Branched-chain amino acids improve glucose metabolism in rats with liver cirrhosis

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It has long been recognized that cirrhosis is associated with impaired glucose metabolism, with the majority of patients (60–80%) being glucose intolerant and 10–15% eventually developing overt diabetes mellitus (24). The characteristics of glucose intolerance in cirrhotic patients are unusual, because although these patients exhibit fasting hypoglycemia, they also have postprandial hyperglycemia and continuous hyperinsulinemia (11). Despite being hyperinsulinemic, many cirrhotic individuals have impaired glucose tolerance or are overtly diabetic.

Hyperinsulinemia in the face of hyperglycemia suggests the presence of insulin resistance (6, 26, 30). In this condition, it is believed that postprandial glucose uptake is not carried out promptly in insulin-sensitive tissues, such as skeletal muscle and adipose tissue, and that glycogen synthesis is not promoted in skeletal muscle or liver. Indeed, there is considerable evidence that liver and skeletal muscle glycogen content is lower in cirrhotic patients than in healthy individuals (29). Under these conditions, even the control of blood glucose during hunger is difficult, with the characteristics of glucose intolerance described above being found in cirrhotic patients. In skeletal muscle and liver, glycogen synthesis is very important and is related directly to blood glucose control. More importantly, it has been reported that glucose intolerance in cirrhotic patients enhances the risk of hepatic insufficiency (4). Two studies in Italy and Japan showed that long-term oral supplementation with branched-chain amino acid (BCAA) in advanced cirrhosis was useful for prevention of progressive hepatic failure, inasmuch as it improved surrogate markers and perceived health status (8a, 9a, 12, 18). In Japan, pharmacological supplementation of BCAA is used widely to ameliorate hypoalbuminemia in patients with decompensated liver cirrhosis. This supplementation involves the use of LIVACT granules administered in four doses, three times a day after meals to provide a 12 g/day dose of BCAA. The weight ratio of leucine to isoleucine to valine in the granules is 2:1:1.2. In the present study, we chose to administer a dose of 1.5 g/kg BCAA to rats, inasmuch as our previous experiments showed that this dose of BCAA in rats achieved and maintained almost the same plasma concentration of BCAA as that measured in patients given 4 g of BCAA mixture.

In a recent report, we showed that leucine stimulated glucose uptake in isolated soleus muscle from normal rats under insulin-free conditions (23), whereas in a study in a myoblast cell line carried out by other investigators, glycogen synthase (GS) was activated by the mammalian target of rapamycin (mTOR) (25). In addition, there is evidence in cultured human muscle cells that total amino acid level regulates GS activity via mTOR (2). Thus we assumed that BCAA may regulate glucose metabolism in cirrhosis, a condition in which blood BCAA levels are decreased. In this study, we examined whether BCAA regulated glucose metabolism in a rat model of cirrhosis, an animal model that has been shown to be glucose intolerant (20, 21). To achieve this objective, we investigated the subcellular location of glucose transporters and the activity of glycogen synthase in skeletal muscles, in which it is possible to carry out some of the substituted functions of glucose metabolism undertaken by the liver.

**MATERIALS AND METHODS**

**Materials.** The mTOR inhibitor rapamycin was purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of the best grade commercially available.

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Animals. The following experimental protocol was reviewed and approved by the Animal Care Committee of Ajinomoto Co., Inc. Male Sprague-Dawley rats, 5 wk of age, were obtained from Charles River Japan (Yokohama, Japan). They were maintained in individual cages in an air-conditioned room (24 ± 1°C) with a 12:12-h light-dark cycle (lights on from 0700 to 1900). The animals were fed a stock pellet diet (CRF-1, Oriental Yeast, Tokyo, Japan). The CCl4 rats were prepared according to the standard method of Kajiwara et al. (12). After 15 wk, plasma albumin, total bilirubin, and alanine aminotransaminase (ALT) levels were determined. A kit was used to measure albumin (Wako Pure Chemicals), and a Dri Chem 5500 was used to measure total bilirubin and ALT (Fuji Medical System, Tokyo, Japan). Rats with an albumin value of 2.8–3.3 g/dl, total bilirubin >1.5 mg/dl, and ALT activity >100 U/l were selected for the experiments.

