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Abstract

To clarify the nature of nitrogen metabolism between branched chain amino acid (BCAA) and glutamine (GIn) in liver failure, we measured arterial plasma concentrations of GIn and ^{15}N uptake to amino-N and amide-N of GIn in normal and D-galactosamine-induced fulminant hepatic failure (FHF) rats after ^{15}N-leucine (Leu) injection. Fifteen, 30 and 60 min after Leu injection, the arterial plasma concentrations of GIn were significantly higher in FHF rats than in controls. The concentrations of amino-^{15}N GIn were also significantly higher in FHF rats than in controls at 5, 15, 30 and 60 min after injection. The concentrations of amide-^{15}N GIn did not significantly differ between FHF and controls at 5, 15 and 30 min. However, at 60 min, the concentration was significantly higher in the FHF rats. The higher uptake of ^{15}N to amino-N of GIn in FHF rats suggests the presence of an enhanced ability to synthesize GIn from Leu in FHF rats. The higher uptake of ^{15}N to amide-N of GIn in FHF rats at 60 min after injection suggests that excessive administration of BCAA to patients with severely impaired urea-cycle capacity suffering with hepatic failure may lead to greater levels of hyperammonemia.

KEYWORDS: stable isotopes, mass fragmentography, fulminant hepatic failure, branched-chain amino acids, glutamine

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A ^{15}N GC/MS Study of *In Vivo* Glutamine Synthesis in Liver Failure Rats

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To clarify the nature of nitrogen metabolism between branched chain amino acid (BCAA) and glutamine (Gln) in liver failure, we measured arterial plasma concentrations of Gln and ^{15}N uptake to amino-N and amide-N of Gln in normal and D-galactosamine-induced fulminant hepatic failure (FHF) rats after ^{15}N-leucine (Leu) injection. Fifteen, 30 and 60 min after Leu injection, the arterial plasma concentrations of Gln were significantly higher in FHF rats than in controls. The concentrations of amino-^{15}N Gln were also significantly higher in FHF rats than in controls at 5, 15, 30 and 60 min after injection. The concentrations of amide-^{15}N Gln did not significantly differ between FHF and controls at 5, 15 and 30 min. However, at 60 min, the concentration was significantly higher in the FHF rats. The higher uptake of ^{15}N to amino-N of Gln in FHF rats suggests the presence of an enhanced ability to synthesize Gln from Leu in FHF rats. The higher uptake of ^{15}N to amide-N of Gln in FHF rats at 60 min after injection suggests that excessive administration of BCAA to patients with severely impaired urea-cycle capacity suffering with hepatic failure may lead to greater levels of hyperammonemia.

Key words: stable isotopes, mass fragmentography, fulminant hepatic failure, branched-chain amino acids, glutamine

Since Fischer *et al.* proposed the plasma amino acids imbalance theory to explain the mechanism of hepatic encephalopathy (1), intravenous administration of branched chain amino acids (BCAA) solution has been commonly used to treat hepatic encephalopathy. The bulk of BCAA are taken up not only by brain tissue but also by extrahepatic peripheral tissues such as skeletal muscle, adipose tissue and splanchnic organs. Among these organs and tissues, the major site that extracts BCAA is skeletal muscle (2, 3), where alanine (Ala) and glutamine (Gln) are synthesized from BCAA and then released into venous blood (4-6). In patients with chronic hepatic encephalopathy, skeletal muscle production of Gln is thought to increase to dispose of excess NH_3 (7, 8). The production rate of alanine has been shown to be significantly lower in fulminant hepatic failure (FHF) rats after intravenous injection of ^{15}N-leucine (9). And the problem of the nitrogen transfer from leucine to glutamine in hepatic failure remains unresolved, because of difficulties in measuring its kinetics using the ^{15}N tracer method. Measuring ^{15}N enrichment of two nitrogen atoms (amino- and amide-N) of Gln was difficult and time consuming until Nissim *et al.* simplified the determination using the GC-MS method (10). With some modification of their method, we previously reported the result of arterial plasma ^{15}N enrichment of amide-N of Gln after intravenous injection of $^{15}NH_4Cl$ into normal rats (11). The aim of the present study is to investigate the nitrogen transfer pathway from Leu to Gln in FHF rats.

