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Role of *N*-Acetylglutamate Concentration and Ornithine Transport into Mitochondria in Urea Synthesis of Rats Given Proteins of Different Quality

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The purpose of this study was to find whether the concentration of *N*-acetylglutamate and ornithine transport into mitochondria would regulate urea synthesis when the dietary protein quality was manipulated. Experiments were done on three groups of rats given diets containing 10 g of gluten, 10 g of casein, or 10 g of whole egg protein/100 g for 10 days. The plasma concentration and urinary excretion of urea, the liver concentration and synthesis of *N*-acetylglutamate, the liver concentrations of glutamate and lysine, and the liver ornithine transport into mitochondria increased with the decrease in quality of dietary protein. A reverse correlation was observed between the activities of urea cycle enzymes, the plasma concentration of arginine, and urinary excretion of urea under these conditions. *N*-Acetylglutamate concentration and ornithine transport into mitochondria in the liver were closely correlated with the excretion of urea. These results suggest that greater *N*-acetylglutamate concentration and ornithine transport into isolated mitochondria in the liver of rats, given the lower quality of protein, stimulate urea synthesis and that the concentrations of glutamate and lysine in the liver are at least partly related to the hepatic *N*-acetylglutamate synthesis and ornithine transport, respectively.

KEYWORDS: Dietary protein quality; urea synthesis; *N*-acetylglutamate; ornithine transport; rats

INTRODUCTION

Shimke (*1*, *2*) has suggested that the concentrations of urea-cycle intermediates were unchanged under conditions affecting the rate of urea excretion (e.g., ingestion of a high-protein diet) and concluded that the activities of various urea-cycle enzymes were regulatory factors of urea synthesis. However, many investigators have previously reported that there was an increase in urinary urea excretion without a comparable increase in the enzyme activities when the diet containing high quality protein was replaced by the isonitrogenous diet with low-quality protein (*3*−*5*).

Urea formation has been shown to be stimulated by adding *N*-acetylglutamate and ornithine in vivo (*6*), in perfused liver (*7*−*9*), and in isolated hepatocytes (*10*) when substrates for urea production were present in excess. *N*-Acetylglutamate, an essential activator of carbamyl phosphate synthetase (EC 6.3.4.16), plays a critical role in the regulation of urea synthesis in mammalian liver (*11*, *12*). Shigesada and Tatibana (*13*) have demonstrated that *N*-acetylglutamate synthetase (EC 2.3.1.1) catalyzed the acetylation of glutamate in liver. This enzyme is activated specifically by arginine. Thus, the supply of *N*-acetylglutamate and ornithine may limit the rate of urea synthesis.

The purpose of this present study was to discover the mechanism by which the dietary protein quality affects urea synthesis. In our previous report (*5*), correlations between the liver concentration of ornithine and urea excretion were not found when the dietary protein quality was manipulated. However, as ornithine transcarbamylase (EC 2.1.3.3) is distributed in hepatic mitochondria, ornithine synthesized by arginase (EC 3.5.3.1) must be transported into the mitochondria. Cohen et al. (*14*) and Zollner (*15*) suggested that, under physiological conditions, the transport of ornithine into hepatic mitochondria limits the rate of urea production and that lysine inhibited the ornithine transport system. Three questions were considered in the present study: (1) whether the dietary protein quality might control the synthesis and concentration of *N*-acetylglutamate in the liver, (2) whether an elevated transport of ornithine into mitochondria in rats fed the low-quality protein might result in increased urea synthesis than that in those animals given the high-quality protein, and (3) whether arginine or glutamate might

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Table 1. Composition (g/100 g of diet) of Experimental Diets

ingredient	10% gluten	10% casein	10% whole egg protein
whole egg protein[a]			10.0
casein		10.0	
gluten	10.0		
cornstarch[b]	25.1	25.1	25.1
sucrose[b]	50.2	50.2	50.2
corn oil	5.0	5.0	5.0
AIN-93G mineral mix[c]	3.5	3.5	3.5
AIN-93VX vitamin mix[c]	1.0	1.0	1.0
cellulose[b]	5.0	5.0	5.0
choline chloride	0.2	0.2	0.2

[a] Supplied by Taiyo Kagaku, Yokkaichi, Japan. [b] Supplied by Oriental Yeast, Tokyo, Japan. [c] Supplied by Nihon Nosan K. K., Yokohama, Japan (*34*).

control the *N*-acetylglutamate synthesis and lysine might control the ornithine transport when the dietary protein quality was manipulated. We therefore examined the hepatic synthesis and concentrations of *N*-acetylglutamate, the hepatic concentrations of glutamate and lysine, the activity of carbamyl phosphate synthetase and ornithine transport into mitochondria in the liver, and the plasma concentration of arginine in rats given proteins of different quality. Argininosuccinate synthetase (EC 6.3.4.5) has been known to be a rate-limiting enzyme in the urea cycle. In the present study, we also determined the hepatic activity of argininosuccinate synthetase.

