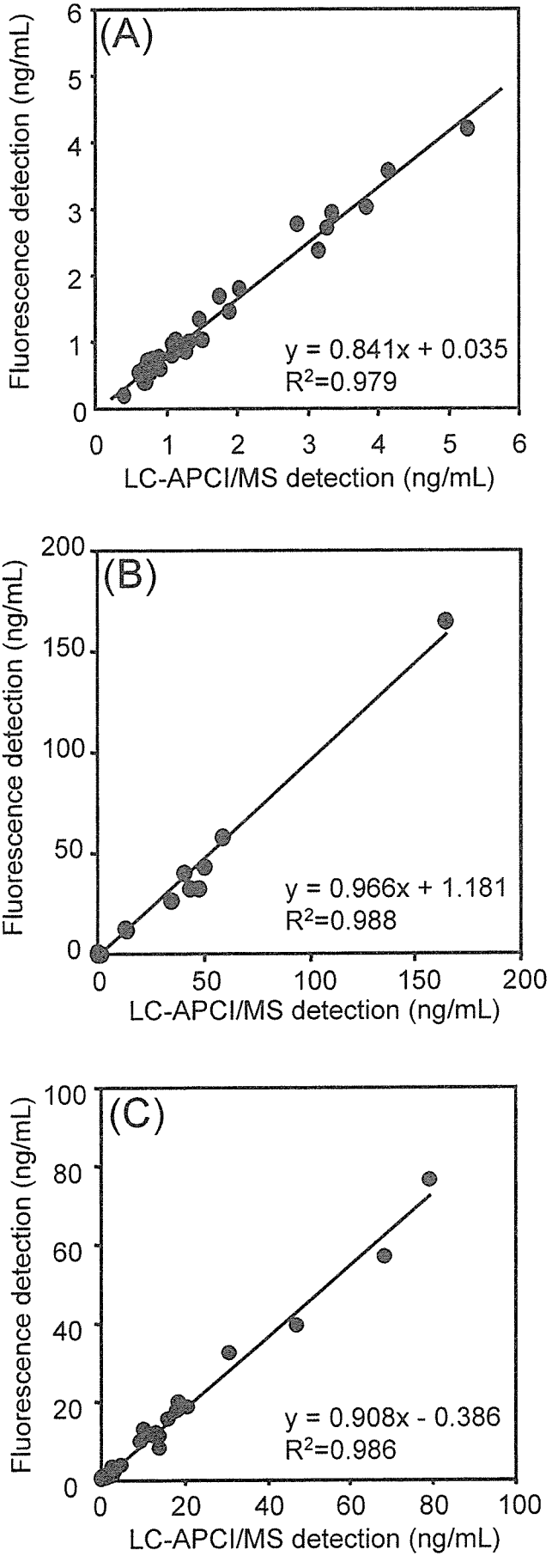
Fig. 1


(A)
Fluorescence detection (ng/mL)
LC-APCI/MS detection (ng/mL)
y = 0.841x + 0.035
R²=0.979
(B)
Fluorescence detection (ng/mL)
LC-APCI/MS detection (ng/mL)
y = 0.966x + 1.181
R²=0.988
(C)
Fluorescence detection (ng/mL)
LC-APCI/MS detection (ng/mL)
y = 0.908x - 0.386
R²=0.986

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Determination of Fat-Soluble Vitamins in Human Plasma, Breast Milk and Food Samples – Application in Nutrition Survey for Establishment of "Dietary Reference Intakes for Japanese" –

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SUMMARY

Dietary habits are an important risk factor for lifestyle-related diseases. To carry out a nutrition survey of fat-soluble vitamins, we developed determination methods of fat-soluble vitamins using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry or high-performance liquid chromatography with fluorescence detection. In these methods, stable isotope-labeled compounds or vitamin K analogs with a saturated side-chain were used as internal standards. These methods have high sensitivity and sufficient accuracy, and we applied them in a nutrition survey about the status of fat-soluble vitamins in Japanese women. Plasma concentrations of 25-hydroxyvitamin D_3 [25(OH)D_3] and 25-hydroxyvitamin D_2 [25(OH)D_2] in healthy postmenopausal women (n=98) were 20.5 ± 7.9 and 0.4 ± 1.4 ng/mL, respectively. A significant negative correlation in plasma levels between 25(OH)D and parathyroid hormone was observed. For vitamin K homologs, plasma levels of phylloquinone (PK), menaquinone-4 (MK-4) and menaquinone-7 (MK-7) in Japanese women of various ages (n=1409) were 1.03 ± 0.90, 0.12 ± 0.28 and 6.71 ± 13.6 ng/mL, respectively. The mean total vitamin K intake of Japanese young women was about 230 μg/day, and 94 % of participants met the Adequate Intake of vitamin K for women aged 18-29 y in Japan, 60 μg/day. Moreover, we determined fat-soluble vitamins in breast milk collected from Japanese lactating women and revealed that the contents of all-*trans*-retinol, vitamin D_3, 25(OH)D_3, α-tocopherol, PK and MK-4 in breast milk were 0.39 ± 0.14 μg/mL, 0.10 ± 0.15 ng/mL, 0.08 ± 0.04 ng/mL, 3.96 ± 1.84 μg/mL, 3.56 ± 2.19 and 1.77 ± 0.68 ng/mL, respectively.

Key words—fat-soluble vitamins, vitamin D, vitamin K, nutrition survey

INTRODUCTION

In Japan, lifestyle-related diseases have been increasing with the advent of the aging society and it is acknowledged that dietary habits are an important risk factor for these diseases. Thus, a nutrition survey aimed at humans is needed as well as a study of the bioavailability, physiological function and metabolism of nutrients to obtain scientific information for the primary prevention of lifestyle-related diseases through the improvement of dietary habits and nutrition. We especially focused on vitamins D and K which are important fat-soluble vitamins for the prevention of osteoporosis.

It is well recognized that plasma or serum levels of 25-hydroxyvitamin D [25(OH)D] reflect the nutritional status of vitamin D in humans. Vitamin D is metabolized to 25(OH)D in the liver and subsequently to the active form of vitamin D, 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D], or the inactive form of vitamin D, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D], in the kidney. In addition, it was demonstrated that vitamin D and its metabolites are also metabolized to their respective C-3 epimers [1-5]. Vitamin D_3, which is the form of vitamin D synthesized by vertebrates including humans, and vitamin D_2, which is the major naturally occurring form in plants, are both metabolized in a similar fashion. 25(OH)D binds to vitamin D-binding protein (DBP) in the blood and is the most abundant circulating metabolite of vitamin D with a concentration of 20-50 ng/mL under normal conditions [6]. Thus, the plasma or serum concentration of 25(OH)D is considered to be a good indicator of the cumulative effects of exposure to sunlight and dietary intake of vitamin D. Plasma or serum 25(OH)D concentration can be measured by high-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector [7], competitive protein-binding assay (CPBA) [8], radioimmunoassay (RIA) [9] and enzyme immunoassay (EIA) [10]. In recent years, RIA and EIA have been widely used in many laboratories and hospitals because of their superior simplicity, rapidity and accuracy; however, these methods require high-quality control to ensure reliable results [11-15]. Moreover, conventional RIA measures 25(OH)D along with 24,25$(OH)_2$D because their antibodies exhibit 100 % cross-reaction with 24,25$(OH)_2$D.

