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Branched-Chain Amino Acid Deficiency Stabilizes Insulin-Induced Vascular Endothelial Growth Factor mRNA in Hepatocellular Carcinoma Cells

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ABSTRACT

Abnormal sugar metabolism is closely related to chronic liver diseases, including hepatocellular carcinoma (HCC). We previously reported that fasting hyperinsulinemia is a poor prognostic factor for HCC patients. A recent large-scale study has shown that long-term administration of branched chain amino acids (BCAA) reduces the risk of HCC development in obese cirrhotic patients who have been diagnosed with diabetes mellitus, although the mechanism by which it does so is unclear. In this study, we analyzed the expression of vascular endothelial growth factor (VEGF) in HepG2 cells under high-insulin culture conditions, and examined the effect of BCAA on VEGF expression. VEGF secretion was significantly increased by 200 nM of insulin under BCAA deficient conditions, but it was repressed by the addition of BCAA. BCAA activated the mTOR pathway and increase HIF-1α expression under high-insulin culture conditions, however quantitative PCR analysis showed that insulin-induced expression of VEGF mRNAs (VEGF121 and VEGF165) decreased 2 h after the addition of BCAA. The half-lives of both VEGF121 and 165 mRNAs were shortened in the presence of BCAA compared to the absence of BCAA. Therefore it is thought that BCAA regulate VEGF expression mainly at the post-transcriptional level. We also examined which of the Valine, Leucine, and Isoleucine components of BCAA were essential for VEGF mRNA degradation. All three BCAA components were required for acceleration of insulin-induced VEGF mRNA degradation. These results suggest that administration of BCAA may downregulate VEGF expression in patients who have hyperinsulinemia and are in the process of developing HCC. J. Cell. Biochem. 113: 3113–3121, 2012.

KEY WORDS: BCAA; HCC; VEGF; HYPERINSULINEMIA; DECAY; STABILITY

Hepatocellular carcinoma (HCC) is the fifth most frequent malignant neoplasm in the world [Bosch et al., 2004]. The rising incidence of HCC has been extensively reported in the United States [El-Serag and Mason, 2000], Japan, and several other countries [Yu and Yuan, 2004]. In recent years, much interest has centered on the relationship between abnormal sugar metabolism and liver disease including HCC, because of its association with non-alcoholic fatty liver disease (NAFLD) including its severe form, non-alcoholic steatohepatitis (NASH) [Marchesini et al., 1999]. We have reported that the development of liver fibrosis is closely associated

Abbreviations: HCC, hepatocellular carcinoma; BCAA, branched chain amino acids; VEGF, vascular endothelial growth factor; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; HIF, hypoxia inducible factor; PI-3K, phosphoinositide 3-kinase; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; FBS, fetal bovine serum; ELISA, enzyme linked immunosorbent assay; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; mTOR, mammalian target of rapamycin; qPCR, quantitative polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; aa, amino acid.

Additional supporting information may be found in the online version of this article.

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with insulin resistance in HCV infected patients [Taura et al., 2006]. The combined data indicate that it is likely that insulin resistance in chronic liver disease triggers hyperinsulinemia and may modulate the biological characteristics of HCC cells. Indeed, Komura et al. [2007] have reported that insulin therapy for coexisting diabetes mellitus is an independent risk factor for HCC recurrence after a curative resection. To examine the relevance of hyperinsulinemia to the progression of HCC, we retrospectively studied a total of 140 patients, who were newly diagnosed with HCC at the Department of Gastroenterology and Hepatology in Nagasaki University Hospital [Miuma et al., 2009]. In that study, we reported that fasting hyperinsulinemia is a risk factor that is associated with a poor prognosis at the early stage of HCC and with a high-recurrence rate at the curative stage of HCC. Hyperinsulinemia alone is also thought to be a risk and a poor prognosis factor for patients with HCC.

A recent large-scale study has reported that administration of branched chain amino acids (BCAA) improves glucose intolerance and hyperinsulinemia in cirrhotic patients [Muto et al., 2005]. It has also been reported that in obese cirrhotic patients, who have been diagnosed with diabetes mellitus, the risk of developing HCC is significantly reduced following long-term administration of BCAA [Muto et al., 2006]. This report is very interesting, because it shows that a close association exists between insulin resistance due to hyperinsulinemia and BCAA, and that this association contributes to the progression of HCC in cirrhotic patients. There have been only a few reports to date regarding the suppression of liver cancer progression by BCAA. Murata and Moriyama [2007] showed that isoleucine prevents tumor growth in a mouse liver metastatic model of colon cancer through inhibition of vascular endothelial growth factor (VEGF). Yoshiji et al. [2010] reported that BCAA exerts a chemopreventive effect against HCC, which is associated with the suppression of VEGF expression and hepatic neovascularization in obese diabetic rats. Both of these reports suggest an anti-angiogenesis activity of BCAA or Isoleucine through suppression of VEGF expression. However, the mechanism by which BCAA administration suppresses VEGF expression remains unclear.

