TABLE 3 The classifications of FOSHU

Classification	Content
FOSHU	These are allowed to use labels that inform consumers who ingest the food for specific health purposes that their purpose may be achieved by consuming the product.
"Qualified" FOSHU	These are allowed to use qualified or conditional labels that inform consumers who ingest the food for specific health purposes that their purpose may be achieved by consuming the product. With the aim of promoting the provision of proper information to people, it was decided to allow health claims with some conditions (qualified) under the FOSHU system for food products that do not have sufficient scientific evidence required in the course of current licensing examination procedures but are considered to have certain efficacy.
"Standardized" FOSHU	These are the FOSHU for which a license/approval is granted on the basis of compliance with the separately prescribed standards
"Reduction of Disease-Risk" FOSHU	A FOSHU with a label containing any indication of a reduction of risks of developing certain diseases. At this moment, foods containing calcium or folic acid are permitted. However, in issuing an approval, the label will be required to contain a sufficient warning that the relevant disease has many risk factors and that sufficient exercise is also required for healthy life The label also will be required to contain a sufficient warning for excessive intake.

The government entrusts the training or education of the professionals to private sectors but does not nationally license them. To address this problem, the National Institute of Health and Nutrition is creating a database of evidence-based information on the effectiveness, safety, and interactions with drugs of "health foods" that are manufactured and marketed mainly in Japan. Any person can access the database on the website (10).

Many "health foods" that are unknown even to specialists in the area are marketed at shops, mail-ordered, sold door-to-door, and imported privately over the Internet system in Japan. Some of these can carry illegal health claims, and others can contain medicinal or chemical components with adverse effects. A warning should be given to remove these foods from the market by the central or local government. Thus, more priority should be given to the safety, rather than the effectiveness, of such products. The National Institute of Health and Nutrition operates a network center to which information on adverse effects of such "health foods" is sent by monitors, most of whom are dietitians and pharmacists. If several cases with a specific adverse effect are referred to the center, the cause or the "health food" can be easily identified. The information is then passed on to consumers through the monitors, who are the professionals in **Figure 5**. It is expected that through this feedback system, large-scale epidemics from unsafe additives will be preventable.

Construction of a safety/effectiveness information network

A major, recent problem with health foods is the presence of unreliable information on television, in magazines, and on the Internet. Reliable knowledge and information on health foods must be provided to consumers so that health foods can be used safely and with confidence. Therefore, it is necessary to provide information to consumers through each medium concerning *1*) basic knowledge about health foods and the laws concerning them, *2*) scientifically based information on safety/effectiveness of health foods and food elements, and *3*) reports of health disturbances associated with health foods around the world. This information should be prepared and provided from the consumer's point of view. Also necessary, is an intelligent system that can effectively determine health problems that may occur in high-risk groups, when certain health foods are consumed.

To execute the above-stated plans, the MHLW and the National Institute of Health and Nutrition of Japan have begun to build an information network system to collect and accumulate information related to health foods. Information about product safety is provided specifically from the consumer's point of view, and all information is based on scientific reports. This information has been posted on the homepage (10: http://hfnet.nih.go.jp/ in Japanese) of the National Institute of Health and Nutrition since July 2004. Although the information on this Web page can be accessed rapidly and effectively, some consumers may have difficulty understanding the material. Thus, we have provided 2 methods of accessing information, as shown in Figure 5. First, the precise method provides each consumer with individual attention through professionals such as dietitians, pharmacists, medical doctors, and researchers with direct conversation. Second, the rapid and effective method provides information through the Internet using the homepage (10). The former can provide information correctly and exactly, and the latter can give information quickly and effectively. In addition, we have created a members' page, which is for professionals such as dietitians, pharmacists, medical doctors, and nutritionists who can apply to be members. On the members' page, it is possible to exchange information between

TABLE 4 Reduction of disease-risk FOSHU

Functional ingredient	Health claim approved	Precautions in ingestion
Calcium Daily intake of calcium from the FOSHU products should be between 300 and 700 mg.	This product contains adequate calcium Intake of a proper amount of calcium contained in healthy meals with appropriate exercise may support healthy bones of young women and reduce the risk of osteoporosis when aged.	Diseases are generally caused by various factors Excessive ingestion of calcium will not eliminate the risk of developing osteoporosis.
Folic acid Daily intake of calcium from the FOSHU products should be between 400 and 1000 μg	This product contains adequate folic acid. Healthy meals containing an appropriate amount of folic acid may support healthy fetal development in pregnant women and allow them to bear healthy babies by reducing the risk of neural tube defects such as spina bifida.	Diseases are generally caused by various factors. Excessive ingestion of folic acid will not eliminate the risk of giving birth to a child with a neural tube defect.

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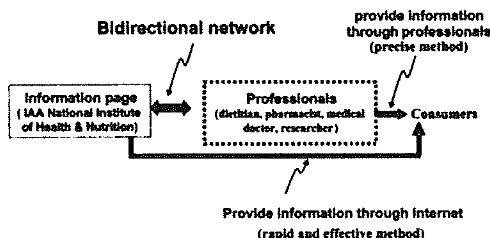

FIGURE 5 Two methods for providing information to consumers. The first is a precise method through professionals, and the second is a rapid and effective method via the Internet using the homepage.

and among members, who can log on as permitted by the National Institute of Health and Nutrition, to receive additions/revisions of the provided information in the homepage and to obtain new scientific publications related to health foods. It is also possible to receive case reports resulting from the use of health foods in our country. The network between the professionals and our Institute is bidirectional, which is helpful for developing our system and providing up-to-date information for consumers. The content currently provided on the homepage includes *1*) fundamental knowledge of health foods and functional foods, FHC, evaluated by MHLW, *2*) current and past health problems associated with the intake of functional foods and health foods, *3*) information on the ingredients and foods that have received recent attention, and *4*) a database of scientifically proven information on foods and food ingredients.

The use of health foods is an international trend; thus, the importation of illegal health foods from overseas is increasing, and health food problems have become increasingly complicated. Our monitoring system can help to cope with this situation. The information that we have obtained is from scientific publications and is therefore reliable, although the understanding of existing information may change when new, reliable information appears. In the future, we hope to continue with the collection and accumulation of this important information.

The current Japanese system for regulation of health foods, designated FHC, consists of 2 categories: FNFC and FOSHU. The label FNFC may be freely used if a product satisfies the standard for the minimum and maximum levels per daily portion consumed. FOSHU are those that contain dietary ingredients that have beneficial effects on the physiological functions of the human body, to maintain and promote health and improve health-related conditions. Health claims on FOSHU correspond to other function claims of the Codex Alimentarius or structure/function health claim in the United States. However, "reduction of disease-risk" claims are not allowed, except for calcium and folic acid. In terms of approval, the Food Safety Commission examines the safety of the product, and the Pharmaceutical Affairs and Food Sanitation Council evaluates its effectiveness. Subsequently, the MHLW individually approves claims, which allows the manufacturer to officially carry the claim and special FOSHU logotype on their product. To make the system beneficial for consumers, training for experts who are able to educate, teach, and counsel about "health foods" is extremely important.

To maintain and improve the health status of people and to prevent chronic noncommunicable diseases, Japanese authorities recommend a diet balanced with staple foods, such as steamed rice, fish, meat, chicken, eggs, and tofu, and side dishes including vegetables as key dietary elements. Healthy individuals should obtain all necessary energy, nutrients, and nonnutritious components from regular meals. They should never take "quasi-drugs" containing vitamins and minerals and "health foods" including FHC in place of their daily diet and ordinary meals.

Other articles in this supplement include references (11–20).

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Review

Foods with Health Claims in Japan

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The Japanese Ministry of Health, Labour and Welfare (MHLW) established the "Foods for Specified Health Uses (FOSHU)" designation in 1991 as a regulatory system for the approval of health claims on labels that refer to the physiological effects of foods or food components on the human body. This labeling system has been revised twice, most recently in 2005. Thus, "Food with Health Claims" (FHC) in Japan consists of two categories. One is "Foods with Nutrient Function Claims" (FNFC), for which health claims on labels can be freely used if the product satisfies the standard for the minimum and maximum levels per daily portion of consumption; the other is FOSHU. FOSHU are foods that contain beneficial dietary ingredients that maintain or promote human health, or that improve risk factors for lifestyle-related diseases. In the present system, FOSHU health claims are individually reviewed and approved on the basis of scientific evidence. In addition to the existing FOSHU, three new types of classifications were established in February 2005: the new regulatory standards of "Standardized FOSHU" and "Qualified FOSHU" and the new health claim "Reduction of disease risk".

Keywords: health claim, FOSHU, FNFC, Japan

Introduction

Since the early 1980s, functionalities of food or food components has become an active area of research and development in Japan, and support for many of these investigations is provided the Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT). The Japanese scientific academy defined functional food as a food which has a tertiary or physiologically active function. The tertiary function of food is a physiological function, such as the regulation of a physical condition or the prevention of certain kinds of diseases. Thus, functional foods are assessed as foods with tertiary function. Research supported by MEXT has provided evidence of a number of physiological functions of foods or food components (Arai, 1996).

Consumption of functional foods has also increased rapidly in recent years due to increased public interest in the prevention of lifestyle-related diseases and improvement of general health against the background of a rapidly aging society. Increasing numbers of consumers now consider health issues to be the primary factor in their purchasing decisions, and manufactures emphasize the health-related characteristics of their products using labels in order to enhance their sales. Therefore, an appropriate regulatory system of labeling for functional foods is important for both consumers and producers. Since consumers can acquire various information such as the content of functional components and usage of foods from labels, it is imperative that the health claims on food labels are based on scientific evidence.

Food labels with health claims should generally meet the following conditions: 1) be compliant with the national nutritional goal and public health policy, 2) be based on the proven usefulness of the food in supplying nutrition or in a specified health effect, 3) be based on acceptable scientific data and use simple and intelligible expressions, 4) offer appropriate information to the consumer, 5) contain any relevant warnings or cautions, including appropriate intake, to prevent adverse effects on health due to overdoses, 6) comply with the Food Sanitation Law, Health Promotion Law, and other related laws, and 7) avoid confusion with drugs and the implication of prevention, treatment and diagnosis of human disease.

