

## Supplementation with Branched-chain Amino Acids Inhibits Azoxymethane-induced Colonic Preneoplastic Lesions in Male C57BL/KsJ-*db/db* Mice

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**Abstract Purpose:** Obesity and related metabolic abnormalities, including insulin resistance and activation of the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, are risk factors for colon cancer. Supplementation with branched-chain amino acids (BCAA) reduces the risk of liver cancer in cirrhotic patients who are obese, and this has been associated with an improvement of insulin resistance. The present study examined the effects of BCAA on the development of azoxymethane (AOM)-initiated colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) mice that were obese and had hyperinsulinemia.

**Experimental Design:** Male *db/db* mice were given 4 weekly s.c. injections of AOM (15 mg/kg of body weight) and then they were fed a diet containing 3.0% BCAA or casein, a nitrogen content – matched control diet, for 7 weeks.

**Results:** Feeding with BCAA caused a significant reduction in the number of total aberrant crypt foci and  $\beta$ -catenin accumulated crypts, both of which are premalignant lesions of the colon, compared with the control diet – fed groups. BCAA supplementation caused a marked decrease in the expression of IGF-IR, the phosphorylated form of IGF-IR, phosphorylated glycogen synthase kinase 3 $\beta$ , phosphorylated Akt, and cyclooxygenase-2 proteins on the colonic mucosa of AOM-treated mice. The serum levels of insulin, IGF-I, IGF-II, triglyceride, total cholesterol, and leptin were also decreased by supplementation with BCAA.

**Conclusion:** BCAA supplementation in diet improves insulin resistance and inhibits the activation of the IGF/IGF-IR axis, thereby preventing the development of colonic premalignancies in an obesity-related colon cancer model that was also associated with hyperlipidemia and hyperinsulinemia. BCAA, therefore, may be a useful chemoprevention modality for colon cancer in obese people.

Colorectal cancer (CRC) is a major health problem worldwide. Recent evidence indicates that the risk of CRC is elevated in patients with metabolic syndrome, also called insulin resistance syndrome, which is commonly associated with obesity and related metabolic abnormalities (1, 2). Obesity is the main determinant of insulin resistance and hyperinsulinemia, which is also a possible risk factor for CRC (3). CRC occurs more frequently in patients with diabetes mellitus, a condition associated with hyperinsulinemia (4, 5). Insulin has growth-

promoting properties in CRC cells, and exogenous insulin injection stimulates the growth of CRC precursors in rodent models (6–8). In addition, elevated circulating levels of insulin causes alterations in the insulin-like growth factor (IGF)/IGF-I receptor (IGF-IR) axis, which is involved in the development and progression of CRC (9, 10). Therefore, increased insulin resistance and abnormalities in the IGF/IGF-IR axis might be a critical target to prevent the development of obesity-related malignancies, including CRC. For instance, (-)-epigallocatechin gallate, the major biologically active component of green tea, inhibited the development of colonic premalignant lesions in an obesity-related colon cancer that was associated with improvement in insulin resistance and inhibition of the IGF/IGF-IR axis (11).

Diet supplementation with branched-chain amino acids (BCAA; leucine, isoleucine, and valine) has been suggested to improve protein malnutrition in patients with liver cirrhosis (12). Recent studies have revealed that BCAA is useful for both preventing progressive hepatic failure and improving event-free survival in patients with chronic liver diseases, such as liver cirrhosis, and these beneficial effects are associated with the improvement of insulin resistance by BCAA (13–15). In addition, oral supplemental treatment with BCAA can reduce the risk of hepatocellular carcinoma in cirrhotic patients who

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Received 8/10/08; revised 1/8/09; accepted 1/23/09; published OnlineFirst 4/14/09.

**Grant support:** Ministry of Education, Science, Sports and Culture of Japan Grants-in-Aid Nos. 18790457 (M. Shimizu) and 17015016 (H. Moriwaki).

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doi:10.1158/1078-0432.CCR-08-2093

## Translational Relevance

Obesity and related metabolic abnormalities, including insulin resistance and the activation of the insulin-like growth factor (IGF)/IGF-I receptor axis, are associated with colorectal cancer (CRC) development. Therefore, the prevention of CRC by targeting the dysregulation of energy homeostasis might be a promising strategy for obese people who are at increased risks of CRC. We believe that this study is novel and clinically relevant because this article is the first report indicating that supplementation with branched-chain amino acids (BCAA) effectively suppressed the development of azoxymethane-induced putative precursor lesions of colonic adenocarcinoma in C57BL/KsJ-*db/db* mice that are obese and developed diabetes mellitus. Our studies indicate that this suppressing effect of BCAA was associated with improvement of hyperlipidemia and hyperleptinemia. BCAA supplementation could also improve insulin resistance and exert a depressant effect on the IGF/IGF-IR axis. The current findings suggest the possibility of using BCAA as a chemopreventive agent for obesity-related malignancies.

are obese (with a body mass index  $\geq 25$ ; ref. 16). Obesity, hyperinsulinemia, and diabetes mellitus are possible risk factors for hepatocellular carcinoma, which commonly develops in cirrhotic livers (16–18). Based on these findings, BCAA supplementation in diet may also reduce the risk of other obesity-related human malignancies, including CRC, by improving insulin resistance. However, no detailed studies on whether BCAA can prevent the development of obesity-related CRC have yet been conducted.

