Effect of Branched-Chain Amino Acid on $^{15}$N Incorporation into Liver and Skeletal Muscle Proteins Following $[^{15}$N]-$\text{Ammonium Chloride Administration to Carbon Tetrachloride-Intoxicated Rats}$

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Summary The effect of branched-chain amino acid (BCAA) on protein synthesis and nitrogen metabolism in liver and skeletal muscle was evaluated by an intraperitoneal injection of $[^{15}$N]ammonium chloride ($^{15}$NH$_4$Cl) to carbon tetrachloride (CCl$_4$)-intoxicated rats. The $^{15}$NH$_4$Cl was bolusly injected at a dose of 6 mg/100 g body weight one hour after an amino acid solution containing leucine and valine (150 mM each, abbreviated as BCAA), phenylalanine and alanine (150 mM each, PA), 120 mM leucine and 30 mM valine (Leu-rich) or 120 mM valine and 30 mM leucine (Val-rich) was administered intragastrically at a dose of 2.5 ml each/100 g body weight. The $^{15}$N-enrichment in the protein fraction of the liver was higher in CCl$_4$-BCAA group than CCl$_4$-PA group. The Leu-rich solution was found more effective in enhanced incorporation of $^{15}$N into liver and skeletal muscle proteins. The disappearance rate of $[^{15}$N]urea from the plasma, which was influenced by the synthesis from $^{15}$NH$_3$ and the excretion into urine, was much faster in the CCl$_4$-BCAA group than CCl$_4$-PA group. In Leu-rich group, both $^{15}$N incorporation into non-protein fraction of skeletal muscle and disappearance rate of plasma urea-$^{15}$N were greater than those in Val-rich group. The results suggest that BCAA, particularly leucine, has beneficial effects on protein synthesis and ammonium detoxification in liver-injured rats.

Key Words branched-chain amino acid, nitrogen-15, $[^{15}$N]ammonium chloride, protein synthesis, liver-injured rat

In cirrhotic patients, low levels of plasma branched-chain amino acids (BCAA: valine, leucine, and isoleucine) have been reported (1). BCAA administration to cirrhotic patients can normalize plasma amino acid imbalance (2), enhance protein synthesis, and enhance nitrogen disposal.
synthesis (3), decrease protein catabolism (4), lower blood ammonia levels and improve hepatic encephalopathy (5). BCAA is oxidized mainly in skeletal muscle and provides energy for muscle (6, 7) especially in cirrhotic patients with impaired glucose metabolism (8). BCAA metabolism in the skeletal muscle is related to alanine and glutamate formation (9, 10) which is followed by glutamine production; during these metabolic processes, ammonia is detoxified in skeletal muscle (11). Cirrhotic patients, to whom a BCAA-enriched nutritional product (Aminolevan R) was administered, showed increases in plasma prealbumin levels and improvement of nitrogen balance (12). However, the proper composition of leucine, valine, and isoleucine among BCAA for obtaining greater effects has not been studied in detail.

In the present communication, the effects of prior administration of BCAA on 15N uptake by skeletal muscle and liver and on 15N incorporation into these tissue proteins and plasma urea were investigated by an injection of [15N]ammonium chloride (15NH₄Cl) to carbon tetrachloride (CCl₄)-intoxicated rats.

**MATERIALS AND METHODS**

Female Sprague-Dawley rats (CLEA Japan Inc., Tokyo), weighing about 175 g each, were used throughout this study. The rats were maintained *ad libitum* for one week on a standard diet (CE-2; CLEA Japan Inc.) containing 23.6 g protein, 4.4 g fat, and 52.7 g carbohydrate per 100 g. Rats were fasted overnight, and a 20% CCl₄ (Katayama Chemical Industries Co., Ltd., Tokyo) solution in liquid paraffin was force-fed in stomach at a dose of 0.5 ml/100 g body weight to induce acute liver injury. Control rats were administered an equal volume of liquid paraffin alone. The animals were fasted for another 24 h (water *ad libitum*) and used for the following experiments.

