ORIGINAL

Soy protein diet prevents hypermethioninemia caused by portacaval shunt in rats

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Abstract : In hepatic disorders, abnormal plasma amino acid profiles are observed. In this study, we examined whether soy protein isolate (SPI) improved plasma methionine concentration in the model animals. Portacaval shunt (PCS) increased alanine aminotransferase (ALT) activity and methionine concentration in blood of rats fed a 40% casein diet supplemented with 0.6% methionine (casein-M diet). A 40% SPI diet supplemented with 1.28% methionine (SPI-M diet), which contained the same amount of methionine as that in 40% casein-M diet, normalized plasma ALT activity and methionine level in PCS rats. These effects of a SPI diet may be due to its amino acid composition, since an amino acid mixture diet mimicking a 40% SPI-M diet was also effective to hypermethioninemia of PCS rats. To find key enzymes for the beneficial effect of soy protein, we examined effects of a 40% SPI-M or casein-M diet on the activities of three methionine-metabolizing enzymes in liver of PCS rats. A SPI-M diet stimulated only the activity of cystathionine γ-lyase, compared with a casein-M diet. A SPI diet has a preventive effect on hypermethioninemia, at least in part, by stimulating cystathionine γ-lyase activity in liver and may be used for nutritional management of liver disorders with hypermethioninemia. J. Med. Invest. 53 : 255-263, August, 2006

Keywords **:** *cystathionine γ-lyase, methionine, portacaval shunted rats, soy protein diet*

INTRODUCTION

In patients with hepatic failure, abnormal profiles of plasma amino acids have occasionally been observed ; low levels of branched-chain amino acids (BCAA) and high levels of tryptophan and methionine (1-4). These abnormal plasma amino acid profiles seriously affect clinical conditions of patients with liver cirrhosis. For example, deficiency of BCAA induces severe encephalopathy, and the BCAA supplementation decreases frequency of complications of cirrhosis (5, 6). Methionine and its metabolites have been reported to play a role as an augmenter or intensifier of toxic effects of ammonia and fatty acids in these patients, leading to severe encephalopathy (7, 8). However, few

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diets are available to normalize the plasma amino acid pattern in patients with liver cirrhosis.

Rats operated an end-to-side portacaval shunt (PCS rats) have a plasma amino acid pattern similar to that observed in patients with chronic liver diseases. Benjamin and Steele reported that PCS operation increased plasma methionine levels in rats fed a 60% casein diet, suggesting that this portal-systemic shunting is a useful animal model for liver cirrhosis (9, 10). They also suggested that this abnormal metabolism of the sulfur-containing amino acids might be improved by diets containing a low amount of sulfur amino acids (10). In fact, several lines of investigations have shown that vegetable protein diets had beneficial effects on clinical conditions of cirrhotic patients (11, 12) due to the lower contents of sulfur amino acid, compared to those of animal protein.

In the present study, we examined how soy protein isolate (SPI) improved increased plasma methionine concentration in PCS rats. Unexpectedly, a 40% SPI diet supplemented with methionine at a level equal to that in a 60% casein diet, significantly decreased plasma and liver methionine concentrations in PCS rats. In addition, we found that among methionine-metabolizing enzymes examined, only cystathionine γ-lyase (EC 4.4.1.1) in liver of PCS rats was significantly activated by a methionine-supplemented diet as well as an unsupplemented SPI diet, compared with casein diets. Our present results suggest that soy protein has a stimulatory effect on methionine catabolism, and that a SPI diet can be used as a dietary measure to prevent hypermethioninemia in chronic liver diseases.

MATERIALS AND METHODS

Animals and PCS operation

Male Wistar/ST rats (6-week-old) were purchased from Japan SLC Inc. (Shizuoka). They were individually housed in a cage at a room kept at 23 ±1°C and lighted from 8 : 00 to 20 : 00. Rats were allowed free access to a 20% casein-based purified diet (Table 1) and water for several days. When their body weight reached 200 g, rats were subjected to PCS operation under ethylether anesthesia, according to the method of Funovics *et al*. (13) with slight modifications. Briefly, we tied a purse-string suture opposite to the right renal vein with a stay suture holding the vena cava up. The portal vein was tied off at the bifurcation with silk. The clamp was placed at the junction of the gastroduodenal vein and the portal vein. The portal vein was fed through the Teflon button and tied after eversion with hemostatic forceps. With the stay suture to retract the vena cava and a clamp, a small elliptical opening was made in the inferior vena cava. The portal vein was inserted into the vena cava, and the purse-string was tied. One day later, the PCS rats were divided and allowed free access to each experimental diet and water for 14 days.

