Effect of Quality and Quantity of Dietary Protein on 4E-BP1 and S6K1 Phosphorylation of Brains in Aged Rats

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Summary We have shown that the rate of brain protein synthesis in aged rats depended on the quality and quantity of dietary protein consumed. The purpose of this study was to determine whether the quality and quantity of dietary protein affected the phosphorylation of eukaryotic initiation factor (eIF) 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 kinase (S6K1) and regulated the brain protein synthesis. Two experiments were done on three groups of 24-wk-old male rats given diets containing 20% casein, 20% gluten, or 20% gelatin (Experiment 1), and 20% casein, 5% casein or 0% casein (Experiment 2) for 10 d. The phosphorylation of S6K1 in both the cerebral cortex and cerebellum, and the phosphorylation of 4E-BP1 in the cerebral cortex declined with a decrease of quality and quantity of dietary protein. The phosphorylation of 4E-BP1 in the cerebellum did not differ among groups. The results suggest that the ingestion of a higher quality and quantity of dietary protein stimulates the phosphorylation of 4E-BP1 and S6K1 in the brain and increases the brain protein synthesis in the aged rats.

Key Words dietary protein, 4E-BP1, S6K1, protein synthesis, brain

The metabolic response to dietary proteins, age and hormonal factors includes marked changes in protein synthesis, especially in the liver, muscles and intestine (1–5). Protein synthesis in the brain is also sensitive to the alteration of dietary amino acid composition in young rats (6, 7).

Many investigators have reported that protein synthesis declined in specific tissues (e.g., liver or muscle) and in the whole body throughout development in mammals after weaning (8–10). We demonstrated that the rate of protein synthesis in the brain decreased with age in rats after weaning (11). In many investigations, the protein synthesis and the concentrations of branched-chain amino acids in the brain have been shown to depend on the quality and quantity of dietary protein in aged rats (12–14).

In both liver (15) and skeletal muscle (16), the stimulation of protein synthesis caused by amino acids and protein was reported to be mediated by an increase in the initiation of mRNA translation. Administration of leucine in vivo enhanced muscle protein synthesis through the activation of the binding of mRNA to the 40S ribosomal subunit (17). The mRNA binding step is regulated by initiation factors collectively referred to as eIF4F (18). The eIF4F initiation factors include eIF4E,

eIF4A, eIF4G and eIF3. The most studied example of the regulation of protein synthesis occurring at the mRNA binding step is the reversible sequestration of eIF4E into an inactive complex with the eIF4E binding protein, 4E-BP1. The binding site on eIF4E for 4E-BP1 overlaps with the eIF4G binding site. Thus, the binding of eIF4E to 4E-BP1 precludes the binding of the eIF4E-mRNA complex to the 40S ribosomal subunit (19). The interaction between eIF4E and 4E-BP1 is regulated by phosphorylation of 4E-BP1; specifically hyperphosphorylation prevents the binding while hypophosphorylation is permissive toward the binding. The phosphorylation of ribosomal protein S6 (rpS6) is another mechanism for the regulation of mRNA binding to 40S ribosomal subunits. The rpS6 is located near the mRNA/tRNA binding site on the 40S ribosomal subunit (20) and, therefore, has a potential role in selecting mRNA for the translation. The phosphorylation of rpS6 is mediated by a protein kinase termed S6K1 (20). The activation of S6K1 is also associated with the phosphorylation (17, 21). Anthony et al. (16, 17) and Yoshizawa et al. (22) demonstrated that the oral administration of leucine stimulated the rates of protein synthesis in liver and skeletal muscle concomitant with increased phosphorylation of 4E-BP1 and S6K1. Overall, the available evidence suggests that amino acids such as leucine stimulate the translation initiation by enhancing eIF4F assembly as well as by activating S6K1. However, the role of the initiation phase of mRNA translation in maintaining the rates of brain protein synthesis remains unknown under physiological conditions in

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Abbreviations: 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; GH, growth hormone; S6K1, ribosomal protein S6 kinase.

Table 1. Composition (g/100 g diet) of experimental diets.

Ingredient	20% Gelatin	20% Gluten	20% Casein	5% Casein	0% Casein
Casein ¹			20.0	5.0	
Gluten ¹		20.0			
Gelatin ¹	20.0				
Cornstarch ²	21.8	21.8	21.8	26.8	28.4
Sucrose ²	43.5	43.5	43.5	53.5	56.9
Corn oil ¹	5.0	5.0	5.0	5.0	5.0
AIN-93M mineral mix ³	3.5	3.5	3.5	3.5	3.5
AIN-93VX vitamin mix ³	1.0	1.0	1.0	1.0	1.0
Cellulose ¹	5.0	5.0	5.0	5.0	5.0
Choline chloride ¹	0.2	0.2	0.2	0.2	0.2

¹ Supplied by Wako Pure Chemical Industries, Ltd., Osaka, Japan.