MATERIALS AND METHODS

Chemicals. *N*-Acetylglutamic acid was purchased from Sigma Chemical (St. Louis, MO). $NaH^{14}CO_3$ (1.85GBq/mmol), U-^{14}C-sucrose (17 GBq/mmol), and U-^{14}C-glutamic acid (9.25 GBq/mmol) were obtained from Amersham (Tokyo, Japan). [2,3-^{3}H]Ornithine (1.11 TBq/mmol) was purchased from Moravek Biochemicals (Tokyo, Japan). All other reagents were purchased from Wako Pure Chemical Industries (Osaka, Japan).

Animals and Diets. Young male Wistar rats (110−120 g, Japan SLC, Hamamatsu, Japan) were maintained at 24° with a 12 h light/dark cycle. The rats were transferred to the experimental diets containing 20% gluten, 20% casein, or 20% whole egg protein (**Table 1**) after being fed with a commercial nonpurified diet (MF, Oriental Yeast, Tokyo, Japan) for 4 days. All rats were individually housed and provided free access to food and water. The approval of the Aichi University of Education Animal Care and Use Committee was given for our animal experiments.

Experimental Procedures. Three experiments were done, with 18 rats being divided randomly into three groups. In each experiment, animals were fed the experiment diet for 10 days. On days 8−9, urine was collected for 24 h, filtered, and used for urea analysis. After the experimental period, the rats were decapitated, and the plasma was collected in glass tubes and stored at −20 °C. The livers were quickly removed, weighed, and used.

In Experiment 1, the effects of dietary protein quality on the urinary excretion of urea, the hepatic activities of carbamyl phosphate synthetase and argininosuccinate synthetase, the concentrations of *N*-acetylglutamate and glutamate in the liver, and the concentration of arginine in the plasma were investigated. In experiment 2, the effects of dietary protein quality on the *N*-acetylglutamate synthesis in vivo in the liver were determined. In experiment 3, the effects of dietary protein quality on ornithine transport into isolated mitochondria and the concentration of lysine in the liver were investigated. The plasma and urinary concentrations of urea were measured by the method of Archibald (*16*). The activities of carbamyl phosphate synthetase and argininosuccinate synthetase in the liver were determined according to the method of Schimke (*1*). The *N*-acetylglutamate concentration was measured by the method of McGivan et al. (*17*) described in our previous report (*18*). For measuring the concentrations of free arginine, lysine, and glutamate, liver and plasma were treated with ice-cold 120 mmol/L sulfosalicylic acid to precipitate the protein (*19*). Arginine, glutamate, and lysine were measured using an amino acid analyzer (L-8500; Hitachi, Tokyo, Japan).

Table 2. Time-Dependent Changes of Specific Radioactivities (Bq/μmol) of Free Glutamate in the Liver of Rats Fed Diets with Different Qualities of Protein[a]

time after injection (min)	10% gluten	10% casein	10% whole egg protein	pooled SEM
5	2600	2530	2760	130
15	2370	2290	2530	150

[a] Values are means and pooled SEMs, $n = 6$. Values within each time period were not significantly different ($p > 0.05$).

***N*-Acetylglutamate Synthesis in the Liver.** Radioactive ^{14}C-glutamate was combined with unlabeled glutamate to yield a dose of 1.11 MBq and a concentration of 5 μmol/mL of saline. The rats were each given an intraperitoneal injection at a dose of 1 mL/100 g of body weight. After 15 min, the rats were decapitated. The measurement of *N*-acetylglutamate synthesis involved isolating *N*-acetylglutamate and then radioactivity counting (*20*).

In a preliminary experiment, we determined the hepatic specific radioactivity of glutamate 5 and 15 min after the injection of radioactive glutamate under these experimental conditions by the method of Morita et al. (*21*). The decrease in labeling of free glutamate at 5 and 15 min in the liver was not significant (**Table 2**). Therefore, the synthesis of *N*-acetylglutamate in the liver was calculated for animals killed at a single time point of 15 min after the injection of radioisotope.