Materials and Methods

Male Sprague-Dawley rats, weighing approximately 250 g, were fed a standard diet (CE-2, Clea Japan, Inc., Tokyo, Japan) and water *ad libitum* prior to the experiments. The rats were divided into 2 groups: FHF (n = 26) and the control group (n = 20). To prepare the FHF rats, D-galactosamine was administered intraperitoneally at a dose of 2.0 g per kg of body weight dissolved in 1 ml of physiological saline. The animals were fasted for 16 h prior to the experiments which were performed 48 h after

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the injection of D-galactosamine. The control rats received intraperitoneal injections of physiological saline, and were starved for 16 h prior to the experiments. All animals received bolus injections of ^{15}N-labeled Leu (95.0 atom %) at a dose of 200 mg per kg of body weight dissolved in 2.5 ml of deionized water intravenously via the tail vein. Blood was drawn from the abdominal aorta before and 5, 15, 30 and 60 min after the injection of Leu. Plasma was immediately separated with sodium heparin as an anticoagulant.

Analytical methods. Plasma amino acid concentrations were determined with an Irica Amino Acid Analyzer A-5500E (Irica Kikai Co. Ltd., Tokyo, Japan). Specimens were deproteinized with 5 % sulfosalicylic acid. ^{15}N enrichment in glutamine-amino-N and glutamine-amide-N were determined by gas chromatography and mass spectrometry (GC/MS), according to the methods described by Nissim *et al.* (10), after some modification as presented in our previous report (11).

Plasma sample preparation for GC/MS. Plasma samples (500 μl) were mixed with three times their volumes of 1 M acetic acid. Samples were then applied directly to an ion-exchange column, filled with 2 ml Dowex 50 W cation-exchange resin. Each column was washed with 10 ml distilled water, and the effluent was discarded. The amino acids were eluted with 3.5 ml of 3 M ammonium hydroxide solution. The eluted amino acid fractions were lyophilized to dryness. To remove the last traces of water, the samples were then azeotroped once with 500 μl aliquots of methylene chloride. To the residue of the plasma sample, 500 μl of trifluoroacetic anhydride (TFAA) was added. The vials were capped, then sonicated for 2-3 min at 70 °C in a block heater. After cooling to room temperature, the excess TFAA was removed under a gentle stream of dry nitrogen, and the residue was dissolved in 30 μl of methylene chloride. The same procedure was also applied to the calibration solutions.

Gas chromatography-mass spectrometry analysis. The derivatized plasma samples and calibration samples were analyzed on a GCMS 9020-DF (Shimadzu Co., Kyoto, Japan) interfaced to a SCAP1123 data analyzing system.

Derivatized glutamine was separated from other components on a 5-m × 3-mm glass column packed with 3 % silicone OV-17. GC conditions were as follows: injector temperature, 250 °C; helium flow rate, 20 ml/min; temperature program, 190 °C isothermal for 1 min then 10 °C/min elevation. The MS conditions were as follows: ion source, 150 °C; separator temperature, 250 °C; ionizing energy, 20 eV. To determine amino-^{15}N enrichment of glutamine, mass fragments were monitored at 181 and 182 (m/z) (Figs. 1, 2). For amide-^{15}N enrichment of glutamine, monitored mass fragments were 111 and 112 (m/z) (Figs. 1, 2).

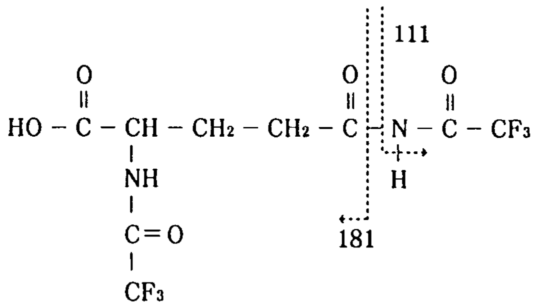

Fig. 1 Glutamine-TFA derivative and its mass fragmentation.

