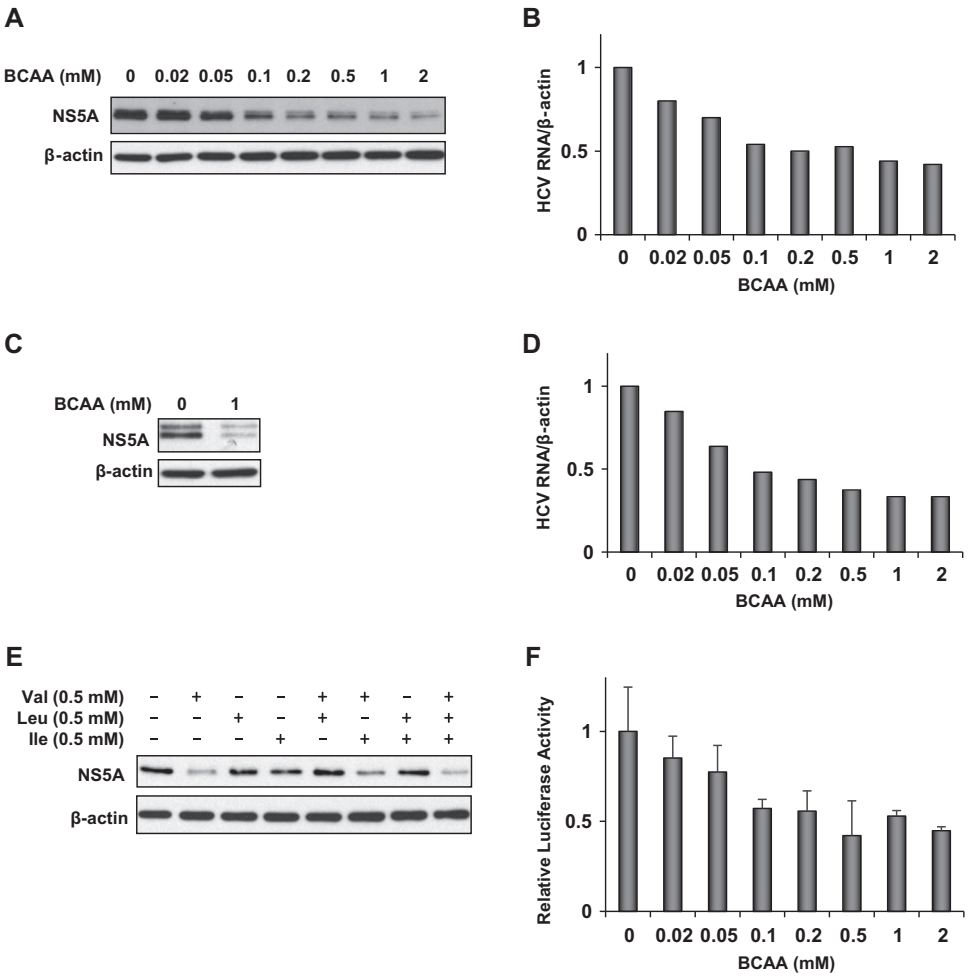

Fig. 1. BCAA limits the abundance of HCV replicon in HCV replicon cells. (A–D). Huh-RepSI (A and B) and 2–3 (C and D) cells were cultured in media for 2 days, with BCAA supplemented at concentrations of 0–2 mM. Total protein or total RNA was recovered and assayed for immunoblot (A and C) or real-time RT-PCR (B and D), respectively. (E) Three BCAAs (0.5 mM each) were added to BCAA-free culture medium of Huh-RepSI. After incubation for 2 days, immunoblot analysis of NS5A and beta-actin were performed. (F) Huh-RepSI cells were transfected with pRLHL, cultured in media with various BCAA concentrations between 0 and 2 mM. After incubation for 2 days, a dual luciferase assay was performed. The ratio of firefly luciferase activity to renilla luciferase activity was then calculated.