Oral glucose tolerance test and insulin measurement with BCAA administration. After 17 h of fasting, the CCl4 rats (450–550 g body wt) were given a compulsory oral 2 or 4 g/kg glucose load at −60 min followed at −30 or 0 min by gavage administration of saline (Cont group) or 1.5 g/kg leucine (Leu group), isoleucine (Ile group), or valine (Val group). Blood glucose concentration was measured at −60, 0, 30, 60, 90, 120, and 180 min using a Dri Chem 5500. Insulin concentration was measured at the same time points using an insulin kit (Mornaga, Tokyo, Japan), except for the 180-min sample.

Glucose uptake in isolated soleus muscle. CCl4 rats were killed by decapitation, and the soleus muscles were isolated immediately from both legs. Glucose uptake in incubated soleus muscles was determined by a procedure we described previously (23).

Preparation of plasma and intracellular membranes from skeletal muscle tissue. Plasma membranes from stock samples of soleus and gastrocnemius muscles (6 g weight) of several CCl4 rats, obtained 30 min after compulsory oral administration of 1.5 g/kg of each BCAA (leucine, isoleucine, valine) or BCAA as LIVACT compositions (2:1:1.2 leucine-isoleucine-valine) or 2 g/kg glucose or saline, were prepared according to previously described methods (15, 22).

GLUT4 and GLUT1 immunoblotting. Rabbit anti-GLUT4 or anti-GLUT1 (Chemicon International) was used to detect GLUT4 and GLUT1 in the plasma membrane. The membrane fractions were also immunoblotted by Na+K+-ATPase antibody (American Research Products). The bands were quantified using Scion Image software and standardized against the intensity of the Na+K+-ATPase bands.

Glycogen content and GS assay. The CCl4 rats were deprived of food for 3 days and then selected randomly for injection via the tail vein with 0.75 mg/kg rapamycin (Wako Pure Chemicals) or an equal volume of vehicle [saline and 2% (vol/vol) ethanol] as described previously (1). A compulsory oral glucose load of 4 g/kg was administered 60 min before 1.5 g/kg of leucine, isoleucine, or saline. After a further 60 min, the soleus muscles were isolated under ether anesthesia for use in subsequent GS and p70 S6 kinase assays. The amount of BCAA administered was equivalent to the average amount of amino acids consumed by rats of this strain during 24 h of free access to food (9) and resulted in plasma total BCAA concentrations of ~2 mM. The CCl4 rats were deprived of food for 3 days but allowed free access to water to deplete skeletal muscle glycogen content, as described previously (19). Soleus muscles of the rats were hydrolyzed with KOH, and the glycogen content of this preparation was determined as described previously (19). A modified filter paper method was used to measure GS activity as described previously, with the results expressed as mean activity per total activity (8).

p70 S6 kinase assay. The activity of p70 S6 kinase was measured in isolated soleus muscle samples according to the method of Hara et al. (10).

Statistics. Values are means ± SE. Differences in mean values were analyzed by Student’s t-test and one-way ANOVA followed by Dunnett and Tukey-Kramer analyses. P < 0.05 was considered significant.

RESULTS

Effects of BCAA on blood glucose and plasma insulin levels in CCl4 rats. After 17 h of fasting, the CCl4 cirrhotic rats were subjected to an OGTT, with measurement of blood glucose and insulin concentrations. We chose a high-dose glucose load of 4 g/kg, inasmuch as we found previously that this was the optimal dose for investigation of muscle glycogen synthesis after 3 days of fasting (data not shown). We checked the optimal timing for BCAA administration in OGTT experiments and concluded that 60 min after the glucose load produced the best results. Blood glucose level in the control group peaked at 0 min, that is, 1 h after the glucose load. The level remained elevated for a further 120 min, despite continuing release of insulin (Fig. 1, A and D). These results indicated a pathological phenotype of impaired glucose tolerance in the CCl4 cirrhotic rats. In the Leu group, blood glucose levels peaked at the same time as in the Cont group, but 30 min after leucine administration, the level decreased significantly (Fig. 1A). Administration of leucine did not appear to stimulate additional insulin release, inasmuch as insulin levels were similar in the Cont and Leu groups at all time points, except 120 min (Fig. 1B).

In the next series of experiments, we examined whether isoleucine or valine affected glucose metabolism in cirrhotic rats. Blood glucose levels in the Ile group were decreased significantly 30 min after administration compared with the Cont group, whereas administration of valine had almost no effect (Fig. 1D). Plasma insulin levels were similar in the Ile, Val, and Cont groups, whereas isoleucine or valine administration did not stimulate insulin release further (Fig. 1E). Because leucine and isoleucine improved glucose levels without additional insulin secretion (Fig. 1, B and E), we assumed that these amino acids improved glucose metabolism without additional insulin secretion.