All animal experiments in the present study were performed in accordance with the guideline principles of The Institutional Animal Care and Over-

Table 1. Composition of casein or SPI diets

Diets	20% Casein	40% Casein	40% Casein-M	40% Casein-MC	40% SPI	40% SPI-M
			(g/kg diet)			
Casein[1]	246.0	492.0	492.0	492.0	-	-
SPI[1]	-	-	-	-	515.0	515.0
Methionine	-	-	6.0	6.0	-	12.8
Cystine	-	-	-	10.8	-	-
α-Corn starch	426.0	262.0	258.0	251.2	247.0	238.2
Sucrose	213.0	131.0	129.0	125.0	123.0	119.0
Oil[2]	50.0	50.0	50.0	50.0	50.0	50.0
Vitamins[3]	10.0	10.0	10.0	10.0	10.0	10.0
Minerals[3]	35.0	35.0	35.0	35.0	35.0	35.0
Cellulose	20.0	20.0	20.0	20.0	20.0	20.0

[1]Casein was purchased from Oriental Yeast (Osaka, Japan) and SPI was a kind gift from Fuji Oil (Osaka, Japan). The nitrogen contents of casein and SPI were analyzed by the Kjeldahl method, and the protein contents were 0.81 and 0.78 g/g, respectively.
[2]Mixture of rape seed oil and soybean oil (1 : 1).
[3]AIN (American Institute of Nutrition)-93 (36).
SPI, soy protein isolate.

sight Committee and were approved by The Committee for the Care and Use of Animals in The University of Tokushima Faculty of Medicine.

Experimental diets

Casein and SPI diets supplemented with methionine or cystine : Since PCS operation itself did not increase plasma methionine level in rats fed a 40% casein diet (Fig. 1), we administered casein diets supplemented with methionine at various doses. A 40% casein diet supplemented with 0.6% methionine (casein-M diet), which had an equal amount of methionine to that in a 60% casein diet, markedly increased plasma methionine level in PCS rats (data not shown). Based on these findings, PCS rats fed this casein-M diet were used as a model for hypermethioninemia.

PCS rats were divided into 5 groups and, one of the following diets was freely accessible to each group : a 40% casein diet, 40% casein-M diet, 40% casein-M diet additionally supplemented with 0.36% cystine (casein-MC diet), 40% SPI diet, or 40% SPI diet supplemented with 1.28% methionine (SPI-M diet). The detailed compositions of these experimental diets were shown in Table 1. A 40% SPI-M diet was designed to contain the same amount of methionine as that in a 40% casein-M diet. In addition, a 40% casein-MC diet contained the same amount of cystine as that in a 40% SPI diet. We measured body weight and food intake of these rats every day. After feeding the experimental diets for 2 weeks, we measured methionine concentrations in plasma, liver and urine, as described below.

Amino acid mixture mimicking casein and SPI : To elucidate that the beneficial effects of SPI on hypermethioninemia in liver diseases are due to its amino acid composition, we administered amino acid mixture diets for 40% casein-M and SPI-M diets, respectively, to PCS rats. The detailed compositions of these amino acid mixture diets were shown in Table 2. These amino acid mixture diets were freely accessible for PCS rats for 2 weeks.

Measurement of methionine concentration

Blood samples were drawn from inferior vena cava and then centrifuged at 1,600 × g for 5 minutes in each experiment. The liver was immediately isolated on the last day of feeding experimental diets. Urine was collected during the last 3 days of the experimental period. Plasma, liver homogenates and urine were deproteinized with 5% sulphosalicylic acid and subjected to an automatic amino acid analyzer (Model A-3300, Irica Instruments Inc., Kyoto, Japan), using a cation exchange column with sulfonate group (2622-SC, 4.6 × 60 mm), after ninhydrine reaction.