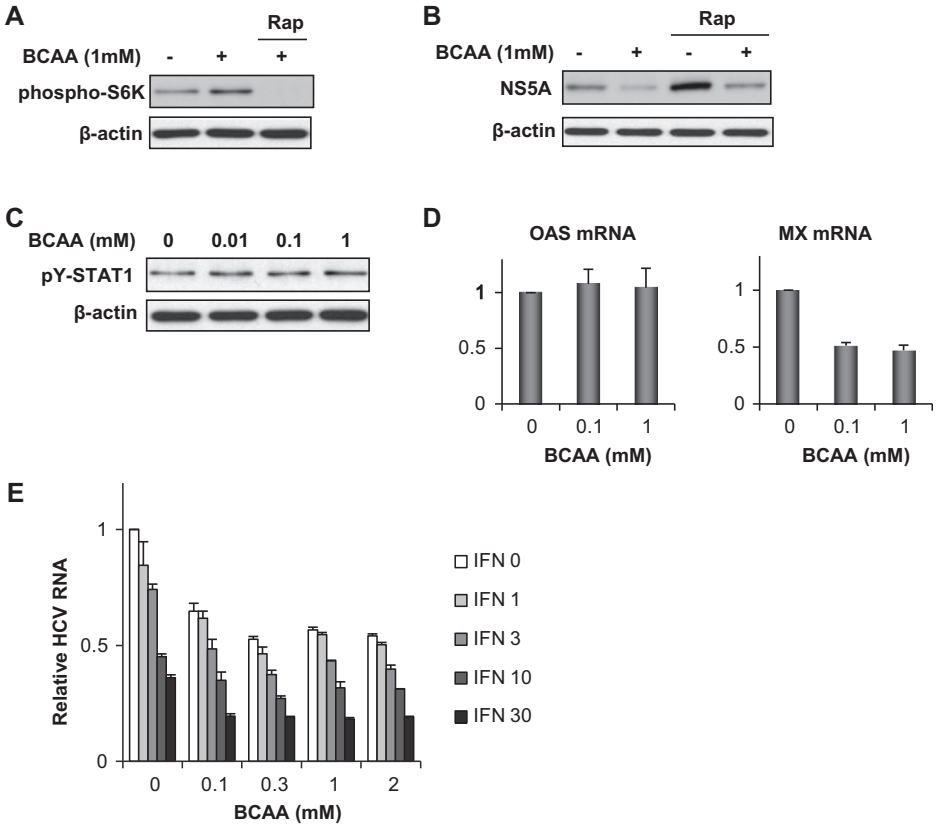

Fig. 2. BCAA-induced suppression of HCV replicon is independent of mTOR or JAK/STAT signaling. (A) Immunoblot of phosphorylated p70 S6 kinase and beta-actin in Huh-RepSI cells cultured in a medium with or without BCAA (1 mM). Rapamycin was added at 100 nM to the BCAA-containing medium. (B) Immunoblot analysis of NS5A and beta-actin in Huh-RepSI cells cultured in a medium with 1 mM BCAA or rapamycin (100 nM). (C) Huh-RepSI cells were incubated in media with various BCAA concentrations (0, 0.01, 0.1, 1 mM), and then, immunoblot analyses of phosphorylated STAT1 (Tyr701) and beta-actin were performed. (D) Huh-RepSI cells were incubated in media with various BCAA concentrations (0, 0.1, 1 mM), and then, a real-time RT-PCR analysis, for expression of OAS and MX, was performed. (E) Huh-RepSI cells were incubated in culture media with various BCAA concentrations (0–2 mM) and IFN-α (0–30 U/ml). HCV RNA abundance was normalized with beta-actin allowing the relative HCV RNA levels to be calculated, setting the HCV RNA level of 0 U/ml IFN-α and 0 mM BCAA as 1. Rap: rapamycin.

manufacturer's suggested protocol. For electroporation, Huh7 cells were washed twice with ice cold phosphate-buffered saline (PBS) and resuspended at a concentration of 10^7 cells/ml in PBS. Subsequently, 10 μg of RNA was mixed with 500 μl of the cell suspension in a cuvette, with a gap width of 0.2 cm (GenePulser II System; Bio-Rad, Hercules, CA, USA). The mixture was immediately subjected to two pulses of current with the intensities of 1.2 kV, 25 μF, and maximum resistance. Following a 10-min incubation at room temperature, the cells were transferred into growth medium.

