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Short Communication

Low bioavailability of dietary epoxyxanthophylls in humans

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Epoxyxanthophylls (epoxide-containing xanthophylls), a group of carotenoids, are ubiquitously distributed in edible plants. Among them, neoxanthin in green leafy vegetables and fucoxanthin in brown algae have been reported to exhibit an antiproliferative effect on several human cancer cells *in vitro*. However, there is little information about the intestinal absorption and metabolic fate of dietary epoxyxanthophylls in humans. To estimate the intestinal absorption of neoxanthin and fucoxanthin in humans, we evaluated the plasma epoxyxanthophyll concentrations before and after 1-week dietary interventions with spinach (*Spinacia oleracea*) and wakame (*Undaria pinnatifida*). The epoxyxanthophylls and their metabolites in the plasma extracts were determined by HPLC after partial purification and concentration with solid-phase extraction cartridges. Even after 1 week of spinach intake (3·0 mg neoxanthin/d), the plasma concentrations of neoxanthin and its metabolites (neochrome stereoisomers) remained very low (about 1 nmol/l), whereas those of β-carotene and lutein were markedly increased. Similarly, the plasma concentration of fucoxanthinol, a gastrointestinal metabolite of fucoxanthin, was $<$ 1 nmol/l after 1 week of wakame intake (6·1 mg fucoxanthin/d). These results indicated that the plasma response to dietary epoxyxanthophylls was very low in humans even after 1-week intake of epoxyxanthophyll-rich diets.

Carotenoids: Neoxanthin: Fucoxanthin: Intestinal absorption

Epidemiological studies have demonstrated an association between the consumption of fruits and vegetables and a lower risk of certain forms of cancer. However, several intervention trials have failed to prove the cancer-chemopreventive effect of β-carotene, a representative carotenoid in fruits and vegetables. In this context, attention has been recently focused on the chemopreventive effect of other phytochemicals in fruits and vegetables, including epoxyxanthophylls (epoxide-containing xanthophylls).

Epoxyxanthophylls are ubiquitously distributed in plant photosynthetic organs and constitute a major fraction of the dietary carotenoids[1]. There are two noteworthy epoxyxanthophylls: neoxanthin (9′-*cis*-neoxanthin), one of the major carotenoids in green leafy vegetables such as spinach (*Spinacia oleracea*)[2], and fucoxanthin, a predominant carotenoid in edible brown algae[3] such as wakame (*Undaria pinnatifida*) and konbu (*Laminaria japonica*), which are popular foodstuffs in East Asia (Fig. 1). These two epoxyxanthophylls have been reported to suppress the proliferation of several cancer cells *in vitro* [4–9] and to inhibit chemically induced carcinogenesis in animal models[10]. Moreover, recent studies have indicated anti-obesity[11,12] and anti-angiogenic[13] effects of fucoxanthin. Thus, these epoxyxanthophylls could have the potential health benefits of preventing cancer and obesity.

In our recent studies[4,5], the intestinal absorption and metabolism of purified neoxanthin and fucoxanthin were demonstrated in animal models, but not in humans. In the present study, to evaluate the bioavailability of neoxanthin and fucoxanthin through the ingestion of vegetables in humans, we measured the levels of these epoxyxanthophylls and their metabolites in human plasma before and after 1-week dietary interventions with cooked spinach and wakame, respectively.

Subjects and methods

Materials

Lutein, neoxanthin (9′-*cis*-neoxanthin) and violaxanthin were isolated from spinach[14,15]. (8′*R*)- and (8′*S*)-Neochrome were prepared from neoxanthin by acid-catalysed epoxide–furanoid rearrangement[5]. Fucoxanthin, fucoxanthinol and amarouciaxanthin A were prepared as reported previously[4]. These xanthophylls (Fig. 1) were identified on the basis of visible absorption spectra, LC-MS, high-resolution MS and NMR spectra. α-Carotene and β-carotene were obtained from Sigma-Aldrich (St Louis, MO, USA). β-Cryptoxanthin and lycopene were purchased from Extrasynthese (Genay, France) and Wako Pure Chemical (Osaka, Japan), respectively. Fresh spinach and dried wakame (dried after heat treatment) were purchased from a local market in Tsukuba (Japan).

