Possible Involvement of Lipids in the Effectiveness of Kombu in Individuals with Abnormally High Serum Triglyceride Levels

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Summary In Japan, Kombu (*Laminaria japonica*), which is a type of seaweed, is considered to be a foodstuff with health-promoting benefits, and Japanese people actively incorporate Kombu into their diets. Previously, we reported that the frequent intake of Kombu reduced the serum triglyceride levels of subjects with abnormally high serum triglyceride levels. In the current human study, we performed metabolomic analysis of serum lipids, and then the molecular species profiles of phosphatidylcholines (PC), phosphatidylethanolamines (PE), lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), and free fatty acids (FFA) were evaluated. As a result, it was found that there were no marked differences between the lipid profiles obtained before and after the intake of Kombu for 4 wk in all subjects. In the subjects with abnormal serum triglyceride levels, the intake of Kombu improved the subjects' molecular species profiles in terms of their serum levels of the diacyl and acyl forms of PC, PE, LPC, and LPE, and FFA. Furthermore, the intake of Kombu also tended to increase the serum levels of both the plasmalogen and plasmalogen forms of PC and PE in these subjects. The lipid alterations observed in our study might be related to the functionality of Kombu. Furthermore, it is important to evaluate the quality of lipids as well as the quantity of lipids in various types of research, including food functionality studies.

Key Words Kombu, triglyceride, phospholipid, plasmalogen, metabolomics

In Japan, Kombu (*Laminaria japonica*), which is a type of seaweed, is considered to be a foodstuff with health-promoting benefits, and Japanese people actively incorporate Kombu into their diets. Kombu contains various active compounds, for example, minerals and vitamins, and it also contains dietary fiber, so it is expected to effects on intestinal regulation, obesity, and cholesterol levels (1–3).

Recently, we investigated how the frequent intake of Kombu affects the body in a human study. As a result, we suggested that the frequent intake of Kombu might reduce the serum triglyceride levels of individuals with abnormally high serum triglyceride levels (4). However, the molecular mechanisms underlying the functionality of Kombu remain to be elucidated. Previously, the relationship between lysophospholipids and obesity was clarified (5). In patients with type 2 diabetes, consuming greater amounts of polyunsaturated fatty acids than carbohydrates or saturated fatty acids was found to be related to lower total mortality and cardiovascular disease mortality, suggesting the importance of increasing the quality of dietary lipids for preventing cardiovascular disease and total mortality in patients with type 2 diabetes (6). In the current human study, we performed

metabolomic analysis of the subjects' serum lipids. First, the serum levels of phosphatidylcholines (PC), phosphatidylethanolamines (PE), lysophosphatidylcholines (LPC), lysophosphatidylethanolamines (LPE), free fatty acids (FFA), acylcarnitines (AC), cholesterol (Cho), and bile acids were measured, and then their molecular species profiles were evaluated.

MATERIALS AND METHODS

Study design and subjects. This human study was approved by the ethics committee at Kobe University Graduate School of Medicine (#1279 and #1369). The human samples were used in accordance with the guidelines of Kobe University Hospital, and written informed consent was obtained from all subjects. The study had a randomized crossover design, and the detailed study design is described in our previous report (4). Roasted Kombu that we used in this study was purchased from Minami Kayabe Fisheries Cooperative Associations (Hokkaido, Japan). The nutrition constituents in roasted Kombu are shown in Table 1. Briefly, during the treatment part of the crossover trial, the subjects consumed 6 g of roasted Kombu (3 g roasted Kombu × 2) every day for 4 wk. Throughout the 8-wk trial, the subjects recorded the contents of their diets and the state of their feces each day. Regarding the contents of their diets,

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no marked differences were observed according to the intake of Kombu for 4 wk. In the state of their feces, it was confirmed the possibility that the intake of Kombu for 4 wk leads to relief from constipation, diarrhea, and

Table 1. Nutrition constituents in roasted Kombu.