2.4. Titration of HCV infectivity

Huh-7.5.1 cells were seeded in 96-well plates at a density of 1×10^4 cells per well 24 h prior to culture media inoculation of the HCV infected cells. Three days after infection, HCV-positive cells were detected with mouse monoclonal antibody that recognized core proteins stained with an Alexa Fluor 488 anti-mouse secondary antibody (Invitrogen, Carlsbad, CA, USA). The infectivity titer was expressed as focus-forming units per mL of supernatant (ffu/mL), expressing the mean number of HCV core-positive foci. The intracellular infectivity and specific intracellular infectivity titer were determined as described previously [16].

3. Results

3.1. BCAA suppresses the amount of HCV replicon

To investigate the role of BCAA in HCV replication, we first examined the effect of BCAA on the HCV replicon. An HCV subgenomic replicon cell line, Huh-RepSI, was incubated in culture medium that contained various concentrations of BCAA (0–2 mM) for 2 days. HCV replicon RNA, as well as the amount of protein, was suppressed by adding BCAA in a dose-dependent manner (Fig. 1A and B). To confirm the effect of BCAA, another replicon cell line, 2–3, carrying a genome-length HCV replicon, was used. In this experiment, suppression of the replicon by BCAA was observed, which is in agreement with the Huh-RepSI assay (Fig. 1C and D). This activity suggested that BCAA possessed the ability to suppress HCV replication.

Three BCAAs exist: valine, leucine, and isoleucine. As previously demonstrated, leucine contains the biological activity to activate mTOR. In addition, we showed that mTOR, which is activated by PI3 kinase/Akt, was able to suppress HCV replication [6]. Therefore, it is possible that the BCAA-mediated suppression of HCV replication was due to leucine. To test this hypothesis, the three amino acids were added independently to BCAA-deficient medium while monitoring the HCV replication level. Unexpectedly, the result refuted the hypothesis (Fig. 1E). Compared to the cells cultured in BCAA-deficient medium, supplementation with only valine efficiently suppressed the HCV replicon, whereas leucine did not; instead, it caused a slight increase. This result showed that BCAA, especially valine, but not leucine, have a suppressive effect on HCV replication.

3.2. BCAA suppresses HCV IRES activity

HCV replication can be controlled by HCV specific translation regulated by IRES, the 5′ UTR region of HCV. Therefore, we next

investigated the effect of BCAA on HCV IRES activity. To do this, we utilized a dicistronic vector, pRLHL, which consists of firefly luciferase driven by HCV IRES and renilla luciferase, translated in a cap-dependent manner (Sup. Fig. 1). Relative HCV IRES activity was evaluated using the ratio of IRES-specific luciferase activity to the cap-dependent luciferase activity. As shown in Fig. 1F, HCV IRES activity was suppressed by BCAA in a dose-dependent manner, which is similar to the result of the replicon abundance (Fig. 1A and B). Thus, the BCAA-mediated suppression of HCV replication is likely due to the inhibition of HCV IRES activity.

3.3. BCAA-mediated suppression of HCV replicon is independent of the mTOR and JAK/STAT pathways

Previous reports have demonstrated that BCAA is capable of activating mTOR [4], and we have reported that mTOR suppresses HCV replication [6]. Therefore, we examined the contribution of mTOR activation on BCAA-mediated suppression of the HCV replicon. Administration of BCAA efficiently phosphorylated p70 S6 kinase, whereas rapamycin completely inhibited its phosphorylation (Fig. 2A). Despite rapamycin enhancing the amount of HCV replicon, BCAA could efficiently suppress it, even in rapamycin-containing medium (Fig. 2B), suggesting that the suppression of the HCV replicon by BCAA is independent of mTOR activation.