Activities of methionine-metabolizing enzymes and other biochemical analyses

We measured activities of methionine-metabolizing enzymes, such as methionine adenosyltransferase (EC 2.5.1.6), cystathionine β-synthase (EC 4.2.1.22) and cystathionine γ-lyase, in liver of PCS rats.

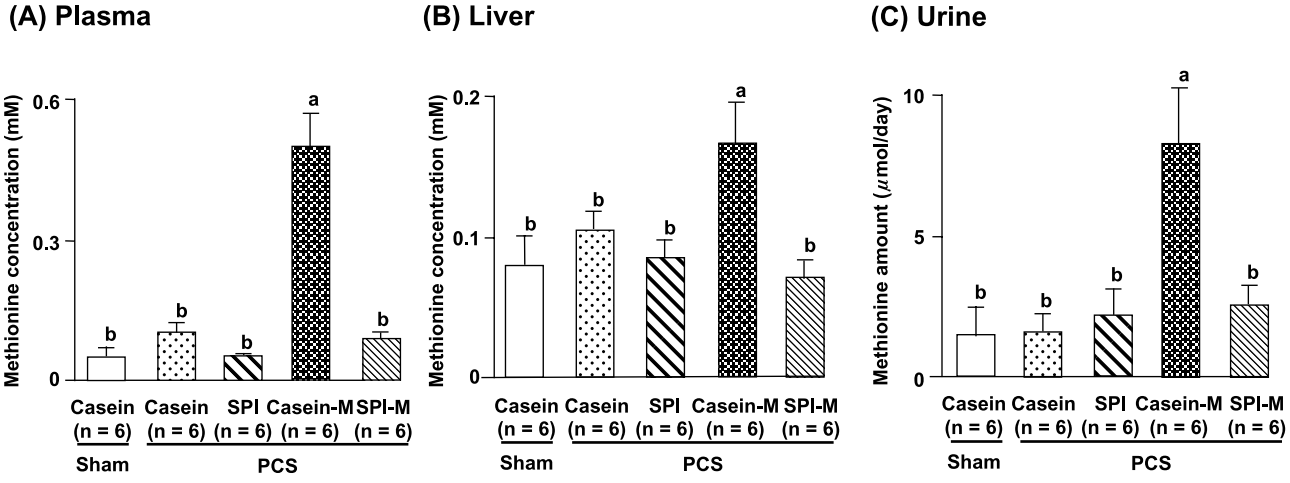

Fig. 1. Amounts of methionine in plasma (A), liver (B) and urine (C) in portacaval shunt (PCS) rats fed experimental diets
Rats were subjected to PCS operation. Normal rats with sham operation were also prepared in parallel. They were allowed free access to the indicated experimental diets (See Table 1) for 2 weeks. Samples were collected and the methionine concentrations were measured as described in Materials and Methods. Values are mean ± SEM, n=6. Means with different superscript letters were determined significantly different ($p<0.05$) by Tukey's test.

Table 2. Composition of amino acid mixture diets

Diet	Amino acid diet-40% casein-M	Amino acid diet-40% SPI-M
Amino acid	(g/kg diet)	(g/kg diet)
Ile	20.5	20.8
Leu	35.4	31.4
Lys	30.3	24.4
Met	17.3	17.3
Cys-Cys	1.8	5.1
Phe	19.4	20.8
Tyr	21.2	15.0
Thr	15.7	14.2
Trp	4.8	5.5
Val	25.6	19.3
His	11.3	10.6
Arg	13.9	30.6
Ala	11.3	16.0
Asp	26.7	47.4
Glu	80.4	76.6
Sly	6.9	16.0
Pro	43.8	21.5
Ser	19.7	19.7
α-Corn starch	320.0	315.2
Sucrose	160.0	157.6
Oil[1]	50.0	50.0
Vitamins[2]	10.0	10.0
Minerals[2]	35.0	35.0
Cellulose	20.0	20.0

[1]Mixture of rape seed oil and soybean oil (1 : 1).
[2]AIN (American Institute of Nutrition)-93 (33).