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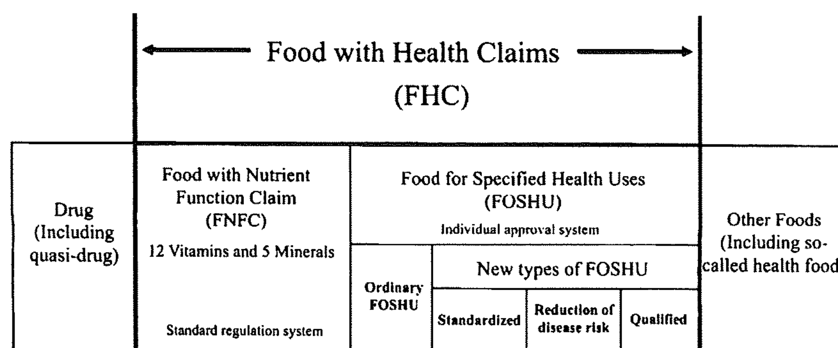

Fig. 1. Classification and class name of Food with Health Claims.

Labeling of nutrient function should be based on internationally recognized findings, i.e., examples of nutrient function claims should include a reference to the Codex Alimentarius and be easy to understand. Food labels with health claims also require attention notices and warnings for intake ranges consistent with safe consumption.

In Japan, the regulatory system to approve health claims on labels was established by the Japanese Ministry of Health, Labour and Welfare (MHLW) in 1991 (Shimizu, 2003; Tanaka, 2004; Ohama, 2006). The current system for regulation of health foods is called "Food with Health Claims" (FHC), the enforcement regulations for which are based on the Food Sanitation Law and the Health Promotion Law (Shokuanhatsu Notification No. 0201002, 2005).

In this paper, we introduce the FHC regulatory system and the principal procedures for its approval in Japan.

Food with Health Claims (FHC) The FHC system, originally established in 2001, was revised on the basis of the "Foods for Specified Health Uses" (FOSHU) system established in 1991; the present regulatory system was finalized in 2005. FHC classifications and class names are illustrated in Figure 1. FHC are defined as foods located between drugs and common foods, including so-called health foods, and are categorized into two classes based on differences in purpose and function: "Foods with Nutrient Function Claims" (FNFC) and FOSHU, in which heath claims are limited to benefits for maintaining or improving health and physical condition. FNFC is the standard regulation system, consisting of 12 vitamins and 5 minerals, while FOSHU is an individual approval system. In the 2005 revisions to FOSHU, three new categories were added: "Standardized FOSHU", "Health Claim for the Reduction of Disease Risk", and "Qualified FOSHU". Once the permission for marketing is granted, the food must carry the logo of approval on the package label (Figure 2). Both labels symbolize "jumping for health".

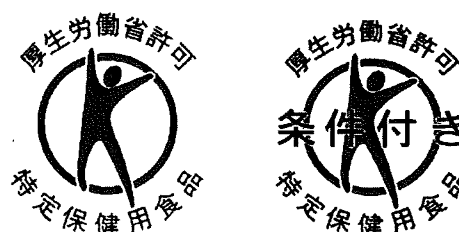

Fig. 2. FOSHU logo of approval, symbolizing "jumping for health".

Application document and procedure for FOSHU approval Applicants for FOSHU approval must submit their documentation to MHLW as listed in Table 1. FOSHU documentation must meet three essential requirements for approval. First, the effectiveness of the functional component should be based on scientific evidence, including human studies. Second, its safety as assessed from historical consumption pattern data, and additional safety studies are required in humans. Third, analytical determination of the functional component responsible for the beneficial physiological function is needed.

The document on the physiological effects on human health should be prepared based on scientific evidence related to not only the effects of the functional component on the physiological functions of humans and animals but also its mechanism of action. In addition, these data should be statistically significant. In order to conduct a proper evaluation, the study must be well designed, that is, utilizing the appropriate biological indices and a sufficient number of subjects to be statistically evaluable.

With respect to the document of safety, both in vivo and in vitro studies should be carried out to assess the safety of intake in humans. Even if the functional component has been

Table 1. Documents required for application for FOSHU designation.

1. Sample of the entire package with labels and health claims.
2. Proof of the clinical and nutritional function of the product and/or its functional component for the maintenance of health.
3. Clinical and nutritional proof of the intake amount of the product and/or its functional component.
4. Documentation concerning the safety of the product and its functional component, including additional human studies and eating experience.
5. Documentation concerning the stability of the product and its functional component.
6. Documentation of the physical and biological characteristics of the product and its functional component.
7. Methods of qualitative/quantitative analytical determination of the functional component, and analytical results on the amount of component in the product.
8. Report on the analysis of the designated nutrient constituents and calorie content of the product.
9. Statement of the production method and equipment of the factory and explanation of the quality control system.

consumed by a number of people for many years, safety data regarding human consumption has to be provided using at least three times the amount of the minimum effective dosage.

The documentation of the methods for the functional components analysis is also required, and must include both qualitative and quantitative analysis.

In addition, a document on the stability of products and functional components should be provided.

It is important that to specify that FOSHU products are designed for healthy individuals and individuals in a preliminary stage or marginal zone between the health and disease stages. Therefore, the physiological effects of FOSHU may be mild compared to medicine for patients (Yamada, 2008).

The Scheme of FOSHU approval is illustrated in Figure 3. The Office of Health Policy on Newly Developed Foods of MHLW initiates the application process for FOSHU in accordance with the receipt of all documents described above. An Expert Committee then evaluates the validity of a FOSHU in either of two sub-committees. One sub-committee assesses products related to lipid metabolism such as cholesterol and neutral fats, obesity, blood pressure, and blood glucose. The other sub-committee evaluates products related to gut condition, mineral absorption, and dental health, among others. After the completion of the evaluation by the Expert Committee, the Pharmaceutical Affairs and Food Sanitation Council and the Food Safety Commission review the *applied* dossier. When the Council determines the application to be appropriate, the MHLW notifies the applicant and requests that the applicant send samples of the product, along with documentation of the analytical method, to the National Institute of Health and Nutrition. The Institute then validates the method and determines the amount of the effective component. After validation and determination of the product has been completed, MHLW makes the decision to grant approval for the product under FOSHU. The applicant is permitted to use the approved health claim on the label with the FOSHU logo.

Health claims for FOSHU FOSHU products have some benefit to human health and physiological functions. As of April 2008, 779 items had been granted FOSHU status, consisting of approximately 70 kinds of effective components. Although it is difficult to classify accurately, the existing health claims for FOSHU generally can be classified as shown below (Saito, 2005; Saito, 2007, Yamada, 2008).

(1) Gastrointestinal (GI) conditions: About half of all FOSHU products have health claims relating to the improvement of GI condition. The effective components are carbohydrates, which can be divided oligosaccharides, dietary fiber and chitosan, and bacteria such as lactic acid bacteria and bifidobacteria. Approved products containing these components can claim that they help increase intestinal bifidobacteria and thus maintain good GI condition.

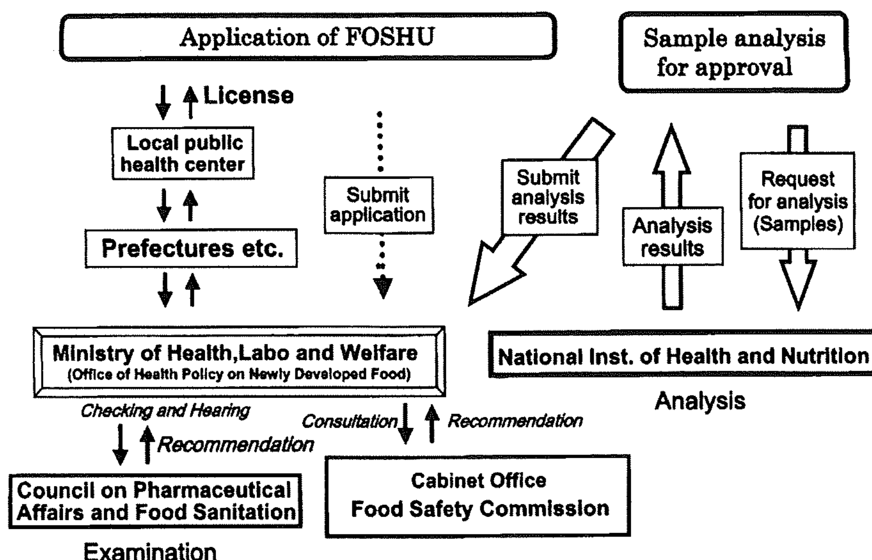

Fig. 3. Scheme of the FOSHU approval.

(2) Blood pressure: Lactotripeptide from fermented milk, dodecapeptide from casein, a group of peptides derived from sardine, dry bonito, soybean protein and salmon, and gamma-aminobutyric acid (GABA), as well as Tochu leaf glycoside (geniposidic acid), are proposed to reduce blood pressure. Approved products containing these components can claim beneficial effects for people with moderately high blood pressure.

(3) Blood glucose: Effective components include indigestible dextrin, wheat albumin, L-arabinose, and guava tea polyphenol. Approved products containing these components can claim that these materials are helpful for those who are concerned about their blood glucose level.

(4) Blood cholesterol: The effective components are soybean protein, chitosan, degraded sodium alginate and phytosterol. Approved products containing these components can claim that they help decrease serum (blood) cholesterol level.

(5) Blood neutral fat: Globin digest and diacylglycerol (DAG) can suppress the elevation of blood neutral fat level after a meal. Therefore, approved products containing these components can claim that they help to reduce postprandial blood triglyceride (neutral fat) levels. In addition, a mixture of eicosapentaenoic acid and docosahexaenoic acid is also believed to decrease blood neutral fat level. This product can claim that beneficial effects for people with moderately high blood triglyceride level.

(6) Body fat accumulation: DAG, structured triacylglycerol combined with medium-chain fatty acids and tea

Table 2. Upper and lower limits of food with nutrient function claims.