The IFN-JAK/STAT signal is known to be an anti-virus pathway, induced under the condition of virus infection. HCV replication is efficiently inhibited by interferon. Therefore, we examined whether BCAA could modify the IFN signal. First, we performed an immunoblot analysis and evaluated the status of STAT1 activation, in the presence or absence of BCAA. However, the phosphorylated STAT1 level was not altered by BCAA in Huh-RepSI cells, and ISG induction was not observed; instead, the expression level of Mx was suppressed by BCAA (Fig. 2C and D). A previous study showed that rapamycin diminished the suppressive effect of IFN-α toward HCV replication via the suppression of ISG induction [17]. Subsequently, we examined the HCV replicon abundance in cells that were cultured in media with various concentrations of BCAA and IFN-α stimuli. Even with the depletion of BCAA, IFN-α efficiently and dose-dependently suppressed HCV replicon abundance. However, IFN-α-induced anti-HCV activity was not augmented by BCAA supplementation (for example, the replicon RNA level decreased to approximately 30% in both BCAA-depleted medium and 2 mM BCAA-supplemented medium) (Fig. 2E). Consequently, BCAA did not influence JAK/STAT activation, and therefore, the suppression of HCV replicon by BCAA may have been independent of the IFN-α-induced signaling pathway.

3.4. BCAA enhances HCVcc production

Next, we examined the impact of BCAA on HCVcc, a system retaining the entire HCV life cycle in a cultured cell. Here, we used HJ3-5(YH/QL), a chimeric HCV of genotype 1a (H77) and 2a (JFH-1). Surprisingly, the results of HJ3-5(YH/QL) were opposite to that of the HCV replicon: HCV abundance was upregulated in a BCAA dose-dependent manner (Fig. 3A). The HCV replicon contains NS3 to NS5B proteins, which are required for HCV RNA genome replication, but not core, E1 and E2 proteins, which are structural proteins required for viral particle formation. The discrepancy in the results between HCV replicon cells and HCVcc-infected cells might be due to differences in virus particle production.

To investigate this discrepancy, we used the wild-type HJ3-5, designated as HJ3-5(wild). As described in the Methods section, HJ3-5(YH/QL) or the HCVcc used in this study, carries two amino acid substitutions at amino acid 361 and amino acid 1251, within E1 and NS3, respectively. These two mutations render the chimeric RNA highly infectious [15]. However, without these mutations, virus particle assembly and consequent virion release from the cells to the medium would not occur. This process is thought to be due to impaired association of the HCV proteins originating from different genotypes, whereas there is no apparent change in the HCV RNA replication level in the cells [15].

We introduced the *in vitro* transcribed genome RNA of HJ3-5(wild) or HJ3-5(YH/QL) into Huh7 cells with electroporation, and then, we examined the effect of BCAA on the cell line. Normally, synthesized HCV RNA introduced into cells executes replication by utilizing HCV proteins encoded in the genome and host factors, resulting in a robust increase that is detectable after 2–3 days. BCAA decreased the abundance of HJ3-5(wild), which was similar to their effect on the HCV replicon (Fig. 3B and C). Thus, HJ3-5(wild), a virus that is defective in virus particle formation, revealed the opposite reaction to BCAA compared to the virus HJ3-5(YH/QL), a virus that is competent in virus particle formation. Together, these findings revealed that although BCAA had the ability to suppress the HCV genome replication, they promoted viral production by enhancing other steps, which included virus assembly, virus particle release and cell re-infection.

3.5. BCAA promotes infectious HCV particle formation, not virus secretion

To further assess the BCAA intracellular mechanisms that influence the HCV life cycle, we adopted a single-cycle virus production assay [18]. We used Huh7-25 cells due to the lack of surface expression of one of the cellular HCV receptors, CD81, thus being non-permissive to HCV infection. Because HCV genome replication or virus production is intact in Huh7-25, we can evaluate viral replication and secretion without the influence of re-infection.