Meanwhile, one of the most common nutritional indicators of vitamin K is the plasma concentration of phylloquinone (PK, vitamin K_1). PK is produced by plants and algae, and the other vitamin K form, menaquinones (MKs, vitamin K_2), is synthesized by bacteria. MKs

comprise a family of molecules distinguished from PK by unsaturated side-chains of isoprenoid units varying in length from 1 to 14 repeats [15]. Vitamin K is a cofactor for an enzyme that converts specific glutamyl residues in several proteins such as plasma clotting factors II (prothrombin), osteocalcin (bone Gla protein) and matrix Gla protein to γ-calboxyglutamyl (Gla) residues. These vitamin K-dependent proteins play crucial roles in blood coagulation and calcification. Several reports indicate an important role for vitamin K in bone health. The administration of vitamin K results in increased bone-mineral density (BMD) and reduced bone resorption in humans [16, 17]. In epidemiological studies, low dietary vitamin K intake was associated with an increased incidence of hip fracture [18, 19]; however, no large-scale nutrition survey of vitamin K has been conducted due to the low plasma concentration of vitamin K. There is still the problem with the accuracy of HPLC with fluorescence detection, which is usually used for the quantitation of plasma vitamin K.

Based on this background, we developed precise assay methods for vitamins D and K using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry (LC-APCI/MS/MS) and HPLC with a fluorescence detector. Then, we applied these methods in a nutrition survey of Japanese women.

Development of Determination Method for Vitamin D

We established a precise and sensitive assay method to determine 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ in human plasma using LC-APCI/MS/MS to provide a gold standard [20]. The method involves the use of deuterated 25(OH)D_3 as an internal standard, which was synthesized in our laboratory. After the addition of the internal standard to 0.1 mL of plasma samples, methanol was added for protein removal. Vitamin D compounds were purified by C_{18} silicagel mini-column and detected by the MS/MS multiple reaction monitoring (MRM) method. The average spiked recoveries from authentic compounds added to normal human plasma samples for 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ were 98-104 %. The average intraassay variation values (relative standard deviation) for 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ were 5.7, 4.5 and 11.4 %, respectively. The average interassay variation values for 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ were 2.5, 5.1 and 9.9 %, respectively. Mean plasma concentrations of 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ in healthy postmenopausal

women (n=98) were 20.5 ± 7.9 (mean ± S.D.), 0.4 ± 1.4 and 0.5 ± 0.7 ng/mL, respectively. The concentrations of 25(OH)D measured by the RIA method using a DiaSorin RIA kit were well correlated with the concentrations of 25(OH)D plus $24,25(OH)_2D_3$ measured by the proposed method, although the RIA method gave slightly higher concentrations than the LC-APCI/MS/MS method. In addition, a significant negative correlation was observed between plasma levels of 25(OH)D and parathyroid hormone (PTH) with the LC-APCI/MS/MS method. In contrast, no significant correlation was observed in plasma levels between 25(OH)D and PTH with the RIA method. Plasma PTH level is an important indicator of vitamin D deficiency or insufficiency. Recently, a negative correlation between plasma 25(OH)D and PTH levels was reported from some cohort studies of healthy subjects [21, 22]. These results suggest that this LC-APCI/MS/MS method would be useful for the evaluation of vitamin D status and provide useful information in the diagnosis of vitamin D insufficiency/deficiency, as well as for the treatment and prevention of osteoporosis with vitamin D.

Development of Determination Method for Vitamin K

We also developed a determination method for vitamin K homologs including PK, MK-4 and MK-7 in human plasma using LC-APCI/MS/MS [23]. As internal standard compounds, ^{18}O-labeled PK, MK-4 and MK-7 were used. After the addition of internal standards to 0.5 mL of plasma samples, vitamin K compounds were extracted with ethanol and hexane. The average spiked recoveries from authentic compounds added to normal human plasma samples for PK, MK-4 and MK-7 were 98-102 %. The average intraassay and interassay variation values for PK, MK-4 and MK-7 were less than 10 %. The quantitation limits for PK, MK-4 and MK-7 were less than 3 pg per injection. Thus, we conclude that this novel LC-APCI/MS/MS method has enough reproducibility and sensitivity to measure vitamin K in human plasma; however, this method does not establish a universal routine assay as it uses an expensive measuring instrument. Therefore, we developed an improved HPLC fluorescence determination method for vitamin K homologs using post-column reduction and synthetic vitamin K analogs with different lengths of the saturated alkyl side-chain as internal standards [24]. Selectivity and reproducibility were increased by optimizing chromatographic conditions

including the mobile phase and excitation wavelength for MK-4 or less polar derivatives, PK and MK-7. The detection limits for PK, MK-4 and MK-7 were less than 4 pg per injection. The recoveries of PK, MK-4 and MK-7 were 93-105 % and the inter- and intraassay variation values of normal human plasma for PK, MK-4 and MK-7 were less than 10 %. The data showed good correlation between the proposed HPLC fluorescence determination method and the LC-APCI/MS/MS method for PK (r^2=0.979), MK-4 (r^2=0.988) and MK-7 (r^2=0.986) (Fig. 1). These results suggest that the improved HPLC fluorescence detection method allows the determination of vitamin K to evaluate the clinical and nutritional status as well as the LC-APCI/MS/MS method. Thus, this method was applied to plasma samples from Japanese women of various ages (n=1409). Plasma levels of PK, MK-4 and MK-7 were 1.03 ± 0.90, 0.12 ± 0.28 and 6.71 ± 13.6 ng/mL, respectively. The plasma levels of PK in elderly women (62.7 ± 10.9 y) were significantly higher than those of high school and junior high school girls. The plasma concentrations of MK-4 have a tendency to increase during periods of growth. In addition, plasma PK and MK-7 concentrations correlated inversely with undercarboxylated osteocalcin (ucOC) in elderly women [25]. The plasma PK or MK-7 concentration required to minimize the ucOC concentration was higher in the group over 70 y, and it decreased progressively for each of the younger age groups. Thus, circulating vitamin K concentrations in elderly people should be kept higher than those in young people.