In previous studies, we have established a useful preclinical animal model to determine the possible underlying mechanisms of how specific agents prevent the development of obesity-related CRC with the use of C57BL/KsJ-*db/db* (*db/db*) mice with obesity, hyperinsulinemia, and hyperleptinemia (19–21). The mice are susceptible to the colonic carcinogen azoxymethane (AOM) because the development of AOM-induced aberrant crypt foci (ACF) and  $\beta$ -catenin-accumulated crypts (BCAC), both of which are putative precursor lesions for colonic adenocarcinoma (22, 23), is enhanced in *db/db* mice compared with *db/+* or *+/+* mice (19, 20). In the present study, we investigated in detail the effects of BCAA on the development of colonic premalignant lesions, ACF and BCAC, in *db/db* mice initiated with AOM, focusing on the improvement of hyperinsulinemia, hyperlipidemia, and hyperleptinemia. In addition, we also determined whether BCAA supplementation in the diet inhibits the activation of the IGF/IGF-IR axis in this animal model.

## Materials and Methods

**Animals, chemicals, and diets.** Four-week-old male homozygous *db/db* mice were obtained from Japan SLC, Inc.. All mice were maintained at the Gifu University Life Science Research Center according to the Institutional Animal Care Guidelines. AOM was purchased from Sigma Chemical Co.. BCAA and casein were obtained from Ajinomoto Co., Ltd.. The BCAA composition (2:1:1.2, leucine/isoleucine/valine) was set at the

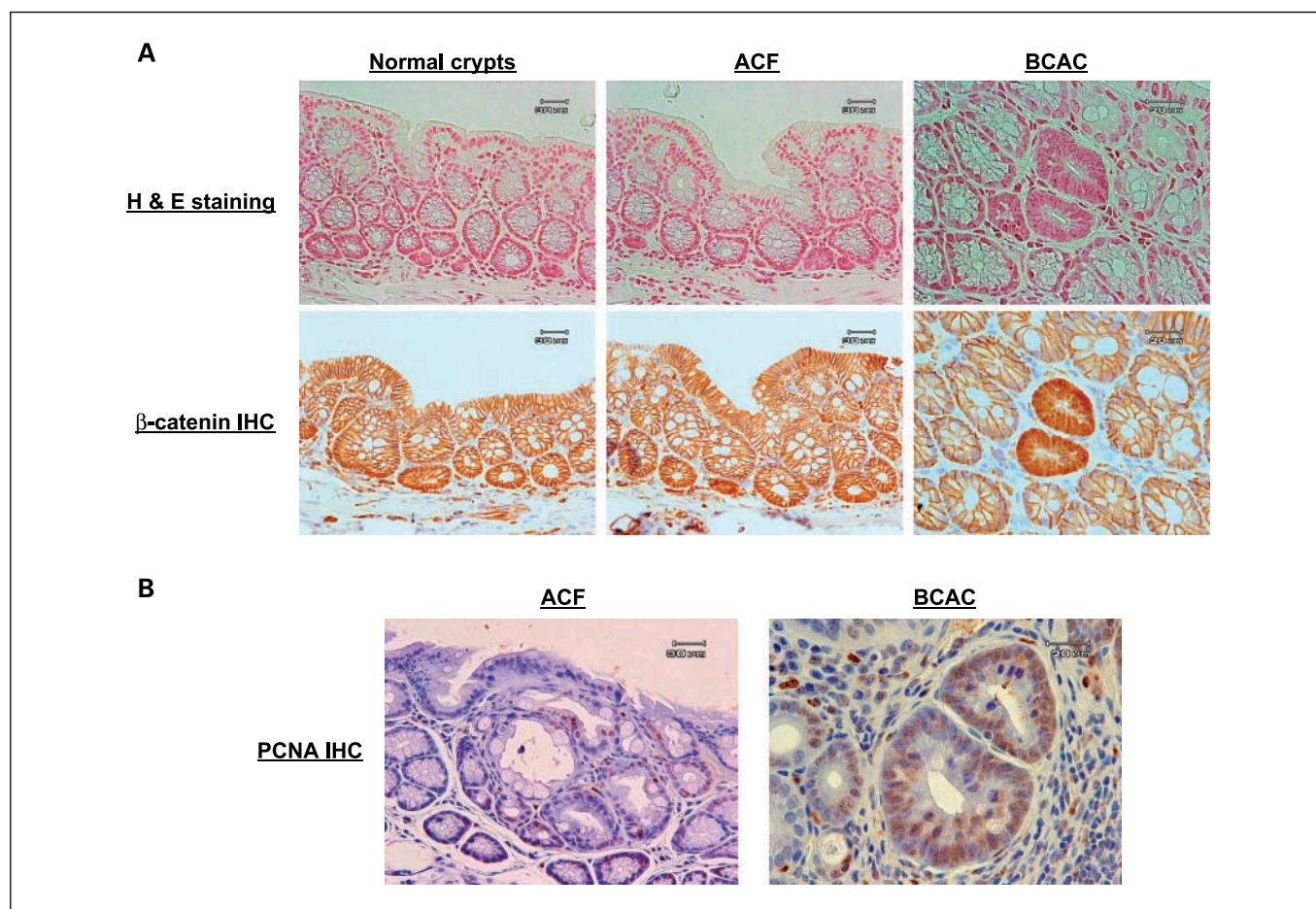
clinical dosage that is used for the treatment of hypoalbuminemia in patients with decompensated liver cirrhosis in Japan.