Angiogenesis is a necessary event for tumor growth and metastasis [Folkman et al., 1989; Weidner et al., 1991]. VEGF is one of the most potent of the angiogenic factors that have been identified [Ahmed et al., 2004; Underiner et al., 2004]. Furthermore previous studies [Ng et al., 2001; Poon et al., 2001] have reported that VEGF is a potent angiogenic factor leading to HCC invasiveness and metastasis. One trigger of VEGF expression is hypoxia caused by an imbalance in oxygen supply and consumption [Knighton et al., 1983; Shweiki et al., 1992]. Hypoxia-induced upregulation of VEGF is considered to be mediated primarily through hypoxia inducible factor (HIF), which is a heterodimeric basic helix–loop–helix transcription factor composed of two subunits, HIF-1α and HIF-1β [Wang et al., 1995; Sharp and Bernaudin, 2004]. Under hypoxic conditions, HIF-1α binds to HIF-1β and forms the HIF complex, which recognizes a consensus hypoxia response element in the VEGF promoter as well as in the promoters of a broad range of other HIF target genes [Hirota and Semenza, 2005]. Some reports suggest that the HIF-1 system is also induced by growth factors such as insulin under non-hypoxic conditions [Jiang et al., 2001; Laughner et al., 2001; Stiehl et al., 2002]. It has been reported that insulin induction of VEGF through HIF-1α mainly occurs through activation of the PI-3K pathway. However, VEGF induction by HIF-1α in HCC cells also involves a MAPK pathway and ROS production [Fukuda et al., 2002; Biswas et al., 2007].

Our aim was to determine the impact of BCAA on the development or progression of HCC in patients with hyperinsulinemia from the aspect of angiogenesis. Therefore, in the present study, we examined the effect of BCAA on VEGF expression in HCC cells cultured under high-insulin conditions.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

Dulbecco's essential medium, fetal bovine serum (FBS) and a solution of human Insulin were obtained from Sigma Chemical Co. (St. Louis, MO). Each of the BCAA components; Valine, Leucine, and Isoleucine, as well as BCAA-free medium, which contained all amino acids except for the BCAA components, were obtained from Ajinomoto Pharmaceuticals Co. (Tokyo, Japan; Table I). Actinomycin D was obtained from Nacalai Tesque Co. (Kyoto, Japan). Human HCC cell lines, HepG2, Huh1, and Huh7 were obtained from the American Type Tissue Culture Collection (ATCC). They were maintained in Dulbecco's essential medium with low glucose containing 10% FBS, 100 mg/ml penicillin G, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere containing 5% CO2.

QUANTIFICATION OF SECRETED VEGF PROTEIN BY ELISA

An equal number of HepG2, Huh-7, and Huh1 (5×10^4 cells per well) were plated in 96-well plates in DMEM containing 10% FBS. After the cells reached 70–80% confluency, the growth medium

TABLE I. Amino Acid Composition of BCAA Free Medium

Amino acid	Concentration (mM)	Amino acid	Concentration (mM)
Glycine	0.4	L-Asparagine	0.4
L-Alanine	0.4	L-Glutamic Acid	0.4
L-Serine	0.4	L-Aspartic Acid	0.4
L-Threonine	0.8	L-Valine	0
L-Cystine 2HCl	0.2	L-Leucine	0
L-Methionine	0.2	L-Isoleucine	0
L-Glutamine	4.0	L-Phenylalanine	0.4
L-Arginine-HCl	0.4	L-Tyrosine	0.4
L-Proline	0.4	L-Tryptophan	0.08
L-Lysine-HCl	0.8	L-HistidineHCl-H2O	0.2

was removed and was replaced with fresh BCAA-free medium without FBS. The cells were then incubated for 20 h, following which the medium was replaced with media with or without 200 nM insulin for 48 h. If BCAA was added, it was added at the concentrations indicated in the text, 30 min before insulin treatment. The medium was then harvested, filtered, and used for measurement of secreted VEGF. VEGF present in the medium was measured using the Quantikine Human VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. In brief, the cells were incubated in a 96-well plate as describe above, and 20 μl of CellTiter96® AQ_{ueous} One Solution Reagent (Promega, Madison, WI,) were added to each well. Following incubation for 2 h at 37°C, the color reaction was recorded at 490 nm using an automated plate reader (Bio-Rad, Melville, NY).

WESTERN BLOTTING

HepG2 cells were seeded in a 60 mm-diameter dish and grown to 80% confluence. The culture medium was then changed to BCAA-free medium without FBS and the cells were grown for a further 20 h. The cells were then treated with or without insulin for various times. If the components of BCAA were added, they were added at the concentrations indicated in the text 30 min before insulin treatment. The cells were then lysed by the addition of lysis buffer for 10 min at 4°C, and insoluble material was removed by centrifugation at 14,000 rpm for 30 min at 4°C. The same amount of protein from each lysate (each with 30 μg total protein, 20 μl/well) was subjected to 15% SDS-polyacrylamide gel electrophoresis. The proteins were transferred onto nitrocellulose membranes, which were then blocked for 1 h using 5% non-fat dried milk in PBS containing 0.1% Tween-20 (PBS-T). The membranes were washed with PBS-T and incubated at 4°C overnight in the presence of individual primary antibodies. The membranes were washed with PBS-T and incubated with sheep anti-mouse IgG or donkey anti-rabbit IgG coupled with horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) for 1 h. The enhanced chemiluminescence system (SuperSignal West Pico Chemiluminescent Substrate; Pierce Chemicals, Rockford, IL) was used for signal detection.