Vitamin K Content of Foods and Dietary Vitamin K Intake in Japanese Young Women

In the current "Dietary Reference Intakes (DRIs) for Japanese", the Adequate Intake (AI) of vitamin K is set at 75 μg for adult men, 60 μg for women aged 18-29 y, and 65 μg for women 30 y and over as a probable sufficient quantity for the maintenance of normal blood clotting. However, the current AI might not be sufficient to maintain bone health. In addition, the assessment of dietary intake of both PK and MKs is incomplete in regions where people habitually eat fermented food, such as Japan. To obtain a closer estimate of dietary intake of PK and MKs in Japanese young women, PK, MK-4 and MK-7 contents in food samples (58 food items) were determined using an improved HPLC method with fluorescence detection. Next, we assessed dietary vitamin K intake in Japanese young women aged 20-23 y (n=125), using the vitamin K contents measured here and the Standard Tables of Food Composition in

Japan [26]. PK was widely distributed in green vegetables and algae, and high amounts were found in spinach and broccoli (raw, 498 and 307 μg/100g wet weight, respectively, unpublished data). Although MK-4 was widely distributed in animal products, overall MK-4 content was lower than PK. Relatively high amounts of MK-4 were found in chicken meat (raw, 27 μg/100 g) and the egg yolk of hen's eggs (raw, 64 μg/100 g). MK-7 was observed characteristically in fermented soybean products such as natto (939 μg/100 g). The mean total vitamin K intake of Japanese young women was about 230 μg/day and 94 % of participants met the AI of vitamin K for women aged 18-29 y in Japan, 60 μg/day. Mean daily intakes of PK, MK-4 and MK-7 (MK-4 equivalent value) were estimated as 155.9 ± 91.1, 16.9 ± 10.4 and 57.4 ± 83.7 μg/day, respectively. The contributions of PK, MK-4 and MK-7 (MK-4 equivalent value) to total vitamin K intake were 67.7, 7.3 and 24.9 %, respectively; therefore, PK from vegetables and algae, and MK-7 from pulses (including fermented soybean foods) were the major contributors to the total vitamin K intake of Japanese young women.

Nutrition Survey on Fat-Soluble Vitamins of Japanese Lactating Women

To estimate an infant's intake of fat-soluble vitamins, we determined their levels in breast milk collected from Japanese lactating women (n=51, age: 30.8 ± 4.4 y, post-partum day: 1.5 ± 1.2 m) by the LC-APCI/MS/MS method using stable isotope-labeled compounds as internal standards. It was reported that the concentrations of vitamin D and its metabolites in human breast milk were very low [27, 28]. Therefore, we used a derivatization method with a Cookson-Type reagent to improve ionization efficiency for the determination of vitamin D and its metabolites in LC-APCI/MS/MS analysis [29]. The contents of all-*trans*-retinol, vitamin D_3, 25(OH)D_3, α-tocopherol, PK, MK-4 and MK-7 in breast milk were 0.39 ± 0.14 μg/mL, 0.10 ± 0.15 ng/mL, 0.08 ± 0.04 ng/mL, 3.96 ± 1.84 μg/mL, 3.56 ± 2.19, 1.77 ± 0.68 ng/mL and 1.19 ± 1.54 ng/mL, respectively (Table 1). Daily intake of vitamin D calculated from an infant's consumption of breast milk, 780 mL/day was 0.47 μg, which did not meet current DRIs (AI, 2.5 μg/day). The concentrations of all-*trans*-retinol, β-carotene, 25(OH)D_3, α-tocopherol, PK and MK-4 in breast milk were positively correlated with lipid content; thus, the secretion of fat-soluble vitamins in breast milk is thought to be highly influenced by lipids.

Summary

We developed reliable determination methods for fat-soluble vitamins and applied them to a nutritional epidemiology study of Japanese. Further large-scale studies will be needed and the obtained data may be useful to maintain and improve health, and to establish DRIs for Japanese.

Acknowledgements

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REFERENCES

1) Bischof, M. G., Siu-Caldera, M. –L., Weiskopf, A. Vouros, P., Cross, H. S., Peterlik, M. and Reddy, G. S. (1998) Differentiation-related pathways of 1α,25-dihydroxycholecalciferol metabolism in human colon adenocarcinoma-derived Caco-2 cells: production of 1α,25-dihydroxy-3-epi-cholecalciferol. *Exp. Cell. Res.*, **241**, 194-201.

2) Masuda, S., Kamao, M., Schroeder, N. J., Makin, H. L. J., Jones, G., Kremer, R., Rhim, J. and Okano, T. (2000) Characterization of 3-epi-1α,25-dihydroxyvitamin D_3 involved in 1α,25-dihydroxyvitamin D_3 metabolic pathway in cultured cell lines. *Biol. Pharm. Bull.*, **23**, 133-139.

3) Kamao, M., Tatematsu, S., Reddy G. S., Hatakeyama, S., Sugiura, M., Ohashi, N., Kubodera, N. and Okano, T. (2001) Isolation, identification and biological activity of 24*R*,25-dihydroxy-3-epi-vitamin D_3: a novel metabolite of 24*R*,25-dihydroxyvitamin D_3 produced in rat osteosarcoma cells (UMR 106). *J. Nutr. Sci. Vitaminol.* (Tokyo), **47**, 108-115.

4) Higashi, T., Ogasawara, A. and Shimada, K. (2000) Investigation of C-3 epimerization mechanism of 24,25-dihydroxyvitamin D_3 in rat using liquid chromatography/mass spectrometry. *Anal. Sci.*, **16**, 477-482.

5) Kamao, M., Tatematsu, S., Hatakeyama, S., Sakaki, T., Sawada, N., Inouye, K., Ozono, K., Kubodera, N., Reddy, G. S. and Okano, T. (2004) C-3 epimerization of vitamin D_3 metabolites and further metabolism of C-3 epimers. *J. Biol. Chem.*, **279**, 15897-15907.

6) Napoli, J. L. and Horst, R. L. (1984) Vitamin D Metabolism. *In Vitamin D: Basic and Applied Aspects* (Kumar, R., Eds.). Martinus Nijhoff, Boston, pp.91-123.

7) Jones, G. (1978) Assay of vitamins D_2 and D_3, and 25-hydroxyvitamins D_2 and D_3 in human plasma by high-performance liquid chromatography. *Clin. Chem.*, **24**, 287-298.

8) Haddad, J. G. and Chyu, K. J. (1971) Competitive protein-binding radioassay for 25-hydroxycholecalciferol. *J. Clin. Endocrinol. Metab.*, **33**, 992-995.

9) Hollis, B. W. and Napoli, J. L. (1985) Improved radioimmunoassay for vitamin D and its use in assessing vitamin D status. *Clin. Chem.*, **31**, 1815-1819.

10) Lind, C., Chen, J. and Byrjalsen, I., (1997) Enzyme immunoassay for measuring 25-hydroxyvitamin D_3 in serum. *Clin. Chem.*, **43**, 943-949.

