Metabolism of branched-chain amino acids in rats with acute hepatic failure: a tracer study using 15N-leucine.

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Abstract

Fulminant hepatic failure (FHF) was produced in rats with intraperitoneal injection of D-galactosamine. Control rats received only physiological saline. 15N-leucine (200 mg/kg of body weight) was injected into the rats via the tail vein. Arterial blood was drawn before and 5, 15, 30 and 60 min after the injection of 15N-leucine. 15N-amino acids were determined quantitatively by gas chromatography and mass spectrometry. The plasma 15N-leucine level decreased logarithmically in the same manner in both groups. This result suggests that leucine is mainly metabolized in extrahepatic tissues. The incorporation of 15N into plasma isoleucine and valine was not significantly different between the groups. Plasma alanine and glutamine concentrations increased in controls and decreased in FHF rates after the injection. The incorporation of 15N into plasma alanine in rats with FHF was significantly later than in controls. This result may suggest that undergoing hyperammonemia causes to form more glutamine from glutamate in extrahepatic sites as the same manner as for chronic hepatic failure. Additionally, insulin levels increased temporarily after the injection of leucine in both groups. This increase may play a role in the decrease in plasma isoleucine and valine concentrations after injection of leucine.

KEYWORDS: stable isotope, gas chromatography and mass spectrometry, acute hepatic failure, branched chain amino acids, muscle metabolism

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METABOLISM OF BRANCHED-CHAIN AMINO ACIDS IN RATS WITH ACUTE HEPATIC FAILURE: A TRACER STUDY USING $^{15}$N-LEUCINE

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Abstract. Fulminant hepatic failure (FHF) was produced in rats with intraperitoneal injection of D-galactosamine. Control rats received only physiological saline. $^{15}$N-leucine (200 mg/kg of body weight) was injected into the rats via the tail vein. Arterial blood was drawn before and 5, 15, 30 and 60 min after the injection of $^{15}$N-leucine. $^{15}$N-amino acids were determined quantitatively by gas chromatography and mass spectrometry. The plasma $^{15}$N-leucine level decreased logarithmically in the same manner in both groups. This result suggests that leucine is mainly metabolized in extrahepatic tissues. The incorporation of $^{14}$N into plasma isoleucine and valine was not significantly different between the groups. Plasma alanine and glutamine concentrations increased in controls and decreased in FHF rats after the injection. The incorporation of $^{15}$N into plasma alanine in rats with FHF was significantly later than in controls. This result may suggest that undergoing hyperammonemia causes to form more glutamine from glutamate in extrahepatic sites as the same manner as for chronic hepatic failure. Additionally, insulin levels increased temporarily after the injection of leucine in both groups. This increase may play a role in the decrease in plasma isoleucine and valine concentrations after injection of leucine.

Key words: stable isotope, gas chromatography and mass spectrometry, acute hepatic failure, branched chain amino acids, muscle metabolism.

There is considerable evidence suggesting that branched chain amino acids (BCAA) are metabolized mainly in muscle (1). Previous investigators have reported that amino groups of BCAA are transaminated and carbon chains are oxidized in the muscle (1, 2). In addition, transaminated amino groups of BCAA are assumed to be used in the de novo synthesis of alanine (3-5). Leucine is a ketogenic BCAA and its carbon chain is a potential precursor of lipid in muscle (6). Chang et al. have shown that leucine inhibits the oxidation of glucose and the decarboxylation of pyruvate in muscle (7). Haymond et al. have recently shown using leucine labelled with $^{15}$N and $^{14}$C that the amino group of leucine is incorporated into alanine (8).

In recent years, amino acid metabolism in patients with hepatic failure has received much attention, and specific plasma amino acid patterns have been reported (9, 10). Plasma BCAA concentrations decrease in patients and experimental animals with chronic hepatic failure (CHF), but they remain normal or increase.
in fulminant hepatic failure. The decrease in BCAA in CHF may result from the increased peripheral utilization of BCAA (11). However, there has been no direct evidence of transamination or utilization of BCAA in muscle of animals with hepatic failure. The present study investigates the metabolism of 15N-leucine in extrahepatic tissues of D-galactosamine-treated rats as a model of fulminant hepatic failure.