First, we studied the replication levels of the infectious virus, JFH-1, in Huh7-25 cells. The full length of the JFH-1 genome RNA

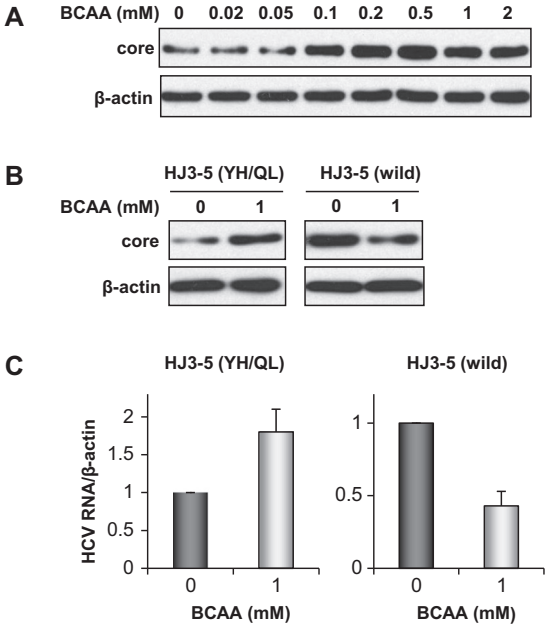

Fig. 3. HCVcc abundance was increased by BCAA. (A) HCVcc-infected Huh7 cells were cultured in media with various BCAA concentrations (0–2 mM). After incubation for 2 days, and an immunoblot analysis of core and beta-actin was performed. (B and C) Synthesized HCV genome RNA of HJ3-5 (YH/QL) or HJ3-5 (wild) was transfected into Huh7 cells via electroporation. After incubation for 24 h, cells were split into 6-well plates and incubated for 2 days in a culture medium with or without 1 mM BCAA. After the cells were harvested, immunoblot analysis of core and beta-actin (B) and real-time RT-PCR analysis (C) were performed.