11) Carter, G. D., Carter, R., Jones, J. and Berry, J. (2004) How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme. *Clin. Chem.*, **50**, 2195-2197.

12) Carter, G. D., Carter, C. R., Gunter, E., Jones, J., Jones, G., Makin, H. L. and Sufi, S. (2004) Measurement of Vitamin D metabolites: an international perspective on methodology and clinical interpretation. *J. Steroid Biochem. Mol. Biol.*, **89-90**, 467-471.

13) Binkley, N., Krueger, D., Cowgill, C. S., Plum, L., Lake, E., Hansen, K. E., DeLuca, H. F. and Drezner, M. K. (2004) Assay variation confounds the diagnosis of hypovitaminosis D: a call for standardization. *J. Clin. Endocrinol. Metab.*, **89**, 3152-3157.

14) Hollis, B. W. (2000) Comparison of commercially available ^{125}I-based RIA methods for the determination of circulating 25-hydroxyvitamin D. *Clin. Chem.*, **46**, 1657-1661.

15) Collins, M. D. and Jones, D. (1981) Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. *Microbial. Rev.*, **45**, 316-354.

16) Sato, Y., Honda, Y., Kuno, H. and Oizumi, K. (1998) Menatetrenone ameliorates osteopenia in disuse-affected limbs of vitamin D- and K-deficient stroke patients. *Bone*, **23**, 291-296.

17) Craciun, A. M., Wolf, J., Knapen, M. H., Brouns, F. and Vermeer, C. (1998) Improved bone metabolism in female elite athletes after vitamin K supplementation. *Int. J. Sports Med.*, **19**, 479-484.

18) Feskanich, D., Weber, P., Willett, W. C., Rockett, H., Booth, S. L. and Colditz, G. A. (1999) Vitamin K intake and hip fractures in women: a prospective study. *Am. J. Clin. Nutr.*, **69**, 74-79.

19) Booth, S. L., Tucker, K. L., Chen, H., Hannan, M. T., Gagnon, D. R., Cupples, L. A., Wilson, P. W., Ordovas, J., Schaefer, E. J., Dawson-Hughes, B. and Kiel, D. P. (2000) Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. *Am. J. Clin. Nutr.*, **71**, 1201-1208.

20) Tsugawa, N., Suhara, Y., Kamao, M. and Okano, T. (2005) Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography-tandem mass spectrometry. *Anal. Chem.*, **77**, 3001-3007.

21) Krall, E. A., Sahyoun, N., Tannenbaum, S., Dallal, G. E., Dawson-Hughes, B. (1989) Effect of vitamin D intake on seasonal variations in parathyroid hormone secretion in postmenopausal women. *Engl. J. Med.*, 321, 1777-1783.

22) Webb, A. R., Pilbeam, C., Hanafin, N., Holick, M. F. (1990) An evaluation of the relative contributions of exposure to sunlight and of diet to the circulating concentrations of 25-hydroxyvitamin D in an elderly nursing home population in Boston. *Am. J. Clin. Nutr.* 51, 1075-1081.

23) Suhara, Y., Kamao, M., Tsugawa, N. and Okano, T. (2005) Method for the determination of vitamin K homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry. *Anal. Chem.*, **77**, 757-763.

24) Kamao, M., Suhara, Y., Tsugawa, N. and Okano, T. (2005) Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. *J. Chromatogr. B*, **816**, 41-48.

25) Tsugawa, N., Shiraki, M., Suhara, Y., Kamao, M., Tanaka, K. and Okano, T. (2006) Vitamin K status of healthy Japanese women: age-related vitamin K requirement for γ-carboxylation of osteocalcin. *Am. J. Clin. Nutr.*, **83**, 380-386.

26) Report of the Subdivision on Resources, The Council for Science and Technology,

Ministry of Education, Culture, Sports, Science and Technology, JAPAN (2005) Standard Tables of Food Composition in Japan, Fifth revised and enlarged edition.

27) Hollis, B. W., Roos, B. A., Draper H. H. and Lambert, P. W. (1981) Vitamin D and its metabolites in human and bovine milk. *J. Nutr.*, **111**, 1240-1248.

28) Takeuchi, A., Okano, T., Tsugawa, N., Katayama, M., Mimura, Y., Kobayashi, T., Kodama, S. and Matsuo, T. (1988) The determination of vitamin D and its metabolites in human breast and cow's milk. *J. Micronutrient. Anal.*, **4**, 193-208.

29) Higashi, T., Awada, D. and Shimada, K. (2001) Simultaneous determination of 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in human plasma by liquid chromatography-tandem mass spectrometry employing derivatization with a Cookson-type reagent. *Biol. Pharm. Bull.*, **24**, 738-743.

FIGURE LEGEND

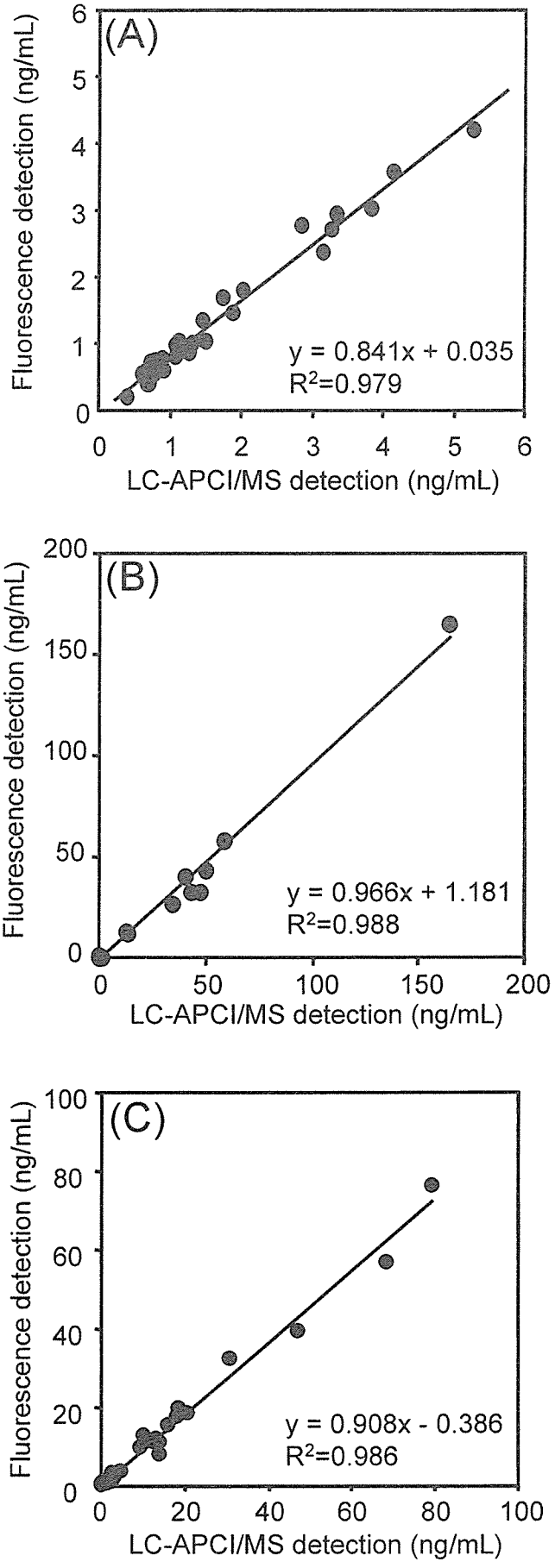
Figure 1. Correlation between the values obtained by HPLC with fluorescence detection using internal standards and those obtained by LC-APCI/MS. (A) PK; (B) MK-4; (C) MK-7.