The 15NH₄Cl (99 atom%, Shoko Tsusho Co., Tokyo) was dissolved in water at a concentration of 6 mg/ml. Urease [EC 3.5.1.5] from Jack beans (Type III) was obtained from Sigma Chemical Co., St. Louis. L-Leucine, L-valine, L-phenylalanine, and L-alanine were purchased from Wako Pure Chemical Industries Co., Ltd., Tokyo.

**Experiment A. Administration of BCAA or PA to CCl₄-intoxicated rats.** Liver-injured rats were divided into three groups of 4 rats each. One group was given nothing but only water *ad libitum* (CCl₄-None group). The second group received a solution containing equal molar (150 mM) of leucine and valine (CCl₄-BCAA group). The third group was given a solution containing equal molar (150 mM) of phenylalanine and alanine (CCl₄-PA group). Each group of rats received intragastrically the respective amino acid solutions at a dose of 2.5 ml/100 g body weight 24 h following CCl₄ administration. One hour following administration of each amino acid solution, 15NH₄Cl was given intraperitoneally at a dose of 6 mg/100 g body weight. All of the animals were housed in individual metabolic cages in a temperature-regulated (24 ± 2°C) light-controlled (light from 0700 to 1900 h) room and received the respective amino acid solutions at intervals of 3 h for

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Blood samples were drawn from the jugular vein under light ether anesthesia in heparin-containing tubes. Plasma was separated and stored at -20°C until it was analyzed for urea-N, amino acid, insulin and glucose. Livers and muscles of hindquarter were thoroughly perfused with chilled saline immediately after the blood samples were drawn, and stored at -20°C. Urine was collected for 24 h following 15NH4Cl administration by using a glass bottle containing 1 ml of 6N HCl in a metabolic cage.

Experiment B. Administration of Leu-rich or Val-rich solution to CCl4-intoxicated rats. Liver-injured rats were divided into the following two groups; each group included 4 rats. One group was administered intraperitoneally a solution rich in leucine (120 mm Leu and 30 mm Val, 0.5 ml/100 g body weight: Leu-rich group) and the other group received a valine-rich solution (30 mm Leu and 120 mm Val, 0.5 ml/100 g body weight: Val-rich group) six times at one-hour intervals for 5 h. The injection of the amino acid solutions was started from the 24th h of CCl4 administration. Immediately after the 2nd administration of the amino acid solution, [15N]ammonium chloride solution was injected as similarly described in Experiment A. All of the rats were individually housed in metabolic cages and received water ad libitum. Urine was collected for 10 h following the 15NH4Cl administration as described in Experiment A.

Analyses. Liver and hindquarter muscle tissues were homogenized in 3 times their weight of saline. The homogenates and plasma were deproteinized with an equal volume of 15% trichloroacetic acid (TCA). After centrifugation at 3,000 rpm for 10 min, the supernates were decanted and analyzed for non-protein nitrogen. TCA precipitates were washed twice with 1 ml of 15% TCA. The residues were suspended in water, and aliquots were analyzed for protein nitrogen. Nitrogen in the protein and non-protein fractions was analyzed by a micro Kjeldahl method, and distilled ammonia was collected into 20 ml of 1% boric acid. The boric acid solution in conical flask was alkalized by 2 ml of 30% NaOH, and the flask was sealed with a rubber cap. The cap was equipped with a small glass tube containing 0.1 ml of 0.1 N HCl which had a small window on the upper lateral side. The alkalized solution was incubated at 40°C for 72 h. Ammonia absorbed into 0.1 N HCl was transferred to a small tube and freeze-dried. White crystals of ammonium chloride in the tube were redissolved in water at nitrogen concentration of 1 mg/ml. This solution was filled in a glass capillary tube (1 cm, φ 1 mm) and dried at 60°C for one hour. The tube was sealed with Xe-He gas according to Dumas method (13), and 15N was assayed spectroscopically (14) using a 15N analyzer (Type N-150, Nihon Bunko Co., Ltd., Tokyo). Plasma urea nitrogen was also collected in 0.1 N HCl using Conway's method (15) with some modification as follows. Plasma free ammonia was eliminated by activated zeolite. The plasma was incubated overnight with urease at room temperature to diffuse ammonia from urea. Plasma amino acid contents were analyzed using a high performance liquid chromatograph amino acid-analyzing system (LC-6A, Shimadzu, Kyoto). Plasma immunoreactive insulin...
(IRI) levels were determined by radioimmunoassay (16) and blood ammonia concentrations were determined by the Amitest meter method (17). All the results were expressed as mean ± SE of the mean, and statistical significance was determined by Student’s t-test.