MATERIALS AND METHODS

Animals and experimental design. Male Sprague-Dawley rats, weighing approximately 250 g, were given food (CE-2, Clea Japan, Inc., Tokyo) and water ad libitum prior to the experiments. The rats were divided into 2 groups: fulminant hepatic failure (n = 25) and control groups (n = 25). D-Galactosamine hydrochloride (Wako Pure Chemical Ind., Osaka) at a dose of 2 g per kg of body weight dissolved in 1 ml of physiological saline was given intraperitoneally, inducing fulminant hepatic failure (12,13). The animals were fasted for 16 h prior to the experiments which were performed 48 h after the injection of D-galactosamine. The control rats received intraperitoneal injections of physiological saline, and were fasted for 16 h prior to the experiments.

All animals received one injection of 15N-labeled leucine (95.0 atom %, Hikari Kogyo Co., Tokyo) 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 leucine. Plasma was separated using EDTA as an anticoagulant. After blood sampling was completed, liver specimens for histological examination were obtained immediately from representative animals. They were fixed in Bouin’s fixative and were stained with hematoxylin eosin, Azan-Mallory, silver reticulin stain and periodic acid Shiff reagent.

Analytical methods. Plasma amino acid concentrations were determined with a JLC/6AH amino acid analyzer (Japan Electron Optics Lab., Tokyo). Specimens were deproteinated with 5% sulfosalicylic acid. Plasma levels of insulin and glucagon were measured by radioimmunoassay. 15N-leucine, -isoleucine, -valine and -alanine were determined according to the method of Gehrke et al. (14, 15), with some modifications as follows:

Preparation of plasma samples. Five ml of 1.2% aqueous picric acid solution were added to 1 ml of plasma. Precipitated protein was separated by centrifugation at 3000 r.p.m. for 10 min. To remove the picrate, the protein free supernatant was passed through a column (10×20 mm) of AG 2-X8 (Bio-Rad Lab., U.S.A.), 200-400 mesh, CI-form. The resin was then washed with 3 ml of 0.02 N HCl. The combined eluate was adjusted to pH 2.0-2.5 with 0.02 N NaOH, and applied to a column (10×60 mm) of Dowex AG 50W-X8, 200-400 mesh, H*-form. After washing with deionized water, amino acids were eluted with 10 ml of 3 N aqueous ammonia. The eluate was immediately evaporated to dryness using a flash evaporator. The free amino acid mixture thus obtained was used for the analysis described below.

Derivatization of amino acids. Moisture in the samples was eliminated by adding 1 ml of dichloromethane and passing a stream of dry pure nitrogen gas at 80°C. This procedure was repeated 3 times. Amino acid methyl esters were prepared with 1 ml of methanol and HCl gas at room temperature for 30 min. Excess solvent was removed under a nitrogen stream. The methyl esters were esterified with 1.0 ml of butanol and HCl gas at 100°C for 2 h. The excess solvent was removed as above. N-Trifluoroacetylation of amino acid butyl esters was performed by the addition of 0.1 ml of dichloromethane and a 0.1 ml of trifluoroacetic acid, and
heating at 150°C for 10 min.

Gas chromatography and mass spectrometry. Gas chromatography was carried out on a GCMS 9020-DF (Shimadzu Co., Kyoto). The data analysing system was SCAP 1123 (Shimadzu Co., Kyoto). The column (0.3 × 100 cm) employed consisted of an unsialinized glass tube packed with chromosorb G (80-100 mesh) coated with 0.5% ethylene glycol adipate (Shinwa Kako Co., Kyoto). The column temperature was programmed from 80°C to 180°C at a rate of 3°C/min. The flow rate of the carrier gas He was 30 ml/min. The conditions for analysis by mass spectrometry were as follows: Ion source, 220°C; separator temperature, 250°C; injection temperature, 200°C; and electron volt 20 eV. 15N enrichment of amino acids was calculated from the peak and average height ratio (Leu m/e 183/182, Ile m/e 183/182, Val m/e 169/168, Ala m/e 141/140) determined by mass chromatography (16, 17).

Recovery. The recovery of amino acids by ion-exchange chromatography has been reported to be over 95% (18), and by ion-exchange chromatography, derivatization and gas chromatography to range from 70 to over 90% (19). Recovery in the present study was examined using human plasma and a known amount of 15N-leucine, and proved to be more than 85%. The correlation of expected value and measured value was shown in Fig. 1.

![Graph showing correlation](image)

Fig. 1. The measured 15N atom % excess of leucine plotted against the calculated 15N atom %, using human plasma and a known amount of 15N-leucine.