Vitamins	Upper limit	Lower limit
Vitamin A (retinol)	600 μg (2000 IU)	135 μg (600 IU)
Vitamin D	5 μg (200 IU)	1.5 μg (60 IU)
Vitamin E	50 mg	2.4 mg
Thiamin	25 mg	0.3 mg
Riboflavin	12 mg	0.33 mg
Niacin	60 mg	3.3 mg
Vitamin B6	10 mg	0.3 mg
Folic acid	200 μg	60 μg
Vitamin B12	60 μg	0.6 μg
Biotin	500 μg	14 μg
Pantothenic acid	30 mg	1.65 mg
Vitamin C	1000 mg	24 mg
Minerals		
Calcium (Ca)	600 mg	210 mg
Iron (Fe)	10 mg	2.25 mg
Zinc (Zn)	15 mg	2.1 mg
Copper (Cu)	6 mg	0.18 mg
Magnesium (Mg)	300 mg	75 mg

catechins can suppress body fat accumulation. Approved products containing these components are permitted to claim that they help suppress body fat accumulation.

(7) Absorption of minerals: Fructo-oligosaccharide, calcium citrate malate and casein phosphopeptide can improve calcium absorption from intestine. Approved products containing these components can claim such effects.

(8) Dental health: Dental health has two categories of health claims. One is anti-cariogenesis, in which some sugar alcohols such as xylitol, maltitol, erythritol, and palatinose are considered to be hypo-cariogenic, while green tea polyphenol is regarded as non-cariogenic. Approved products containing these sugar alcohols can claim that these products are low or non-cariogenic. The other category is tooth health. Compounds such as xylitol and CPP-ACP (casein phosphopeptide-amorphous calcium phosphate compound) can make

Table 3. Nutrient Function Claims for FNFC in Japan.

Nutrient	Nutrition functional claims
Vitamins	
Vitamin A	Vitamin A is a nutrient that helps to maintain vision in the dark.
(β-carotene)	Vitamin A is a nutrient that helps maintain skin and mucosa in a healthy state.
Vitamin D	Vitamin D is a nutrient that promotes calcium absorption in the gut intestine and aids in the development bone.
Vitamin E	Vitamin E is a nutrient that prevents lipid in the body from oxidizing through its antioxidant effect and helps maintenance of healthy cells.
Thiamin	Thiamin is a nutrient that helps energy production from carbohydrate and maintain skin and mucosa in a healthy state
Riboflavin	Riboflavin is a nutrient that helps maintain skin and mucosa in a healthy state.
Niacin	Niacin is a nutrient that helps maintain skin and mucosa in a healthy state.
Vitamin B6	Vitamin B6 is a nutrient that helps energy production from protein and helps maintain skin and mucosa in a healthy state.
Folic Acid	Folic acid is a nutrient that helps red cell formation.
	Folic acid is a nutrient that contributes the normal growth of the fetus.
Vitamin B12	Vitamin B12 is a nutrient that helps red cell formation.
Biotin	Biotin is a nutrient that helps maintain skin and mucosa in a healthy state.
Pantothenic acid	Pantothenic acid is a nutrient that helps maintain skin and mucosa in a healthy state.
Vitamin C	Vitamin C is a nutrient that helps maintain skin and mucosa in a healthy state, and has an antioxidant effect.
Minerals	
Calcium (Ca)	Calcium is a nutrient, which is necessary in the development of bone and teeth.
Iron (Fe)	Iron is a nutrient, which is necessary in red blood cell formation.
Zinc (Zn)	Zinc is a nutrient required to maintain healthy sense of taste.
	Zinc is a nutrient that helps maintain skin and mucosa in a healthy state.
	Zinc is a nutrient that is involved in protein and nucleic acid metabolism and helps heath maintenance.
Copper (Cu)	Copper is a nutrient that helps red cell formation.
	Copper is a nutrient that helps normal functions of enzymes in the body and bone formation.
Magnesium (Mg)	Magnesium is a nutrient required for bone and tooth formation. Magnesium is a nutrient that helps normal functions of enzymes in the body and energy production and that is required to maintain normal blood.

teeth strong and healthy. Approved products containing these components can claim such effects.

(9) Bone health: Vitamin K2, soy isoflavone, milk basic protein and fructo-oligopeptides are regarded as promoters of bone calcification. Approved products containing these components can claim such effects.

The health claims on FOSHU must not express, specify or validate any medical terminology used for human health such as "prevent", "cure", "treat", or "diagnose".

Foods with Nutrient Function Claims (FNFC) MHLW enacted a new regulation system of "Food with Health Claims (FHC)" in 2001. FHC consists of two categories, FOSHU and FNFC. Twelve vitamins including vitamins A, B1, B2, B6, B12, C, E, D, biotin, pantothenic acid, folic acid and niacin, as well as calcium and iron, have been standardized as FNFC. In 2003, three minerals, zinc, magnesium and copper, were added to FNCN. The upper and lower levels for the daily intake of 12 vitamins and 5 minerals have been set as shown in Table 2. The minimum daily level per portion for consumption of the products is 30% of the standard values for the nutrition labeling as per the 2005 edition of the Japanese dietary reference intakes (DRIs) (MHLW, 2005). The maximum level is set as the maximum amount of nutrient items in quasi drugs.

Nutrition Function Claims Nutrient function claims are approved according to the guidelines of the 1997 Codex Alimentarius. These claims have been widely accepted by scientific experts, are based on scientific evidence, are applied to existing foods or supplements internationally, and are readily understood by the general public (Table 3).

Attentions and warnings should be also provided on labels for every nutrient, including information concerning excess intake of the product. The recommended consumption should also be provided.

Conclusion

The current Japanese system for regulation of "health foods", called "Food with Health Claims" (FHC) consists of two categories: "Foods with Nutrient Function Claims" (FNFC) and "Foods for Specified Health Uses" (FOSHU). The FNFC label can be freely used if a product satisfies the standard for the minimum and maximum levels per daily consumption.

FOSHU products contain specified dietary ingredients that have physiologically beneficial effects for human health, that maintain and promote health, or that improve health-related conditions. In terms of approval, the Food Safety Commission examines the safety of the product and the Pharmaceutical Affairs and Food Sanitation Council evaluates its effectiveness. Subsequently, the MHLW approves individual claims, which allow the manufacturer to officially display the health claims and the FOSHU logo on their products.

In order to maintain and improve human health and to prevent lifestyle related diseases, Japanese authorities recommend the intake of a well-balanced diet. Healthy individuals should obtain all necessary energy, nutrients and non-nutritious components from regular meals.

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Bilobalide in ginkgo biloba extract is a major substance inducing hepatic CYPs

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Abstract

In a search for substances related to the marked induction of hepatic cytochrome P450 (CYP) by ginkgo biloba extract (GBE), mice were given either GBE (1000 mg kg^{-1}) or fractions of GBE for 5 days. The content and activity of CYPs were induced markedly by a bilobalide-rich fraction, but not by flavonoid-rich fractions. The level of induction by the bilobalide-rich fraction was almost the same as that induced by the unfractionated GBE, suggesting that bilobalide is largely responsible for the CYPs induction. To confirm these findings, mice were given various doses of bilobalide (10.5, 21 and 42 mg kg^{-1}), or GBE (1000 mg kg^{-1}, containing bilobalide at 42 mg kg^{-1}). Treatment with bilobalide induced CYPs markedly and in a dose-dependent manner, and the level of induction was quite similar between bilobalide (42 mg kg^{-1}) and GBE. Treatment with GBE and with bilobalide greatly induced pentoxyresorufin O-dealkylase activity. These findings indicate that bilobalide is the major substance in GBE that induces hepatic CYPs.

Introduction

Ginkgo biloba extract (GBE) is a popular herbal medicine, and is used mostly by the elderly population for the treatment of cerebral insufficiency, dementia and intermittent claudication (Le Bars et al 1997; Blumenthal 1998; Pittler & Ernst 2000). It has been recognized that GBE exerts many pharmacological effects with few adverse reactions. The adverse reactions include headache, gastric symptoms, diarrhoea and allergic skin reactions, but reports of these are rare (De Smet 2002; Ernst 2002).

Recently, adverse reactions to herbal remedies as the result of herb–drug interactions have received a great deal of attention. For example, St John's wort (*Hypericum perforatum*) induces hepatic cytochrome P450 (CYP)3A4 activity and thereby reduces the efficacy of therapeutic drugs such as ciclosporin, indinavir and digoxin (Roby et al 2000). People who take GBE tend to be elderly because of the reported beneficial effects (De Smet 2002; Ernst 2002) but GBE–drug interactions are a matter of concern. In previous studies, we found that feeding GBE to rats increased markedly the concentration of hepatic CYP, the expression of various CYP mRNAs and the activity of some enzymes (Shinozuka et al 2002; Umegaki et al 2002). Moreover, we reported that pretreatment of rats with GBE attenuated the efficacy of co-administered drugs such as tolbutamide (Sugiyama et al 2004a), nicardipine (Kubota et al 2003) and phenobarbital (Kubota et al 2004). Similar findings in rats were reported by other investigators using EGb761, a well-known standardized GBE extract (Chatterjee et al 2005; Zhao et al 2006). In contrast to these rat studies, reports of GBE–drug interactions in clinical studies are inconsistent, and many of them showed no interaction (Markowitz et al 2003; Mauro et al 2003; Jiang et al 2005; Greenblatt et al 2006; Lu et al 2006; Wolf 2006).

GBE is a natural plant product that contains many different chemicals. Most of the commercially available GBE products are standardized according to the content of ginkgo flavonol glycosides (glycosidic derivatives of quercetin, kaempferol, isorhamnetin) and terpenoids (ginkgolides A, B, C, and bilobalide), which comprise 22–27% and approximately 5–7% of GBE, respectively (Blumenthal 1998; DeFeudis 1998). GBE products contain 0.5–1% organic acids, such as vanillic acid and *p*-hydroxybenzoic acid. The exact constituents of GBE may

vary among the products. Without identifying a substance that induces hepatic CYPs, it is difficult to elucidate the GBE–drug interactions. For this purpose, cell-free studies using human and rat hepatic microsomes have been performed; however, there are discrepancies between the results of in-vitro and in-vivo studies (Gaudineau et al 2004; Sugiyama et al 2004c; Mohutsky et al 2006). To our knowledge, there has been little research intended to identify a substance in GBE that relates to the induction of hepatic CYPs in-vivo.