Activity of methionine adenosyltransferase in liver was measured according to the method of Akerman *et al*. (14) with slight modifications. Activities of liver cystathionine β-synthase and cystathionine γ-lyase were determined by the methods of Kashiwamata *et al*. (15) and Greembeg (16), respectively. Protein concentration of liver homogenates was determined by the Lowry method (17). Concentrations of plasma ammonia, total protein and albumin were measured as described previously (18-20). Plasma alanine aminotransferase (ALT, EC 2.6.1.2) activity was determined by the spectrophotometric method of Henry *et al*. (21).

Statistical analysis

All data were statistically evaluated by analysis of variance (ANOVA) with StatFlex software (Version 5 ; ARTECH Co., Tokyo, Japan) and were expressed as mean±SEM, n=6-8. One-way ANOVA was used to determine the significant effects of experimental diets on the measured variables. Individual differences between groups were assessed with Tukey's test. Differences were considered significant at $P < 0.05$.

RESULTS

Nutritional status and blood biochemical analysis

Among PCS rats fed 4 experimental diets, such as casein, casein-M, SPI and SPI-M diets, there were no significant differences in food intake, body weight gain, protein efficiency ratio (PER), plasma total protein and albumin, as shown in Table 3. These findings indicated that experimental diets did not change nutritional status of PCS rats.

PCS operation has been reported to induce atrophy of liver and hyperammoninemia (22). Consistent with this report, liver wet weight of PCS rats fed a 40% casein diet significantly decreased to 70% of that of sham-operated rats (normal rats) fed a 40% casein diet (Table 3). Furthermore, PCS operation significantly increased plasma ammonia concentration and ALT activity 3.5-and 2.4-folds, respectively, in rats fed a 40% casein diet (Table 3). SPI diet significantly suppressed ALT activity increased by PCS operation, but it hardly changed liver wet weight and plasma ammonia concentration in PCS rats.

Beneficial effects of soy protein diets on plasma methionine levels in PCS rats

PCS operation alone did not increase methionine concentrations in blood and liver of rats fed a 40% casein or SPI diet (Fig. 1 A and B). Therefore, we fed a 40% casein-M diet, a methionine-supplemented diet, to PCS rats, and the diet significantly increased plasma and hepatic methionine levels in PCS rats. Interestingly, any increases in methionine levels in blood and liver were not observed in PCS rats fed a 40% SPI-M diet, which contained the equal amount of methionine to that in a 40% casein-M diet (Fig. 1 A and B). These findings were consistent with the result that a SPI-M diet, but not a casein-M diet, suppressed plasma ALT activity increased by PCS operation (Table 3). Thus, it is assumed that even the methionine-supplemented SPI diet has a beneficial action on abnormal amino acid profile associated with liver dysfunction. In contrast, urinary methionine excretion was increased in PCS rats fed a casein-M diet, compared with sham-operated or PCS rats fed a casein diet (Fig. 1 C). However, urinary methionine excretion in PCS rats fed a SPI or SPI-M

diet was not increased, indicating that this increased urinary excretion in casein-M diet group was due to hypermethioninemia.

Effects of SPI diets on methionine-metabolizing enzymes in liver

To elucidate the mechanism of a beneficial action of SPI diets on plasma and hepatic methionine levels, we measured activities of methionine catabolism enzymes in rat liver (Table 4). PCS operation significantly increased the activity of hepatic methionine adnenosyltransferase in rats fed a 40% casein diet, while it did not affect the activities of cystathionine γ-lyase or cystathionine β-synthase. Methionine supplementation to a casein diet further increased methionine adenosyltransferase activity. However, neither SPI nor methionine-supplemented SPI diets changed the activity of this enzyme increased by the PCS operation and methionine supplementation. Interestingly, cystathionine γ-lyase was sensitive to only SPI diets. SPI and methionine-supplemented SPI diets significantly stimulated the activity of cystathionine γ-lyase, whereas PCS and the methionine supplementation to a casein diet did not change it. The activity of cystathionine β-synthase was hardly changed by the PCS operation, diets and methionine supplementation.