Comparison of effects of leucine and isoleucine on glucose tolerance. The findings described above showed that leucine and isoleucine improved glucose tolerance in CCl4 rats, whereas valine did not. To compare the blood glucose-lowering effects of leucine and isoleucine, we performed another series of OGTTs on the CCl4 rats. In these experiments, we chose a high-dose glucose load of 4 g/kg to clarify the difference between the effect of leucine and the effect of isoleucine. At 30 min after administration of leucine or isoleucine, blood glucose levels in both groups were significantly decreased. Although blood glucose levels in the Leu group remained unchanged after administration of leucine or isoleucine, blood glucose levels in both groups were significantly decreased. Although blood glucose levels in the Leu group remained unchanged from 30 to 120 min, levels in the Ile group decreased continuously even after 60 min (Fig. 2A). Changes in plasma insulin levels were similar in the three groups (Fig. 2B). These results suggested that glucose levels were decreased to a greater extent by isoleucine than by leucine (Fig. 2C).

Glucose uptake in soleus muscle of CCl4 rats treated with BCAA. The blood glucose-lowering activities of leucine and isoleucine are not mediated by additional insulin secretion. Therefore, we assumed that the main action of BCAAs in tissues is to stimulate glucose consumption. Muscle is one of the major glucose-consuming tissues in the body. We also showed previously that, in normal rats, leucine directly promotes glucose uptake in isolated muscle (23). The following series of experiments were performed to examine the effects of leucine and isoleucine on glucose uptake in isolated soleus...
muscle from CCl4 rats and also to elucidate the mechanism of this glucose-lowering effect. Figure 3 demonstrates that leucine and isoleucine, but not valine, promoted glucose uptake in isolated soleus muscle from CCl4 rats. Although the blood glucose-lowering effects of leucine and isoleucine seemed to improve insulin sensitivity (Figs. 1, C and F, and 2C), which was indicated as glucose-to-insulin area under the curve ratio in OGTT experiments, both amino acids were able to stimulate glucose uptake in the absence of insulin. These results suggested that isoleucine and leucine would act directly to stimulate glucose uptake as well as insulin secretion.

Effects of BCAA administration on translocation of GLUT1 and GLUT4 to the plasma membrane. On the basis of the above finding, we next examined whether orally administered BCAAs had any direct effects on the muscle of cirrhotic rats. Transporter-mediated uptake of glucose is the rate-limiting determinant of glucose utilization in skeletal muscle (3). GLUT4 is the most abundant glucose transporter isoform in skeletal muscle and adipose tissue (16, 27, 28) and is responsible for the majority of insulin-dependent glucose uptake in these tissues (14). It has long been recognized that insulin promotes the rapid translocation of GLUT4 from intracellular membrane compartments to the plasma membrane (16, 27, 28). GLUT1 protein is expressed in almost all tissues but is a minor isoform in skeletal muscle. To examine whether leucine and isoleucine have any effects on translocation of GLUT4 and GLUT1, we used immunoblotting to measure the amount of these transporters in skeletal muscle membrane fractions. The data were standardized against activity for Na\(^+\)-K\(^+\)-ATPase, a protein localized constitutively at the plasma membrane (see Fig. 5C). Muscle samples were isolated from rats treated with BCAA, glucose, and BCAA + glucose. Additional adminis-
trations of BCAAs augmented the glucose-induced plasma membrane translocation of GLUT4 in skeletal muscle of CCl₄ rats (Fig. 4). Moreover, oral administration of leucine or isoleucine alone, but not valine, promoted translocation of the GLUT4 protein to the plasma membrane, similar to that observed with administration of glucose (Fig. 5A). GLUT1 translocation was also promoted by administration of leucine, isoleucine, or glucose alone (Fig. 5B). The plasma insulin levels increased significantly after administration of glucose or leucine, but not isoleucine (Fig. 5D).