Constituents	Amount/100 g
Moisture (g)	5.4
Protein (g)	5.6
Fat (g)	1.8
Ash (g)	17.7
Saccharide (g)	41.8
Dietary fiber (g)	27.7
Energy (kcal)	158

Energy of roasted Kombu was calculated according to amount of nutrition constituents. Amount of sodium (g) in roasted Kombu was 2.11 g/100 g of roasted Kombu.

hard stools as shown in our previous report (4). Before the start of the trial (0 wk), and at 4 and 8 wk after the start of the trial, blood biochemistry tests; medical interviews; and measurements of height, weight, pulse rate, and blood pressure were performed. Blood samples were collected in the morning using the standard venous blood sampling protocol, and the separated serum was subjected to lipid metabolomic analysis. Sera used at this study were the same as sera collected and used in our previous report (4), so the detailed protocols and results of this human study including blood biochemistry tests; medical interviews; and measurements of height, weight, pulse rate, and blood pressure can be checked in our previous report (4).

Lipid metabolomics. To analyze the serum levels of PC, PE, LPC, LPE, FFA, AC, Cho, and bile acids, lipid metabolomics was performed. First, 10 μ L of serum was mixed with 80 μ L of methanol and 10 μ L of 0.8 nmol/mL PC 12:0–12:0 (Avanti Polar Lipids, AL) as an internal standard in methanol. After being vortexed,

(A)

(B)

(C)

Fig. 1. Overview of the results of the LC/MS-based lipid analysis. The differences between the data obtained before (Pre) and after (Post) the intake of Kombu for 4 wk (Post/Pre) (A, C) or between the groups with abnormal and normal serum triglyceride (TG) levels (abnormal/normal) (B) are shown. The vertical axis shows the total unsaturation number, and the number indicates the number of double bonds. The horizontal axis shows lipid groups and their variants. Acyl (sn1 or sn2), Diacyl, P (plasmalogen), and E (plasmalogen) indicate the binding forms of fatty acids. The length of each bar indicates the total number of carbon atoms, and the longer bar indicate the larger total number of carbon atoms in each molecule. The color of each bar reflects whether the level of the metabolite had increased (red) or decreased (blue). N.D.: not detected, AC: acylcarnitine, FFA: free fatty acids, LPC: lysophosphatidylcholines, LPE: lysophosphatidylethanolamines, PC: phosphatidylcholines, PE: phosphatidylethanolamines, Cho: cholesterol. Cho include 4-cholesten-3-one and 5 α -cholestan-3-one (left and right circles, respectively). Primary bile acids include chenodeoxycholic and cholic acid (left and right circles, respectively). Secondary bile acids include ursodeoxycholic acid and lithocholic acid (left and right circles, respectively). Conjugated bile acids include taurocholic acid, taurochenodeoxycholic acid, glycocholic acid, and glycodeoxycholic acid (first, second, third, and forth circles from the left, respectively). (A) The results for the subjects that completed the trial ($n=48$) (Post/Pre; The red indicates that that each lipid level detected after the intake of Kombu is higher than before the intake of Kombu.); (B) The results for the subjects with abnormal ($n=9$) and normal ($n=39$) serum triglyceride levels (abnormal/normal; The red indicates that each lipid level in the subjects with abnormal serum triglyceride levels is higher than the subjects with normal serum triglyceride levels.); (C) The results for the subjects with abnormal serum triglyceride levels ($n=9$) (Post/Pre; The red indicates that that each lipid level detected after the intake of Kombu in the subjects with abnormal serum triglyceride levels is higher than before the intake of Kombu in the subjects with abnormal serum triglyceride levels.).