² Supplied by Oriental Yeast Co., Ltd., Tokyo, Japan.

³ Supplied by Nihon Nosan K.K., Yokohama, Japan (41).

aged rats. The possible effects of 4E-BP1 and S6K1 phosphorylation on the brain protein synthesis in aged rats are of nutritional importance in understanding the role of protein nutrition in the brain function in mammals.

The purpose of our present study was to determine the mechanism by which the quality and quantity of dietary protein affect the brain protein synthesis in aged rats. Two questions were considered in the present study: 1) whether the quality and quantity of dietary protein control the phosphorylation of 4E-BP1 in the brains and regulate the brain protein synthesis in aged rats, and 2) whether the phosphorylation of S6K1 might regulate the protein synthesis in the brains when the quality and quantity of dietary protein was manipulated. Therefore, we examined the phosphorylation of 4E-BP1 and S6K1 in the cerebral cortex and cerebellum of aged rats. Gelatin and gluten are known to be lower quality protein than casein because of a deficiency in several essential amino acids (e.g. tryptophan) in gelatin, and a deficiency in lysine in gluten, and also because of lower nitrogen retention (23). In particular, as the gelatin has less of many essential amino acids, the body weight decreased in young and aged rats fed a 20% gelatin diet (7, 13). Thus, in this experiment, 20% gelatin, 20% gluten and 20% casein diets were chosen to investigate the mechanism by which the quality of dietary protein altered the brain protein synthesis.

MATERIALS AND METHODS

Chemicals. The polyclonal antibodies of S6K1 and 4E-BP1 were purchased from Santa Cruz Biotechnology (CA, USA). All other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Animals and diet. Male 24-wk-old Wistar rats (Japan SLC, Inc., Hamamatsu, Japan) were housed at 24°C in a room with a 12-h light/dark cycle. The rats were transferred to the experimental diets after being fed the 20% casein diet for 10 d. The experimental diets contained 20% gelatin, 20% gluten or 20% casein (Experiment 1, Table 1), or 0, 5 or 20% casein (Experiment 2, Table 1). All animals were individually housed and given free

Table 2. Time-dependent changes of phosphorylation states on 4E-BP1 and S6K1 in the cerebral cortex of rats treated with meal-feeding.¹

Time after ingestion (h)	4E-BP1 (%) ²	S6K1 (%) ³
0	24.1 ± 3.5 ^b	34.3 ± 2.2 ^{cd}
1	15.7 ± 4.0 ^b	26.2 ± 3.6 ^d
2	22.9 ± 2.3 ^b	40.3 ± 4.3 ^c
3	49.9 ± 2.7 ^a	66.0 ± 2.3 ^a
4	30.6 ± 3.6 ^b	60.6 ± 4.0 ^a
5	31.2 ± 3.1 ^b	49.9 ± 1.5 ^b

¹ Values are mean ± SE, *n* = 6. Means with different superscript letters are significantly different (*p* < 0.05).

² Data indicate the amount of 4E-BP1 in γ -phosphorylated form, expressed as the percentage of the total 4E-BP1.

³ Data indicate the amount of S6K1 in γ -phosphorylated form, expressed as the percentage of the total S6K1.

access to food and water. The approval of Aichi University of Education Animal Care and Use Committee was given for our animal experiments.

Experimental design. Two experiments were done, with 18 rats being divided randomly into three groups. Yoshizawa et al. (22) reported that 4E-BP1 and S6K1 phosphorylation in the liver and skeletal muscle were affected very rapidly after feeding the test diet. In a preliminary experiment, we also demonstrated that the phosphorylation of 4E-BP1 and S6K1 in the brains increased significantly after only a 3-h feeding period of the 20% casein diet (Table 2). Therefore, in the present study, the change in 4E-BP1 and S6K1 phosphorylation was measured after only one 3-h feeding period of the test diet. After all rats were fed the 20% casein diet for 10 d (only one 3-h feeding period per day, from 9:00–12:00), they were given the experimental diets for 1 d (only one 3-h period). After a 3-h feeding period, the rats were decapitated and brain regions (24) were quickly removed. In Experiment 1, the effects of the quality of dietary protein on 4E-BP1 and S6K1 phosphorylation in the cerebral cortex and cerebellum were