Rabbit polyclonal anti-human phospho-mTOR, rabbit polyclonal anti-human phospho-p70S6K, rabbit monoclonal anti-human phospho-eIF4EBP1, rabbit polyclonal anti-human HIF-1α, and rabbit polyclonal anti-human β-Actin were obtained from Cell signaling Technology (San Diego, CA). All western blotting was performed at least in duplicate.

QUANTITATIVE PCR

The mRNAs of VEGF variants were quantified using quantitative polymerase chain reaction (qPCR). Total RNA was isolated from cell lines using the GenElute (TM) Mammalian Total RNA Miniprep Kit (Sigma Chemical Co.) according to the manufacturer's instructions. cDNA was synthesized from 1 μg of RNA using the QuantiTect Reverse Transcription kit (QIAGEN, Valencia, CA), and random hexamers. The cDNA was stored at −20°C until further analysis. Quantification of messenger RNA (mRNA) was performed using TaqMan or SYBR Green real-time PCR and the LightCycler (TM) 2.0 for Real-Time PCR system (Roche Applied Science, Indianapolis, IN). TaqMan qPCR of each VEGF isoform, without cross-reaction, was performed using the QuantiTect Probe PCR kit (QIAGEN). A common forward primer 5′-ATCTTCAAGCCATCCTGTGTGC-3′ and fluorescent hybridization probe 5′-AGTGTGTGCCCACTGAGGAGTCC-3′, both based on exon 3 sequences, were used. Each splice variant was amplified using specific reverse primers that spanned the variant specific exon boundaries: exon 5/8 for VEGF121 (5′-TGCGCTTGTCACATTTTTCTTG-3′), exon 5/7 for VEGF165 (5′-CAAGGCCCACAGGGATTTTC-3′), and exon 6/7 for VEGF189 (5′-CACAGGGAACGCTCCAGGAC-3′).

SYBR Green real-time PCR was performed for human GAPDH (forward primer 5′-TGAAGGTCGGAGTCAACGGATTTGGTCGTA-3′, reverse primer 5′-ATCTCGCTCCTGGAAGATGGTGATGGGATT-3′. To confirm specific amplification by the SYBR Green PCR, a dissociation curve analysis was performed for each primer pair, and both non-RT negative controls and water controls were used for these analyses. The amounts of loaded cDNA were normalized using GAPDH as an endogenous control. Differential gene expression was calculated by evaluation of the threshold cycle (Ct), and relative quantification was calculated using the comparative Ct method. All experiments were performed at least in duplicate.

RNA KINETICS

After HepG2 cells were treated with actinomycin-D (5 μg/ml) they were then incubated with or without 0.8 mM BCAA and 200 nM insulin. VEGF121 and VEGF165 mRNA were prepared at 0, 1, 2, 4, 6, and 8 h after actinomycin-D treatment and their mRNA levels were quantified using qPCR. The RNA quantities are expressed as a percentage of the mRNA level at the time point when actinomycin-D was added (0 h), and are referred to as the "percentage of RNA remaining." Degradation curves were estimated for these mRNAs using GraphPad Software (San Diego, CA).

STATISTICAL ANALYSIS

Values are expressed as means ± SD. Multiple comparisons were done using one-way ANOVA. Intergroup comparisons were done using Bonferroni's correction for multiple comparisons. A level of $P < 0.05$ was considered statistically significant.

RESULTS

BCAA SUPPRESSES INSULIN-INDUCED VEGF EXPRESSION IN HCC CELL LINES

HepG2, Huh7, and Huh1 cells were exposed to 200 nM of insulin in BCAA-free medium with or without 0.8 mM of BCAA for 48 h. VEGF secreted from the cells was then measured using an ELISA. In BCAA-free medium, insulin increased VEGF secretion in all HCC cell lines 1.4- to 2.3-fold compared to VEGF secretion in the absence of insulin (Fig. 1A). When 0.8 mM of BCAA was added to the BCAA-free medium, insulin-induced VEGF secretion was significantly suppressed in the HepG2 cells, but was only slightly suppressed in the Huh7 and Huh1 cells (Fig. 1A). We next assayed the effect of exposure of HepG2 cells to various concentrations of BCAA, in the presence or absence of insulin for 48 h, on VEGF secretion and on