RESULTS

Experiment A. Effects of BCAA or PA administration on \(^{15}\text{NH}_4\text{Cl}\) metabolism in CCl\(_4\)-intoxicated rats

The effect of CCl\(_4\)-liver injury and amino acid administration on serum insulin, blood glucose, and plasma amino acid levels was examined just prior to \(^{15}\text{NH}_4\text{Cl}\) injection (Table 1). The serum IRI levels remarkably increased in liver-injured rats, and no difference was recognized among the three groups (CCl\(_4\)-treated). Blood glucose levels were insignificantly lower in CCl\(_4\)-BCAA group than in CCl\(_4\)-None or CCl\(_4\)-PA group. Plasma levels of leucine and valine in CCl\(_4\)-BCAA group elevated evidently, but other amino acids did not show any significant change. In CCl\(_4\)-PA group, not only the phenylalanine but also the tyrosine level increased markedly.

Figure 1 shows \(^{15}\text{N}\)-enrichment in protein and non-protein fractions of liver and skeletal muscle tissues 5 h following \(^{15}\text{NH}_4\text{Cl}\) administration. The \(^{15}\text{N}\)-enrichment in protein fraction of liver was significantly higher in CCl\(_4\)-BCAA group than CCl\(_4\)-PA group, while \(^{15}\text{N}\)-enrichment in non-protein fraction of liver was insignificantly higher in CCl\(_4\)-PA group than in CCl\(_4\)-BCAA group. Significant difference in protein fraction of skeletal muscle was recognized between CCl\(_4\)-BCAA group and CCl\(_4\)-None group. However, in both protein and non-protein fractions of skeletal muscle, no difference of \(^{15}\text{N}\)-enrichment was observed between

Table 1. Serum immunoreactive insulin, blood glucose, and plasma amino acid levels before \(^{15}\text{NH}_4\text{Cl}\) administration in control and CCl\(_4\)-intoxicated rats.

|                  | Control          | CCl\(_4\)                |
|------------------|-----------------|--------------------------|
|                  | None            | BCAA                     | PA                      |
| IRI (μU/ml)      | 14.0 ± 0.5      | 74.8 ± 11.7              | 80.9 ± 18.2             | 70.7 ± 23.0          |
| Glucose (mg/100ml) | 108 ± 12        | 97 ± 14                  | 61 ± 12                 | 88 ± 6              |
| Amino acid (nmol/ml) |               |                          |                         |                     |
| Leucine          | 139.8 ± 19.6    | 273.0 ± 43.1*            | 1,344.7 ± 479.2**       | 204.0 ± 39.2        |
| Valine           | 162.6 ± 20.7    | 285.1 ± 41.5*            | 1,711.0 ± 333.7**       | 277.0 ± 47.3        |
| Isoleucine       | 83.8 ± 10.4     | 88.1 ± 18.2              | 61.9 ± 9.8              | 122.5 ± 19.2        |
| Phenylalanine    | 58.3 ± 12.1     | 138.5 ± 39.5             | 114.6 ± 38.5            | 1,226.8 ± 274.8**   |
| Tyrosine         | 55.6 ± 11.7     | 317.2 ± 87.8*            | 206.4 ± 103.9           | 601.3 ± 50.0**      |
| Methionine       | 49.8 ± 6.0      | 178.1 ± 42.8*            | 118.0 ± 24.8            | 110.9 ± 20.7        |

M ± SE, n=4. *p<0.05, compared with control group; **p<0.01, compared with CCl\(_4\)-None group.