Table 3. Effect of the quality of dietary protein on the phosphorylation states of 4E-BP1 and S6K1 in the brain regions of aged rats.¹

	20% Gelatin	20% Gluten	20% Casein
Food intake (g/d)	13.0±0.7	14.5±0.6	13.6±0.5
Tissue weight (g/100 g of body weight)			
Cerebral cortex	0.100±0.004	0.110±0.005	0.104±0.004
Cerebellum	0.089±0.002	0.087±0.003	0.085±0.002
S6K1 phosphorylation (%) ²			
Cerebral cortex	10.9±2.6 ^b	17.8±3.0 ^b	50.0±6.1 ^a
Cerebellum	14.0±3.0 ^b	15.4±2.3 ^b	54.7±3.8 ^a
4E-BP1 phosphorylation (%) ³			
Cerebral cortex	36.5±2.6 ^b	39.3±3.0 ^b	55.6±2.3 ^a
Cerebellum	38.5±8.2	23.8±6.5	21.8±3.8

¹ Values are mean±SE, *n*=6. Means with different superscript letters are significantly different (*p*<0.05). Final body weights were from 317 to 332 g.

² Data indicate the amount of S6K1 in γ -phosphorylated form, expressed as the percentage of the total S6K1.

³ Data indicate the amount of 4E-BP1 in γ -phosphorylated form, expressed as the percentage of the total 4E-BP1.

investigated. In Experiment 2, the effects of the quantity of dietary protein on 4E-BP1 and S6K1 phosphorylation in the cerebral cortex and cerebellum were determined.

Sample collection. Tissues were homogenized in 7 volumes of buffer [20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonia acid (HEPES) at pH 7.4, 100 mM KCl, 0.2 mM EDTA, 2 mM ethylene glycol-bis-(β -aminoethyl ether)-*N*, *N*,*N'*,*N'*-tetraacetic acid (EGTA), 1 mM dithiothreitol, 50 mM NaF, 50 mM β -glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine and 0.5 mM sodium vanadate] by the use of a Dounce homogenizer. The homogenate was centrifuged at 10,000 $\times g$ for min at 4°C (15).

Examination of 4E-BP1 phosphorylation state. An aliquot of the 10,000 $\times g$ supernatant was boiled for 10 min and then centrifuged at 10,000 $\times g$ for 30 min at 4°C. The resulting supernatant was mixed with an equal volume of 2 $\times$ SDS sample buffer, and the diluted sample was subjected to electrophoresis on 15% polyacrylamide gel. The samples were then subjected to a protein immunoblot analysis using 4E-BP1 polyclonal antibodies, as described previously (25).

Phosphorylation of S6K1. An aliquot of the 10,000 $\times g$ supernatant was combined with an equal volume of 2 $\times$ SDS sample buffer, and the diluted sample was subjected to electrophoresis on 7.5% polyacrylamide gel. The samples were then subjected to a protein immunoblot analysis using S6K1 polyclonal antibodies, as described previously (26).

Statistical analysis. The means and SE are reported. Duncan's multiple range test was used to compare means after one-way ANOVA (27, 28). Differences were considered significant at *p*<0.05.

RESULTS

Effect of the quality of dietary protein on the phosphorylation of 4E-BP1 and S6K1 in the brains (Experiment 1)

The food intake and relative weights of brain regions did not differ among the three groups (Table 3). 4E-BP1

Fig. 1. Phosphorylation state of S6K1 in the cerebral cortex (A) and cerebellum (B) of aged rats fed the 20% gelatin (GEL), 20% gluten (GLU) or 20% casein (CAS) diet. Tissue extracts were subjected to SDS-polyacrylamide gel electrophoresis and the phosphorylation state of S6K1 was determined by protein immunoblot analysis. S6K1 is resolved into three bands on SDS-polyacrylamide gels, with the top band (γ -band) corresponding to the most highly phosphorylated species. The more rapidly migrating bands are designated α and β . Data in the table indicate the amount of S6K1 in the γ -phosphorylated form, expressed as a proportion of the total S6K1.

and S6K1 are resolved into multiple electrophoretic forms on SDS-polyacrylamide gels, termed α , β , γ , representing differentially phosphorylated forms of the protein. The γ form is the most highly phosphorylated

form and exhibits the lowest electrophoretic mobility. Therefore, in the present study, the phosphorylation and 4E-BP1 and S6K1 were expressed as the percent of γ form in the 4E-BP1 and S6K1 respectively. The phosphorylation of S6K1 in the cerebral cortex and cerebellum was markedly lower in rats fed the 20% gelatin diet

or 20% gluten diet than in those fed the 20% casein diet (Fig. 1, Table 3). The 4E-BP1 phosphorylation in the cerebral cortex also decreased significantly with the 20% gelatin diet or 20% gluten diet compared with the 20% casein diet. The phosphorylation of 4E-BP1 in the cerebellum was not different among the groups (Fig. 2, Table 3).