Table 1. Concentrations of fat-soluble vitamins in human milk and estimated infant's intake

Vitamin	Compound	Concentration in human milk[1]	Estimated infant's intake[2]
A	all-*trans*-retinol	0.39 ± 0.14 (μg/mL)	335 μg RE/day[3]
	β-carotene	0.05 ± 0.04 (μg/mL)	
D	vitamin D_3	0.10 ± 0.15 (ng/mL)	0.47 μg/day[4]
	vitamin D_2	0.09 ± 0.19 (ng/mL)	
	25(OH)D_3	0.08 ± 0.04 (ng/mL)	
	25(OH)D_2	0.003 ± 0.002 (ng/mL)	
E	α-tocopherol	3.96 ± 1.84 (μg/mL)	3.09 mg/day
K	PK	3.56 ± 2.19 (ng/mL)	4.79 μg/day[5]
	MK-4	1.77 ± 0.68 (ng/mL)	
	MK-7	1.19 ± 1.54 (ng/mL)	

[1] Values are the means ± S.D., n=51.
[2] The product of the concentrations of fat-soluble vitamins in human milk and infant's consumption of human milk (780 mL/day).
[3] The sum of all-*trans*-retinol and β-carotene expressed as retinol equivalent (RE) value.
[4] The sum of vitamin D and vitamin D equivalent 25(OH)D [25(OH)D x 5, vitamin D conversion factor of 25(OH)D=5].
[5] The sum of PK, MK-4 and MK-4 equivalent MK-7 (MK-7 content x 444.7/649).

Fig. 1


(A)
Fluorescence detection (ng/mL)
LC-APCI/MS detection (ng/mL)
y = 0.841x + 0.035
R²=0.979
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Note

Increase in *S*-Adenosylhomocysteine Content and Its Effect on the *S*-Adenosylhomocysteine Hydrolase Activity under Transient High Plasma Homocysteine Levels in Rats

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Summary The objective of this study was to examine how transient high plasma homocysteine (Hcy) levels affect the metabolism of Hcy, the activity and expression of *S*-adenosylhomocysteine (SAH) hydrolase which catalyzes both SAH hydrolysis and SAH synthesis. Wistar ST rats (males) were cannulated in the right jugular vein for intravenous infusion of physiological saline or DL-Hcy solutions (15 and 30 mg/mL) for 1 h at 1.1 mL/h/rat. The content of *S*-adenosylmethionine (SAM), SAH-synthetic activity of SAH hydrolase and the expression of SAH hydrolase mRNA in liver extracts showed no significant difference in the Hcy infused groups as compared to the Control group. On the other hand, the contents of hepatic SAH in the Hcy infused groups were dose-dependent and significantly higher than that of the Control group. Thus, this study showed that hepatic SAH increased without any increase in the SAH-synthetic activity and the expression of SAH hydrolase mRNA under transient high plasma Hcy levels after intravenous infusion of Hcy.

Key Words plasma homocysteine, *S*-adenosylhomocysteine, *S*-adenosylhomocysteine hydrolase, methionine metabolism

Homocysteine (Hcy) is an intermediate of the methionine metabolic pathway, and it occupies a branch point of two metabolic pathways, the remethylation pathway to methionine and the transsulfuration one to cysteine. These two pathways are coordinated by *S*-adenosylmethionine (SAM), which acts as an allosteric inhibitor of the methylentetrahydrofolate reductase (EC 1.1.1.68) and as an activator of cystathionine β-synthase (EC 4.2.1.22; CBS) (*1*). SAM is converted to *S*-adenosylhomocysteine (SAH) by some transmethylation reactions, and then SAH is hydrolyzed by SAH hydrolase (EC 3.3.1.1), a reversible enzyme which catalyzes SAH-hydrolytic reaction to Hcy and adenosine, and SAH-synthetic reaction from Hcy and adenosine. In general, equilibrium of the SAH hydrolase reaction favors the direction of SAH synthesis, while the SAH-hydrolytic reaction predominates under physiological conditions. Predominant SAH hydrolysis is maintained by rapid removal of Hcy. However, if it is difficult for sufficient Hcy to be removed from this cycle due to CBS deficiency or inhibition of methionine synthase, it means that the methionine cycle can not maintain its balance. The balance of the methionine cycle is maintained intricately by methionine metabolites. Therefore disturbed methionine metabolism induces several diseases, e.g. hyperhomocysteinemia.

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An increase of plasma Hcy is caused by nutritional deficiencies or genetic mutations. Vitamin B_6 (B_6) deficiency (*2*, *3*) and folic acid deficiency (*4*) were reported as the factors affecting Hcy accumulation. Furthermore, the relationship between elevated plasma Hcy and accumulated SAH was observed in several studies using rats (*5*, *6*). Elevated plasma Hcy and accumulated SAH are likely to be caused by disturbed methionine metabolism. We reported in our previous study that the elevation of plasma Hcy and accumulation of hepatic SAH were observed in B_6-deficient rats (*7*). In B_6 deficiency, the transsulfuration pathway is suppressed due to the fact that B_6 is a coenzyme of CBS and γ-cystathionase. Therefore, abnormal methionine metabolism is caused by B_6 deficiency. Moreover, the increase in SAH-synthetic activity of SAH hydrolase was also observed in B_6 deficiency (*7*), the cause of which was not clarified. It was surmised that the elevation of Hcy, a substrate of SAH-synthetic reaction of SAH hydrolase, might induce the increase of SAH-synthetic activity of SAH hydrolase.

In this experiment, we examined the SAH content and the SAH-synthetic activity of SAH hydrolase in the liver of rats with transiently elevated plasma Hcy levels after intravenous infusion of Hcy.