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Fig. 1. $^{15}$N-enrichment in protein (●●●) and non-protein (■■■) fractions of liver and skeletal muscle tissues 5h after $^{15}$NH$_4$Cl was administered to control and CCl$_4$-injured rats. CCl$_4$-BCAA and CCl$_4$-PA groups were administered the solution containing equal molar (150 mM) of leucine and valine, or phenylalanine and alanine at a dose of 2.5 ml/100 g body weight, respectively, one hour before $^{15}$NH$_4$Cl was administered. Results are expressed as mean ± SE. Asterisks show significant difference (* p<0.05, ** p<0.02).

Table 2. Plasma urea-N concentration and $^{15}$N-enrichment one hour following $^{15}$NH$_4$Cl administration to control and CCl$_4$-intoxicated rats.

| Rat            | Urea-N (mg/100 ml) | $^{15}$N-enrichment (atom\% excess) |
|----------------|--------------------|-------------------------------------|
| Control        | 18.7 ± 0.8         | 4.08 ± 0.18                         |
| CCl$_4$-None   | 12.3 ± 1.0         | 3.77 ± 0.18                         |
| CCl$_4$-BCAA   | 16.4 ± 2.6         | 3.30 ± 0.46                         |
| CCl$_4$-PA     | 19.8 ± 3.1         | 2.53 ± 0.42                         |

M ± SE, n=4, ** p<0.01, * p<0.05.

CCl$_4$-BCAA group and CCl$_4$-PA group.

Plasma urea-N concentration and $^{15}$N-enrichment one hour following $^{15}$NH$_4$Cl administration are presented in Table 2. Plasma urea-N concentration in CCl$_4$-None group decreased evidently as compared to control group, and elevated significantly in CCl$_4$-PA group as compared to CCl$_4$-None group. The $^{15}$N-enrichment in plasma urea was significantly lower in CCl$_4$-PA group than CCl$_4$-
Fig. 2. Effect of prior administration of amino acids on disappearance of plasma urea-\(^{15}\)N following \(^{15}\)NH\(_4\)Cl administration to \(\text{CCl}_4\)-injured rats. Control rats were administered liquid paraffin alone. \(\text{CCl}_4\)-BCAA and \(\text{CCl}_4\)-PA groups were administered the respective solution containing an equal molar (150 mM) of leucine and valine or phenylalanine and alanine at a dose of 2.5 ml/100 g body weight, respectively, one hour before \(^{15}\)NH\(_4\)Cl was administered.

Table 3. Urinary excretion of total nitrogen and \(^{15}\)N-enrichment in the urinary nitrogen during 24 h following \(^{15}\)NH\(_4\)Cl administration to \(\text{CCl}_4\)-treated rats.

| \(\text{CCl}_4\)-treated rats | Total nitrogen (mg) | \(^{15}\)N-enrichment (atom\% excess) |
|-----------------------------|---------------------|-------------------------------------|
| \(\text{CCl}_4\)-BCAA       | 91.2 ± 0.2          | 1.63 ± 0.10                        |
| \(\text{CCl}_4\)-PA         | 71.4 ± 17.0         | 1.17 ± 0.14                        |

\(M \pm SE, n=4, *p<0.05.\)

None group.

The disappearance rate of plasma urea-\(^{15}\)N following \(^{15}\)NH\(_4\)Cl injection was calculated as \(K=0.693/t^{1/2}\) (Fig. 2). The rate was greatest in the control group \((K=0.330)\) and depressed to 0.154 in \(\text{CCl}_4\)-None group. BCAA administration increased the rate to 0.257, and PA administration diminished the rate to 0.079. The \(^{15}\)N-enrichment in the urinary total nitrogen was higher in \(\text{CCl}_4\)-BCAA group than \(\text{CCl}_4\)-PA group (Table 3). No significant difference in urinary excretion (24 h) of total nitrogen was observed between the BCAA group and PA group.