RESULTS

Fig. 2 shows the typical liver histology of the rats with fulminant hepatic failure (FHF). Massive hepatic necrosis is shown. Plasma leucine concentrations and isotopic enrichment in both groups after the injection are shown in Fig. 3. Before the injection of 15N-leucine, the plasma leucine concentration of rats with FHF was 3 times higher than controls. After the injection, it rose from 548 to 3125 nmol/ml in FHF rats and from 160 to 2079 nmol/ml in controls. It decreased almost logarithmically from 5 min to 60 min in both groups. 15N enrichment of
leucine decreased at the same manner as the plasma leucine concentrations in both groups (Table 1). There was no significant difference between the groups during the course of the study.

Before the injection of $^{15}$N-leucine, the plasma isoleucine concentration of FHF rats was 3 times higher than controls (Fig. 4). After the injection, it remained at almost the same value up to 5 min, and then decreased from 70 nmol/ml at 5 min

Fig. 2. Liver specimens from a rat with fulminant hepatic failure. Azan-Mallory, × 100.

Fig. 3. (A) Plasma leucine concentrations before and after the injection of $^{15}$N-leucine. (B) Isotopic enrichment of $^{15}$N-leucine after the injection of $^{15}$N-leucine. Vertical lines indicate the standard deviation of the mean. FHF: Fulminant hepatic failure.
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Table 1. **H^N-Enrichment of Plasma Leucine, Isoleucine, Valine and Alanine after the Injection of H^N-Leucine**

<table>
<thead>
<tr>
<th>Group</th>
<th>Minutes after loading</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine</td>
<td>Control</td>
<td>69.7 ± 5.3</td>
<td>44.2 ± 6.5</td>
<td>28.1 ± 0.9</td>
<td>14.6 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>FHF</td>
<td>70.9 ± 0.9</td>
<td>48.9 ± 4.6</td>
<td>30.1 ± 4.6</td>
<td>14.8 ± 0.6</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>Control</td>
<td>21.1 ± 2.6</td>
<td>22.0 ± 3.6</td>
<td>12.1 ± 5.0</td>
<td>4.8 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>FHF</td>
<td>15.8 ± 3.0</td>
<td>25.2 ± 1.5</td>
<td>14.6 ± 7.5</td>
<td>5.9 ± 1.8</td>
</tr>
<tr>
<td>Valine</td>
<td>Control</td>
<td>7.3 ± 2.4</td>
<td>12.2 ± 1.3</td>
<td>9.7 ± 2.5</td>
<td>5.0 ± 2.4</td>
</tr>
<tr>
<td></td>
<td>FHF</td>
<td>5.5 ± 0.8</td>
<td>9.2 ± 1.0</td>
<td>10.8 ± 2.9</td>
<td>6.8 ± 0.7</td>
</tr>
<tr>
<td>Alanine</td>
<td>Control</td>
<td>2.7 ± 1.9</td>
<td>8.3 ± 1.9</td>
<td>9.6 ± 1.4</td>
<td>7.3 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>FHF</td>
<td>0.6 ± 1.0</td>
<td>0.1 ± 0.1</td>
<td>8.3 ± 4.6</td>
<td>10.1 ± 1.8</td>
</tr>
</tbody>
</table>

Mean ± SD  
FHF: Fulminant hepatic failure  
*: p < 0.05  **: p < 0.01  
Each group consists of different 3 rats

![Graph 1](image1)

![Graph 2](image2)

**Fig. 4.** Plasma amino acid concentrations before and after the injection of \(^{15}N\)-leucine. Vertical lines indicate the standard deviation of the mean. •: fulminant hepatic failure. ○: control.

to 26 nmol/ml at 60 min in controls. In FHF rats, it remained steady from 0 min to 15 min and decreased from 267 nmol/ml at 15 min to 71 nmol/ml at 60 min. \(^{15}N\) incorporation into isoleucine was observed at 5 min (Table 1). Isotopic enrichment remained steady from 5 min to 15 min and decreased from 15 min to 60 min in controls. It rose to a peak level at 15 min and decreased during the 15 to 60 min period in FHF rats. There was no significant difference between them.