**Experimental procedure.** The animal experiment was approved by the Institutional Committee of Animal Experiments of Gifu University. A total of 54 male *db/db* mice were divided into 6 groups. At 5 wk of age, the mice in groups 1 to 3 were s.c. injected with AOM (15 mg/kg of body weight) weekly for 4 wk. As controls, the mice in groups 4 to 6 were given s.c. injections of saline. Groups 1 (12 mice) and 4 (6 mice) were fed a basal diet, corticotropin-releasing factor (CRF)-1 (Oriental Yeast Co., Ltd.), throughout the experiment. Groups 3 (12 mice) and 6 (6 mice) were given a basal diet containing 3.0% BCAA (weight for weight) for 7 wk, starting 1 wk after the last injection of AOM. The BCAA concentration (3.0%) was determined by the previous study, which indicated the same intake to improve insulin resistance in C57BL/6J mice (24). The mice in groups 2 (12 mice) and 5 (6 mice) were given a basal diet containing 3.0% casein (weight for weight). The casein-fed groups were served as nitrogen content-matched controls for the BCAA-treated groups to eliminate the possibility that the nitrogen content itself affects the promotion or the prevention of colonic premalignant lesions. At the termination of the study (16 wk of age), the mice were sacrificed by CO<sub>2</sub> asphyxiation to analyze the number of colonic ACF and BCAC.

**Counting the number of ACF and BCAC.** The ACF and BCAC were determined according to the standard procedures described previously (20, 21, 25). ACF are defined as single or multiple crypts that have altered luminal openings, exhibit thickened epithelia, and are larger than adjacent normal crypts (22). BCAC, which have high frequency mutations in the  $\beta$ -catenin gene, show histologic dysplasia with a disruption of the cellular morphology and an accumulation of this protein (Fig. 1A; ref. 23). BCAC do not have a typical ACF-like appearance because the lesion is not recognized on the mucosal surface like ACF and is only identified in the histologic sections of en face preparations. Both of these lesions are utilized as biomarkers to evaluate a number of agents for their potential chemopreventive properties (26). After the colons were fixed flat in 10% buffered formalin for 24 h, the mucosal surface of the colons were stained with methylene blue (0.5% in distilled water), and then the number of ACF were counted under a light microscope. Thereafter, the distal parts (5 cm from the anus) of the colon were cut to count the number of BCAC. To identify BCAC intramucosal lesions, the distal part of the colon (mean area, 0.7 cm<sup>2</sup> per colon) was embedded in paraffin, and then a total of 20 serial sections (4- $\mu$ m thick each) per colon were made by an en face preparation (20, 21, 25). For each case, 2 serial sections were used to analyze BCAC.

**Histopathology and immunohistochemical analyses for  $\beta$ -catenin and PCNA.** Three serial sections were made from paraffin-embedded tissue blocks. Two sections were subjected to H&E staining for histopathology and  $\beta$ -catenin immunohistochemistry to count the number of BCAC. The other section was used for the proliferating cell nuclear antigen (PCNA), a G<sub>1</sub>-to-S phase marker, immunohistochemistry to estimate the cell proliferative activity in the colonic mucosa. Immunohistochemical analyses for  $\beta$ -catenin and PCNA were done with the labeled streptavidin-biotin method (LSAB kit; DAKO) as previously described (20, 21). Anti- $\beta$ -catenin antibody (1:1,000 final dilution) was obtained from Transduction Laboratories (catalogue no. 610154). Anti-PCNA antibody (1:100 final dilution) was from Santa Cruz Biotechnology, Inc. (sc-7907). Negative control sections were immunostained without the primary antibody. PCNA-positive cells in the colonic mucosa, which seemed normal by H&E staining, were counted and expressed as a percentage of the total number of normal crypt cells. The PCNA labeling index (%) was determined by counting at least 200 crypt cells in each mouse (a total of 1,000 crypt cells per group). Two experienced pathologists (Y. Hirose and T. Tanaka) immunohistologically determined the BCAC and PCNA-positive cells.