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Changes in blood ammonia concentrations following intraperitoneal $^{15}$NH$_4$Cl administration in Leu-rich (■) and Val-rich (□) group. Data are expressed as mean ± SE of 4 rats.

**Experiment B. Effects of Leu-rich and Val-rich solution on $^{15}$NH$_4$Cl metabolism in CCl$_4$-intoxicated rats**

Blood ammonia concentrations measured to assess the effects of $^{15}$NH$_4$Cl injection on blood ammonia level showed only small increase, 122 ± 43 μg/100 ml in Leu-rich group and 120 ± 53 μg/100 ml in Val-rich group, one hour following $^{15}$NH$_4$Cl administration, and no difference was recognized between the two groups (Fig. 3). The changes in plasma amino acid concentrations after leucine and valine were administered intraperitoneally to Leu-rich group or Val-rich group are shown in Fig. 4. Val-rich group, plasma valine concentrations greatly increased over the leucine levels one hour following Val-rich solution, but in Leu-rich group, the plasma leucine levels increased only slightly one hour after the administration of Leu-rich solution. Plasma isoleucine levels showed little change in both Leu-rich group and Val-rich group.

Changes in $^{15}$N-enrichment in protein and non-protein fractions of liver and skeletal muscle following $^{15}$NH$_4$Cl administration are illustrated in Fig. 5. In liver tissue, significantly high $^{15}$N uptake into protein was recognized in Leu-rich group 2 h following $^{15}$NH$_4$Cl injection. The $^{15}$N-enrichment in non-protein fraction of liver reached the highest level within one hour following $^{15}$NH$_4$Cl injection and decreased rapidly in Leu-rich group and slowly in Val-rich group. In skeletal muscle tissue, significantly higher $^{15}$N-enrichment in protein fraction was also recognized in Leu-rich group compared with Val-rich group. In the non-protein fraction of skeletal
Fig. 4. Changes in plasma Leu (■), Val (○), and Ile (▲) concentrations after administration of leucine and valine in Leu-rich group (-----) and Val-rich group (------). Arrows indicate the injection of leucine and valine (♀), and 15NH₄Cl (♀). Results are expressed as mean ± SE.

Fig. 5. Changes in ¹⁵N-enrichment in liver and skeletal muscle tissues following ¹⁵NH₄Cl administration to CCl₄-injured rats. Lines indicate the ¹⁵N-enrichment in non-protein fraction of Leu-rich group (■) and Val-rich group (□). Columns indicate the ¹⁵N-enrichment in the protein fraction of Leu-rich (■) and Val-rich (□) group. Data are expressed as mean ± SE of 4 rats. Asterisks show significant difference between Leu-rich group and Val-rich group (**p < 0.01, *p < 0.05).

Muscle tissue, ¹⁵N-enrichment in Leu-rich group increased to 1.8 times that in Val-rich group 2 h following ¹⁵NH₄Cl injection.

The disappearance rate of [¹⁵N]urea from plasma was faster in Leu-rich group (K = 0.231) than in Val-rich group (K = 0.156) (Fig. 6). However, there was no significant difference in the urinary excretion of ¹⁵N between Leu-rich group and Val-rich group (Table 4).
DISCUSSION

In the present investigation, nutritional significance of leucine and valine was comparatively evaluated in liver-injured rats. Leucine is a ketogenic and valine a glycogenic amino acid. Therefore, phenylalanine, a ketogenic amino acid, and alanine, a glycogenic amino acid, were used for control amino acids.

Administration of BCAA resulted in an increase of $^{15}$N uptake into liver and skeletal muscle proteins in CCl$_4$-treated rat, but phenylalanine and alanine did not. Increase of $^{15}$N-enrichment in protein fractions suggests the activation of protein synthesis in the tissues tested. These results may support the beneficial role of BCAA on protein synthesis as already suggested in previous reports (18, 19).