In this study, we prepared fractionated GBE samples and identified a substance that induces hepatic CYPs in an animal study. To minimize the amount of test samples, we used mice instead of rats, which were used in our previous studies (Shinozuka et al 2002; Umegaki et al 2002; Sugiyama et al 2004a). The results of this study provide important indications for the elimination of GBE–drug interactions and for the standardization of GBE products in the market.

Materials and Methods

Materials

Powdered ginkgo biloba extract (GBE) was supplied by Tama Seikagaku-Kogyo Co. (Tokyo, Japan). GBE contained 24.9% flavonoids and 10.6% total terpene, which consisted of 2.9% ginkgolide A, 1.4% ginkgolide B, 2.1% ginkgolide C and 4.2% bilobalide. Resorufin, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, testosterone, 6β-hydroxytestosterone, corticosterone, *p*-nitrophenol, 4-nitrocatechol, 7-ethoxycoumarin, quercetin, ginkgolide A, ginkgolide B, ginkgolide C and bilobalide were purchased from Sigma-Aldrich (St Louis, MO). (*S*)-Warfarin and 7-hydroxywarfarin were obtained from Ultrafine (Manchester, UK). NADPH was obtained from Oriental Yeast (Tokyo, Japan). All other reagents were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan).

Preparation of fractionated GBE samples

Powdered GBE (100 g) was dissolved in water (2000 mL). Diethyl ether (2000 mL) was added and the mixture was shaken in a separating funnel to prepare a diethyl ether extract, which was evaporated under vacuum to dryness (fraction 1, yield 12.11 g). Then, the aqueous layer was extracted with ethyl acetate (2000 mL) to prepare fraction 2 (yield 15.81 g). The resulting aqueous layer was dried, acetone (1000 mL) was added and the mixture was stirred for 4 h, then filtered to obtain the acetone-soluble matter, which was evaporated to dryness (fraction 3, yield 21.79 g). Methanol was added to the acetone-insoluble residue (1000 mL), and the methanol-soluble matter was prepared as described for fraction 3 (fraction 4, yield 38.15 g). Deionized water (500 mL) was added to the methanol-insoluble residue; the mixture was stirred, filtered and dried (fraction 5, yield 8.02 g). Finally, 0.05% NaOH (500 mL) was added to the insoluble residue; the mixture was stirred, filtered and dried (fraction 6, yield 1.84 g). Terpenoids (ginkgolide A, ginkgolide B, ginkgolide C and bilobalide) in the samples were determined by HPLC with an evaporative light-scattering detector (Shimadzu, Kyoto) according to the method of Tang et al (2003). For the analysis of flavonoids (quercetin, isorhamnetin, kaempferol), samples were treated with 6% HCl in 60% aqueous methanol at 90 °C for 60 min to hydrolyse flavonoid glycosides, and the resulting flavonoid aglycones were determined by HPLC with a UV detector measuring absorbance at 360 nm (Shimadzu, Kyoto, Japan). The HPLC conditions were as follows: column material, L-column ODS (4.6 mm × 250 mm; Chemical; Inspection & Testing Institute, Tokyo, Japan); temperature, 40°C; flow-rate, 1 mL min^{-1}; mobile phase, 0.5% citric acid–acetonitrile–isopropanol (100:35:4, by vol.). Typical chromatograms of unfractionated GBE sample are shown in Figure 1.

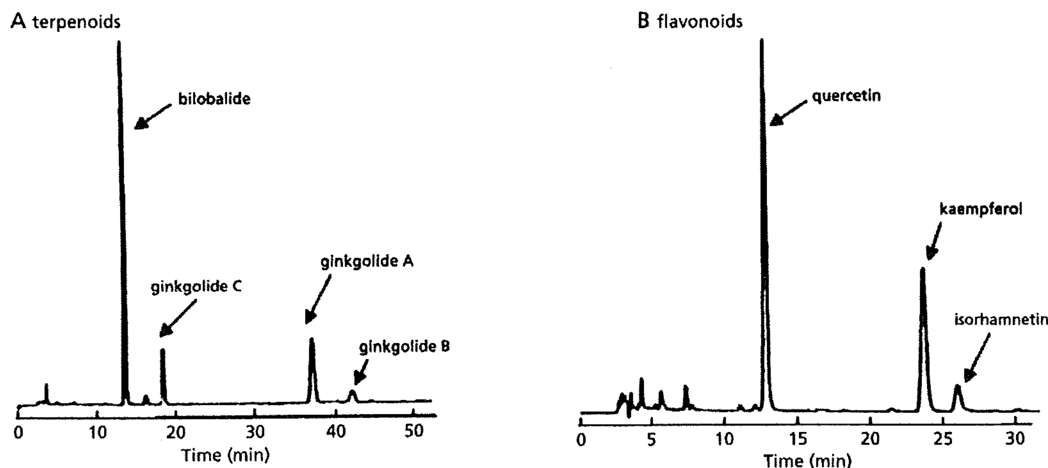

Figure 1 HPLC chromatogram of terpenoids (A) and flavonoids (B) in unfractionated GBE sample. Terpenoids (ginkgolide A, ginkgolide B, ginkgolide C, bilobalide) were detected by HPLC with evaporate light-scattering detector. Flavonoids aglycones (quercetin, isorhamnetin, kaempferol) were detected by HPLC with a UV detector (360 nm). Detailed conditions are shown in the text.

Animal experiments

Male ICR mice, 5 weeks old (Japan Clea, Tokyo, Japan), were kept at a constant temperature (23 ± 1°C) with a 12-h light–dark cycle in polypropylene cages with free access to laboratory feed (CE2; Japan Clea) and tap water. Mice were given the test samples orally (GBE, various fractions of GBE, or bilobalide) suspended in 0.5% carboxymethylcellulose or vehicle for 5 days. One day after the last administration, mice were anaesthetized with pentobarbital and sacrificed. The liver was removed immediately and weighed. All procedures were in accordance with the National Institute of Health and Nutrition guidelines for the Care and Use of Laboratory Animals, and approved by the ethical committee.

Preparation of microsomes from the liver and analysis of CYP enzyme activity

The liver was rinsed with 0.9% (w/v) NaCl, and homogenized in 50 mmol L^{-1} Tris-HCl buffer (pH 7.4) containing 0.25 mol L^{-1} sucrose. The homogenate was centrifuged at 10 000 *g* at 4°C for 30 min. The supernatant was centrifuged at 105 000 g at 4°C for 60 min. The pellet was washed once with 50 mmol L^{-1} Tris-HCl buffer (pH 7.4) containing 0.25 mol L^{-1} sucrose by centrifugation at 105000 *g* at 4°C for 60 min, and the concentration and activity of CYP were analysed. The CYP content was quantified by the method of Omura & Sato (1964), and the activity of various CYP enzymes were determined by HPLC methods as described (Umegaki et al 2002). The subtypes of CYP enzymes examined and the corresponding CYPs were: ethoxyresorufin O-deethylase, CYP1A1; methoxyresorufin O-demethylase, CYP1A2; pentoxyresorufin O-dealkylase, CYP2B; (*S*)-warfarin 7-hydroxylase, CYP2C9; p-nitrophenol hydroxylase, CYP2E1; and testosterone 6*β*-hydroxylase, CYP3A.

Protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL).

Statistical analysis

The data are presented as the mean and standard deviation (s.d.) for the individual groups. Statistical analysis of the data was carried out by analysis of variance with Fisher's PLSD post-hoc test. $P < 0.05$ was considered to be significant. These statistical analyses were performed with Stat View 5.0 (ASA Institute Inc., Cary, NC).

Results

A preliminary study showed that oral administration of GBE to mice at doses of 100 mg kg^{-1} and 1000 mg kg^{-1} induced an increase of the hepatic content and activity of CYPs in a dose-dependent manner, as observed in the earlier study with rats (Umegaki et al 2002). Thus, in this mouse study, screening for a substance that induced hepatic CYPs in GBE was performed at a dose of 1000 mg kg^{-1}. As shown in Table 1, GBE was separated into fractions 1–6. Typical substances were terpenoids in fractions 1 and 2 and flavonoids in fractions 3 and 4. In particular, fraction 1 contained the greatest amount of bilobalide (80% of the GBE). For 5 days, mice were given either GBE (1000 mg kg^{-1}) or a sample of one of the fractions, the dose of which was equivalent to the amount contained in GBE (1000 mg kg^{-1}). No significant influence of body weight or liver weight was observed due to the administration. Induction of an increased CYP content was greatest for fraction 1 and lower for fractions 3–6 (Figure 2). The level of induction was comparable between fraction 1 and GBE. Also, the activity of various CYPs was highest with fraction 1, and the level was almost the same as that induced by GBE (Table 2). These findings suggested that bilobalide is the major component of GBE that induces hepatic CYPs. To confirm this hypothesis, mice were given various doses of bilobalide (10.5, 21 and 42 mg kg^{-1}), or GBE (1000 mg kg^{-1}) that contained bilobalide at a dose of 42 mg kg^{-1}. The administration of bilobalide induced an increase in the content and activity of CYPs in a dose-dependent manner (Figure 3, Table 3). The induction levels of the CYPs at the 42 mg kg^{-1} dose of bilobalide and GBE were quite similar. GBE markedly induced pentoxyresorufin O-dealkylase activity, as reported previously (Umegaki et al 2002), and marked induction of pentoxyresolfin O-dealkylase by the administration of bilobalide was detected in this study. The enzyme induction by bilobalide at the dose of 10.5 mg kg^{-1} was 13.8 times higher than in the untreated control (Table 3).

Table 1 Major constituents in fractionated samples of ginkgo biloba extract (GBE)

Fraction No.	Amount in GBE (%)	Content in fractionated samples from GBE						
		Flavonoids			Terpenoids			
		Quercetin	Kaempferol	Isorhamnetin	Ginkgolide A	Ginkgolide B	Ginkgolide C	Bilobalide
1	12.11	2.93	0.42	0.34	7.64	5.36	3.33	15.33
2	15.81	12.46	12.65	0.31	5.18	1.77	6.94	3.56
3	21.79	15.42	10.92	2.60	ND	ND	0.1	ND
4	38.15	14.05	9.32	1.74	ND	ND	ND	ND
5	8.02	6.53	4.24	0.78	ND	ND	ND	ND
6	1.84	2.43	2.52	0.06	ND	ND	ND	ND

ND, not detected.