Ornithine Transport into Isolated Mitochondria in the Liver. Ornithine transport into isolated mitochondria was measured according to the method of McGivan et al. (*22*). Liver mitochondria were prepared according to the method of Novicoff and Hews (*23*). After hepatic mitochondria (3 mg of protein/mL) were preincubated with 2 mM sodium phosphate, 3 mM succinate, 10 mM sucrose, 1 μg/mL oligomycin, 0.5 mM aminooxyacetate, 225 mM choline chloride, and 10 mM Tris-morpholinopropanesulfonate (pH 7.0) at 20 °C for 2 min, 5 mM ^{3}H-ornithine (18.5 kBq/mL) was added together with ^{14}C-sucrose (9.25 kBq/mL), which was used as a marker of the extramitochondrial space. After a further 2 min, the pellets and supernatants were separated by centrifugation, acidified, and assayed for radioactivity (LS 5000TD; Beckman Japan, Tokyo, Japan).

Statistical Analyses. The means and pooled SEMs are reported. Duncan's multiple-range test was used to compare means after one-way ANOVA (*24*, *25*). Differences were considered significant at $p < 0.05$.

RESULTS

Experiment 1. The rats fed the 10% gluten diet gained less body weight and had less food intake than the other two groups, which did not differ. Compared with the rats fed the 10% whole egg protein or 10% casein diets, rats fed the 10% gluten diet had relative liver weights which were significantly lower. Urinary excretion, the plasma concentration of urea, and hepatic concentrations of *N*-acetylglutamate increased significantly with the 10% casein diet and still more with the 10% gluten diet as compared with the 10% whole egg protein diet (**Table 3**). The liver concentration of glutamate in rats given the 10% gluten diet was significantly greater than that in rats given the 10% casein or 10% whole egg protein diets. The activities of carbamyl phosphate synthetase and argininosuccinate synthetase, urea cycle enzymes, were proportional to the dietary protein quality. Compared with the case of the rats fed the 10% whole egg protein, the plasma concentration of arginine was significantly lower in rats fed the 10% gluten or 10% casein diets. The liver concentration of *N*-acetylglutamate was correlated to the excretion of urea ($r = 0.885$, $p < 0.001$). The correlation

Table 3. Effects of the Quality of Dietary Protein on the Activity of Hepatic Urea Cycle Enzymes, the Liver Concentrations of *N*-Acetylglutamate and Glutamate, and Plasma and Urine Urea Concentrations in Rats[a]

	10% gluten	10% casein	10% whole egg protein	pooled SEM
initial body weight (g)	131.8	132.0	131.7	1.1
body weight gain (g/10 days)	15.5[b]	33.1[a]	36.2[a]	2.0
food intake (g/day)	18.4	18.5	19.3	0.7
liver weight (g/100 g of body weight)	3.96[b]	4.38[a]	4.41[a]	0.12
urinary urea (mmol/day)	7.61[a]	5.25[b]	3.69[c]	0.50
plasma urea (mmol/L)	4.37[a]	3.56[b]	2.67[c]	0.15
liver argininosuccinate synthetase[b] (U/g of liver)	0.131[c]	0.155[b]	0.188[a]	0.07
liver carbamyl phosphate synthetase[c] (U/g of liver)	0.363[c]	0.410[b]	0.471[a]	0.017
liver *N*-acetylglutamate (nmol/g of liver)	19.8[a]	16.6[b]	14.1[c]	0.4
liver glutamate (μmol/g of liver)	4.38[a]	3.65[b]	3.22[b]	0.16
plasma arginine (mmol/L)	0.21[b]	0.19[c]	0.28[a]	0.007

[a] Values are means and pooled SEMs, $n = 6$. Means with different superscript letters are significantly different ($p < 0.05$). [b] Unit of enzyme activity: mmol of urea produced per hour. [c] Unit of enzyme activity: mmol of citrulline produced per hour.