**Glycogen content in soleus muscle in normal and CCl₄ rats.**

Skeletal muscle is one of the main tissues for storing energy in the body, with glycogen being synthesized from excess glucose as a future fuel reserve. It is well known that cirrhotic patients have low glycogen stores, not only in liver but also in muscle (11, 24). In animals receiving normal feed, we found that glycogen content in isolated soleus muscles and liver was significantly lower in CCl₄ than in normal rats (Fig. 6A). The next experiments were performed with the aim of evaluating the effects of BCAA on glycogen synthesis. As shown above, leucine and isoleucine acted directly on muscle to promote glucose uptake and improve glucose intolerance after an oral glucose load in cirrhotic rats. Glucose uptake and glycogen synthesis, a major use of glucose, are thought to be essential for normal postprandial glucose homeostasis. We measured GS activity in isolated muscles from CCl₄ rats fasted for 3 days. At −60 min these animals were given a glucose load, at time 0 leucine or isoleucine was administered, and soleus muscles were isolated at 60 min, when the difference in blood glucose levels between controls and the BCAA group was greatest. GS activity in isolated soleus muscle from the CCl₄ rats was upregulated significantly by the administration of leucine, but not isoleucine (Fig. 6B). We also found that glycogen content in soleus muscle tended to be greater in the Leu than in the Cont group (data not shown).

**Effects of rapamycin on GS and p70 S6 kinase activity in soleus muscle and blood glucose and plasma insulin levels after leucine administration.** To elucidate the mechanism of leucine-induced GS activity (Fig. 6B), we investigated mTOR signaling, a pathway that is thought to be a cellular sensor for amino acids. Although leucine stimulated GS activity, it also induced strong phosphorylation of p70 S6 kinase, a downstream target of mTOR protein kinase (Fig. 7, A and B). GS activity and p70 S6 kinase phosphorylation were inhibited by pretreatment with rapamycin. Blood glucose and plasma insu-
in glucose uptake is mediated by a novel signal independent of insulin concentrations were not altered by pretreatment with rapamycin (Fig. 7, C and D).

**DISCUSSION**

BCAA mixtures have been used extensively in Japan as a prescription drug for the treatment of hypoalbuminemia in patients with decompensated liver cirrhosis. Impaired glucose metabolism occurs frequently in patients with cirrhosis and is thought to be among the risk factors that contribute to the serious complications associated with the disorder. The present studies were planned and carried out to evaluate whether BCAA supplementation improved the impaired glucose metabolism associated with cirrhosis. These investigations were based on findings from several clinical studies that BCAA supplementation improved insulin tolerance and glucose tolerance in patients with liver cirrhosis (10a, 11a, 26b).

We investigated the effects of BCAA on glucose metabolism in rats with CCl4-induced cirrhosis and found that leucine and isoleucine had characteristic pharmacological effects on skeletal muscle that led to improved glucose metabolism. Our data showed that, after a glucose load in CCl4 rats, administration of leucine or isoleucine reduced blood glucose level without increasing insulin secretion. A possible mechanism to explain these changes is enhancement of glucose uptake via upregulation of mTOR signaling. First, we showed previously that leucine upregulates glucose uptake directly in isolated skeletal muscle in a rapamycin-resistant manner (23), with this action being blocked by phosphatidylinositol 3-kinase (PI3K) and PKC inhibitors. In our previous experiment, the effect of leucine was blocked completely by LY-294002 (a specific inhibitor of PI3K) and GF-109203X (a specific inhibitor of PKC). These results suggest that leucine promotes glucose uptake in skeletal muscle via the PI3K and PKC pathways. The present study demonstrates that isoleucine and leucine have similar effects on glucose uptake and translocation of GLUT4 and GLUT1.

We anticipate that GLUT4 translocation induced by BCAAs may be mediated by PI3K and atypical PKC signals, because both of these factors are thought to be involved in the translocation of GLUT4 (5, 7, 23). Using isolated soleus muscles from CCl4 rats, we observed results similar to those seen in normal rats (data not shown). Additionally, we confirmed previously that isoleucine and leucine promoted GLUT4 translocation without activation of Akt phosphorylation, a well-known reaction downstream of PI3K signaling that leads to induction of GLUT4 translocation by insulin. Therefore, further detailed analyses are required to clarify how BCAAs induce the signaling for GLUT4 translocation (unpublished observations). These additional studies also need to examine other energy status-regulated signals, such as AMP kinase. On the other hand, there are several reports indicating that insulin and a change in energy status result in upregulation of GLUT1 mRNA and translocation of the transporter to the plasma membrane (13, 23a, 26a, 31). Inasmuch as BCAAs have similar effects, it is possible that these compounds act as novel signal inducers of an alternative pathway. The mechanism of GLUT1 induction by leucine and isoleucine, however, remains unclear and requires clarification in future studies.