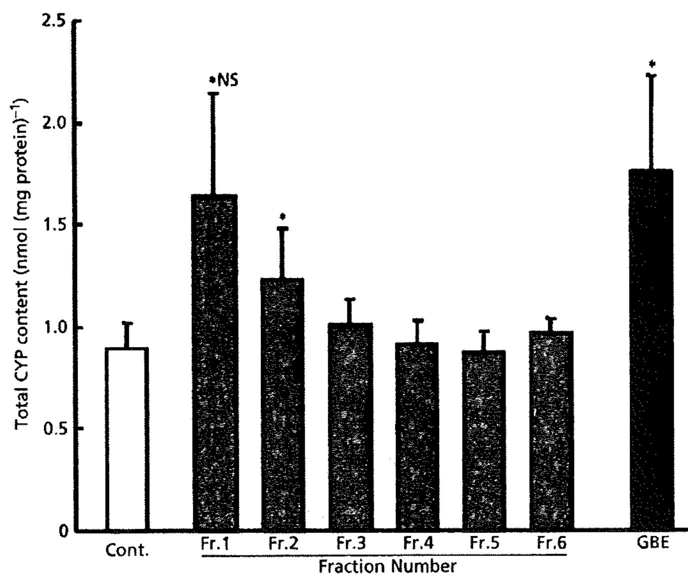

Figure 2 Hepatic CYP content in mice given either ginkgo biloba extract (GBE) or its fractionated samples. Mice were given either GBE (1000 mg kg^{-1}) or fractionated GBE samples (at the same amount of each constituent in GBE) for 5 days. The dose of the fractionated samples was 121 mg kg^{-1} in fraction 1, 158 mg kg^{-1} in fraction 2, 218 mg kg^{-1} in fraction 3, 382 mg kg^{-1} in fraction 4, 80 mg kg^{-1} in fraction 5 and 18 mg kg^{-1} in fraction 6. Each value is expressed as the mean ± s.d. for 5 mice. *$P < 0.05$ vs control; ^{NS}not significant vs GBE (1000 mg kg^{-1}).

Table 2 Activity of hepatic CYPs in mice given either ginkgo biloba extract (GBE) or its fractionated samples

	Control	**Fractionated GBE**						**GBE 1000 mg kg^{-1}**
		Fraction 1	Fraction 2	Fraction 3	Fraction 4	Fraction 5	Fraction 6	
Dose (mg kg^{-1})	0	121	158	218	382	80	18	
	Activity (pmol (mg protein)$^{-1}$ min^{-1})							
Ethoxyresorufin O-deethylase (CYP1A1)	42.9±8.6	123.6±37.3 [2.9]*^{NS}	64.8±21.6 [1.5]	56.9±11.8 [1.3]	35.6±15.4 [0.8]	31.4±7.7 [0.7]	33.0±8.9 [0.8]	101.1±39.3 [2.4]*
Methoxyresorufin O-demethylase (CYP1A2)	35.4±8.7	75.5±12.8 [2.1]*^{NS}	46.6±14.1 [1.3]	37.2±5.7 [1.1]	30.4±6.7 [0.9]	25.8±3.8 [0.7]	23.2±4.4 [0.7]	58.8±17.8 [1.7]*
Pentoxyresorufin O-dealkylase (CYP2B)	6.8±2.3	113.5±41.2 [16.7]*^{NS}	34.8±13.4 [5.1]*^{NS}	8.7±3.3 [1.3]	6.4±1.3 [0.9]	5.5±1.4 [0.8]	8.1±1.7 [1.2]	116.1±32.0 [17.1]*
(s)-Warfarin 7-hydroxylase (CYP2C9)	1.12±0.22	3.25±1.32 [2.9]*^{NS}	1.79±0.54 [1.6]	1.52±0.48 [1.4]	1.11±0.31 [1.0]	1.04±0.13 [0.9]	1.41±0.31 [1.3]	3.24±1.42 [2.9]*
p-Nitrophenol hydroxylase (CYP2E1)	4710±549	6146±1069 [1.3]*^{NS}	4304±946 [0.9]	5316±740 [1.1]	5004±778 [1.1]	4967±888 [1.1]	5082±570 [1.1]	6619±1096 [1.4]*
Testosterone 6β-hydroxylase (CYP3A)	2560±433	5249±1724 [2.1]*^{NS}	3464±550 [1.4]	2428±586 [0.9]	2434±528 [1.0]	2092±171 [0.8]	3147±257 [1.2]	5509±2110 [2.2]*

Mice were given either GBE (1000 mg kg^{-1}) or fractionated GBE samples (Fractions 1–6) at the same amount of each constituent in GBE for 5 days. Each value is expressed as the mean ± s.d. for 5 mice. Number in brackets indicates the increase ratio to control. *$P < 0.05$, vs control; ^{NS}not significant vs GBE (1000 mg kg^{-1}).

Discussion

As GBE is a natural product that gives a variety of products with different compositions, identification of a substance that induces hepatic CYPs is critical for standardizing GBE products and to avoid GBE–drug interactions, which are suggested by studies in animals (Kubota et al 2003, 2004; Sugiyama et al 2004a; Zhao et al 2006) and man (Yin et al 2004; Uchida et al 2006). Various studies have been performed to identify the active substance in GBE, but no clear data have been obtained so far. In this study, we tried to

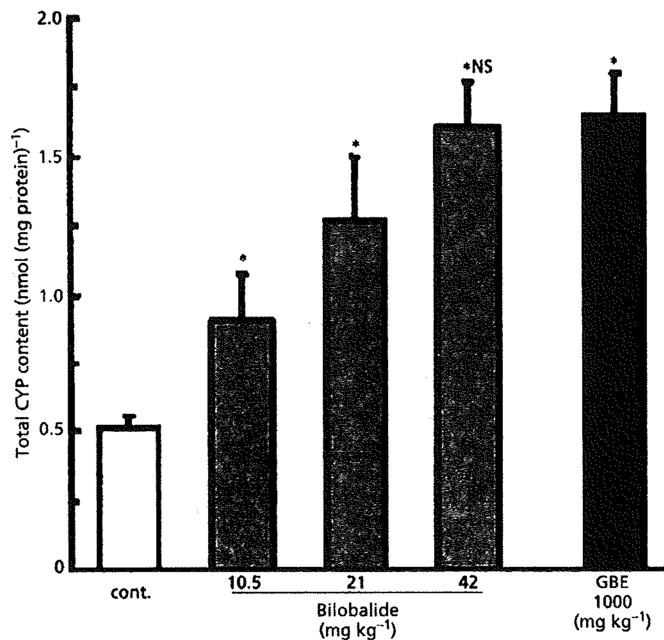

Figure 3 Hepatic CYP content in mice given bilobalide or ginkgo biloba extract (GBE). Mice were administered either bilobalide (10.5, 21, 42 mg kg^{-1}) or GBE (1000 mg kg^{-1}; 42 mg kg^{-1} as bilobalide) for 5 days. Each value is expressed as the mean ± s.d. for 5 mice. *$P < 0.05$ vs control; ^{NS}not significant vs GBE (1000 mg kg^{-1}).

Table 3 Activity of hepatic CYPs in mice given bilobalide or ginkgo biloba extract (GBE)

	Control	Bilobalide			GBE
Dose (mg kg^{-1})	0	10.5	21	42	1000 mg kg^{-1}
	Activity (pmol (mg protein)$^{-1}$ min^{-1})				
Ethoxyresorufin O-deethylase (CYP1A1)	41.4 ± 5.6	96.5 ± 24.9 [2.3]*	133.2 ± 20.9 [3.2]*^{NS}	147.0 ± 24.3 [3.6]*^{NS}	147.0 ± 40.7 [3.6]*
Methoxyresorufin O-demethylase (CYP1A2)	31.4 ± 3.0	76.9 ± 14.9 [2.4]*	108.6 ± 23.3 [3.5]*^{NS}	136.7 ± 15.2 [4.4]*^{NS}	124.7 ± 25.7 [4.0]*
Pentoxyresorufin O-dealkylase (CYP2B)	6.1 ± 1.2	84.3 ± 12.6 [13.8]*	98.6 ± 6.7 [16.2]*^{NS}	113.8 ± 2.6 [18.7]*^{NS}	105.6 ± 13.5 [17.3]*
(s)-Warfarin7-hydroxylase (CYP2C9)	1.07 ± 0.22	2.31 ± 0.76 [2.2]*	3.18 ± 0.49 [3.0]*^{NS}	4.41 ± 0.87 [4.1]*^{NS}	3.83 ± 1.41 [3.6]*
p-Nitrophenol hydroxylase (CYP2E1)	5253 ± 111	6594 ± 558 [1.3]*	7093 ± 504 [1.4]*^{NS}	8107 ± 509 [1.5]*^{NS}	7919 ± 1329 [1.5]*
Testosterone6β-hydroxylase (CYP3A)	2242 ± 247	3596 ± 629 [1.6]*	4422 ± 616 [2.0]*	6603 ± 1144 [2.9]*	7875 ± 120 [3.5]*

Mice were administered either bilobalide (10.5, 21, 42 mg kg^{-1}) or GBE (1000; 42 mg kg^{-1} as bilobalide) for 5 days. Each value is expressed as the mean ± s.d. for 5 mice. Number in brackets indicates the increase ratio to control. *$P < 0.05$ vs control; ^{NS}not significant vs GBE (1000 mg kg^{-1}).

identify the substance that induces hepatic CYPs in an animal study.