Table 4. Effects of the Quality of Dietary Protein on the Hepatic Synthesis of *N*-Acetylglutamate and the Plasma Concentration of Urea in Rats[a]

	10% gluten	10% casein	10% whole egg protein	pooled SEM
body weight gain (g/10 day)	13.0[b]	43.1[a]	45.7[a]	2.4
food intake (g/day)	18.1	19.2	20.7	1.2
liver weight (g/100 g of body weight)	3.23[b]	3.97[a]	4.12[a]	0.09
plasma urea (mmol/L)	4.37[a]	3.56[b]	2.67[c]	0.15
liver *N*-acetylglutamate synthesis (Bq/g of liver)	26.3[a]	21.2[b]	15.8[c]	1.0

[a] Values are means and pooled SEMs, $n = 6$. Means with different superscript letters are significantly different ($p < 0.05$). Initial body weight of rats is from 140 to 156 g.

Table 5. Effects of the Quality of Dietary Protein on the Ornithine Transport into Isolated Mitochondria, the Concentration of Lysine in the Liver, and the Plasma Urea Concentration[a]

	10% gluten	10% casein	10% whole egg protein	pooled SEM
initial body weight (g)	131.8	135.3	135.1	1.1
body weight gain (g/10 day)	15.2[b]	41.6[a]	47.2[a]	2.2
food intake (g/day)	18.4	18.5	19.2	0.8
liver weight (g/100 g of body weight)	3.93[b]	4.25[a]	4.50[a]	0.10
plasma urea (mmol/L)	4.27[a]	3.36[b]	2.76[c]	0.16
ornithine transport (nmol/mg of protein)	2.46[a]	2.00[b]	1.22[c]	0.10
liver lysine (μmol/g of liver)	0.34[c]	1.40[b]	1.64[a]	0.03

[a] Values are means and pooled SEMs, $n = 6$. Means with different superscript letters are significantly different ($p < 0.05$).

between the concentrations of *N*-acetylglutamate and glutamate was significant ($r = 0.801$, $p < 0.001$).

Experiment 2. As in experiment 1, the group fed the 10% gluten diet grew less than the group fed the 10% casein diet or the 10% whole egg protein diet. The plasma concentration of urea and hepatic synthesis of *N*-acetylglutamate increased significantly with the 10% casein diet and still more with the 10% gluten diet as compared with the 10% whole egg protein diet (**Table 4**).

Experiment 3. The concentration of plasma urea elevated gradually with the decreasing quality of the dietary protein. The dietary protein quality affected ornithine transport into isolated hepatic mitochondria (**Table 5**). Compared with the case of the rats fed the 10% whole egg protein diet, the ornithine transport into mitochondria and the concentration of lysine in the liver were significantly higher in rats fed the 10% gluten diet or the 10% casein diet (**Table 5**). The correlation between the plasma concentration of urea and the ornithine transport into hepatic isolated mitochondria was significant ($r = 0.870$, $p < 0.001$).

DISCUSSION

Das and Waterlow (*3*) and we (*5*) reported that an increase in excretion of urea occurred without a concomitant change in the activities of urea cycle enzymes when a diet containing high-quality protein was replaced by an isonitrogenous diet with low-quality protein. The purpose of the present experiments was to elucidate the mechanism by which the dietary protein quality alters urea synthesis. In the present study, the activities of carbamyl phosphate synthetase and argininosuccinate synthetase, urea cycle enzymes, were proportional to the dietary protein quality, while urea excretion was greater in the group given the diet with low-quality protein (**Table 3**). Therefore, the results suggest that regulation of urea synthesis by dietary protein quality may not be attributable to changes in activities of urea cycle enzymes, thus corrobolating the findings of Das and Waterlow (*3*).

N-Acetylglutamate is a physiological activator of carbamyl phosphate synthetase (*11*, *26*). Variation in the *N*-acetylglutamate concentration has correlated well with urea synthesis in intact animals (*13*, *20*, *27*, *28*) and in isolated hepatocytes (*10*, *29*). Many investigators have indicated that a change in *N*-acetylglutamate synthesis was involved in regulating the liver concentration of *N*-acetylglutamate (*20*, *26*). Therefore, we assumed that *N*-acetylglutamate concentration and synthesis might be more important than carbamyl phosphate synthetase activity in regulating urea synthesis when the dietary protein quality was changed. Urinary excretion of urea and liver

concentration and synthesis of *N*-acetylglutamate increased with the decrease in dietary protein quality (**Tables 3** and **4**). The higher synthesis of liver *N*-acetylglutamate in rats fed the 10% gluten or 10% casein diets enhanced the *N*-acetylglutamate concentration and stimulated urea synthesis in these groups. Therefore, the changes in *N*-acetylglutamate synthesis may have controlled *N*-acetylglutamate concentration and been one of the factors affecting urea synthesis in this study.