**Protein extraction and western blot analysis.** Total proteins were extracted from the scraped mucosa from the remaining colon of the AOM-treated mice (groups 1 to 3), and equivalent amounts of proteins



**Fig. 1.** Histopathology and immunohistochemical expression of  $\beta$ -catenin and PCNA proteins in ACF and BCAC. *A*, representative photographs of ACF and BCAC induced by AOM in *db/db* mice. Top, H&E staining; bottom,  $\beta$ -catenin immunohistochemistry. Left, normal crypts; middle, ACF; right, BCAC. The localization of the accumulated  $\beta$ -catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells in BCAC. *B*, immunohistochemical pattern of PCNA protein in ACF and BCAC. The nuclear expression of the PCNA protein significantly increased in BCAC compared with ACF and surrounding normal crypts. Bar, 20 or 30  $\mu$ m, respectively.

(40  $\mu$ g per lane) were examined by a western blot analysis with the use of the primary antibodies for IGF-1R, phosphorylated IGF-1R (p-IGF-1R), phosphorylated glycogen synthase kinase 3 $\beta$  (p-GSK-3 $\beta$ ), Akt, phosphorylated Akt (p-Akt), cyclooxygenase-2 (COX-2), and glyceraldehyde-3-phosphate dehydrogenase as described previously

(11, 27, 28). An antibody to glyceraldehyde-3-phosphate dehydrogenase served as a loading control. The intensities of the blots were quantified with the NIH Image software version 1.62. The intensities of the blots found at the CRF-fed mice in each antibody was set at 1, and the changes in expression were shown as the fold difference.

**Table 1.** Body, liver, kidney, and white adipose tissue weights of the experimental mice

Group no.	Treatment	Diet	No. of mice	Final body weight (g)	Body length (cm)	BMI	Absolute organ weight		
							Liver	Kidney	White adipose tissue
1	AOM 15 mg/kg	CRF-1	12	49.7 $\pm$ 8.3 <sup>*, †</sup>	9.25 $\pm$ 0.77	0.58 $\pm$ 0.05	2.64 $\pm$ 0.76	0.38 $\pm$ 0.04 <sup>†</sup>	2.67 $\pm$ 0.64
2	AOM 15 mg/kg	Casein	12	51.7 $\pm$ 4.8	9.43 $\pm$ 0.37	0.58 $\pm$ 0.02	2.75 $\pm$ 0.48 <sup>‡</sup>	0.41 $\pm$ 0.04	2.74 $\pm$ 0.37
3	AOM 15 mg/kg	BCAA	12	50.3 $\pm$ 5.0 <sup>§</sup>	9.47 $\pm$ 0.25	0.56 $\pm$ 0.04	2.58 $\pm$ 0.64 <sup>§</sup>	0.40 $\pm$ 0.05	2.51 $\pm$ 0.42
4	Saline	CRF-1	6	58.1 $\pm$ 2.5	9.63 $\pm$ 0.22	0.63 $\pm$ 0.02	3.35 $\pm$ 0.72	0.45 $\pm$ 0.06	3.02 $\pm$ 0.32
5	Saline	Casein	6	58.0 $\pm$ 2.1	9.70 $\pm$ 0.18	0.62 $\pm$ 0.01	3.87 $\pm$ 1.04	0.44 $\pm$ 0.04	2.70 $\pm$ 0.38
6	Saline	BCAA	6	58.5 $\pm$ 2.5	9.63 $\pm$ 0.17	0.63 $\pm$ 0.01	3.83 $\pm$ 0.86	0.44 $\pm$ 0.01	2.60 $\pm$ 0.35

\*Mean  $\pm$  SD.

<sup>†</sup>Significantly different from group 4 ( $P < 0.05$ ).

<sup>‡</sup>Significantly different from group 5 ( $P < 0.05$ ).

<sup>§</sup>Significantly different from group 6 ( $P < 0.05$ ).

**Table 2.** Effects of BCAA on AOM-induced ACF and BCAC formation in the experimental mice

Group no.	Treatment	Diet	No. of mice	Length of colon (cm)	Total no. of ACFs per colon	Total no. of BCACs/cm <sup>2</sup>
1	AOM 15 mg/kg	CRF-1	12	12.4 ± 1.4*	85.9 ± 8.1	11.7 ± 8.4
2	AOM 15 mg/kg	Casein	12	12.5 ± 0.5	83.4 ± 11.2	8.3 ± 3.9
3	AOM 15 mg/kg	BCAA	12	12.0 ± 0.7	54.5 ± 8.6 <sup>†,‡</sup>	4.2 ± 6.7 <sup>§</sup>
4	Saline	CRF-1	6	12.5 ± 1.0	0	0
5	Saline	Casein	6	11.5 ± 0.7	0	0
6	Saline	BCAA	6	11.3 ± 0.5	0	0

\*Mean ± SD.  
<sup>†</sup>Significantly different from group 1 ( $P < 0.001$ ).  
<sup>‡</sup>Significantly different from group 2 ( $P < 0.001$ ).  
<sup>§</sup>Significantly different from group 1 ( $P < 0.05$ ).