As shown by our results, it is suggested strongly that bilobalide is the major substance in GBE that induces hepatic CYPs. The GBE used in our study contained 42 mg of bilobalide in 1000 mg GBE, and induction levels of CYPs by 42 mg kg^{-1} bilobalide and those by 1000 mg GBE were quite similar. Induction of CYPs by bilobalide has been reported by

other investigators. Sasaki et al (1997) has reported that administration of bilobalide to mice at 30 mg kg^{-1} (per os) for 4 days increased the amount of hepatic drug-metabolizing enzymes. Chang et al (2006a) has observed the induction of CYP2B1 mRNA, and the respective enzyme activity, by bilobalide in primary cultured rat hepatocytes. In our previous studies, we showed that administration of GBE to rats increased markedly the expression of CYP2B mRNA, and the content and activity of enzyme (i.e. pentoxyresorufin O-dealkylase (CYP2B)) (Shinozuka et al 2002; Umegaki et al 2002). As indicated in Tables 2 and 3, the activity of pentoxyresorufin O-dealkylase was highest in bilobalide-rich fraction, and was markedly induced by bilobalide. The GBE used in our studies contained higher amount of bilobalide as shown in Figure 1. Taken together, from these findings it is reasonable to conclude that bilobalide is a major substance in GBE inducing hepatic CYPs. Marked induction of CYPs was not observed for GBE samples that were rich in flavonoids (i.e. fractions 3 and 4). As reported previously (Sugiyama et al 2004c), feeding quercetin, a major flavonoid in GBE, for 1 week at a dose of 0.125% in the diet did not induce hepatic CYPs in rats. These findings indicate that the involvement of flavonoids in GBE in induction of hepatic CYPs is highly unlikely or very low. On the other hand, ginkgolide A and B in GBE may be also responsible for the induction of CYPs, because both compounds are also rich in the fraction 1, which showed the highest induction of CYPs (Figure 2, Table 2). In a cell culture study, it has been reported that ginkgolide A in GBE increased CYP3A mRNA and CYP3A-mediated enzyme activity (Rajaraman et al 2006), although the effect of GBE can not be explained only by ginkgolide A. The content of ginkoglide A and B in GBE is lower than that of biloblide (Figure 1), therefore the contribution of ginkgolide A and B to the induction of CYPs by GBE may be very low. Further detailed study will be needed to clarify the exact contribution of ginkgolide A and B to the induction of CYPs by GBE.

The lack of an effect of bilobalide on hepatic CYPs has been reported in cell-free enzyme studies using microsomes from rats or man (Kuo et al 2004; Sugiyama et al 2004c; von Moltke et al 2004; Chang et al 2006b). Studies performed in a cell-free enzyme system are very effective for screening for a substance in GBE that influences hepatic CYPs, but the results are not always applicable to the in-vivo situation. This discrepancy in the effect of GBE in-vivo and in-vitro has been pointed out by several studies (Gaudineau et al 2004; Sugiyama et al 2004c; Mohutsky et al 2006). In the cell-free study using microsomes, an unphysiological situation, such as concentration or form of the substances, might contribute to the difference in results obtained in the in-vivo situation. Direct interaction between enzymes and substances examined in the cell-free study may give rise to misunderstanding of the real phenomena that occur the in-vivo situation. GBE–drug interactions have been reported in clinical studies, and the results are inconsistent. The interaction of GBE (280–360 mg daily for 12–28 days) with omeprazole (Yin et al 2004) and tolbutamide (Uchida et al 2006) has been demonstrated. It should be mentioned that these findings are similar to those obtained in animal studies (Kubota et al 2003; Sugiyama et al 2004a; Zhao et al 2006) but the GBE–drug interaction, particularly the pharmacodynamic interaction, is very slight. On the other hand, the lack of interaction of GBE (120–240 mg, for less than 14 days) with flurbiprofen and tolbutamide (Greenblatt et al 2006; Mohutsky et al 2006), dextromethorphan and alprazolam (Markowitz et al 2003), S-warfarin (Jiang et al 2005), digoxin (Mauro et al 2003) and acetylsalicylic acid (Wolf 2006) has been reported. It is reasonable to speculate that the dose (in particular bilobalide in GBE) and periods of treatment with GBE are related to the inconsistent results of GBE–drug interaction in most of the clinical studies and the animal studies. Generally, the recommended dose of standardized GBE is less then 240 mg per day, and positive interaction data are found at doses higher than this. In animal studies, marked induction of hepatic CYPs was detected with a high dose (>10 mg kg^{-1}) of GBE, equivalent to more than 600 mg per day in man. A difference of hepatic drug-metabolizing enzymes between species is well known. Thus, species difference of CYPs may be involved, in part, in the different findings of GBE–drug interactions between man and rats.

There are many chemicals in GBE but it is not clear which is (or are) responsible for the beneficial effect of GBE in cases such as cerebral insufficiency and peripheral vascular disease (Le Bars et al 1997; Blumenthal 1998; Pittler & Ernst 2000). The marked effect of bilobalide on hepatic CYPs shown in this study indicates that bilobalide is a biologically active substance within GBE, and may be related to the beneficial effect of GBE. In fact, several studies have shown a neuroprotective effect of bilobalide in-vivo (Chandrasekaran et al 2003; Mdzinarishvili et al 2007). The reported half-life of bilobalide in blood is approximately 2 h in rats and man (Biber & Koch 1999; Mauri et al 2001), indicating it is eliminated easily from blood. These data agree well with the results of our previous study (Sugiyama et al 2004b), in which continuous and excess GBE administration to rats induced hepatic CYPs, and discontinuation of the treatment led to the recovery of the normal level of CYPs within 1 week. Therefore, GBE–drug interactions are not of particular concern when healthy subjects are using the recommended dose (< 240 mg daily) of standardized products. Even in patients who have taken GBE and worry about an interaction with a drug, the interaction could be rapidly preventable by discontinuation of GBE administration.

In conclusion, we identified that bilobalide in GBE is a major substance that induces hepatic CYPs in this animal study, in which absorption, metabolism and excretion of the substances in GBE can be evaluated. The results will be helpful when considering the detail of interactions between GBE and co-administered drugs in the clinical field.

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Antioxidant Properties of 2-*O*-β-D-Glucopyranosyl-L-ascorbic Acid

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The antioxidant activity of a provitamin C agent, 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (AA-2βG), was compared to that of 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) and ascorbic acid (AA) using four *in vitro* methods, 1,1-diphenyl-picrylhydrazyl (DPPH) radical-scavenging assay, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$)-scavenging assay, oxygen radical absorbance capacity (ORAC) assay, and 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced erythrocyte hemolysis inhibition assay. AA-2βG slowly and continuously scavenged DPPH radicals and $ABTS^{\bullet+}$ in roughly the same reaction profiles as AA-2G, whereas AA quenched these radicals immediately. In the ORAC assay and the hemolysis inhibition assay, AA-2βG showed similar overall activities to AA-2G and to AA, although the reactivity of AA-2βG against the peroxyl radical generated in both assays was lower than that of AA-2G and AA. These data indicate that AA-2βG had roughly the same radical-scavenging properties as AA-2G, and a comprehensive *in vitro* antioxidant activity of AA-2βG appeared to be comparable not only to that of AA-2G but also to that of AA.

Key words: 2-*O*-β-D-glucopyranosyl-L-ascorbic acid; antioxidant activity; *in vitro* radical-scavenging assay; moderate and long-lasting radical-scavenging reaction; stable vitamin C derivative

L-Ascorbic acid (AA), known as vitamin C, plays key roles in many biological processes, such as collagen formation, carnitine synthesis, and iron absorption.[1,2] In addition, it is an important antioxidant in food and biological systems,[3] but it is very unstable in aqueous solution. Hence, Yamamoto *et al.* developed a stable AA derivative, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G).[4–7] AA-2G is highly stable under the conditions mentioned above, but once it is administered into the body, it is readily hydrolyzed to free AA by mammalian α-glucosidase. Thus AA-2G exhibits inherent vitamin C activity, such as antiscorbutic activity, *in vivo*, and only AA was detected in the blood in an experiment on oral administration of AA-2G to rats.[8–12] AA-2G has been approved by the Japanese government as a quasi-drug principal ingredient in skin care products and as a food additive.

We have found that AA-2G *per se*, which had been though to no biological activity, exerted radical-scavenging activity toward unnatural model radicals, such as the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical[13–16] and the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$).[15,17] The chemical properties of AA-2G as a radical scavenger were largely different from those of AA, in that the reaction rate with these model radicals of AA-2G was far slower than that of AA, but the long-term radical scavenging ability per molecule of AA-2G was superior to that of AA under optimal conditions. We also found recently that the radical-scavenging activity of AA-2G was biological relevant using a cell-based antioxidant assay system, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH)-induced erythrocyte hemolysis inhibition assay.[18] These data suggest that AA-2G can be used not only as a stable source of AA but also as a useful antioxidant in the food and cosmetics fields.

Recently, a stereoisomer of AA-2G, 2-*O*-β-D-glucopyranosyl-L-ascorbic acid (AA-2βG), was isolated from lycium fruit, a popular traditional Chinese food.[19] The chemical structures of AA, AA-2G, and AA-2βG are shown in Fig. 1. AA-2βG is also a stable AA derivative, and has been found to have vitamin C activity using osteogenic disorder Shionogi (ODS) rats.[20] Differently from AA-2G, it was reported that oral administration of

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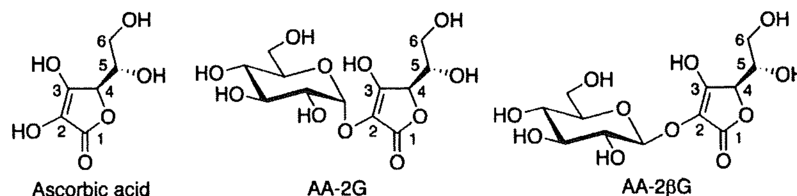

Fig. 1. Chemical Structures of AA, AA-2G, and AA-2βG.

AA-2βG to rats increased the level of AA-2βG as well as AA in the blood of the portal vein, probably due to lesser distribution of β-glucosidase than of α-glucosidase.[19] Thus, if AA-2βG *per se* possesses antioxidant activity as dose AA-2G, it might contribute to antioxidant defense in the body. As far as we know, there have been no reports on the antioxidant activities of AA-2βG. Hence in this study we investigated the antioxidant activity of AA-2βG *per se in vitro*, and compared it to those of AA-2G and AA. Two methods utilizing unnatural model radicals, DPPH radical-scavenging assay and $ABTS^{\bullet+}$-scavenging assay, and two methods utilizing a peroxyl radical, oxygen radical absorbance capacity (ORAC) assay and AAPH-induced erythrocyte hemolysis inhibition assay, were employed.