Our data also suggest that there may be another mechanism to account for the effects of leucine on glucose metabolism in skeletal muscle, namely, promotion of glycogen synthesis via activation of mTOR signaling (Figs. 6 and 7). We found that GS activity in soleus muscles was augmented by leucine, but not by isoleucine, and was inhibited completely by pretreatment with rapamycin (Fig. 7, A and B). We assume that differences in the potential of leucine and isoleucine to activate...
Fig. 5. Effects of single BCAAs on GLUT1 and GLUT4 translocation to plasma membrane in skeletal muscle of CCl4 rats. After 17 h of fasting, 1.5 g/kg BCAA and 2 g/kg glucose or vehicle (saline) were administered at 0 min. After 60 min, skeletal muscles were isolated from the rats. A and B: immunoblots for GLUT4 and GLUT1, (4.2 and 7.5 μg of protein, respectively). Each band was detected in 3 rat samples and scanned and quantified by Scion Image beta 4.02. C: Na⁺-K⁺-ATPase was detected as a plasma membrane marker (protein content of each application = 7.5 μg). D: plasma insulin concentration at the time of soleus muscle collection. *P < 0.05 compared with Fast (Dunnett’s multiple comparison test).

Fig. 6. Effects of orally administered Leu and Ile on glycogen synthase (GS) activity in CCl4 rats after glucose load. A: glycogen content in isolated soleus muscle and liver measured in normal and CCl4 rats. Values are means ± SE of 5 rats. *P < 0.05 compared with normal rats (Student’s t-test). B: after 3 days of fasting, CCl4 rats were treated with 4 g/kg glucose at 60 min and 1.5 g/kg Leu, Ile, or saline (Cont) at 0 min. After 60 min, soleus muscles were isolated from each of the CCl4 rats, and GS activity was measured. *P < 0.05 compared with Cont (Student’s t-test).
The mTOR pathway may be the reason for these variable effects on GS, inasmuch as there is evidence that isoleucine has little effect on mTOR signal activity, in contrast to leucine, which results in strong activation of the pathway (17). However, it has also been shown in rat primary hepatocytes that leucine activates mTOR signaling but does not increase GS activity (17). Taken together, these studies suggest that there are tissue specificities for regulation of mTOR signaling and glycogen synthesis.

The results of our OGTTs with a 2 g/kg glucose load suggested that the blood glucose-lowering action of leucine was weaker than that of isoleucine (Fig. 2). It also appeared that the differences in reduction of blood glucose levels caused by leucine and isoleucine in the later stage of the OGTT were greater than those in the early stage of the test. We assumed that these differences reflected the potential of leucine and isoleucine to activate the mTOR pathway. In a recent report, mTOR signals were demonstrated to inhibit insulin signals by inducing Ser/Thr phosphorylation of insulin receptor substrate-1 (32). Among the amino acids, leucine has been shown to be the most effective activator of mTOR (1). These results may be explained by inhibition of the endogenous insulin signal by leucine via mTOR activation during the late stage of the OGTT. Therefore, the early reduction in blood glucose was caused by endogenous insulin and leucine, whereas the later effects were caused predominantly by leucine in the absence of insulin signaling. Inasmuch as isoleucine was a weaker activator of the mTOR signal in skeletal muscle (1), endogenous insulin would have maintained low blood glucose levels, even after administration of isoleucine. To explain the results shown in Fig. 2, we need to determine the mechanism that accounts for the greater effect on glucose tolerance of isoleucine than of leucine.

In conclusion, this study in a rat model of cirrhosis found that administration of leucine and isoleucine, but not valine, decreased blood glucose levels by enhancing glucose uptake as
a result of increased translocation of GLUT4 and GLUT1 to the plasma membrane. Our data indicated another possible mechanism by which leucine reduces blood glucose that involves increased use of intracellular glucose and activation of GS via mTOR signaling. It is important to determine the molecular mechanism of signal transduction used by leucine and isoleucine to regulate glucose transport, inasmuch as it is distinct from that of insulin (Fig. 8). It is therefore possible in cirrhosis that leucine and isoleucine may partially substitute for insulin in the regulation of glucose transport and also improve glucose metabolism by promoting glycogen synthesis and glucose uptake. Recently, several preliminary clinical studies in Japan indicated that BCAA supplementation given as LIVACT granules has beneficial effects on abnormal glucose metabolism in cirrhotic patients (10a, 11a, 26b). Our results provide one possible explanation for these novel pharmacological actions of BCAAs.

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