the mixture was kept on ice for 10 min and then centrifuged at $16,000 \times g$ for 5 min at 4°C . The resultant supernatant was collected in a new vial and then subjected to liquid chromatography/mass spectrometry (LC/MS)-based lipid analysis. The lipid analysis was performed using a Nexera LC system (Shimadzu Co., Kyoto, Japan) equipped with two LC-30AD pumps; a DGU-20As degasser; an SIL-30AC autosampler; a CTO-20AC column oven; and a CBM-20A control module, which was coupled to an LCMS-8040 triple quadrupole mass spectrometer (Shimadzu Co.). The targeted lipids were separated using an octadecylsilylated silica column (InertSustain C18, 100×2.1 mm, $3 \mu\text{m}$; GL Sciences, Tokyo, Japan) attached to a guard column (10×3 mm, $5 \mu\text{m}$; GL Sciences). The mobile phase consisted of A: 20 mM ammonium acetate in water and B: methanol. The flow rate was 0.4 mL/min , and the column oven temperature was 40°C . The gradient program for mobile phase B was as follows: 0 min, 80%; 13 min, 98%; 30 min, 98%; 30.1 min, 80%; and 35 min, 80%. The target lipids were detected using multiple reaction monitoring, and the lipid database for multiple reaction monitoring was prepared based upon the metabolites' physicochemical properties and/or their spectral similarity with the data in public/commercial spectral libraries.

Statistical analysis. Statistical comparisons were carried out using the Mann-Whitney U test or Wilcoxon signed-rank test. In all cases, p -values of <0.05 were considered to indicate a significant difference. The analyses were performed using the default conditions of JMP9 (SAS Institute Inc., Cary, NC) or Ekuseru-Toukei 2010 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

RESULTS

In our human study, 61 Japanese healthy adults (22 males, 39 females; age range: 34–86 y; mean age: 66.7 y) were recruited from among the general public. However, 9 subjects withdrew before the trial started. The remaining subjects ($n=52$) were randomly divided into group A ($n=26$), which took Kombu in the last 4 wk, and group B ($n=26$), which took Kombu in the first 4 wk, but 4 of the subjects withdrew during the trial. Thus, 48 subjects (20 males, 28 females; age range: 39–86 y; mean age: 65.9 y) participated in the trial until its completion. The detailed protocols and results of this human study were described in our previous report (4), and sera used at this study were the same as sera collected and used at our previous report (4).

First, we compared the lipid profiles obtained before and after the intake of Kombu for 4 wk (Fig. 1A). AC, Cho, and bile acids were not detected in the subjects' sera, maybe due to below the detection limit or defect peak shapes. As for PC, PE, LPC, LPE, and FFA, we did not detect any marked alterations in their levels that were induced by the intake of Kombu. In our previous study, the intake of Kombu for 4 wk significantly reduced the serum triglyceride levels of subjects with abnormal serum triglyceride levels (4). Therefore, we decided to focus on the effects of Kombu on subjects with

Fig. 2. Volcano plot of lipid species. (A) Comparisons between the serum levels of the lipids detected in the subjects with abnormal ($n=9$) and normal ($n=39$) serum triglyceride levels were performed using the Mann-Whitney U -test. X axis: abnormal to normal ratio on a $\log_2$ scale (The positive value indicates that each lipid level in the subjects with abnormal serum triglyceride levels is higher than the subjects with normal serum triglyceride levels.); Y axis: $-\log_{10}(p\text{-values})$ (The horizontal dashed red line represents a p -value of 0.05.). (B) Comparisons between the serum levels of the lipids detected before (Pre) and after (Post) the intake of Kombu for 4 wk in the subjects with abnormal serum triglyceride levels ($n=9$) were performed using the Wilcoxon signed-rank test. X axis: Post to Pre ratio on a $\log_2$ scale (The positive value indicates that each lipid level detected after the intake of Kombu is higher than before the intake of Kombu.); Y axis: $-\log_{10}(p\text{-values})$ (The horizontal dashed red line represents a p -value of 0.05.). LPC: lysophosphatidylcholines, LPE: lysophosphatidylethanolamines, PC: phosphatidylcholines, PE: phosphatidylethanolamines, FFA: free fatty acids.