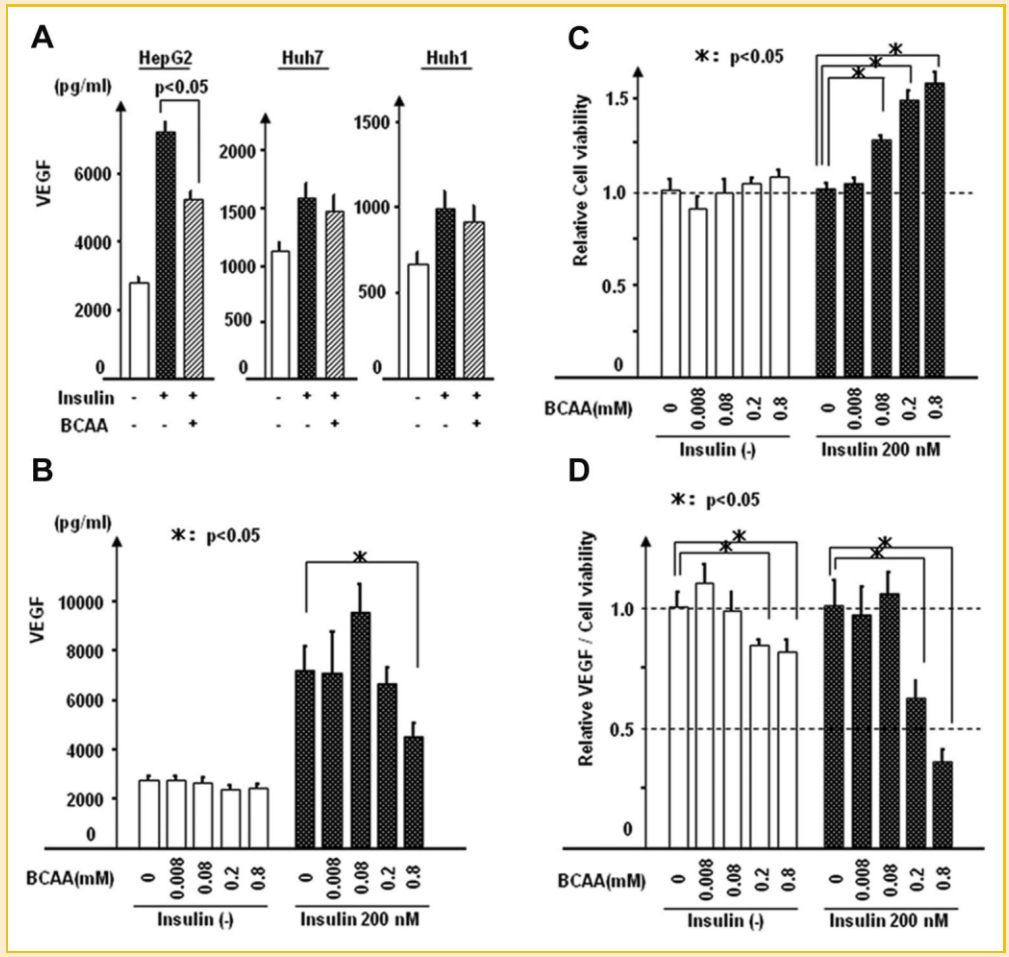

Fig. 1. Vascular endothelial growth factor (VEGF) secretion from HepG2, Huh-7, and Huh-1 cells after insulin treatment with or without BCAA. A: HepG2, Huh7, and Huh1 cells were exposed to BCAA-free medium with or without insulin (200 nM), or with insulin plus BCAA (0.8 mM) for 48 h, and VEGF secretion was analyzed by ELISA. Data represent the mean ± SD for four separate experiments. B,C: HepG2 cells were exposed to various BCAA concentrations with or without insulin for 48 h. VEGF secretion (B), and cell viability (C), were analyzed using an ELISA and an MTS assay, respectively. Data represent the mean ± SD for four separate experiments. D: The ratio of VEGF secretion to cell viability was then calculated. Data represent the ratio of VEGF secretion to cell viability over control value (the ratio in BCAA free medium), which was arbitrarily set to one with or without insulin, respectively, and represent the mean ± SD for four separate experiments.

cell viability. As the BCAA concentration was increased, insulin-induced VEGF secretion was significantly suppressed (Fig. 1B). In contrast, cell viability was significantly increased with increasing BCAA concentrations under high-insulin culture conditions (Fig. 1C). The ratio of VEGF secretion to cell viability was significantly decreased as the concentration of BCAA increased, either in the presence or absence of insulin. However, the decrease in this ratio was stronger in the presence of insulin (Fig. 1D).