Effect of the quantity of dietary protein on the phosphorylation of 4E-BP1 and S6K1 in the brain (Experiment 2)

The food intake and relative weights of brain regions did not differ among the experimental groups (Table 4). The phosphorylation of S6K1 in the cerebral cortex and cerebellum was significantly lower in rats fed the 0% casein or 5% casein diets than in those fed the 20% casein diet (Fig. 3, Table 4). The 4E-BP1 phosphorylation in the cerebral cortex also decreased significantly with the 0% casein or 5% casein diet compared with the 20% casein diet. The phosphorylation of 4E-BP1 in the cerebellum was not affected by the dietary protein quantity (Fig. 4, Table 4).

DISCUSSION

More research concerning age-related changes in brain composition and function (e.g., nutrient metabolism), is necessary to understand the modulating effects of nutritional factors (29). Direct evidence that the supply of amino acid influenced the neuronal protein synthesis was provided by the study of Parks et al. (30). In previous studies, we found that the rate of protein synthesis in the brain decreased with the decrease in dietary protein in aged rats (12). In older rats, we also reported that the rate of protein synthesis in the brain of animals given the gluten or gelatin diets is lower than in animals given the casein diet (13). The purpose of the present experiments was to elucidate the mechanism by which dietary protein affects the brain protein synthesis in aged rats. In aged rats, we demonstrated that the higher quality and quantity of dietary protein improved the polysomal profile in the brain (31). Many investigations suggested that the polysome profile in tissues rep-

Fig. 2. Phosphorylation state of 4E-BP1 in the cerebral cortex (A) and cerebellum (B) of aged rats fed the 20% gelatin (GEL), 20% gluten (GLU) or 20% casein (CAS) diet. 4E-BP1 was resolved into three electrophoretic forms on SDS-polyacrylamide gels. The more rapidly migrating bands are designated α and β , the more slowly migrating band γ . Data in the table indicate the amount of 4E-BP1 in the γ form, expressed as the percentage of the total 4E-BP1.

Table 4. Effect of the quantity of dietary protein on the phosphorylation states of 4E-BP1 and S6K1 in the brain regions of aged rats.¹

	0% Casein	5% Casein	20% Casein
Food intake (g/d)	13.4±0.9	14.1±0.7	15.1±1.1
Tissue weight (g/100 g of body weight)			
Cerebral cortex	0.100±0.004	0.107±0.005	0.105±0.003
Cerebellum	0.085±0.003	0.082±0.002	0.084±0.002
S6K1 phosphorylation (%) ²			
Cerebral cortex	18.5±1.2 ^b	16.9±2.2 ^b	50.5±10.1 ^a
Cerebellum	16.5±3.6 ^b	13.7±3.9 ^b	54.5±3.5 ^a
4E-BP1 phosphorylation (%) ³			
Cerebral cortex	21.5±1.2 ^b	37.5±2.2 ^b	55.4±2.5 ^a
Cerebellum	38.4±7.5	38.7±8.9	20.8±6.8

¹ Values are mean±SE, n=6. Means with different superscript letters are significantly different ($p<0.05$). Final body weights were from 318 to 333 g.

² Data indicate the amount of S6K1 in γ -phosphorylated form, expressed as the percentage of the total S6K1.

³ Data indicate the amount of 4E-BP1 in γ -phosphorylated form, expressed as the percentage of the total 4E-BP1.

Fig. 3. Phosphorylation state of S6K1 in the cerebral cortex (A) and cerebellum (B) of aged rats fed the 0% casein (0C), 5% casein (5C) or 20% casein (20C) diet.

resents the changes in the translational phase of protein synthesis (4, 5, 7, 32). However, there is little information on the mechanism by which the dietary protein affects the RNA translation in the brains of aged rats. Therefore, we hypothesized that the phosphorylation of 4E-BP1 and S6K1 in the brain regions decreased in aged rats given a lower quality or quantity of dietary protein.