Importance of amino acid composition in SPI diets

We finally examined effects of amino acid mixture diets mimicking a 40% casein-M or SPI-M diet on plasma methionine concentration in PCS rats, to elucidate what factors in the SPI diet contributed to the normalization of plasma methionine concentration. Two-week-administration of an amino acid mixture diet mimicking a 40% SPI-M diet (amino acid diet-SPI-M) did not significantly change food intake, body weight gain or protein efficiency ratio of PCS rats, compared with that of an amino acid mixture diet mimicking a 40% casein-M diet (amino acid diet-casein-M) (data not shown). Expectedly, an amino acid diet-SPI-M significantly decreased plasma methionine concentration (Fig. 2) and ALT activity (data not shown). Except for plasma ALT activity and methionine

Table 3. Effects of experimental diets on general nutritional status and plasma biochemistry in PCS rats

Rats	Sham	PCS			
Diet	Casein	Casein	SPI	Casein-M	SPI-M
Food intake (g/day)	17.0±0.7	16.7±0.5	16.0±0.5	15.5±0.5	16.9±0.2
BW gain (g/day)	9.7±0.7[a]	5.8±0.4[b]	5.2±0.6[b]	5.0±0.4[b]	6.3±0.2[b]
PER	1.45±0.10[a]	0.87±0.04[b]	0.80±0.06[b]	0.81±0.05[b]	0.93±0.03[b]
Total protein (g/dl)	5.8±0.1	5.4±0.2	5.1±0.1	5.4±0.1	5.2±0.1
Albumin (g/dl)	3.2±0.1[a]	2.8±0.1[a]	2.7±0.1[a]	2.8±0.1[a]	2.5±0.1[b]
Liver wet weight (g)	11.8±0.5[a]	7.6±0.3[b]	6.4±0.3[b]	7.8±0.4[b]	7.5±0.3[b]
Ammonia (mg/dl)	96.7±13.4[a]	334.8±18.4[b]	323.2±46.4[b]	318.5±27.4[b]	284.7±24.1[b]
ALT (IU/l)	34.6±2.6[a]	83.1±6.9[b]	46.4±5.6[a]	82.9±13.5[b]	43.4±3.9[a]

Values are expressed as mean ± SEM, n=6.
Means with different superscript letters within each column were determined significantly different ($p<0.05$), by Tukey's test.
ALT, alanine aminotransferase ; BW, body weight ; PCS, portacaval shunt ; PER, protein efficiency ratio ; SPI, soy protein isolate.

Table 4. Effects of experimental diets on hepatic methionine-metabolizing enzyme activities in PCA rats

Rats	Sham	PCS			
Diet	Casein	Casein	SPI	Casein-M	SPI-M
Methionine adenosyltransferase	262.3 ± 11.3[a]	369.9 ± 33.3[b]	456.8 ± 41.0[c]	463.0 ± 36.9[c]	404.4 ± 28.5[c]
Cystathionine γ-lyase	95.6 ± 9.5[a]	89.1 ± 4.6[a]	121.8 ± 12.9[b]	85.4 ± 6.2[a]	106.9 ± 8.4[b]
Cystathionine β-synthase	19.3 ± 1.0	22.1 ± 1.0	24.5 ± 1.8	28.9 ± 2.6	22.1 ± 1.1

Values are expressed as mean ± SEM, n = 6.
Means with different superscript letters within each column were determined significantly different ($p<0.05$), by Tukey's test.
PCS, portacaval shunt ; SPI, soy protein isolate.

concentration, the values of blood biochemistry in PCS rats were similar between these amino acid mixture diet groups (data not shown). The increase in plasma methionine concentration (Fig. 2) and ALT activity (data not shown) in rats fed an amino acid diet-casein-M were comparable to those in rats fed a casein-M diet.

Soy protein contains a high amount of cystine, and the higher ratio of cystine to methionine in soy protein, compared to that of casein, has been reported to contribute to the beneficial effects of soy protein (23, 24). Accordingly, we examined the effect of the increased ratio of cystine to methionine in the diet on plasma methionine concentration of PCS rats (Fig. 3). No difference was observed in food intake among PCS rats fed casein and SPI diets supplemented with methionine or methionine plus cystine (data not shown). However, a 40% casein diet with the same cystine-to-methionine ratio as that of a 40% SPI-M diet failed to decrease plasma methionine concentration, while a 40% SPI-M diet significantly decreased it (Fig. 3). These results suggested that the cystine content of a SPI-M diet was not responsible for its inhibitory effect on hypermethioninemia.