Materials and Methods

Reagents. Somnopentyl® was purchased from

Schering-Plough Co. (Kenilworth, USA). SAH, SAM, adenosine and L-Hcy thiolactone were purchased from Sigma Chemicals Co. (St.Louis, USA). DL-Hcy was purchased from Nacalai Tesque Inc. (Kyoto, Japan). SBD-F (7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate) and 1-heptanesulfonic acid sodium salt were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol (HPLC grade) was purchased from Kanto Kagaku Co. (Tokyo, Japan).

Animal and diets. Male Wistar ST rats, from 8 to 10 wk old, were obtained from Japan SLC, Inc. (Hamamatsu, Japan). The rats had free access to MF chow diet and water during the experimental period. The rats were housed in a temperature-controlled room (23±1°C) with alternating 12 h cycle of light (light: 6:00 a.m. to 6:00 p.m.). The experiments were performed in accordance with the governmentally legislated guidelines in Japan on the proper use of laboratory animals (1980), and our experiments were approved by the ethical committee of the Faculty of Applied Biological Sciences in Gifu University.

Surgical operation. The right jugular vein was cannulated for intravenous infusion of physiological saline or Hcy solution, and the surgical method followed was of Kraegen et al. (*8*). After a 3 d recovery term from surgery, the rats were used for the following infusion study.

Homocysteine infusion. DL-Hcy was dissolved in physiological saline at the concentration of 15 (15-Hcy group) or 30 (30-Hcy group) mg/mL, and infused at a rate of 4 mL/h/kg body weight using a syringe pump (Model IVAC P3000, IMI Co., Ltd., Saitama, Japan) for 1 h. Transient high plasma Hcy levels were achieved by the same amount of Hcy infusion (*9*). The rats in the Control group were given physiological saline at the same infusion rate as the Hcy groups. Rats were randomly divided into three groups ($n=7$) before intravenous infusion of Hcy. The body weights (g) were 284±7, 284±5, and 283±4 (means±SE) in the Control, 15-Hcy, and 30-Hcy groups, respectively.

Sample preparation. Under Somnopentyl® anesthesia, blood samples were drawn from the abdominal aorta with a heparinized syringe, and then rat livers were immediately excised and weighed. Plasma was obtained by centrifugation at 2,000×*g* at 4°C for 20 min. Plasma and liver were stored at −20°C until analyses.

Assay of plasma Hcy and cysteine. Hcy in plasma was measured by the method of Yamaguchi et al. (*10*).

Assay of SAM and SAH. SAM and SAH were measured by the method of She et al. (*11*).

Assay of hepatic SAH hydrolase. The SAH-synthetic activity of SAH hydrolase was measured using a modified method of She et al. (*11*). Liver was homogenized with 5 volumes of 0.25 M sucrose/3.3 mM $MgCl_2$/2 mM glutathione (reduced form)/50 mM Tris-HCl buffer (pH 7.4). The homogenate was centrifuged at 100,000×*g* (4°C) for 1 h. The supernatant was used for assay of SAH hydrolase.

For the assay of the SAH-synthetic activity of SAH hydrolase, the incubation mixture consisted of 100 mM potassium phosphate buffer (pH 7.3), 2 mM dithiothreitol, 5 mM L-Hcy thiolactone, 100 μM adenosine and 50 μM liver extract (1 mg protein/mL) in a total volume of 0.3 mL. After incubation at 37°C for 10 min, the reaction was stopped by adding 50 μL of 3 N perchloric acid. The content was mixed and then centrifuged at 2,000×*g* for 10 min. The supernatant was filtered through a Millipore membrane (0.45 μM) and applied to HPLC for analysis of SAH. SAH-synthetic activity of SAH hydrolase was estimated by formed SAH.

Extraction of total RNA. Total RNA in each homogenate was isolated by the acid guanidium-phenol-chloroform method, using TRIzol (Invitrogen Corporation, Carlsbad, CA, USA). RNA concentration was determined by absorption at 260 nm using HITACHI U-2001 spectrophotometer (Tokyo, Japan).

Assay for expression of SAH hydrolase mRNA. Expression of SAH hydrolase mRNA was determined by RT-PCR using a TaKaRa One Step RNA PCR Kit (Takara Bio Inc., Otsu, Japan). The RT-PCR partly followed the method of Ohmori et al. (*12*). The primer of SAH hydrolase used was as follows: the upstream primer sequence was 5′-AAGCTGCCATGGAGGGCTACGA-3′ and the downstream primer sequence was 5′-GATGGCAGCTGGAAGGTGAAGG-3′. For the primer of β-actin (used as an invariant control), β-actin RT-PCR Primer set (Toyobo Co., Ltd., Osaka, Japan) was used.

The samples were amplified by 30 PCR cycles, where each consisted of denaturation at 94°C for 60 s, annealing at 58°C for 60 s and extension at 72°C for 75 s. Each PCR product was resolved by electrophoresis on 1% agarose gel stained with ethidium bromide, and photographed under UV light. Band intensity was evaluated by the NIH image program, which was developed by U.S. National Institutes of Health.

Statistical analysis. The statistical difference among mean was estimated at $p<0.05$ according to ANOVA and Scheffe's test (Excel Statistics 2006 for Windows, Social Survey Research Information Co., Ltd., Tokyo, Japan).

Results and Discussion

Hcy is an important intermediate of methionine metabolism because it occupies a branch point in the metabolism. Therefore its metabolites and activity of relevant enzyme would change under disturbed Hcy metabolism. Elevated plasma Hcy and the accumulation of SAH were observed in rats with disturbed methionine metabolism (*5*, *6*). This suggests that metabolic situations in which plasma Hcy is increased by intravenous infusion of Hcy is more likely to induce the accumulation of SAH.

In this study, intravenous infusion of Hcy was performed to make transient high plasma Hcy levels in rats. Plasma Hcy concentration after intravenous infusion of physiological saline or Hcy solution is shown in Fig. 1. The 15-Hcy and 30-Hcy groups showed significant increase in plasma Hcy concentrations. Plasma Hcy concentrations in the 15-Hcy and 30-Hcy groups were approximately 46 times and 73 times higher as

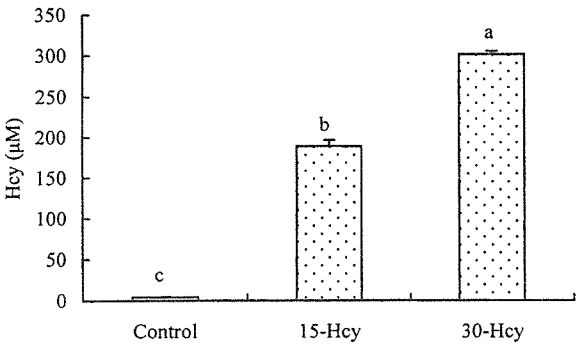

Fig. 1. Concentration of homocysteine in plasma. After 1 h infusion of physiological saline or each concentration of Hcy solution, plasma Hcy concentration was measured by HPLC following the method described by Yamaguchi et al. (*10*). Values without a common superscript letter are significantly different at $p<0.05$. Values are mean±SE for seven rats.