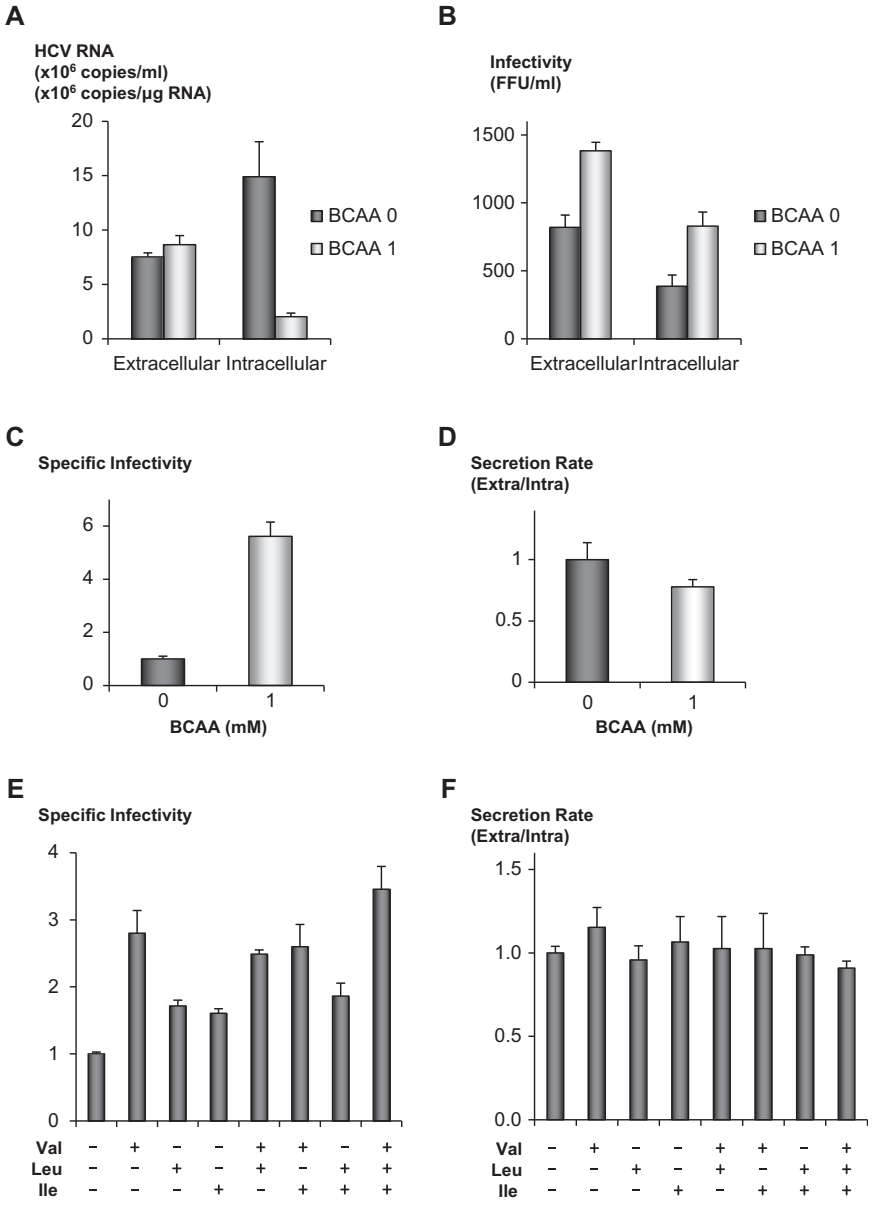

Fig. 4. Single-cycle virus production assay indicates a promoting effect of BCAA on virus formation. (A) Huh7-25 cells were transfected with *in vitro*-transcribed RNA of JFH-1, incubated in media with or without BCAA, followed by the RNA levels in the media or in the cells being calculated using the real-time quantitative RT-PCR method. (B) Infectivities in the media or in the cell lysates were measured. (C) Specific infectivities were calculated by dividing the infectivities by the HCV RNA amounts. (D) Secretion rates were calculated by dividing extracellular infectivities by intracellular infectivities. The data were presented as ratios defining the value of BCAA at 0 mM as 1. (E and F) Specific infectivities and secretion rates in the presence of valine (0.5 mM), leucine (0.5 mM), or isoleucine (0.5 mM). The data were presented as ratios defining the value with no BCAA as 1.

was translated *in vitro* and transfected into the Huh7-25 cells. The cells were cultured in media, with or without 1 mM of BCAA, with the RNA levels being monitored using quantitative RT-PCR. As observed in the experiment of replicon cells or virus particle formation-deficient viruses, the intracellular RNA level of HCV was suppressed by the presence of BCAA (Fig. 4A). However, the levels of extracellular HCV RNA were similar. Despite the suppression of intracellular HCV RNA levels by BCAA-containing medium, the infectivity titer of the intracellular virus in the cells treated with 1 mM BCAA was significantly higher than that of the cells with 0 mM BCAA (Fig. 4B). Extracellular infectivity titers were similar to those of intracellular infectivity. The specific infectivity of intracellular virus was calculated by dividing the infectivity titer by the HCV RNA level and this revealed that cultivation of the cells in a medium of 1 mM BCAA resulted in a 5.6-fold higher specific virus infectivity than that of 0 mM BCAA (Fig. 4C). Next, we measured virus secretion rates by dividing extracellular infectivity titers by intracellular infectivity titers. There was a minimal difference between infectious virus particle secretions (Fig. 4D). Thus, these results indicated that the infectious virion production was promoted in the BCAA-supplemented medium, although the virus RNA replication was suppressed.

In the study using replicon cells, valine was shown amino acid responsible for regulating HCV RNA replication (Fig. 1E). Finally, we assessed the effect of individual BCAA on virus production. HCV infected cells were cultured in media containing each amino acid at 0.5 mM or a combination of them and subsequently specific infectivity and secretion rate were examined (Fig. 4E and F). Among the three BCAAs, valine promoted infectious virus production most effectively, while leucine and isoleucine promoted infectious virus production modestly. Secretion rates did not show a significant difference.