BOTH BCAA AND INSULIN ACTIVATE THE mTOR PATHWAY AND INCREASE THE EXPRESSION OF HIF-1α

Previous reports showed that insulin-induced VEGF expression under non-hypoxic conditions involves activation of the PI-3K pathway, which is followed by activation of mTOR, P70S6K, and eIF4BP1 and results in the expression of HIF-1α [Fukuda et al., 2002; Stiehl et al., 2002]. It has also been shown that BCAA stimulates mTOR and activates signals that regulate protein translation and synthesis [Ijichi et al., 2003]. To determine the involvement of these signaling pathways in the above BCAA/insulin effects on HepG2 cells, we therefore examined the phosphorylation of PI-3K/m-TOR signaling proteins at 2 h, and the expression of HIF-1α at 6 h after BCAA and/or insulin treatment of HepG2 cells, using western blotting. As shown in Figure 2, the expression of phosphorylated mTOR, phosphorylated P70S6K, phosphorylated eIF4BP1, and HIF-1α was upregulated by BCAA or insulin treatment alone. Additionally, these changes were enhanced by combined treatment with both BCAA and insulin. These results indicate that BCAA and insulin synergistically activate intracellular signaling pathways that induce HIF-1α protein expression, although they had opposing effects on VEGF expression (Fig. 1).

A HIGH-INSULIN CONCENTRATION AUGMENTS, WHEREAS BCAA SUPPRESSES, THE EXPRESSION OF THREE MAJOR VARIANT VEGF mRNAs

We next analyzed the effect of BCAA and/or insulin treatment on the mRNA expression level of VEGF using qPCR. It is known that

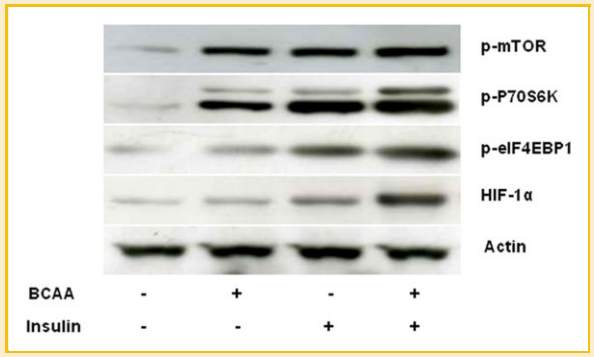

Fig. 2. Effects of BCAA and insulin on the expression of phosphorylated mTOR, phosphorylated p70S6K, phosphorylated eIF4EBP-1, and HIF-1α. HepG2 cells were exposed to BCAA-free medium with or without BCAA (0.8 mM) and/or insulin (200 nM). The expression of phosphorylated mTOR, phosphorylated p70S6K, and phosphorylated eIF4EBP-1 in cell lysates was analyzed after 2 h incubation by Western blotting using the appropriate antibodies. The expression of HIF-1α was similarly analyzed following 6 h incubation. Actin was blotted as a loading control.

there are many VEGF isoforms, that are derived from a single gene by alternative splicing. The three major isoforms of VEGF are VEGF121 (121 aa), VEGF165 (165 aa), and VEGF189 (189 aa) [Park et al., 1993; Neufeld et al., 1994; Takahashi and Shibuya, 2005]. It has been reported that the majority of HCC abundantly express VEGF121 and VEGF165, and that high-VEGF165 expression is related to poor prognosis of HCC patients [Jeng et al., 2004a]. Prior to analysis of VEGF mRNA expression in cells, we confirmed the reliability of the specific primer pairs and probes designed to assay VEGF121, VEGF165, and VEGF189 mRNA expression (Supplementary Figure). HepG2 cells were then incubated for various times with 200 nM of insulin in BCAA-free medium with or without 0.8 mM of BCAA, and the expression of VEGF mRNAs in the cells was then analyzed using qPCR. As shown in Figure 3, VEGF165 mRNA was the most abundant, and VEGF121 mRNA was the second-most abundant VEGF mRNA in HepG2 cells. VEGF189 mRNA was only weakly expressed. Insulin-induced expression of all three VEGF mRNAs decreased significantly 2h after BCAA treatment and remained low over the next 14 h. Since it takes 6 h to induce detectable levels of HIF-1α following BCAA treatment it is likely that the suppression of VEGF mRNA expression by BCAA is independent of the HIF-1 system.

BCAA DECREASE THE STABILITY OF INSULIN-INDUCED VEGF mRNA

The above data suggested that BCAA and insulin modulate intracellular signaling that regulates HIF-1α expression in a coordinated manner, but that BCAA antagonizes the effect of insulin on induction of the expression of VEGF mRNA. We therefore further analyzed the effects of BCAA and insulin on the post transcriptional regulation of VEGF mRNAs in these cells by determination of their effect on the half-life of VEGF mRNAs following inhibition of transcription using actinomycin-D. HepG2 cells were treated with actinomycin-D (5 μg/ml), and were incubated with insulin (200 nM) with or without BCAA (0.8 mM) for 0, 1, 2, 4, 6, and 8 h. The level of VEGF121 and VEGF165 mRNAs at these time points (termed "the remaining mRNA") was analyzed using q-PCR. Insulin treatment increased the stability of VEGF121 and VEGF165 mRNA in BCAA-free medium, respectively (Fig. 4). The addition of BCAA significantly decreased the stability of VEGF121 or VEGF165