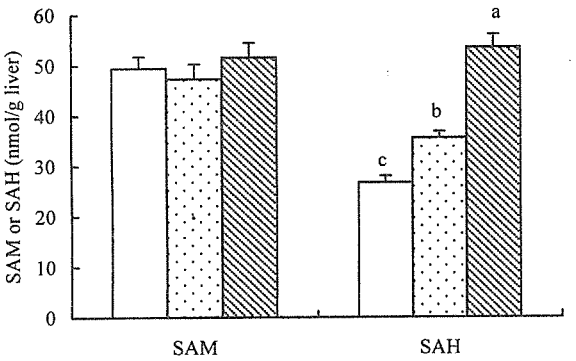

Fig. 2. Contents of SAM and SAH in liver. After 1 h infusion of physiological saline or each concentration of Hcy solution, rats were dissected and livers were excised immediately. SAM and SAH in livers were measured by HPLC following the method of She et al. (*11*). White bars: Control group, dotted bars: 15-Hcy group (15 mg/mL Hcy), hatched bars: 30-Hcy group (30 mg/mL Hcy). Values without a common superscript letter are significantly different at $p<0.05$. Values are mean±SE for seven rats.

compared to that in the Control group, respectively. This result showed that the elevated plasma Hcy concentration was achieved by intravenous infusion of Hcy.

As shown in Fig. 2, the content of hepatic SAH in the 15-Hcy and 30-Hcy groups were significantly higher than that in the Control group. There was a positive correlation ($r=0.877$, $p<0.001$) between the amount of infused Hcy and SAH content in liver. On the other hand, the content of SAM in liver did not increase by Hcy infusion (Fig. 2). Fu et al. (*9*) and Kloor et al. (*13*) reported significantly increased SAH in examined tissues of the rats received Hcy infusion, while SAM was not significantly different as compared to the Control group. The results of our study were similar to their reports. The content of SAM in liver might not be affected by the elevated plasma Hcy unlike the case of

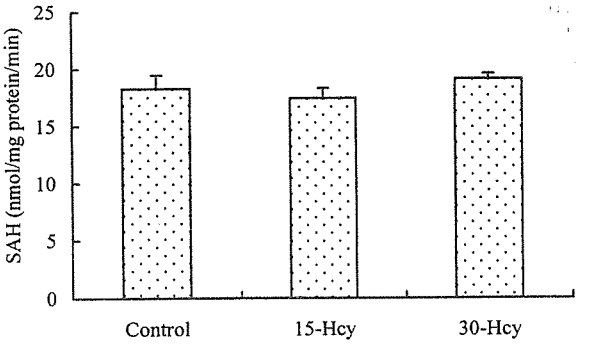

Fig. 3. Activity of SAH hydrolase in liver. SAH hydrolase activity was measured by HPLC following the modified method described by She et al. (*11*). SAH-synthetic activity of SAH hydrolase was estimated by increased SAH. Values are means±SE for seven rats.

SAH. Moreover, a decrease in the SAM/SAH ratio, which is used as an indicator of methylation capacity, was significant under transient high plasma Hcy levels (1.85±0.09, 1.33±0.08, and 0.96±0.02 in the Control, 15-Hcy, and 30-Hcy groups, respectively). The decrease of the ratio indicated a block in methylation reactions (*14*). Therefore, the accumulation of SAH might be caused by the SAH-synthetic reaction.

Under physiological conditions, SAH hydrolysis works predominantly, in which Hcy and adenosine are removed efficiently via the remethylation and transsulfuration pathways. In contrast, when nutritional deficiencies (*2–4*) and genetic mutations (*5, 6*) induced plasma Hcy elevation, the SAH-synthetic reaction was surmised to be favored. However, under these conditions, the SAH-synthetic activity of SAH hydrolase was not examined. In this study, the SAH-synthetic activity of SAH hydrolase in liver extracts was assayed under transient high plasma Hcy levels (Fig. 3). SAH-synthetic activity of SAH hydrolase in liver extracts showed no significant difference among the three groups. In addition, the ratio of SAH hydrolase mRNA versus β-actin mRNA in liver extracts (0.81±0.06, 0.76±0.02, and 0.89±0.04 in the Control, 15-Hcy, and 30-Hcy groups, respectively) showed no significant difference among the three groups. Thus SAH hydrolase mRNA in liver extracts could not be upregulated by transiently elevated plasma Hcy concentration. These results indicate that the accumulation of hepatic SAH is likely to be caused by a SAH-synthetic reaction although its activity was unchanged in liver.

In our previous study when B_6-deficient rats were used, an increase in the SAH-synthetic activity of SAH hydrolase was observed under elevated plasma Hcy and accumulated hepatic SAH (*7*). However, in this study using normal rats, transient high plasma Hcy induced the accumulation of SAH without any change in SAH-synthetic activity of SAH hydrolase. In this study, Hcy rapidly decreased from plasma after discontinuance of intravenous Hcy infusion (data not shown), which showed that further metabolism of Hcy was working

properly. SAH-synthetic activity of SAH hydrolase may change under conditions of abnormal methionine metabolism in which remethylation and transsulfuration pathways are disturbed. Our next concern is the effects of long-term infusion of Hcy on methionine metabolism including the SAH-synthetic activity of SAH hydrolase.

In conclusion, this study showed that there was an increase in hepatic SAH without any increase in SAH-synthetic activity of SAH hydrolase in liver extracts under transient high plasma Hcy levels.