The plasma valine concentration was 635 nmol/ml in FHF rats and 167 nmol/ml in controls before the injection of \(^{15}N\)-leucine (Fig. 4). After the injection, it increased slightly, and then decreased between 5 and 60 min in controls. It remained
steady between 0 and 15 min and decreased during the 15 to 60 min period in FHF rats. $^{15}$N incorporation into valine was observed at 5 min in both groups (Table 1). Isotopic enrichment of valine rose to a peak level at 15 min, and decreased from 15 min to 60 min in controls. It rose to peak level at 30 min, and decreased during the 30 to 60 min period in FHF rats. Isotopic enrichment was significantly low in FHF rats at 15 min.

The plasma alanine concentration was significantly higher in FHF rats before the injection of $^{15}$N-leucine (Fig. 4). After the injection, it increased from 521 nmol/ml at 0 min to 648 nmol/ml at 15 min and decreased to 426 nmol/ml at 60 min in controls. The value decreased from 6387 nmol/ml at 0 min to 1540 nmol/ml at 60 min in FHF rats. $^{15}$N incorporation into alanine was observed at 5 min and rose to a peak level at 30 min in controls (Table 1). However, incorporation was observed at 30 min in FHF rats. There was a significant difference between both groups. Enrichment of alanine with $^{15}$N was very low at 5 min and extremely low at 15 min after the injection of $^{15}$N-leucine in FHF rats.

The plasma glutamine concentration in FHF rats was 6 times higher than in controls before the injection of $^{15}$N-leucine (Fig. 4). It rose from 692 nmol/ml at 0 min to 866 nmol/ml at 5 min, and decreased to 557 nmol/ml at 60 min in controls. It decreased from 4297 nmol/ml at 5 min to 1749 nmol/ml at 60 min in FHF. An accurate estimate of $^{15}$N enrichment of glutamine and glutamate could not be made with the present method because these column works could not separate glutamine and glutamate.

Plasma levels of insulin and glucagon were measured to verify the hormonal effects on alanine metabolism. Plasma insulin levels in FHF rats were higher than in controls before the injection of $^{15}$N-leucine (Table 2). After the injection, the

| Table 2. Plasma Levels of Insulin and Glucagon Before and After the Injection of $^{15}$N-Leucine |
|----------------------------------|---------------|---------------|---------------|---------------|---------------|
|                                  |               | 5             | 15            | 30            | 60            |
|                                  | Group         | Before        |               |               |               |
| Insulin (μu/ml)                  | Control       | 12.2          | 54.1          | 44.4          | 51.0          | 9.4           |
|                                  | FHF           | 7.9           | 65.6          | 20.6          | 19.5          | 7.7           |
|                                  |               | 15.2          | 39.8          | 17.6          | 23.4          | 26.5          |
|                                  |               | 8.1           | 37.0          | 27.4          | 8.8           | 16.3          |
| Glucagon (pg/ml)                 | Control       | 76            | 95            | 62            | 71            | 40            |
|                                  | FHF           | 46            | 116           | 39            | 82            | 59            |
|                                  |               | 202           | 87            | 57            | 98            | 49            |
|                                  |               | 111           | 101           | 127           | 134           | 66            |

FHF: Fulminant hepatic failure
Each group consists of different 2 rats at each time.
levels rose to a peak level at 5 min, and decreased during the 5 to 60 min period in both groups. However, the increase was more remarkable in controls. Plasma glucagon levels were higher in FHF rats than in controls before the injection (Table 2). After the injection, the levels increased slightly at 5 min, and decreased at 60 min in controls. In FHF rats, the value decreased between 0 and 60 min. The reaction tendency after the injection was not clear in the case of glucagon.

DISCUSSION

Leucine is known to be a ketogenic amino acid. Rosenthal et al. have shown that leucine utilization in adipose tissue and muscle combined is 7 times greater than in liver of normal subjects (6). Furthermore, Hagenfeldt et al. have shown that leucine is taken up by muscle tissue (55 %) and by the splanic region (25 %) during continuous intravenous infusion (20). The carbon chain of leucine is thought to be oxidized to acetyl CoA in the muscle (2, 6).