Materials and Methods

Chemicals. AA, sodium fluorescein, and AAPH were purchased from Wako Pure Chemical Industries (Osaka, Japan). AA-2G and AA-2βG were gifts from Hayashibara Biochemical Laboratories (Okayama, Japan) and Suntory (Osaka, Japan) respectively. DPPH was from Aldrich Chemical (Milwaukee, WI). ABTS and horseradish peroxidase (HRP; type VI-A, essentially a salt-free 1,310 units/mg solid) were from Sigma Chemical (St. Louis, MO). H_2O_2 (30%) was from Nacalai Tesque (Kyoto, Japan). Sheep erythrocytes were from Nihon Seibutsu Zairyou Center (Tokyo). Reagents were used without further purification. All water used was Milli-Q grade.

DPPH radical-scavenging assay. The radical-scavenging activities of AA-2βG, AA-2G, and AA against DPPH radical were assessed as described in previous papers.[14,15] Briefly, DPPH (100 μM) was mixed with an antioxidant (20 μM) in 60% ethanol/40% citric acid-sodium citrate buffer (10 mM, pH 3–6). The reaction was carried out under an atmosphere of argon at 25 °C. Changes in the absorbance at 524 nm due to scavenging of the DPPH radical were measured with a spectrophotometer (Shimadzu UV-1200, Kyoto, Japan).

$ABTS^{\bullet+}$-Scavenging assay. The radical-scavenging activities of AA-2βG, AA-2G, and AA against $ABTS^{\bullet+}$ were assessed as described in a previous paper.[17] Briefly, $ABTS^{\bullet+}$ (100 μM) generated with an ABTS/H_2O_2/HRP system was mixed with an antioxidant (20 μM) in citric acid-sodium citrate buffer (50 mM, pH 3–6). The reaction was carried out under an atmosphere of argon at 25 °C. Changes in the absorbance at 730 nm due to the scavenging of $ABTS^{\bullet+}$ were measured with a spectrophotometer.

Stoichiometric studies for DPPH radical-scavenging assay and $ABTS^{\bullet+}$-scavenging assay. The numbers of DPPH radicals and $ABTS^{\bullet+}$ scavenged by each antioxidant were calculated by following equation:

$$RSA(n) = (\Delta A_{120}/A_0) \times [radical]/[antioxidant]$$

where RSA(n) is radical-scavenging activity factor n in moles of radicals scavenged by each mol of antioxidant; ΔA_{120} is the absorbance difference between the reaction solution and control at 120 min; A_0 is the initial absorbance of the control; [radical] is the DPPH radical and $ABTS^{\bullet+}$ concentration (100 μM); and [antioxidant] is the AA-2βG, AA-2G, and AA concentration (20 μM).

ORAC assay. ORAC assay for AA-2βG, AA-2G, and AA was carried out as described by Gillespie *et al.*[21] with a slight modification. Briefly, fluorescein (60 nM), antioxidant (12.5 μM), and AAPH (18.75 mM) were incubated in 200 μl of KH_2PO_4-K_2HPO_4 buffer (75 mM, pH 7.0) at 37 °C in a 96-well plate (Costar no. 3615). The fluorescence (an excitation wavelength of 485 nm and an emission wavelength of 520 nm) was monitored every 1 min for 1 h by Powerscan HT (DS Pharma Biomedical, Osaka, Japan). The area under the curve (AUC) was calculated as

$$AUC = 0.5 + f_1/f_0 + f_2/f_0 + f_3/f_0 + f_4/f_0 + \cdots + f_{58}/f_0 + f_{59}/f_0$$

where f_0 is the initial fluorescence reading at 0 min and f_i is the fluorescence reading at i min. The net AUC was obtained by subtracting the AUC of the blank from that of a sample.

AAPH-Induced erythrocyte hemolysis inhibition assay. Sheep erythrocytes were washed 2 times with phosphate-buffered saline (PBS: 150 mM NaCl, 8.1 mM Na_2HPO_4, and 1.9 mM NaH_2PO_4, pH 7.4), and resuspended in PBS at a 10% (v/v) suspension. The erythrocyte suspension (1.6 ml) and an antioxidant in PBS (125 μM, 12.8 ml) were mixed into a flat-bottomed

test tube (30 × 120 mm), and incubated in a water bath shaker (Taitec, Saitama, Japan) at 37 °C for 5 min. An ice-cold AAPH/PBS solution (400 mM, 1.6 ml) was injected into the above mixture to initiate hemolysis. An aliquot of this mixture was periodically withdrawn and centrifuged, and the absorbance at 524 nm of the supernatant was measured with a spectrophotometer. The degree of hemolysis (%) was determined from the concentrations of hemoglobin in the supernatant. The value of 100% hemolysis was determined from the supernatant by adding 9 volumes of water to 1 volume of 10% (v/v) erythrocytes.

Results

DPPH radical-scavenging assay and ABTS•+-scavenging assay

The DPPH radical is a relatively stable radical that dose not exist in nature. The deep purple color of the DPPH radical disappears when the radical is reduced, and so decreases in the radical can easily be monitored with a spectrometer.[22] Hence DPPH assay is one of the most common methods of assessing antioxidant activities. ABTS$^{\bullet+}$ is also a relatively stable unnatural radical. It bears a blue-green color, and decoloration assay can be performed in a manner similar to DPPH assay.[22]

A time-course study of the DPPH radical-scavenging reactions of AA-2βG, AA-2G, and AA was carried out in 60% ethanol/40% citrate buffer (pH 3) for 2 h (Fig. 2), since AA-2G reacted with the DPPH radical more effectively at pH 3 than at pH 6, as reported in previous papers.[14,15] AA-2βG showed the same reaction curve as AA-2G. AA-2βG and AA-2G continuously reacted with the DPPH radical for 2 h, whereas AA rapidly scavenged the DPPH radical, within 1 min.

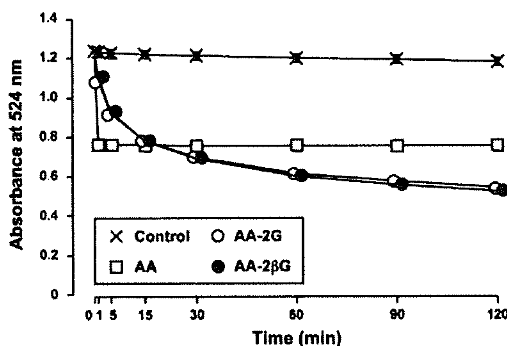

Fig. 2. Time Course of DPPH Radical-Scavenging Reaction of AA-2βG, AA-2G, and AA.

AA-2βG, AA-2G, or AA (20 μM) and the DPPH radical (100 μM) were incubated in 60% ethanol/40% citrate buffer (10 mM, pH 3) at 25 °C. Changes in absorbance at 524 nm due to scavenging of the DPPH radical were monitored at the indicated times. Values are means ± S.D. of three separate experiments. Absence of S.D. bar means that the S.D. bar is within the symbol.

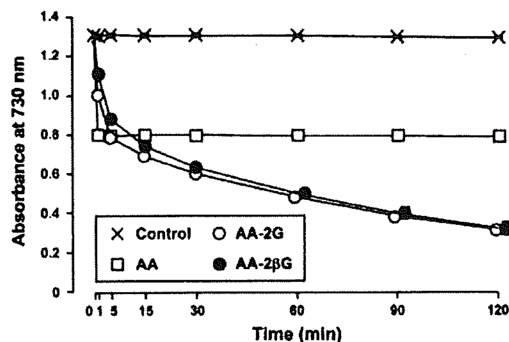

Fig. 3. Time Course of ABTS•+-Scavenging Reaction of AA-2βG, AA-2G, and AA.

AA-2βG, AA-2G, or AA (20 μM) and ABTS•+ (100 μM) were incubated in citrate buffer (50 mM, pH 6) at 25 °C. Changes in absorbance at 730 nm due to scavenging of ABTS•+ were monitored at the indicated times. Values are means ± S.D. of three separate experiments. Absence of S.D. bar means that the S.D. bar is within the symbol.

The reactions of AA-2βG, AA-2G, and AA toward ABTS$^{\bullet+}$ were measured in citrate buffer (pH 6) for 2 h (Fig. 3), since AA-2G was found to react with ABTS$^{\bullet+}$ more effectively at pH 6 than at pH 3.[15,17] AA-2βG scavenged the ABTS$^{\bullet+}$ in a manner similar to AA-2G. The reaction of these two AA derivatives proceeded for 2 h, whereas the reaction of AA was completed within 1 min.

We have found that the reactivity of AA-2G against the DPPH radical is largely affected by the pH of the reaction mixture.[14,15] Hence the effects of pH on the long-term reaction stoiciometry of AA-2βG in the DPPH radical- and ABTS$^{\bullet+}$-scavenging assays were compared to those of AA-2G and AA (Table 1). The stoichiometric factor RSA(n) was defined as the number of radicals consumed per molecule of each antioxidant in a 2-h reaction. AA-2βG showed almost the same profiles as AA-2G. The RSA(n) of these two AA derivatives against the DPPH radical in a pH range of 3 to 4 was greater than that of AA, while their scavenging abilities sharply decreased with increasing pH, and disappeared at pH 6. On the other hand, their RSA(n) values against ABTS$^{\bullet+}$ were little affected by pH changes, and were superior to that of AA at all pH levels. The RSA(n) values of AA were about 2 in all cases examined.