4. Discussion

In the present study, we investigated the role of BCAA in the HCV life cycle and discovered that these amino acids suppress HCV genome replication but promote viral particle formation. Thus far, many reports have indicated that various cellular factors are involved in the regulation of HCV. In particular, intracellular signaling pathways are important modulators for HCV genome replication [5–10]. BCAA, specifically leucine, were demonstrated to have a role in activating the mTOR pathway, leading to protein synthesis such as upregulation of albumin [4] and HGF production [19]. Recently, mTOR was reported to be involved in IFN-α signaling [17]. IFN-α induced phosphorylation of STAT1 was diminished by rapamycin (but not by LY294002, a PI3 kinase inhibitor). Consequently, rapamycin inhibited the IFN-stimulated regulatory element. Although we demonstrated that BCAA can activate mTOR (Fig. 2A), the inhibition of mTOR revealed that it was not the main pathway for the BCAA suppression of HCV replication. BCAA supplementation did not change the STAT1 phosphorylation status, nor did it induce ISG expression, indicating that the JAK/STAT pathway was not relevant for the suppression of HCV replication. Considering that leucine, the factor required for mTOR activation, did not actually take part in regulation of the HCV replicon (Fig. 1E), it was not surprising that mTOR was shown to not be the responsible molecule.

Very recently, Honda et al. demonstrated that STAT1 phosphorylation was increased by BCAA in a dose-dependent manner [20]. They showed that BCAA increased the phosphorylation levels of STAT1, Foxo3a and p70 S6 kinase leading to downregulation of Socs3 expression and HCV replication. The range of BCAA concentration examined in the present study was between 0 and 2 mM. We ranged the concentration of BCAA between 0 and 2 mM because its concentration in blood is approximately 1.6 mM after oral administration of 5 g of BCAA. However, in the Honda et al. study, the BCAA concentration at which STAT1 was efficiently phosphorylated was more than 4 mM, whereas at 2 mM or less, no obvious increase in phospho-STAT1 was observed. Therefore, we may have detected no change in phospho-STAT1 due to BCAA levels used in this study. Thus, BCAA may be capable of suppressing HCV genome replication at a low concentration by inhibiting HCV IRES activity while decreasing virus replication by augmentation of IFN signaling at high concentrations.

Although BCAA suppressed replication of HCV replicon, they increased HCVcc production in infected cells. The life cycle of HCV has many steps that are required to achieve infection, such as attachment to the cell surface, endocytosis of the virus, uncoating and releasing genome RNA, RNA replication, polyprotein synthesis and processing, viral assembly, and release of progeny virus. Among these, the HCV replicon system only represents the steps of genome RNA replication and non-structural protein synthesis in the cells, and BCAA affects these by impairing protein synthesis via suppression of HCV IRES activity. However, HCVcc replication requires all of these steps. We assumed that the increase of HCVcc due to BCAA indicated that some step(s) must be upregulated by BCAA to the extent of overcoming the decreased genome replication. The study of particle formation-deficient viruses suggested that virus assembly or some downstream step in the virus life cycle was critical for the augmentation of HCVcc by BCAA. A single-cycle virus production assay indicated that the production of an infectious virus was prominent in the presence of BCAA, while virus secretion was not strongly affected. Although HCV genome replication was suppressed by BCAA, more infectious virus particles were secreted into the media, and they could have re-infected the Huh7 cells. We suggest that the abundant infectious HCV in BCAA-supplemented medium causes amplification of the virus during repetition of such re-infection, which leads to an accumulation of HCV in the cells, and thus, the abundance of HCV RNA in the cells with BCAA medium overcomes that without BCAA. Further investigation is needed on the detailed mechanisms defining how BCAA regulates HCV particle formation. Clarification of this process could contribute to new insights into HCV replication and could also suggest a basis for treatment of HCV patients.

Acknowledgments and disclosures

We thank Stanley Lemon for providing plasmids pRLHL and pHJ3-5, as well as Charles Rice for Huh7.5.

K. Takehana is an employee of Ajinomoto Pharmaceutical Co., Ltd. All other authors declare no conflict of interest.

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to T. Tak.) and a Grant-in-Aid for Research on Hepatitis from the Ministry of Health, Labour and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.06.051.

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