Subjects

The participants were five healthy volunteers (three women and two men) recruited from the National Food Research Institute (Tsukuba, Japan). They had a mean age of 37·4 (range 30–50) years, a weight of 58·2 (SD 15·8) kg and a

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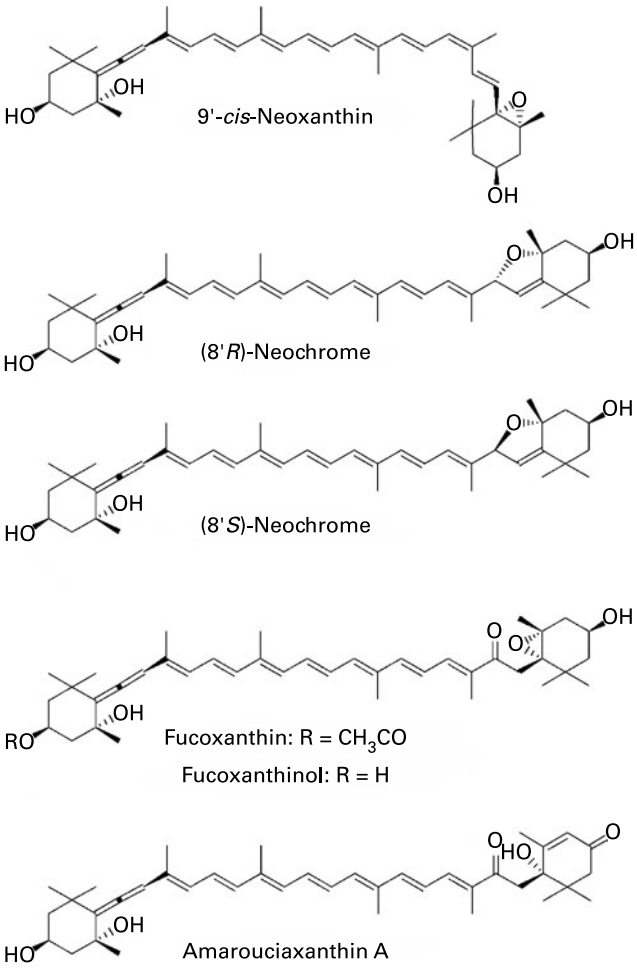

Fig. 1. Structure of neoxanthin, fucoxanthin and their metabolites.

BMI of 21·7 (SD 4·1) kg/m^2, and were considered healthy as assessed by medical history and regular medical checkup. None of the participants had taken any carotenoid-containing supplement during the year before the study started or during the study period. The present study was approved by the Ethics Committee of the National Food Research Institute and signed informed consent was obtained from each participant.

Study design

The study consisted of four consecutive 1-week periods (week 1 to week 4). Throughout the study, the participants were instructed to consume their normal diets, except that they were asked to restrict their consumption of dark green and yellow-orange vegetables (< 100 g/d) during week 1 (wash-out) and week 2 (stir-fried spinach intervention period), and to consume no brown algae-containing foodstuff during week 3 (wash-out) and week 4 (stir-fried wakame intervention period), because brown algae are the exclusive dietary source of fucoxanthin. Participants were supplied with stir-fried spinach as a dish of lunch every day between 11.30 and 12.30 hours during week 2 and were similarly supplied with stir-fried wakame during week 4. Blood was taken by venepuncture into heparinised tubes from overnight-fasting participants between 08.30 and 09.00 hours on the first day of week 2 and week 4, and on the day after the final day of week 2 and week 4. Blood samples were centrifuged at 1000 ***g*** for 15 min at 4°C. The plasma was collected and stored at − 80°C until the analyses were performed.