**Clinical chemistry.** At sacrifice, blood samples were collected from the AOM-treated mice (groups 1-3) to measure the serum concentrations of insulin, leptin, triglyceride, total cholesterol, IGF-I, IGF-II, and BCAA. The serum triglyceride, total cholesterol, and BCAA levels were assayed as described previously (20, 29). The serum insulin, leptin, IGF-I, and IGF-II were determined by an enzyme immunoassay according to the manufacturer's protocol (R&D Systems).

**Statistical analysis.** The results were presented as the mean ± SD and were analyzed with the use of the GraphPad InStat software program version 3.05 (GraphPad Software) for Macintosh. Differences between groups were analyzed by one-way ANOVA or, as required, by two-way ANOVA. When ANOVA showed a statistically significant effect ( $P < 0.05$ ), comparisons of each experimental group with the control group were then made with the use of the Tukey-Kramer multiple comparisons test. The differences were considered significant when the two-tailed  $P$  was  $<0.05$ .

## Results

**General observations.** As shown in Table 1, the average body weights of groups 1 (CRF-1) and 3 (BCAA) in the AOM-injected mice at the termination of this experiment were smaller than those of the saline-injected groups 4 (CRF-1;  $P < 0.05$ ) and 6 (BCAA;  $P < 0.05$ ). The mean liver weights in the AOM-treated groups 2 (casein) and 3 (BCAA) were significantly lower than those in the saline-treated groups 5 (casein;  $P < 0.05$ ) and 6 (BCAA;  $P < 0.05$ ). Among CRF-1-fed mice, the mean kidney weight in the AOM-treated group 1 was also significantly lower than that of the saline-treated group 4 ( $P < 0.05$ ). No significant difference was observed in the body length, body mass index, and mean white adipose tissue weight among the experimental mice. A histopathologic examination also

revealed no alteration, thus suggesting the absence of toxicity of BCAA in the liver and kidney of the mice in groups 3 and 6 (data not shown).

**Effects of BCAA supplementation on AOM-induced ACF and BCAC formations in db/db mice.** Table 2 summarizes the total number of ACF and BCAC (Fig. 1) in the mice of all groups. ACF and BCAC developed in the colons of all the mice that received AOM (groups 1 to 3) but not in the colons of the mice that did not receive AOM (groups 4 to 6). Dietary supplementation with BCAA significantly decreased the number of total ACF compared with those of the CRF-1-fed (37% reduction;  $P < 0.001$ ) and casein-supplemented groups (35% reduction;  $P < 0.001$ ). Compared with the CRF-1-fed group, the administration of BCAA also significantly reduced the number of total BCAC (64% reduction;  $P < 0.05$ ).

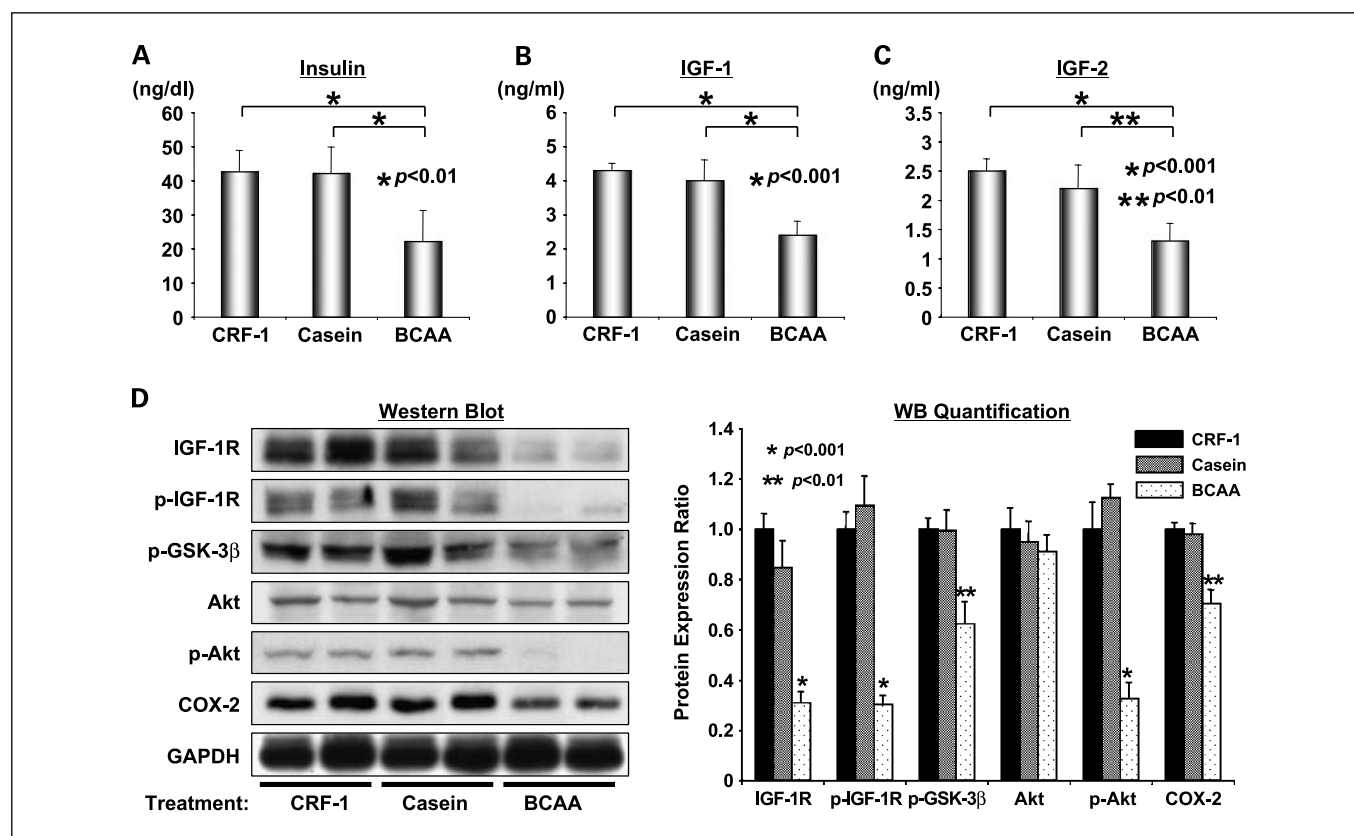
**Effects of BCAA supplementation on the serum levels of BCAA in AOM-treated db/db mice.** Because the colonic premalignant lesions developed only in the AOM-injected mice (Table 2), the following experiments were done among the mice that received AOM (groups 1 to 3). BCAA supplementation caused a significant increase in the serum concentrations of total BCAA (valine, isoleucine, and leucine;  $1736 \pm 179$  nmol/mL) compared with the CRF-1-fed ( $882 \pm 160$  nmol/mL;  $P < 0.001$ ) and casein-supplemented groups ( $853 \pm 51$  nmol/mL;  $P < 0.001$ ). These findings suggest that supplementation with 3.0% BCAA is sufficient to raise the serum concentration of BCAA.