abnormal serum triglyceride levels. First, separating the abnormal and normal subjects of serum triglyceride levels was performed according to the standard range of serum triglyceride levels in the blood biochemistry test (4), and we compared the lipid profiles and lipid levels of subjects with abnormal ($n=9$) and normal ($n=39$) serum triglyceride levels prior to Kombu intake (Fig. 1B;

Supplemental Online Material, Table S1). As a result, we found that various LPC and LPE exhibited higher serum levels in the abnormal subjects than in the normal subjects, and these results were confirmed in a volcano plot of the lipid species (Fig. 2A). Most of the PC and PE with diacyl linkages also displayed higher serum levels in the abnormal subjects, although PC, including unsaturated fatty acids with more double bonds, demonstrated lower serum levels in the abnormal subjects. In addition, both the plasmalogen and plasmenyl forms of PC and PE exhibited lower serum levels in the abnormal subjects than in the normal subjects. Regarding FFA, saturated FFA tended to display higher levels in the abnormal subjects, and unsaturated FFA with more double bonds, tended to exhibit lower serum levels in the abnormal subjects.

Next, we compared the lipid profiles and lipid levels detected before and after the intake of Kombu for 4 wk in the subjects with abnormal serum triglyceride levels ($n=9$) (Fig. 1C; Supplemental Online Material, Table S2). As a result, we found that in the subjects with abnormal serum triglyceride levels the intake of Kombu improved the subjects' molecular species profiles; i.e., their serum levels of most of the PC with diacyl linkages, PE with diacyl linkages, LPC with acyl linkages, LPE with acyl linkages, and FA improved. The intake of Kombu also tended to increase the serum levels of both the plasmalogen and plasmenyl forms of PC and PE in these subjects. The volcano plot of the lipid species (Fig. 2B) also showed that the intake of Kombu reduced the serum levels of most LPC and LPE.

DISCUSSION

Kombu has been used as a traditional foodstuff in Japan for centuries, so evaluations of the functionality of Kombu in human studies are meaningful. In a previous human study, we found that the frequent intake of Kombu reduced the serum triglyceride levels of subjects with abnormally high serum triglyceride levels (4). Recently, it has been suggested that the quality of lipids; i.e., the type of lipids consumed, is more likely to be related to disease states than the quantity of lipids. For example, the patients with type 2 diabetes consuming higher amounts of polyunsaturated fatty acids compared with carbohydrates or saturated fatty acids had the lower total mortality and cardiovascular disease mortality (6). Moreover, the types of dietary lipids consumed were shown to influence susceptibility to enteric damage, whereas the total number of dietary lipid calories consumed did not affect susceptibility to enteric damage (7). In addition, a relationship between lysophospholipid metabolism and obesity has been reported to exist (5). Therefore, we performed metabolomic analysis of serum lipids, and then the molecular species profiles of serum PC, PE, LPC, LPE, and FFA were evaluated, focusing on the quality of the lipids.

LPC and LPE are produced from PC and PE, respectively, by phospholipases (PLA), and PLA2 has been reported to be related to the pathogenesis of obesity (8). Previous studies have also shown that it is hard to induce obesity using a high-fat diet or to induce insu-