Among three BCAAs, leucine was expected to have the most potential effect on
regulation of protein metabolism (20). The present study showed that Leu-rich solution accelerated $^{15}$N uptake into proteins in liver and skeletal muscle of CCl$_4$-intoxicated rat. Tischler et al. already reported that increase in intracellular levels of leucine stimulate protein synthesis (21). The observation on plasma amino acid levels (Fig. 4) shows that leucine increased clearly in Leu-rich group but only small increase was observed in Val-rich group.

As leucine enhances insulin secretion (22), the increased incorporation of $^{15}$N into liver and skeletal muscle proteins in Leu-rich group may be attributed to the insulin effect. Plasma IRI levels in CCl$_4$-intoxicated rat were higher than control rats, but there was no significant difference among the three group of CCl$_4$-intoxicated rats.

Exogenous $^{15}$NH$_3$ was expected to be detoxified by urea synthesis in liver. Plasma urea-$^{15}$N concentration indeed elevated rapidly following $^{15}$NH$_4$Cl administration and the level in CCl$_4$-None group was diminished to 60%, when it was compared to control group in the first hour of $^{15}$NH$_4$Cl administration. Disappearance rate of $^{15}$N-urea from plasma delayed in CCl$_4$-PA group and BCAA administration enhanced the rate (Fig. 2). These results support the clinical efficacy of BCAA also as a nitrogen source for cirrhotic patients with disturbed nitrogen metabolism.

The $^{15}$NH$_3$, which were not incorporated into urea, may be detoxified by glutamine synthesis in skeletal muscle and other tissues. Administration of BCAA, especially Leu-rich solution, to CCl$_4$-treated rats induced a rapid decrease in plasma urea-$^{15}$N. The rapid decrease of plasma urea-$^{15}$N can be explained by two factors. Accelerated excretion of urea-$^{15}$N into urine is considered from the results that higher $^{15}$N-enrichment of urinary nitrogen was recognized in CCl$_4$-BCAA group than CCl$_4$-PA group (Table 4). Rapid incorporation of $^{15}$NH$_3$ into glutamic acid and other amino acids (11) is another explanation for these observations. High incorporation of $^{15}$N into non-protein fraction of skeletal muscle was recognized in Leu-rich group. Therefore, the rapid decrease of plasma urea-$^{15}$N may contribute to the high $^{15}$N incorporation into skeletal muscle.

There are many reports suggesting that leucine has more potential effect than valine for protein synthesis or protecting protein degradation in muscle (21, 23). On the other hand, some reports revealed that only the infusion of valine significantly increased muscle protein synthesis (24) or had a potential effect on ammonium detoxification (25). It was pointed out, however, that the cirrhotic patient has valine intolerance (26, 27). In the present report, plasma valine levels in liver-injured rats reached the extremely high levels after valine-rich solution was administered. The result indicates that a leucine-rich solution is desirable for liver-injured animals. However, there is no general agreement on the desirable proportions of BCAA for obtaining more nutritional efficacies. Isoleucine was not included in the present BCAA solution to compare the efficacy of leucine with that of valine on protein synthesis and ammonia detoxification. The transamination rate of leucine is about 5-fold that of valine (28). Bolus administration of leucine stimulates the activity of
branched-chain α-keto acid dehydrogenase and diminishes other branched-chain amino acids, valine and isoleucine, concentrations (29). Therefore it may be necessary to contain valine and isoleucine in leucine-rich BCAA solution in order to prevent plasma amino acid imbalance. In some reports (30), in contrast, negative effects of BCAA for stimulating protein synthesis in liver cirrhosis were discussed. However, it was concluded from the present results that BCAA, especially a leucine-rich mixture, was an effective nitrogen source for protein synthesis and ammonia detoxification in liver-injured animals.

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