**Effects of BCAA supplementation on the serum levels of total cholesterol, triglyceride, and leptin in AOM-treated db/db mice.** As shown in Table 3, the serum levels of total cholesterol in the BCAA-supplemented mice were significantly lower than

**Table 3.** Serum levels of total cholesterol, triglyceride, and leptin in AOM-treated db/db mice

Group no.	Treatment	Diet	No. of mice	Total cholesterol (mg/dL)	Triglyceride (mg/dL)	Leptin (ng/dL)
1	AOM 15 mg/kg	CRF-1	12	185 ± 34*	244 ± 49	117 ± 18
2	AOM 15 mg/kg	Casein	12	186 ± 40	229 ± 40	133 ± 32
3	AOM 15 mg/kg	BCAA	12	141 ± 48 <sup>†,‡</sup>	187 ± 48 <sup>†</sup>	99 ± 23 <sup>§</sup>

\*Mean ± SD.  
<sup>†</sup>Significantly different from group 1 ( $P < 0.05$ ).  
<sup>‡</sup>Significantly different from group 2 ( $P < 0.05$ ).  
<sup>§</sup>Significantly different from group 2 ( $P < 0.01$ ).



**Fig. 2.** The effect of BCAA supplementation on the serum levels of insulin, IGF-I, and IGF-II, and on the expression levels of the IGF-IR, p-IGF-1R, p-GSK-3 $\beta$ , Akt, p-Akt, and COX-2 proteins in AOM-treated *db/db* mice. *A* to *C*, the serum concentration of insulin (*A*), IGF-I (*B*), and IGF-II (*C*) were measured by an enzyme immunoassay. Bars, SD of triplicate assays. *D*, total proteins were extracted from the scraped colonic mucosa, and equivalent amounts of proteins were examined by a western blot analysis as described in Materials and Methods. Lanes, protein samples from two different mice in each group (*left*). The intensities of blots were quantitated by densitometry (*right*). Repeat western blots gave similar results. Values, mean  $\pm$  SD. \*,  $P < 0.001$  and \*\*,  $P < 0.01$ : significant differences obtained by comparison with CRF-1-treated or casein-treated mice, respectively.

those in the CRF-1-fed ( $P < 0.05$ ) and casein-supplemented mice ( $P < 0.05$ ). The mice supplemented with BCAA showed a significant decrease in the serum levels of triglyceride compared with the CRF-1 fed ( $P < 0.05$ ). The serum leptin level of group 3 (BCAA) was also significantly lower than that of group 2 (casein;  $P < 0.01$ ).

**Effects of BCAA supplementation on the serum levels of insulin, IGF-I, and IGF-II in AOM-treated *db/db* mice.** Supplementation with BCAA caused a significant decrease in the serum levels of insulin (Fig. 2A) compared with the CRF-1-fed ( $P < 0.01$ ) and casein-supplemented mice ( $P < 0.01$ ). Similarly, there was a significant decrease in the serum levels of both IGF-I (Fig. 2B) and IGF-II (Fig. 2C) in BCAA-supplemented mice compared with the CRF-1-fed ( $P < 0.001$  for each comparison) and casein-supplemented mice ( $P < 0.001$  and  $P < 0.01$ , respectively).