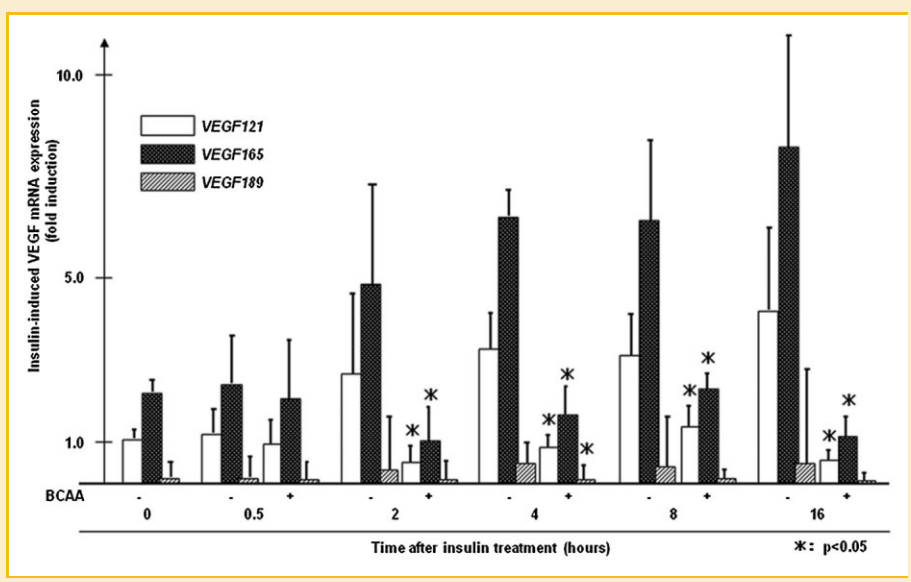

Fig. 3. Time course analysis of the mRNA expression of the three major isoforms of VEGF after insulin treatment with or without BCAA. HepG2 cells were exposed to insulin (200 nM) in BCAA free medium with or without BCAA (0.8 mM). The mRNA expression of VEGF121, VEGF165, and VEGF189 in the cells was then analyzed at the indicated times using quantitative PCR. Data represent fold induction of VEGF mRNA expression over control value (VEGF121 mRNA at 0 h), which was arbitrarily set to one, and represent the mean ± SD for four separate experiments. ($^*P < 0.05$ for VEGF121, VEGF165, and VEGF189 mRNA level of BCAA(+)Insulin(+) group compared with BCAA(−)Insulin(+) group at each time).

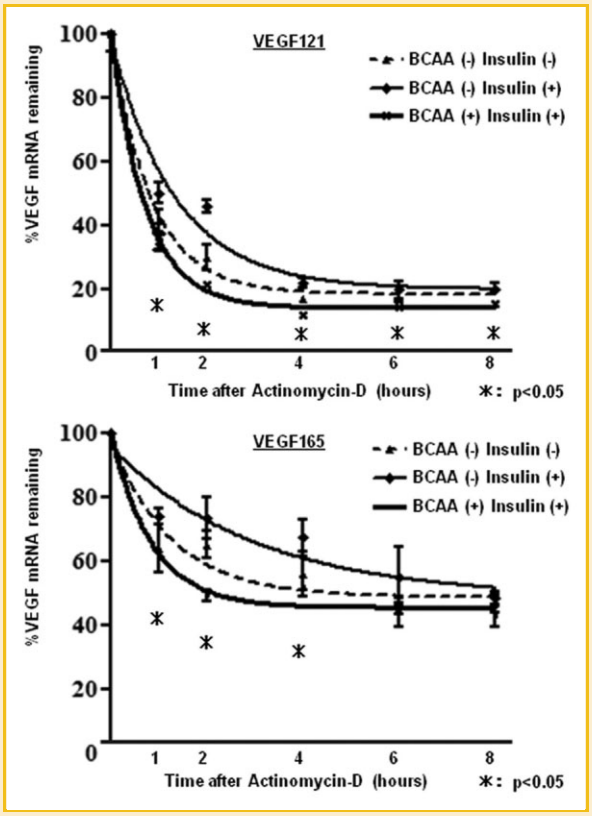

Fig. 4. VEGF121 and VEGF165 mRNA stability after actinomycin-D treatment with or without BCAA and insulin. HepG2 cells were incubated in BCAA-free medium for 20 h. The medium was then changed to fresh BCAA-free medium, or to medium with insulin (200 nM) with or without BCAA (0.8 mM), and actinomycin-D (5 μg/ml) was added to all cultures. mRNA was prepared 0, 1, 2, 4, 6, and 8 h after actinomycin-D treatment and the levels of VEGF121 and VEGF165 mRNA remaining at each time point were analyzed using quantitative PCR. Data represent mRNA expression over control value (VEGF 121 and VEGF 165 mRNA at 0 h), which was arbitrarily set to 100%, and represent the mean ± SD for four separate experiments. Degradation curves were constructed based on a model of exponential decay. (*$P < 0.05$ for VEGF121 and EGF165 mRNA level of BCAA(+)Insulin(+) group compared with BCAA(−)Insulin(+) group at each time).

mRNA, respectively. These data suggested that BCAA decreased VEGF mRNA stability at high-insulin concentrations.