REFERENCES

1) Selhub J. 1999. Homocysteine metabolism. *Annu Rev Nutr* **19**:217–246.
2) Smolin LA, Benevenga NJ. 1982. Accumulation of homocysteine in vitamin B_6 deficiency: a model for the study of cystathionine β-synthase deficiency. *J Nutr* **112**: 1264–1272.
3) Smolin LA, Benevenga NJ. 1984. Factors affecting the accumulation of homocyst(e)ine in rats deficient in vitamin B_6. *J Nutr* **114**: 103–111.
4) Huang R-FS, Hsu YC, Lin HL, Yang FL. 2001. Folate depletion and elevated plasma homocysteine promote oxidative stress in rat livers. *J Nutr* **131**: 33–38.
5) Caudill MA, Wang JC, Melnyk S, Pogribny IP, Jernigan S, Collins MD, Santos-Guzman J, Swendseid ME, Cogger EA, James SJ. 2001. Intracellular *S*-adenosylhomocysteine concentrations predict global DNA hypomethylation in tissues of methyl-deficient cystathionine β-synthase heterozygous mice. *J Nutr* **131**: 2811–2818.
6) Choumenkovitch SF, Selhub J, Bagley PJ, Maeda N, Nadeau MR, Smith DE, Choi SW. 2002. In the cystathionine β-synthase knockout mouse, elevations in total plasma homocysteine increase tissue *S*-adenosylhomocysteine, but responses of *S*-adenosylmethionine and DNA methylation are tissue specific. *J Nutr* **132**: 2157–2160.
7) Isa Y, Tsuge H, Hayakawa T. 2006. Effect of vitamin B_6 deficiency on *S*-adenosylhomocysteine hydrolase activity as a target point for methionine metabolic regulation. *J Nutr Sci Vitaminol* **52**: 302–306.
8) Kraegen EW, James DE, Bennett SP, Chisholm DJ. 1983. In vivo insulin sensitivity in the rat determined by euglycemic clamp. *Am J Physiol* **245**: E1–E7.
9) Fu W, Dudman NPB, Perry MA, Young K, Wang XL. 2000. Interrelations between plasma homocysteine and intracellular *S*-adenosylhomocysteine. *Biochem Biophys Res Commun* **271**: 47–53.
10) Yamaguchi A, Fukushi M, Mizushima Y, Sato M, Shimizu Y, Kikuchi Y, Takasugi N. 1989. High-performance liquid chromatographic measurement of total homocysteine and total cysteine in blood. *Rinsho Shoni Igaku* **37**: 109–113 (in Japanese).
11) She QB, Nagao I, Hayakawa T, Tsuge H. 1994. A simple HPLC method for the determination of *S*-adenosylmethionine and *S*-adenosylhomocysteine in rat tissues: The effect of vitamin B_6 deficiency on these concentrations in rat liver. *Biochem Biophys Res Commun* **205**: 1748–1754.
12) Ohmori O, Hirano H, Ono T, Abe K, Mita T. 1996. Down-regulation of *S*-adenosylhomocysteine hydrolase in the active methyl transfer system in the brain of genetically epileptic E1 mice. *Neurochem Res* **21**: 1173–1180.
13) Kloor D, Delabar U, Mühlbauer B, Luippold G, Osswald H. 2002. Tissue levels of *S*-adenosylhomocysteine in the rat kidney: effects of ischemia and homocysteine. *Biochem Pharmacol* **63**: 809–815.
14) Hoffman DR, Marion DW, Conatzer WE, Duerre JA. 1980. *S*-Adenosylmethionine and *S*-adenosylhomocysteine metabolism in isolated rat liver. Effects of L-methionine, L-homocysteine, and adenosine. *J Biol Chem* **255**: 10822–10827.

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Effect of Vitamin B_6 Deficiency on *S*-Adenosylhomocysteine Hydrolase Activity as a Target Point for Methionine Metabolic Regulation

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Summary The objective of this study was to clarify the relationship between the accumulation of *S*-adenosylhomocysteine (SAH) and the change in the SAH hydrolase activity in vitamin B_6 (B_6). Male Wistar rats were fed a control diet (control and pair-fed groups) or B_6-free diet (B_6-deficient group) for 5 wk. Although the SAH-synthetic activity of SAH hydrolase significantly increased in the B_6-deficient group, SAH-hydrolytic activity of SAH hydrolase showed no significant difference in the liver among the three groups. On the other hand, SAH hydrolase mRNA in the liver did not show any significant change. Thus, the accumulation of SAH would be due to the increased SAH-synthetic activity of SAH hydrolase. The disturbed methionine metabolism by B_6-deficiency, such as a significant increase of plasma homocysteine, might induce the activation of SAH hydrolase in the direction of SAH synthesis.

Key Words Vitamin B_6, *S*-adenosylhomocysteine hydrolase, *S*-adenosylhomocysteine, homocysteine, methionine metabolism

Vitamin B_6 (B_6) is known as a coenzyme related to amino acid metabolism, including methionine. Methionine is metabolized to *S*-adenosylmethionine (SAM) by L-methionine *S*-adenosyltransferase (EC 2.5.1.6) (*1*, *2*). SAM is a methyl donor for transmethylation in vivo, involving protein, histones, DNA and RNA as cellular methyl acceptors (*3*). SAM is converted to *S*-adenosylhomocysteine (SAH) by some transmethylation reactions, and then SAH is hydrolyzed to homocysteine (Hcy) by SAH hydrolase (EC 3.3.1.1). Hcy was reported to be an independent risk factor for cardiovascular diseases and arterial sclerosis (*4*), so it is important to remove Hcy from the metabolic pathway. Hcy is catabolized by cystathionine β-synthase (EC 4.2.1.22; CBS) and γ-cystathionase (EC 4.4.1.1), both of which require PLP as a coenzyme. This route is called the transsulfuration pathway. On the other hand, Hcy is remethylated to methionine and this route is called the remethylation pathway. This pathway requires betain, folic acid and vitamin B_{12}. Under normal physiological conditions, the remethylation pathway predominates rather than the transsulfuration pathway (5). Additionally, it was reported that appropriate intake of vitamin B_{12}, folic acid and B_6 is important to avoid Hcy accumulation (*6*, *7*).

SAH hydrolase plays an important role in the metabolism of SAH. SAH is reversibly hydrolyzed to adenosine and Hcy by SAH hydrolase. Thermodynamically, an equilibrium of SAH hydrolase reaction favors the direction of SAH synthesis. However, under physiological conditions, SAH hydrolase directs toward the hydrolysis of SAH because adenosine and Hcy are rapidly catabolized.

Our previous report showed the accumulation of SAH and an increase of the SAH-synthetic activity of SAH hydrolase in the liver and thymus of B_6-deficient rats (*8*). This indicated the possibility that SAH accumulated in the liver and thymus due to a dominant increase in the SAH-synthetic activity of SAH hydrolase. Since SAH hydrolase is a reversible enzyme, it is needed to examine whether the SAH-hydrolytic activity of SAH hydrolase was also activated or SAH hydrolase, per se, increased during B_6 deficiency. Therefore, we measured the activity of SAH hydrolase in both directions and examined the expression of SAH hydrolase mRNA to clarify the mechanism of SAH accumulation during B_6 deficiency.

MATERIALS AND METHODS

Reagents. Somnopentyl® was purchased from Schering-Plough Co. (NJ, USA). SAH, SAM, adenosine and L-homocysteine thiolactone were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). SBD-F (7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate) and 1-heptanesulfonic acid sodium salt were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Methanol and acetonitrile were purchased from Kanto Kagaku Co. (Tokyo, Japan). Other reagents were purchased from Nacalai Tesque Inc. (Kyoto, Japan) unless otherwise indicated.

Animal and diets. Male Wistar rats, 4-wk old and weighing 80–100 g, obtained from Japan SLC, Inc. (Hamamatsu, Japan) were acclimatized on AIN-76 diet

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