N-Acetylglutamate synthetase (EC 2.3.1.1) has been shown to catalyze the acetylCoA-dependent acetylation of glutamate in liver mitochondria (*13*). This enzyme is activated by arginine (*20*, *30*). Shigesada et al. (*20*) have demonstrated that an arginine treatment stimulated the hepatic synthesis of *N*-acetylglutamate in vivo and in isolated hepatocytes. Therefore, we examined the effect of dietary protein quality on the concentrations of hepatic glutamate and plasma arginine. The changes in free arginine concentration are not considered to be the factor leading to the greater synthesis of liver *N*-acetylglutamate in rats fed the low-quality protein (**Table 3**). The concentration of free arginine may not have regulated urea synthesis in the present study. On the other hand, the greater concentration of hepatic glutamate in the groups given the 10% gluten or 10% casein diets increased the *N*-acetylglutamate synthesis in these groups (**Table 3**). The glutamate is the substrate of *N*-acetylglutamate synthesis. The changes in concentration may have controlled the *N*-acetylglutamate concentration in the present investigation. Measurement of *N*-acetylglutamate synthetase activity and acetylCoA concentration in the liver should be included in further studies of the effect of dietary protein quality on urea synthesis.

Ornithine was an intermediate of the urea cycle. Katunuma et al. (*31*) reported that an elevated hepatic ornithine concentration could be involved in activating the urea cycle under the physiological conditions. However, as ornithine transcarbamylase is distributed in hepatic mitochondria, ornithine synthesized by arginase must be transported into the mitochondria. The K_m value (1.8 mM) of ornithine transcarbamylase for ornithine was reported to be markedly greater than the mitochondrial concentration of ornithine (*32*). Zollner (*15*) suggested that citrulline formation was dependent on the rate of ornithine transport into hepatic mitochondria in hepatocytes when ammonia was supplied and that the transport of ornithine limited the rate of urea production. An indication for limitation of urea synthesis by ornithine transport was reported by our previous study (*33*) when thyroid status was manipulated. The thyroid hormone treatment to the hypothyroid rats decreased the excretion of urea and the ornithine transport into mitochondria in the liver. Therefore, we assumed that higher urea production in the group given the low-quality protein might be due to the elevation of ornithine transport into hepatic mitochondria. In the present experiment, not only a higher concentration of *N*-acetylglutamate but also ornithine transport into mitochondria in the liver may have induced a greater synthesis of urea in the groups fed diets containing the low-quality protein (**Table 5**). In the previous report (*5*), we demonstrated that the dietary protein quality did not affect the hepatic concentration of ornithine. The changes in ornithine transport into mitochondria in the liver, not ornithine concentration, may have regulated urea synthesis in this study.

Many investigators indicated that ornithine was transported into mitochondria on a specific carrier (*15*, *22*). Zollner (*15*) and McGivan et al. (*22*) suggested that lysine abolished the binding of ornithine to the outer surface of the mitochondrial membrane and decreased the rate of ornithine transport in the hepatocytes and isolated hepatocytes. Gluten is known to be a lower quality protein than casein or whole egg protein because of deficiency in lysine. Therefore, we assumed that lysine concentration regulated ornithine transport into mitochondria. The hepatic concentration of free lysine decrreased significantly with the casein diet and markedly more with the gluten diet as compared with the whole egg protein diet (**Table 5**). The lower concentration of liver lysine may induce greater ornithine transport into mitochondria in rats fed the low-quality protein. The regulation of urea synthesis may be partly mediated through the inhibition of ornithine transport by lysine when the dietary protein quality is manipulated. On the other hand, as lysine was not added in vitro in this study, the dietary protein quality may affect the regulation of ornithine transport directly. However, little documentation of the effect of dietary protein quality on the carrier system for ornithine transport mediated by the lysine or direct ornithine transport is available. This is another possibility to consider in further examination of the mechanism by which the dietary protein quality alters urea synthesis.

These results suggest that the greater *N*-acetylglutamate concentration and ornithine transport into isolated mitochondria in the liver of rats given the lower quality of protein stimulate urea synthesis and that the concentrations of glutamate and lysine in the liver are at least partly related to the hepatic *N*-acetylglutamate synthesis and ornithine transport, respectively.

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