**Effects of BCAA supplementation on the expression levels of IGF-IR, p-IGF-1R, p-GSK-3 $\beta$ , p-Akt, and COX-2 proteins, and on cell proliferative activity in the colonic mucosa of AOM-treated *db/db* mice.** Hyperinsulinemia and abnormal activation of the IGF/IGF-IR axis play a critical role in obesity-related CRC development (3, 6–10). Therefore, the effects of BCAA on the levels of IGF-IR and the phosphorylated (i.e., activated) form of IGF-IR proteins, and cell proliferation were examined in the colonic mucosa of AOM-treated mice. As shown in Fig. 2D, western blot analyses showed that BCAA supplementation

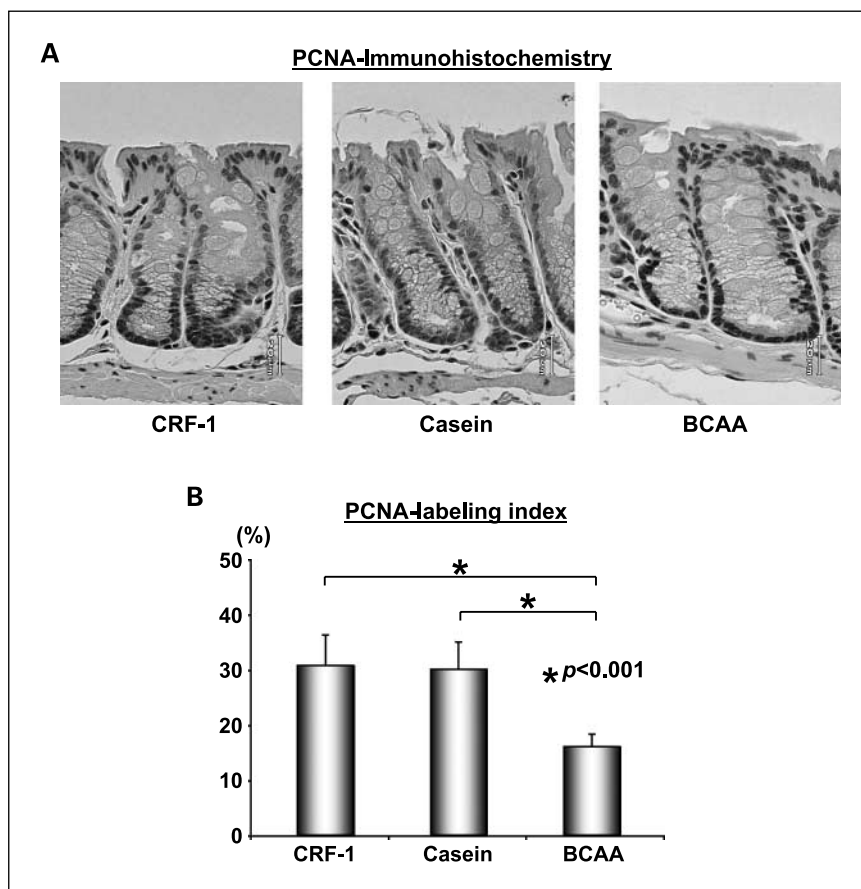
caused a decrease in the levels of IGF-IR ( $P < 0.001$  for each comparison) and p-IGF-1R ( $P < 0.001$  for each comparison) proteins compared with the CRF-1-fed and casein-supplemented mice. Supplementation with BCAA also decreased the expression levels of the phosphorylated (i.e., inactivated) form of GSK-3 $\beta$  ( $P < 0.01$  for each comparison), the phosphorylated (i.e., activated) form of Akt ( $P < 0.001$  for each comparison), and COX-2 ( $P < 0.01$  for each comparison) proteins compared with the control groups. The finding that BCAA supplementation inhibited the phosphorylation of Akt is considered to be significant because the activation of this protein is one of the critical targets in the constitutive activation of the IGF/IGF-IR axis in colorectal carcinogenesis (30).

In addition, as shown in Fig. 3, the PCNA labeling index of nonlesional crypts in the BCAA-supplemented mice was significantly smaller than that of the CRF-1-fed and casein-supplemented mice ( $P < 0.001$  for each comparison), thus indicating that BCAA supplementation significantly inhibits cell proliferation in the colonic mucosa of the AOM-treated *db/db* mice.

## Discussion

The present study clearly indicated that dietary supplementation with BCAA effectively suppressed the development of putative precursor lesions, ACF and BCAC (Fig. 1), for CRC

**Fig. 3.** The effect of BCAA supplementation on colonic epithelial cell proliferation in AOM-treated *db/db* mice. **A**, immunohistochemical staining of the normal crypts in the colon of AOM-treated *db/db* mice with anti-PCNA antibody. Sections of the colon were analyzed from CRF-1 – fed, casein-supplemented, and BCAA-supplemented mice, respectively. They were stained with anti-PCNA monoclonal antibody as described in Materials and Methods. Representative photographs from each group are shown. Bar, 20  $\mu$ m. **B**, PCNA labeling index in the normal crypts in the colon of AOM-treated *db/db* mice. Bars, SD of triplicate assays.