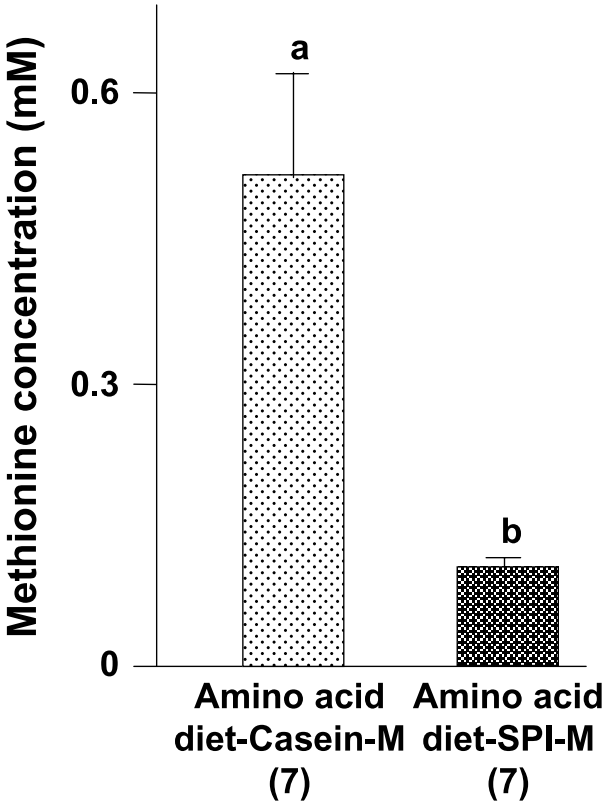

Fig. 2 Plasma methionine concentration in portacacal shunt (PCS) rats fed amino acid mixture diets

Rats were subjected to PCS operation. They were allowed free access to the indicated amino acid mixture diets (See Table 2) for 2 weeks. Blood was collected, and the methionine concentration was measured, as described in Materials and Methods. Values are mean±SEM, n=6. Means with different superscript letters were determined significantly different ($p<0.05$) by Tukey's test.

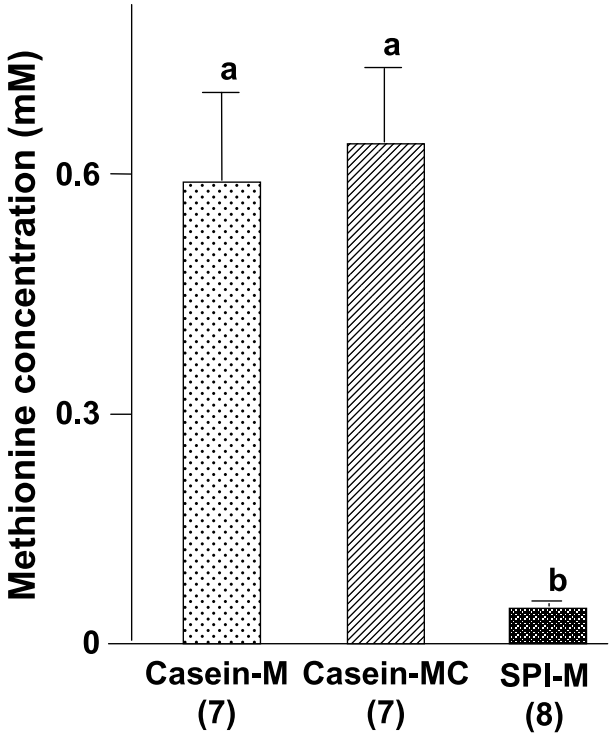

Fig. 3 Effect of the increased ratio of cystine to methionine in diets on plasma methionine concentration in portacaval shunt (PCS) rats

Rats were subjected to PCS operation. They were allowed free access to the indicated experimental diets, casein-M, casein-MC and soy protein isolated (SPI)-M (See Table 1), for 2 weeks. Blood was collected and the methionine concentration was measured as described in Materials and Methods. Values are mean±SEM, n=6. Means with different superscript letters were determined significantly different ($p<0.05$) by Tukey's test.