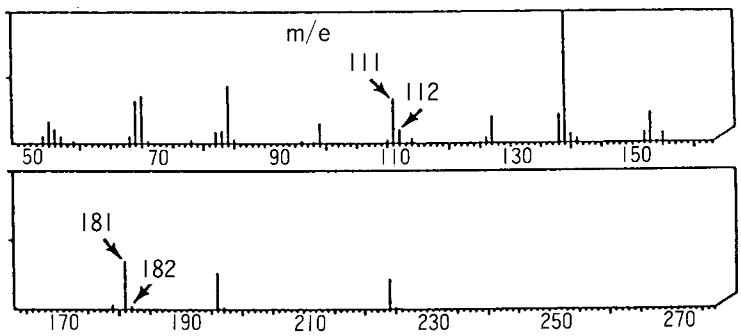

Fig. 2 Mass spectrogram of glutamine-TFA derivative.

Results

Calibration solutions. Calibration solutions for plasma enrichment with amino-^{15}N glutamine were prepared by adding 156.8, 78.4 and 39.2 nmol/ml of 95 atom % amino-^{15}N glutamine to an equal volume of fresh heparinized plasma samples containing 691.5 nmol/ml glutamine to achieve a final enrichment of 17.5, 9.6 and 5.1 atom % excess, respectively (Fig. 3). Calibration solutions for plasma enrichment with amide-^{15}N glutamine were similarly prepared to achieve a final enrichment of 9.7, 5.1 and 2.6 atom % excess, respectively. Calibration curves showed good linearity (Fig. 3).

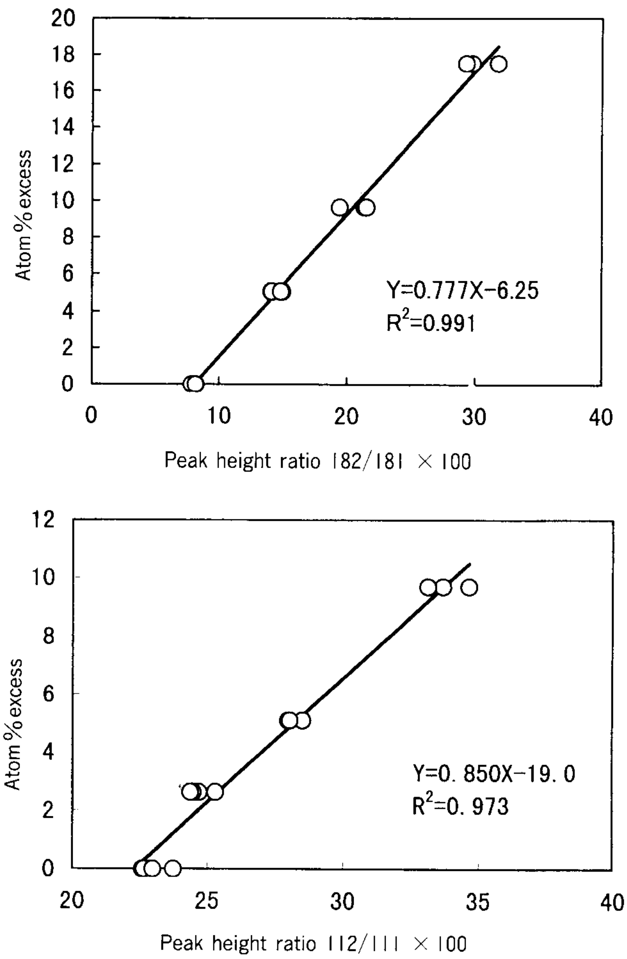

Fig. 3 Calibration of amino- and amide-^{15}N glutamine. Upper panel: amino-^{15}N glutamine; lower panel: amide-^{15}N glutamine.