Diets

For week 2, fresh spinach leaves (200 g/serving) were boiled, coarsely chopped (about 5 cm) and stir-fried with olive oil (15 ml) and salt. For week 4, dried wakame (6 g/serving) was soaked in water for 10 min and drained well, yielding rehydrated wakame that swelled to about fifteen times its dry weight. Thereafter, the wakame was chopped and stir-fried with olive oil and salt in the same way as for spinach leaves. For the carotenoid analysis, cooked spinach and wakame were milled well and the duplicate portions were extracted with dichloromethane–methanol (2:1, v/v). The carotenoid content in the extract was measured by HPLC as reported previously[5]. The stir-fried spinach contained 8·6 mg β-carotene, 13·4 mg lutein, 6·5 mg violaxanthin and 3·0 mg neoxanthin per serving, and the stir-fried wakame contained 6·1 mg fucoxanthin and 0·2 mg β-carotene per serving.

Analysis of epoxyxanthophylls in plasma

Because of their low concentrations in plasma, the epoxyxanthophylls were partially purified and concentrated by solid-phase extraction before HPLC analysis. In brief, after the plasma (1·0 ml) was mixed with methanol (2·0 ml), carotenoids were extracted twice with dichloromethane (4·0 ml). The extracts were combined and dried up *in vacuo*. The residue was dissolved in hexane–diethyl ether (9:1, v/v) and applied to a Bond Elut ALN solid-phase extraction cartridge (100 mg; Varian, Palo Alto, CA, USA), which was pretreated with hexane. After the cartridge was washed with 1·0 ml hexane–diethyl ether (9:1, v/v), the epoxyxanthophylls were eluted with 1·0 ml diethyl ether–ethanol (4:1, v/v). The eluate was dried up *in vacuo*, re-dissolved in methanol–water (85:15, v/v) and filtered with a membrane filter (0·2 μm). A sample of the filtrate was then subjected to HPLC analysis. The solid-phase extraction procedure yielded > 78 % recovery for all epoxyxanthophylls tested. HPLC analyses were carried out with an Agilent 1100 System equipped with a photodiode array detector (Agilent Technologies, Santa Clara, CA, USA). A TSK-gel Super-ODS column (2 × 100 mm; Tosoh, Tokyo, Japan) attached to a Super-ODS guard column (2 × 10 mm; Tosoh) was used with a mobile phase of methanol–water (85:15, v/v) containing 0·1 % ammonium acetate at a flow rate of 0·2 ml/min.

Analysis of lipophilic carotenoids in plasma

For the analysis of major carotenoids in plasma (α-carotene, β-carotene, cryptoxanthin, lutein and lycopene), carotenoids were extracted with dichloromethane and analysed by HPLC without further purification. In brief, after plasma (0·1 ml) was mixed with PBS (0·4 ml) and methanol (1·0 ml), carotenoids were extracted twice with dichloromethane (2·0 ml). The extracts were combined and dried up *in vacuo*. The residue was dissolved in acetone and subjected to HPLC analysis.

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HPLC analyses were carried out with the same apparatus as described above with a TSK-gel ODS-80Ts column (2 × 250 mm; Tosoh) attached to an ODS-S1 guard column (2 × 10 mm; Tosoh). Analytes were eluted with methanol–ethyl acetate (70:30, v/v) containing 0·1 % ammonium acetate at a flow rate of 0·2 ml/min. Cryptoxanthin concentration was expressed as the combined value of α- and β-cryptoxanthin because of the insufficient separation of these two carotenoids in the HPLC chromatogram.

Statistics

Values are presented as mean values and standard deviations. A paired *t* test was performed to evaluate the changes in plasma carotenoids using the StatView version 4.5J statistical software (Abacus Concepts, Berkeley, CA, USA). The probability value of $P<0{\cdot}05$ was considered to be significant.

Results

Spinach carotenoids

Although we could not accurately quantify the epoxyxanthophylls because of their low concentration and coelution with unknown compounds in the HPLC chromatograms, the plasma concentrations of neoxanthin and its gastrointestinal metabolites (neochrome stereoisomers) were below the limit of quantification (1 nmol/l) after the first wash-out period and slightly increased after the 1-week spinach intervention in week 2 (Fig. 2 (A)). However, the concentrations remained below the limit of quantification in all participants. Similarly, the plasma concentrations of violaxanthin, another major epoxyxanthophyll in spinach, and its gastrointestinal metabolites (stereoisomers of auroxanthin and luteoxanthin)[5] were below the limit of quantification (1 nmol/l) even after the spinach intervention (data not shown). On the other hand, the plasma concentrations of β-carotene and lutein were markedly increased in all participants during week 2, whereas those of α-carotene, cryptoxanthin and lycopene, which were absent in spinach, were not changed significantly (Table 1).