A DEFICIT OF ONE COMPONENT OF BCAA ENHANCED THE EXPRESSION OF VEGF mRNA AT HIGH-INSULIN CONCENTRATIONS

BCAA consist of the three amino acids, Valine, Leucine, and Isoleucine. We examined which component, or combination of BCAA components, suppresses insulin-induced expression of VEGF mRNAs. HepG2 cells were exposed to insulin (200 nM) in BCAA-free medium containing Valine, Leucine, or Isoleucine at 0.8 or 2.4 mM concentrations. The concentration of 2.4 mM was used to adjust the Fischer ratio, which represents the ratio of branched-chain amino acids to aromatic amino acids. The cells were alternatively exposed to insulin (200 nM) and BCAA containing all three components, each at a concentration of 0.8 mM. Following incubation for 4 h, the mRNA expression of VEGF121 and VEGF165 was analyzed by qPCR. The results of this experiment are shown in Figure 5A. BCAA-free medium supplemented with individual amino acids at 0.8 mM concentration, or even at 2.4 mM concentration, could not suppress the expression of VEGF mRNAs at a high-insulin concentration. In contrast, the combination of the three components of BCAA, each at 0.8 mM concentration, strongly suppressed insulin-induced mRNA expression of both VEGF isoforms. We next analyzed the effect of combining two of the components of BCAA on insulin-induced upregulation of VEGF mRNAs. The cells were therefore exposed to insulin (200 nM) in BCAA-free medium supplemented with Valine and Leucine, Valine and Isoleucine, or Leucine and Isoleucine, each at a concentration of 0.8 or 1.2 mM. The cells were also exposed to insulin (200 nM) in media containing the three components of BCAA, each at a concentration of 0.8 mM. As seen for the single supplementation, double supplementation using any combination of the three components of BCAA, with each component at either 0.8 or 1.2 mM, did not suppress insulin-induced expression of VEGF mRNAs (Fig. 5B). These data indicate that all three components of BCAA are needed to accelerate the degradation of VEGF mRNAs at a high-insulin concentration, and thus, a deficiency in any of the components of BCAA stabilizes VEGF mRNA induced by a high-insulin concentration.

DISCUSSION

In this study, we demonstrated that treatment of HepG2 cells with BCAA suppresses insulin-induced VEGF secretion particularly at high-insulin concentrations. Individually BCAA and insulin similarly activate the mTOR pathway and increase the expression of HIF-1α. However, BCAA inhibits insulin-induced expression of VEGF mRNA by decreasing the stability of insulin-induced VEGF mRNAs.

We found that all three of the components of BCAA are required for this inhibitory effect. Neither individual components, nor any combination of two components, are inhibitory. In our experiments with complete BCAA, the concentration of Valine, Leucine, and Isoleucine was set at 0.8 mM each, to yield a Fischer ratio of 3.0. It has been reported that the mean value of the Fischer ratio is 3.5 in healthy controls, 2.7–3.0 in chronic hepatitis, 1.5 in compensated cirrhosis, and 1.1 in decompensated cirrhosis [Kano et al., 1991]. Based on these data we consider that the concentration of BCAA used in our experiments is appropriate for analysis of HCC cell metabolism. Indeed, the suppressive effect of BCAA on insulin-induced VEGF secretion was strengthened as the BCAA concentration was increased to give a Fischer ratio of 3.0 (Fig. 1D).

Several studies [Poon et al., 2001; Kaseb et al., 2009; Schoenleber et al., 2009] have reported a close association between the level of VEGF in tissue or serum and the development and progression of HCC, in which angiogenesis plays an important role. Park et al. [2000] have reported that the expression of VEGF gradually increases with the stepwise development of HCC. Therefore, BCAA administration has the potential to modulate the early stage of hepatocarcinogenesis, rather than the advanced stage of HCC, through the suppression of VEGF secretion.