Changes in glutamine, amino-^{15}N-glutamine and amide-^{15}N-glutamine concentrations. Plasma Gln concentrations showed a slight decrease in control rats after ^{15}N-Leu injection, but there was no decrease or significant increase at 60 min in the FHF rats (Table 1). Comparing the two groups, a higher plasma Gln concentration was observed in the FHF group from 15 min to 60 min after Leu injection.

Higher plasma concentrations of amino-^{15}N-Gln were observed in the FHF group during the study period.

Plasma concentrations of amide-^{15}N-Gln changed similarly in both groups until 30 min but at 60 min a higher concentration was observed in the FHF rats. Comparing the two groups, only the concentration at 60 min was significantly higher in the FHF group.

Discussion

In this study, a higher concentration of amino-^{15}N-Gln after ^{15}N-Leu injection was observed in the FHF group as compared with that in the controls. This result probably indicates that in hepatic failure, peripheral tissues play an important role in eliminating high blood NH_3 by synthesis and release of Gln after uptake of Leu and blood NH_3. These facts support the previously reported results of Ganda *et al.* (7) and Morimoto *et al.* (11, 12).

Table 1 Changes in glutamine, amino-^{15}N- and amide-^{15}N-glutamine concentrations

Time after injection (min)	Gln conc (nmol/ml)		Amino-^{15}N Gln conc (nmol/ml)		Amide-^{15}N Gln conc (nmol/ml)	
	Control	FHF	Control	FHF	Control	FHF
0	629 ± 21	647 ± 105	−0.04 ± 0.44	0.59 ± 0.93	3.45 ± 4.36	6.60 ± 3.84
5	671 ± 70	837 ± 283	6.39 ± 1.40	12.56 ± 3.22**	12.39 ± 1.94	12.95 ± 7.33
15	553 ± 38	1,042 ± 239**	22.36 ± 6.39	42.26 ± 9.81**	17.65 ± 1.44	20.00 ± 9.83
30	482 ± 45	816 ± 269**	21.89 ± 5.01	36.89 ± 13.41*	15.52 ± 2.23	19.66 ± 5.79
60	483 ± 44	1,890 ± 588**	17.14 ± 4.56	83.43 ± 25.72**	8.94 ± 0.91	35.10 ± 15.64**

Gln conc: glutamine concentration; FHF: fluminant hepatic failure. **$P < 0.01$, *$P < 0.05$, Mean ± SD.

Another important finding in the present study is that a higher arterial plasma concentration of amide-^{15}N Gln was observed at 60 min after ^{15}N-Leu in the FHF group than in the control group. In many animal species, the gut, especially the small intestine, actively takes up arterial Gln and releases NH_3, alanine, citrulline and proline to portal blood after removing two nitrogen atoms from Gln (13–17). In like fashion, we believe that the amino-^{15}N-Gln synthesized and released from peripheral tissue after taking up ^{15}N-Leu was metabolized in the rat's gut, then $^{15}NH_3$ was produced *de novo*. This $^{15}NH_3$ is supposed to be transported through the portal vein to the liver and utilized for urea synthesis. However, as Morimoto *et al.* (12) previously reported, since the capacity of the urea cycle is impaired in hepatic failure, a larger amount of $^{15}NH_3$ appears to be transported again into extrahepatic peripheral tissues to form amide-^{15}N-labeled Gln. According to these results, BCAA administration may cause a worsening of hepatic failure due to nitrogen over-loading, if reserve capacity and blood supply to the liver is not accurately assessed.

Considering the above observations, some peculiarities in previously reported clinical studies can be more easily understood. For example, Weber *et al.* reported a decrease in blood NH_3 after administering BCAA solution to patients with barely stable liver cirrhosis (18). Also a transient elevation of blood NH_3 was reported in liver cirrhosis patients after oral administration of BCAA (19). Moreover, in fulminant hepatitis patients, Takahashi *et al.* noted that the use of BCAA solution was a significant factor in deterioration of the prognosis (20). Thus, the metabolic relationship of BCAA and blood NH_3 during severe liver failure has not yet been thoroughly elucidated. Future studies should quantitatively evaluate their metabolic relationships in each organ using a method such as perfusion.

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