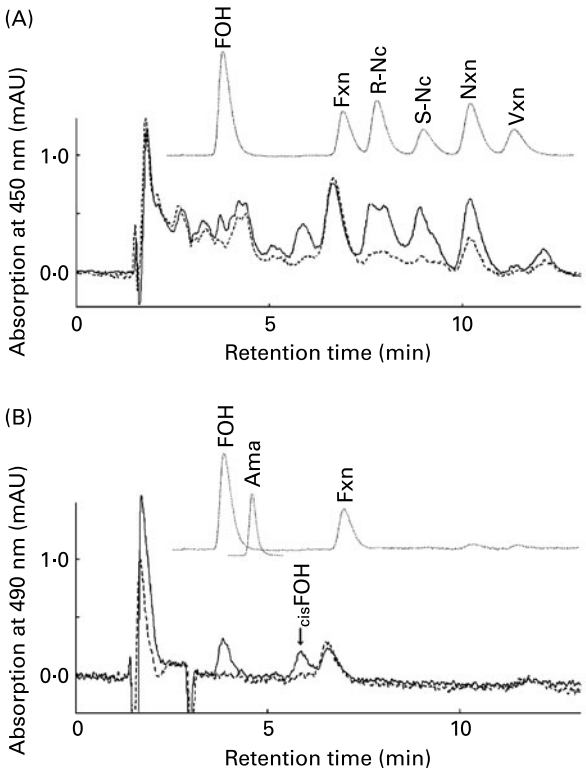

Fig. 2. Representative HPLC chromatograms of human plasma before (- - -) and after (—) 1-week intake of spinach (A) or wakame (B). The participant was supplied every day with stir-fried spinach (200 g fresh weight; 3·0 mg neoxanthin (Nxn)) or stir-fried wakame (6 g dry weight; 6·1 mg fucoxanthin (Fxn)). The upper traces (···) in both (A) and (B) indicate the chromatograms of authentic carotenoid standards (fucoxanthinol (FOH), Fxn, (8′*R*)-neochrome (R-Nc), (8′*S*)-neochrome (S-Nc), Nxn, violaxanthin (Vxn) and amarouciaxanthin A (Ama)). cisFOH indicates a *cis*-isomer of fucoxanthinol[25]. mAU, milli absorbance units.

Fucoxanthin in wakame

Fucoxanthinol, an intestinal metabolite of fucoxanthin, was not detected in the plasma after the second wash-out period, but found in the plasma of all participants after the 1-week wakame intervention in week 4 (Fig. 2 (B)). However, its concentration was very low (0·8 (SD 0·4) nmol/l), close to the limit of quantification (1 nmol/l). In addition, neither amarouciaxanthin A, a metabolite of fucoxanthinol found in mice and HepG2 cells[4], nor intact fucoxanthin was detected in the plasma before and after the wakame intervention.

Discussion

To date, there have been only a few reports on the bioavailability of food epoxyxanthophylls, despite their abundance in foodstuffs and their potential health benefits. In the present study, we determined plasma epoxyxanthophyll concentrations

Table 1. Plasma concentrations (nmol/l) of α-carotene, β-carotene, cryptoxanthin, lutein and lycopene before and after 1-week intake of spinach or wakame*

(Mean values and standard deviations of five participants)

	Before		After		Response†	
Carotenoid	Mean	SD	Mean	SD	Mean	SD
Spinach intake						
α-Carotene	200	75	171	64	−28	24
β-Carotene	2061	1153	2539	1130	+478‡	250
Cryptoxanthin§	418	255	384	217	−35	61
Lutein	860	424	1080	384	+221‡	125
Lycopene	1240	741	950	468	−290	297
Wakame intake						
α-Carotene	184	74	178	69	−6	17
β-Carotene	2403	1432	2136	1375	−268‡	159
Cryptoxanthin§	389	290	400	301	−11	72
Lutein	971	531	821	537	−150	171
Lycopene	1367	880	1149	537	−218	476