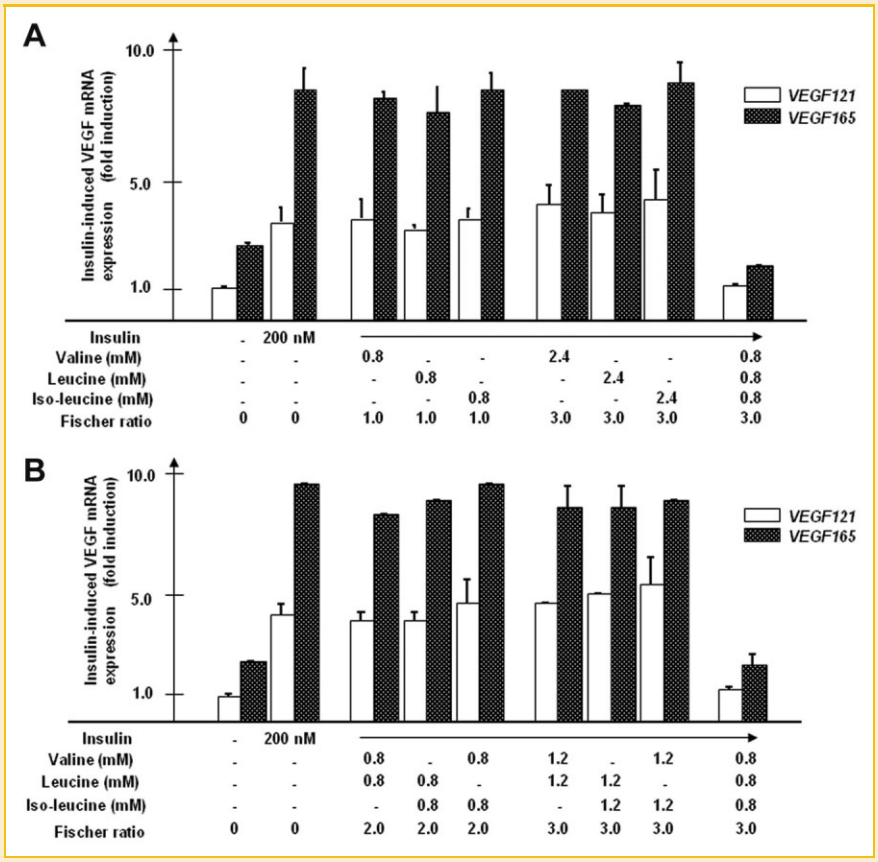

Fig. 5. The mRNA expression of VEGF 121 and VEGF 165 after insulin treatment with or without Valine, Leucine, and/or Isoleucine. A. HepG2 cells were exposed to BCAA-free medium with or without insulin (200 nM) in the presence or absence of individual amino acids, Valine, Leucine, or Isoleucine each at a concentration of 0.8 or 2.4 mM. The cells were alternatively exposed to insulin (200 nM) with BCAA (containing 0.8 mM each of all three amino acids). After 4 h incubation the mRNA expression of VEGF121 and VEGF165 was analyzed using quantitative PCR. B. HepG2 cells were exposed to BCAA free medium with or without insulin (200 nM) in the presence or absence of two amino acids, either Valine and Leucine, Valine and Isoleucine, or Leucine and Isoleucine, each at a concentration of 0.8 or 1.2 mM. The cells were alternatively exposed to insulin (200 nM) with BCAA (containing 0.8 mM each of all three amino acids). After 4 h incubation the mRNA expression of VEGF121 and VEGF165 was analyzed using quantitative PCR. The Fischer ratio, (ratio of branched-chain amino acids to aromatic amino acids) is shown below. Data represent fold induction of VEGF mRNA expression over control value (VEGF121 mRNA in BCAA free medium without insulin), which was arbitrarily set to one, and represent the mean ± SD for four separate experiments.

In our study, BCAA suppressed insulin-induced expression of all isoforms of VEGF mRNAs in a similar manner, including that of VEGF165 mRNA, whose expression has been reported to be linked to poor prognosis of HCC patients [Jeng et al., 2004b]. These results indicate that the process of alternative splicing of the VEGF gene is unaffected by BCAA.

It is a well-known fact that a major transcriptional activator of the VEGF gene is HIF1α [Semenza, 2002]. In this study, HIF1α expression was enhanced by combined treatment with both BCAA and insulin. It is likely that BCAA, which was reported to activate mTOR pathways [Ijichi et al., 2003], contribute to upregulate HIF1α expression with additive effect. Nevertheless, secretory VEGF expression was suppressed through the decrease of insulin-induced VEGF mRNA stability by BCAA. This result raises the possibility that the regulation of VEGF mRNA at post-transcriptional level plays an important role in secretory VEGF expression. Regulation of VEGF expression by modulation of its mRNA stability has also been reported to occur during induction of VEGF by hypoxia [von Marschall et al., 2001; Yoo et al., 2006].

Contrary to our expectations, all three components of BCAA are a prerequisite for the decay of VEGF mRNAs. Neither individual components, nor any combination of two components had this inhibitory effect. Interestingly, BCAA suppression was stronger at high-insulin concentrations. This phenomenon is in agreement with the results of a recent large-scale study, which reported that the administration of BCAA reduced the development of HCC in obese cirrhotic patients who had been diagnosed with diabetes mellitus [Muto et al., 2006]. This phenomenon may simply reflect an improvement of a BCAA deficiency rather than a direct effect of added BCAA. In other words, if there is a deficit in even one component of BCAA, VEGF mRNA is not degraded. It is likely that a BCAA deficit is a critical condition for HCC cell survival and that, even in the presence of a growth factor (such as insulin), HCC cells will give priority to the synthesis of proteins that are essential for survival.

In conclusion, we have demonstrated that a deficit of BCAA prevents the degradation of insulin-induced VEGF mRNA. This effect is repressed by supplementation with BCAA. These results

suggest that administration of BCAA to cirrhotic patients exhibiting both hyperinsulinemia and a decreased Fischer ratio has the potential to decrease HCC development or progression through suppression of VEGF expression.

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