lin resistance in group 1B PLA2-IB-deficient mice (9), whereas diet-induced obesity and diabetes were promoted in group 1B PLA2-IB transgenic mice (10). In a genome-wide association study, polymorphisms in the PLA2G1B gene were identified as determinants of central obesity in humans (11). In addition, lysophospholipids inhibited fatty acid oxidation in the livers of mice (12), and LPC promoted triglyceride synthesis in hepatocytes (13). Furthermore, high serum levels of lipoprotein-associated PLA2 were detected in overweight and obese schoolchildren (14). These findings indicate that the LPC produced by PLA2 cause obesity and/or insulin resistance, and possibly increased triglyceride levels. In the current study, various LPC and LPE displayed higher serum levels in the subjects with abnormal serum triglyceride levels than in those with normal serum triglyceride levels, and frequent Kombu intake reduced the serum LPC and LPE levels of the subjects with abnormal serum triglyceride levels. Therefore, Kombu intake might lead to the downregulation of PLA2 activity and/or reductions in LPC levels, resulting in decreased serum triglyceride levels, although detailed investigations are necessary to confirm this. Moreover, the molecular species profiles of each type of phospholipid were analyzed to confirm whether the intake of Kombu causes alterations in molecular species profiles. As a result, it was demonstrated that the intake of Kombu reduced the serum levels of various LPC and LPE in the subjects with abnormal serum triglyceride levels.

Interestingly, the subjects with abnormal serum triglyceride levels exhibited lower serum levels of both the plasmalogen and plasmenyl forms of PC and PE than the subjects with normal serum triglyceride levels, although the abnormal subjects also had higher serum levels of PC and PE with diacyl linkages. In addition, in the subjects with abnormal serum triglyceride levels, the intake of Kombu tended to increase the serum levels of both the plasmalogen and plasmenyl forms of PC and PE, which are known as plasmalogens and seem to be important for human health (15). Previously, it was reported that serum triglyceride levels might be negatively correlated with serum plasmalogen levels, and it was suggested that the latter association might be stronger than the associations between low serum plasmalogen levels and elevated plasma glucose or serum lipid levels (16). In addition, among hyperlipidemic subjects myo-inositol treatment significantly increased the levels of plasmalogens and tended to reduce the levels of small dense low-density lipoproteins and high-sensitivity C-reactive protein, and blood glucose levels (17). On the other hand, no significant increases in the levels of plasmalogens were observed in non-hyperlipidemic subjects (17). These effects of myo-inositol treatment were similar to those of Kombu treatment, and therefore, increased levels of plasmalogens might contribute to improving hyperlipidemia, although detailed and large-scale studies are needed to confirm the role of plasmalogens in the functionality of Kombu.

Kombu contains a variety of active molecules, such as minerals, vitamins, and dietary fibers. Recently, func-

Fig. 3. Summary of this study. TG: triglycerides, PC: phosphatidylcholines, PE: phosphatidylethanolamines, LPC: lysophosphatidylcholines, LPE: lysophosphatidylethanolamines, FFA: free fatty acids.

tionality of dietary fibers has been widely reported. Fucoxanthin and alginic acid, which are famous as dietary fibers in Kombu, were reported to reduce the serum cholesterol levels (18, 19). However, fucoxanthin and alginic acid could not decline serum triglyceride levels (18, 19). In this study, the intake of Kombu could improve the abnormal serum triglyceride levels. Certain molecules except fucoxanthin and alginic acid might be effective, or the synergetic effects of some molecules might be exerted.

In this study, characteristic alterations in the molecular species profiles of each type of phospholipid were induced by Kombu intake (Fig. 3). Unfortunately, the number of samples was too small to allow us to examine the relationship between Kombu intake and hypertriglyceridemia because our previous trial (4) was a pilot study. However, we suggest that the intake of Kombu might reduce LPC and LPE levels and increase PC/PE plasmalogen levels in subjects with abnormal serum triglyceride levels. Taken together, the lipid alterations observed in our study might be related to the functionality of Kombu. In addition, it is important to evaluate the quality of lipids as well as the quantity of lipids. For example, it is known that *n*-3/*n*-6 unsaturated fatty acids are important factors for biological responses, and our study also suggested that plasmalogens of PCs and PEs may be important for the effects of Kombu. Therefore, the quality of lipids should be noted in various types of research, including food functionality studies.

Disclosure of state of COI

The authors declare that they have no conflicts of interest.

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Supporting information

Supplemental online material is available on J-STAGE.

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