S6K1 phosphorylation in the cerebral cortex and cerebellum were highest in those fed on the 20% casein diet, followed by the 20% gluten and 20% gelatin diets in Experiment 1, or by the 5% casein and 0% casein diets in Experiment 2, in that order (Tables 3 and 4). The phosphorylation of 4E-BP1 in the cerebral cortex also decreased significantly in aged rats given a lower quality and quantity of protein. The changes in the phosphorylation of S6K1 and 4E-BP1 in the brains depended on the quality and quantity of dietary protein. Yoshizawa et al. (15) reported that the stimulation of protein synthesis caused by dietary protein was mediated by the increase in the initiation of mRNA translation in the liver. Therefore, in the present study, the quality and quantity of dietary protein may have controlled the phosphorylation of S6K1 and 4E-BP1, and been one of the factors affecting brain protein synthesis in aged rats, thus corroborating the finding of Yoshizawa et al. (15).

In our previous works (7, 14), we demonstrated that most essential amino acids such as branched amino acids, both in blood and in the brain, showed variations

Fig. 4. Phosphorylation state of 4E-BP1 in the cerebral cortex (A) and cerebellum (B) of aged rats fed the 0% casein (0C), 5% casein (5C) or 20% casein (20C) diet.

in accordance with their concentrations in the dietary protein of weaned and aged rats, and that the alterations in the amino acid concentrations in the blood and brain, as well as in the brain protein synthesis, resulted from changes in the quality and quantity of dietary protein. Koie et al. (33) and Lyou et al. (34) reported that the addition of lysine or methionine to a low-gluten diet or to a low-soy protein diet, respectively, increased the protein synthesis rates in the brains of aged rats. Recently, leucine has been shown to be the most potent of the amino acids in enhancing the initiation phase of mRNA translation (17). Yoshizawa et al. (35) demonstrated that leucine administration had an obvious stimulatory effect on 4E-BP1 and S6K1 phosphorylation in both liver and skeletal muscle. Our present experiment showed that, when the quality and quantity of dietary protein were high, the phosphorylation of 4E-BP1 and S6K1 in the brain was improved, which could lead to an improvement in the brain protein synthesis of aged rats. Therefore, the decrease of initiation factors of mRNA translation in the brain resulting from the lower quality or quantity of dietary protein may be due to the dietary-limiting amino acids, which were at low levels in both the blood and brain. Measurement of the role of amino acids on 4E-BP1 and S6K1 phosphorylation in the brain should be included in further studies of the effect of dietary protein on the brain protein synthesis in aged rats.

Several investigators have reported protein synthesis in visceral organs and skeletal muscle by growth hormone (GH). Recent studies have shown that GH may affect many functions related to the central nervous system. Treatment of adult GH-deficient patients with

human GH is reported to improve psychological well being and memory function (36, 37). In the previous study, we indicated that the brain protein synthesis was increased by GH in hypophysectomized aged rats, and that the plasma concentration of GH depended on the quality and quantity of dietary protein (14, 38). Kato (39) suggested that GH might stimulate the translational phase of protein synthesis. The effect of GH treatment on the initiation factors of mRNA translation in the brain of aged rats is another question to consider in a further study.

In the cerebellum, the phosphorylation of 4E-BP1 was not affected by dietary protein. Thus, in the initiation phase of mRNA translation of the cerebellum, S6K1 phosphorylation, rather than 4E-BP1 phosphorylation might be regulated by the quality and quantity of dietary protein in aged rats. Tujioka et al. (40) reported that the ingestion of a lower quality and quantity of dietary protein decreased the concentration and mRNA level of nerve growth factor in the brain of young rats. In the present study, we did not determine the mRNA level in the brain regions. This is another possibility to consider in further studies of the effect of dietary protein on brain protein metabolism in aged rats. On the other hand, whether leucine or GH improves the phosphorylation of 4E-BP1 in not only the cerebral cortex but also the cerebellum remains unknown. Therefore, as mentioned above, the effects of leucine and GH on 4E-BP1 and S6K1 phosphorylation in the cerebral cortex and cerebellum should be determined in detail in further studies.

These results suggest that the ingestion of a higher quality and quantity of dietary protein increases the phosphorylation of 4E-BP1 and S6K1 in aged rats, and that the 4E-BP1 and S6K1 phosphorylation are at least partly related to the mechanism by which the dietary protein affects brain protein synthesis in aged rats.

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