DISCUSSION

Surgical construction of PCS increased plasma ALT activity and ammonia concentration in rats fed a 40% casein diet. In addition, supplementation of methionine to diets induced similar hypermethioninemia to that observed in patients with liver disorders. Based on these findings, we used PCS rats fed a methionine-supplemented casein diet as an animal model for hypermethioninemia in liver diseases. In this model animal, we found that even a methionine-supplemented soy protein diet, which contained a similar amount of methionine to a 40% casein-M diet, significantly decreased plasma methionine concentration and ALT activity in PCS

rats. Our present results suggest that the soy protein diets have beneficial effects on hepatic disorders through an unknown mechanism other than its low amount of sulfur amino acids.

To investigate the mechanism of this beneficial effect of SPI, we examined the effects of amino acid mixture diets or a casein diet with the increased cystine-to-methionine ratio on plasma methionine concentration. An amino acid mixture diet mimicking a 40% SPI-M diet significantly decreased plasma methionine concentration in PCS rats, but an amino mixture diet mimicking a 40% casein-M diet did not. These findings suggest that the amino acid composition contributes to the beneficial effects of SPI diets. In contrast, the cystine-supplemented 40% casein-M diet failed to decrease plasma methionine concentration, suggesting that the higher cystine-to-methionine ratio in the SPI diet did not contribute to the normalization of plasma methionine concentration. Determining the key amino acids in soy protein for its beneficial effect is the next important subject to develop adequate protein diets for patients with hepatic disorders.

In mammals, plasma methionine is mainly catabolized via the transsulfuration pathway in liver (25). Therefore, we investigated the stimulatory effects of SPI diets on the activities of methionine adenosyltransferase, cystathionine β-synthase and cystathionine γ-lyase, in this pathway. Consistent with the previous reports (26, 27), the PCS operation and methionine supplementation to a casein diet significantly increased plasma methionine adenosyltransferase, while they hardly changed the activities of cystathionine β-synthase and cystathionine γ-lyase. However, SPI diets failed to further stimulate the activity of methionine adenosyltransferase, suggesting that the target enzyme of soy protein diets is not this enzyme. We found, to our knowledge, for the first time, that diets containing soy protein had a positive effect on cystathionine γ-lyase, suggesting that the increased activity of this enzyme may stimulate the degradation of methionine in PCS rats. At the 5'-promoter region of mouse cystathionine γ-lyase, the following transcriptional factor-binding consensus elements have been reported to be identified : signal transducers and activators of transcription x(STATx), myeloid zinc finger protein 1 (MZF1), acute myeloid leukaemia-1a (AML-1a), upstream stimulatory factor-1(USF-1), N-Myc, specificity protein 1 (Sp1), heat shock factor 2 (HSF2) and GATA-binding factor 1 (GATA-1)(28). The activation of these transcriptional factors by amino acid composition in diets may be responsible for the mechanism underlying SPI-mediated increase in cystathionine γ-lyase activity. Further examinations are necessary to identify the most important transcriptional factor regulated by SPI.

A SPI diet most effectively increased the activity of hepatic cystathionine γ-lyase among the tested diets (Table 4). This finding led us to consider that depletion of methionine in a SPI diet relative to a casein diet may stimulate expression of cystathionine γ-lyase, because methione depletion upregulates expression of several genes, such as the mRNA (N 6-adenosine)methyltransferase (29), possibly through amino acid response element (ARE), a *cis*-transcriptional element essential for the promoter regulation by amino acid depletion (30). However, there is no report indicating the existence of amino acid response element (ARE) at the 5'-promoter region of cystathionine γ-lyase. Methionine depletion may not be involved in SPI-mediated increase in cystathionine γ-lyase activity.

Cystathionine γ-lyase has been reported to contribute to selenomethionine detoxification and cytosolic glutathione peroxide biosynthesis in mouse liver (31). In addition, hydrogen sulfide (H_2S), an endogenous gaseous mediator that causes vasodilation (32, 33), is generated in various tissues by cystathionine γ-lyase (34). Recently, it has been reported that cystathionine γ-lyase-derived H_2S is involved in the maintenance of portal venous pressure (35). Therefore, an increase in cystathionine γ-lyase activity by a SPI diet in diseased liver contributes to the reduction of hepatocyte abnormalities resulted from PCS operation through the increased production of H_2S. This hypothesis is supported by the evidence that diets containing soy protein significantly suppressed plasma ALT activity, a marker for liver injury. However, further examinations are necessary to elucidate the cystathionine γ-lyase-mediated effects of soy protein.

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