As a report concerning the amino groups of BCAA, Ruderman et al. investigated the isolated rat hindquarter by tissue perfusion with a solution containing leucine (21). Garber et al. investigated the rat epitrochlaris skeletal muscle by incubation with leucine (5). These studies showed that leucine induces the release of glutamine and alanine from the muscle in vitro. Leucine can act as a substrate for glutamate formation by transamination with α-ketoglutarate (5). Furthermore it is thought that glutamate is a common substrate for alanine and glutamine formation (3, 5). This suggests that the amino group of leucine may be a precursor for alanine and glutamine formation (3, 21). In recent years, the study of nitrogen in amino groups has been performed using the tracer method (22). Haymond et al. have shown using 15N and GC-MS that leucine nitrogen is incorporated into alanine in normal dogs and humans (8, 23). In normal dogs, 15N alanine was detected as early as 30 min after the continuous infusion of 15N leucine. Examination of the blood samples from the femoral vein and aorta revealed that 54 % of the leucine taken up by the dog hindlimb transferred its nitrogen to alanine. It could account for 25 % of the observed 15N incorporation into alanine. In humans, the percentage of circulating alanine nitrogen derived from leucine was 12.5 % in arterialized venous plasma samples.

In the present study, plasma leucine concentrations of FHF rats and controls were noted to be much different even prior to the single injection of 15N-leucine. However, 15N enrichment after the injection and the disappearance rate of 15N-leucine were not significantly different between the groups. This result may suggest that 15N-leucine is metabolized at the same rate in both groups and that leucine is mainly metabolized in extrahepatic sites such as muscle and adipose tissue. Plasma alanine and glutamine concentrations increased 5 and 15 min after the injection of 15N-leucine in the control group. This result may support the above mentioned results of in vitro studies (3, 5, 21). Accordingly, the same mechanism
of amino acid metabolism hypothesized from the in vitro studies may be inferred from the results of the present study.

As for alanine, 8.3% of the alanine nitrogen was transaminated from leucine at 15 min in the control group. In FHF rats, the percentage of alanine nitrogen derived from $^{15}$N-leucine was only 0.1% at 15 min. In this way, the incorporation of $^{15}$N from leucine to alanine was noticeably lower in FHF. This low incorporation may be due to the low availability of carbon chain of alanine or to the low rate of transamination to form alanine. The carbon source of alanine is generally believed to be derived from glucose and amino acids. However, at this time there is no agreement as to which takes the major part in alanine synthesis (3, 4, 24). The plasma alanine concentration increases much in FHF (9). Williams et al. have reported that plasma levels of lactate and pyruvate increase in patients with FHF regardless of the etiology of the disease (25). This increase was thought to be a cause for increased alanine concentrations in FHF. In addition, pyruvate did not increase the rate of either alanine or glutamine formation in vitro (5). Thus, in this study, it is hard to assume that pyruvate limits the rate of alanine synthesis.

There are some reports about amino acid metabolism in the muscle during chronic hepatic failure. Ganda et al. (26) and Imler et al. (27) have shown that the release of glutamine from the muscle increased and the release of alanine decreased in humans with liver cirrhosis by measuring blood arterio-venous differences in amino acids. Imler et al. also have shown that the release of glutamine from the muscle increased when NH$_4$Cl was injected into normal rats. As previously mentioned, alanine and glutamine, which are synthesized de novo in the muscle, have the same precursor glutamate. Such de novo synthesis is thought to occur in the stage of liver cirrhosis and hyperammonemia, and it cause increased glutamine release and decreased alanine release (27). Accordingly, the delay or $^{15}$N incorporation into alanine in this study may be explained by the amino flow toward glutamine formation from glutamate induced by hyperammonemia. The same mechanism that operates in chronic hepatic failure may act in FHF.

There are many reports concerning the regulatory effects of insulin and glucagon on amino acid metabolism (28, 29). Berger et al. have shown that insulin reduced the plasma BCAA levels in humans (30). Garber et al. (4) and Ruderman et al. (21) have shown that insulin increases the uptake of glucose to the muscle, but has no effect on the release of alanine and glutamine from the muscle in vitro. Ruderman et al. also have shown that glucagon has no effect on the release of amino acids (21). In this study, insulin increased 5 min after the injection of leucine. This increase may not be related to the change in alanine and glutamine after the injection, and may explain the decrease in plasma isoleucin and valine concentrations after the injection in both groups. Hagenfeldt et al. have reported that plasma isoleucine and valine concentrations declined continually during the injection of leucine into humans (20). Though the plasma insulin level was not measured, their results may explain the decrease in amino acids in this study.
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However, there is no good explanation for the decrease in alanine and glutamine in FHF.

In metabolic tracer studies, there are many factors which affect the results, including blood flow and recycling of the isotope (31). We plan to investigates these factors further. However, it was notable that we found a delay of \[ 15^N \] incorporation from leucine to alanine in FHF rats. More detailed investigation of metabolism of glutamate and glutamine will be expected using this \[ 15^N \] tracer method.

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