ORAC assay and AAPH-induced erythrocyte hemolysis inhibition assay

ORAC assay and AAPH-induced erythrocyte hemolysis inhibition assay utilize a peroxyl radical, which is involved in biologically relevant oxidative processes. ORAC assay measures the inhibition of peroxyl radical-inuced oxidations of a fluorescent probe by antioxidants.[22] The peroxyl radical is continuously generated by thermal degradation of AAPH, and it reacts with

Table 1. Effects of pH on RSA(n) of AA-2βG, AA-2G, and AA against the DPPH Radical and ABTS$^{\bullet+}$

	RSA(n) against DPPH radical				RSA(n) against ABTS$^{\bullet+}$			
	pH 3	pH 4	pH 5	pH 6	pH 3	pH 4	pH 5	pH 6
AA-2βG	2.7 ± 0.0	2.1 ± 0.0	0.8 ± 0.1	0.1 ± 0.0	3.3 ± 0.1	3.4 ± 0.1	3.8 ± 0.1	3.9 ± 0.2
AA-2G	2.6 ± 0.0	2.2 ± 0.0	0.9 ± 0.0	0.2 ± 0.0	3.6 ± 0.0	4.1 ± 0.0	3.7 ± 0.0	3.9 ± 0.0
AA	1.8 ± 0.0	1.9 ± 0.1	1.9 ± 0.0	1.7 ± 0.0	2.0 ± 0.1	1.9 ± 0.1	2.0 ± 0.1	1.9 ± 0.1

In the case against the DPPH radical, the reaction mixture contained AA-2βG, AA-2G, or AA (20 μM) and the DPPH radical (100 μM) in 5.0 ml of 60% ethanol/40% citrate buffer (10 mM, pH 3 6). In the case against ABTS$^{\bullet+}$, the reaction mixture contained AA-2βG, AA-2G, or AA (20 μM) and ABTS$^{\bullet+}$ (100 μM) in 5.0 ml of citrate buffer (50 mM, pH 3 6). The reaction was carried out at 25 °C for 2 h. The number of radicals scavenged per molecule of AA derivatives and of AA is expressed as RSA(n). Results are means ± S.D. (n = 6).

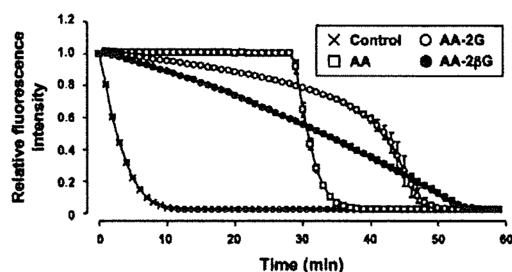

Fig. 4. ORAC Assay for AA-2βG, AA-2G, and AA.

The reaction mixture contained AA-2βG, AA-2G, or AA (12.5 μM), fluorescein (60 nM), and AAPH (18.75 mM) in 200 μl of KH_2PO_4-K_2HPO_4 buffer (75 mM, pH 7.0). They were incubated at 37 °C for 1 h. Changes in the fluorescence intensity of fluorescein were monitored. Values are means ± S.D. of triplicate experiments. Absence of S.D. bar means that the S.D. bar is within the symbol.

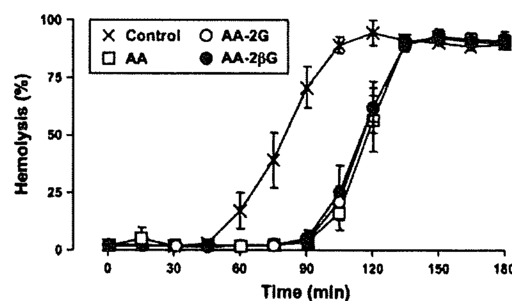

Fig. 5. Inhibition of AAPH-Induced Erythrocyte Hemolysis by AA-2βG, AA-2G, and AA.

Sheep erythrocyte at 1% (v/v) suspension in PBS was incubated with 40 mM of AAPH in the presence of 100 μM of the AA derivatives or AA at 37 °C for 180 min with shaking. Each value is the means ± S.D. of three separate experiments. Absence of S.D. bar means that the S.D. bar is within the symbol.

fluorescein to form nonfluorescent products. AAPH-induced erythrocyte hemolysis inhibition assay is a cell-based *in vitro* antioxidant assay.[23] The oxidation of erythrocyte membranes by AAPH-derived peroxyl radical induces oxidation of lipids and proteins and eventually causes hemolysis, and this hemolysis can be inhibited by antioxidants.[24]

In the ORAC assay, inhibition of fluorescence decay by AA-2βG showed quite different profiles from that by AA-2G and AA (Fig. 4). In the early phase of the reaction, AA-2βG and AA-2G partially suppressed the loss of fluorescence, whereas AA completely inhibited it. The initial inhibition effects were in the order AA > AA-2G > AA-2βG. In the case of AA, fluorescence rapidly decayed after a lag time of about 30 min. On the other hand, AA-2G continued partial inhibition for a longer period. Furthermore, AA-2βG maintained partial inhibition during the whole period. As a result, the remaining fluorescence intensity was reversed in the late phase of the reaction in the order AA-2βG > AA-2G > AA. In this way, the profiles of the fluorescence decay curves of AA-2βG, AA-2G, and AA were largely different. On the other hand, in the ORAC assay, the total extent of inhibition by an antioxidant is expressed by net AUC, which is obtained by subtracting the area under the blank curve from the area under the sample curve. The net AUCs of AA-2βG, AA-2G, and AA were 31.9 ± 0.2, 37.6 ± 0.6, and 31.6 ± 0.7 respectively (means ± S.D. of triplicate experiments). Thus, the overall antioxidant activity of AA-2βG in the ORAC assay was roughly in the same range as that of AA-2G and AA. In the AAPH-induced erythrocyte hemolysis inhibition assay, AA-2βG also inhibited hemolysis to the same extent as AA-2G and AA (Fig. 5).

Discussion

In the present study, we investigated the antioxidant activity of AA-2βG *per se* in comparison to those of AA-2G and AA using four *in vitro* methods. In assays utilizing unnatural model radicals, the DPPH radical-scavenging assay and the ABTS$^{\bullet+}$-scavenging assay, AA-2βG showed reaction properties similar to AA-2G, *i.e.*, slow and long-lasting radical-scavenging as compared to AA (Figs. 2, 3). The effects of pH on the RSA(n) values were also nearly the same as between AA-2βG and AA-2G (Table 1). In addition, the RSA(n) values of these AA derivatives exceeded that of AA under optimal conditions. In the ORAC assay, which utilizes a similar kind of peroxyl radical actually generated *in vivo*, the profiles of the fluorescence decay curves of AA-2βG, AA-2G, and AA were quite different (Fig. 4), but their net AUC, which reflects overall antioxidant activity in the ORAC assay, were roughly in the same range. In the AAPH-induced erythrocyte

hemolysis inhibition assay, a cell-based antioxidant assay utilizing the same radical as in the ORAC assay, AA-2βG showed almost the same inhibition efficacy as AA-2G and AA (Fig. 5). Therefore, AA-2βG had generally similar characteristics to AA-2G in the *in vitro* antioxidant assays, except for the ORAC assay.

Differences between AA-2βG and AA-2G were clearly observed in the ORAC assay (Fig. 4). Initial inhibition efficacy due to AA-2βG was inferior to that due to AA-2G, indicating that AA-2βG was less reactive against the AAPH-derived peroxyl radical than AA-2G. In addition, in the $ABTS^{\bullet +}$-scavenging assay, AA-2βG and AA-2G also exhibited slight but significant differences ($p < 0.001$, unpaired *t*-test), *i.e.*, the amounts of $ABTS^{\bullet +}$ quenched by AA-2βG were lower than those by AA-2G in the early phases of the reaction (1 and 5 min, Fig. 3), and the RSA(n) values of AA-2βG at pH levels 3 and 4 were also lower than those of AA-2G (Table 1). These results are attributable to their stereochemical differences at the C-2 position, the α-glucosidic bond or the β-glucosidic bond (Fig. 1). In this way, the reactivity of AA-2βG toward certain radicals might be inferior to that of AA-2G. However, the net AUC in the ORAC assay (Fig. 4) and the amount of finally quenched $ABTS^{\bullet +}$ (120 min, Fig. 3) were similar as between AA-2βG and AA-2G. Thus their *in vitro* antioxidant activity appeared not to be greatly different.

In AA-2βG and AA-2G the oxidizable hydroxyl group at C-2 position of AA moiety was replaced with the glucosyl group (Fig. 1), and thus AA-2βG and AA-2G can be expected to exert lower radical-scavenging activity than AA. It is true that the reactivity of these AA derivatives toward radicals was always inferior to that of AA (Figs. 2–4), but their reaction stoichiometry against unnatural model radicals was occasionally superior to that of AA (Table 1), and their net AUC in the ORAC assay was comparable to that of AA (Fig. 4). Furthermore, AA-2βG and AA-2G protected erythrocytes from biologically relevant peroxyl radicals to the same extent as AA (Fig. 5). These findings suggest that these AA derivatives act as potent antioxidants comparable to AA in the biological context. Recently, we found that AA-2G scavenged the DPPH radical not *via* AA as an intermediate but *via* a covalent adduct formation with the radical.[16] This unique reaction was the reason AA-2G showed unexpectedly high reaction stoichiometry against the DPPH radical. The results obtained in this study suggest that AA-2βG possesses essentially the same radical-scavenging mechanisms as AA-2G, and we believe that the potent antioxidant activities of AA-2G and AA-2βG comparable to that of AA can be explained by a covalent adduct formation with a radical.

In conclusion, AA-2βG had roughly the same radical-scavenging properties as AA-2G, and the comprehensive *in vitro* antioxidant activity of AA-2βG appeared to be comparable not only to that of AA-2G but also to that of AA. Recently, it was reported that AA-2G and another AA derivative, ascorbic acid 2-phosphate, can protect the skin from UVB/A-induced photodamage *ex vivo* by themselves more effectively than AA.[25] Hence it may be necessary to consider the antioxidant activities of AA derivatives *per se* not only *in vitro* but also *in vivo*. As mentioned in the introduction, *in vivo*, the distribution of β-glucosidase, which hydrolyzes AA-2βG to AA, is limited as compared to that of α-glucosidase, which converts AA-2G to AA. Thus AA-2βG is more likely to remain in an unmetabolized form than AA-2G, and so the antioxidant activity of the unmetabolized AA derivatives *per se* may be more important to AA-2βG than to AA-2G *in vivo*.

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