(Table 2) by improving hyperlipidemia and hyperleptinemia in *db/db* mice (Table 3). The suppressive effect of BCAA in the early phase of obesity-related colorectal carcinogenesis was also associated, most likely, with the improvement of hyperinsulinemia (Fig. 2A) and the inhibition of cell proliferation on the colonic mucosa of experimental mice (Fig. 3). BCAA supplementation has also been reported to significantly decrease the incidence of hepatocellular carcinoma in patients with chronic liver disease if they had a body mass index score  $\geq 25$ , and this effect might be associated with improvement of insulin resistance (15, 16, 31). Thus, BCAA might effectively prevent cancer development, at least in several organs, in obese subjects who are considered to have insulin resistance syndrome (3).

How can BCAA exert chemopreventive effects on obesity-related colorectal carcinogenesis? As described above, insulin resistance might be a critical target of BCAA in this beneficial effect because insulin has oncogenic properties on CRC cells. For instance, insulin stimulates the proliferation of CRC cells and promotes colorectal tumor growth in animal models (6–8). These reports, therefore, suggest that BCAA inhibits the development of colonic premalignant lesions (Table 2) and excessive cell proliferation in the colonic mucosa of AOM-injected *db/db* mice (Fig. 3) by improving insulin resistance (Fig. 2A). Recent studies by others have indicated that BCAA improves glucose tolerance by modulating insulin-independent glucose uptake into skeletal muscle in rodent models (32, 33). An improvement of insulin resistance and glucose tolerance by BCAA has also been shown by certain clinical trials (15, 31).

In addition, it is widely accepted that insulin resistance causes alterations in the IGF/IGF-IR axis, which may be closely associated with the development of CRC (9, 10, 30). For instance, the IGF-IR protein is overexpressed in BCAC compared with the surrounding normal cryptal cells (11). Therefore, the IGF/IGF-IR system is regarded as one of the effective targets with respect to the prevention of CRC (11). Our observations described herein comprise the first report showing that BCAA decreases the serum levels of IGF-I and IGF-II (Fig. 2B and C), thereby inhibiting the expression and activation of IGF-IR on the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). Our findings suggest that not only the improvement of insulin resistance but the inhibition of IGF/IGF-IR activation by BCAA plays a critical role in suppressing obesity-related and diabetes mellitus-related colorectal carcinogenesis.

The present study revealed that BCAA supplementation in the diet prevents the development of BCAC (Table 2), which is characterized by abundant  $\beta$ -catenin protein expression (23) and also accumulates the IGF-IR protein (11) while decreasing the expression levels of p-Akt and p-GSK-3 $\beta$  proteins on the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). Recent *in vitro* studies have indicated that insulin and the IGF/IGF-IR axis stabilize and activate the Wnt/ $\beta$ -catenin pathway, which is involved in the development of CRC (34, 35). GSK-3 $\beta$ , which can be phosphorylated by phosphatidylinositol 3-kinase/Akt via insulin or IGF treatment, is considered to be a key kinase for CRC development because the inactivation of GSK-3 $\beta$  leads to the dissociation of the adenomatous polyposis coli/axin/ $\beta$ -catenin complex and cytosolic  $\beta$ -catenin accumulation (36).

Free accumulated  $\beta$ -catenin translocates into the nucleus and forms a complex with the transcription factor T cell factor, thereby activating the transcription of target genes, including cyclin D1 and c-Myc, and thus contributing to abnormal proliferation and tumor progression (37, 38). Therefore, supplementation with BCAA, which targets insulin-associated and IGF-associated  $\beta$ -catenin accumulation by decreasing the levels of p-Akt and p-GSK-3 $\beta$  proteins (Fig. 2D), might be an effective strategy to prevent the development of CRC.

In addition to the beneficial effects mentioned above, BCAA has other physiologic activities that might be useful to prevent the development of CRC. For instance, supplementation with BCAA is capable of reducing the production of oxidative stress and microinflammation in patients with liver cirrhosis, which possibly leads to a decrease in the occurrence of hepatocellular carcinoma (39). In the current study, BCAA caused a decrease in the expression of the COX-2 protein in the colonic mucosa of AOM-treated *db/db* mice (Fig. 2D). COX-2 is one of the main mediators in the inflammatory signaling pathway and is certainly involved in CRC development; therefore, it might be a critical target for CRC chemoprevention (40). This effect might be explained by the inhibitory effect of BCAA on the IGF/IGF-IR axis because the activation of this axis mediates COX-2 expression (41, 42). Additional studies are required to clarify the direct effects of BCAA on inflammation and their relevance to the antitumor effects of this agent.

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## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

## Acknowledgments

We thank Yukari Nomura for her excellent technical assistance.

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