* All participants were supplied every day with a dish of stir-fried spinach (200 g fresh weight) or stir-fried wakame (6 g dry weight) for 1 week.
† Concentration changes during the 1-week intake of spinach or wakame.
‡ Significantly changed during the 1-week intake of spinach or wakame ($P<0{\cdot}05$; paired *t* test).
§ Combined value of α- and β-cryptoxanthin.

before and after 1-week intake of epoxyxanthophyll-rich diets to estimate the bioavailability of dietary epoxyxanthophylls in humans.

The marked increases of β-carotene and lutein in plasma during week 2 indicated that β-carotene and lutein in spinach were efficiently absorbed. On the other hand, neoxanthin and fucoxanthin were poorly incorporated into blood plasma during the spinach and wakame intervention weeks (week 2 and week 4), respectively. Barua & Olson[16] also reported that no epoxyxanthophyll was detected in human plasma after a single oral dose (10 mg) of purified violaxanthin or lutein 5,6-epoxide dissolved in oil. In addition, Pérez-Gálvez *et al.* [17] reported that neither violaxanthin nor capsanthin 5,6-epoxide was detected in human chylomicrons after the ingestion of paprika oleoresin (2·4 mg violaxanthin and 1·8 mg capsanthin 5,6-epoxide) together with oil. In these two studies, release of epoxyxanthophylls from the food matrix was not a limiting factor for their bioavailability. Taken together, these findings suggest that the intestinal absorption of epoxyxanthophylls from the diet is strictly limited in humans.

As other hydrophobic compounds, carotenoid uptake by intestinal epithelial cells has been thought to occur basically by simple diffusion[18]. Our previous study actually demonstrated that the uptake of various carotenoids by human intestinal Caco-2 cells was dependent on the lipophilicity of the carotenoids[15]. The partition coefficients ($\log P$ values) of β-carotene, lutein, neoxanthin and fucoxanthin were calculated to be 17·6, 14·8, 11·9 and 11·4, respectively[19]. Therefore, the limited absorption of neoxanthin and fucoxanthin may be due in part to their lower lipophilicity. Although the disruption of the food matrix and the incorporation of carotenoids into mixed micelles during gastrointestinal digestion are prerequisite for the intestinal absorption of food carotenoids, the incorporation rate of spinach neoxanthin into the micelles (including neochromes) was comparable with that of lutein and was greater than that of β-carotene in our previous *in vitro* digestion study[20]. Similarly, more than 70 % of fucoxanthin present in wakame could be solubilised as intact fucoxanthin after *in vitro* digestion (data not shown). Hence, the digestion steps before the intestinal uptake would not greatly affect the absorption of the epoxyxanthophylls. In our previous study in which purified neoxanthin and fucoxanthin were administered to mice, significant increases in the metabolites of these epoxyxanthophylls were detected in plasma, in contrast to the present study in which spinach and wakame were used as epoxyxanthophyll sources. Therefore, we cannot exclude the possibility that some components of the spinach or wakame matrix may inhibit the intestinal absorption of epoxyxanthophylls, as dietary fibres are known to reduce the bioavailability of carotenoids[20].

In addition to the simple diffusion theory, recent reports have indicated the involvement of certain receptor-mediated transport systems in the intestinal absorption of lipophilic compounds, including carotenoids[18]. For instance, the scavenger receptor class B type I, a member of the ATP-binding cassette (ABC) transporter super-family, has been shown to form a hydrophobic channel which may facilitate the concentration-gradient-dependent flux of lipophilic compounds[21]. Several reports have indicated that the scavenger receptor class B type I participates in the intestinal carotenoid uptake and transport[22,23]. A recent report also suggested the involvement of ABCG5 transporter gene polymorphisms in the individual plasma responses to dietary carotenoids and cholesterol[24]. Therefore, the absorption and excretion of lipophilic carotenoids through these receptor-mediated mechanisms may contribute to the relatively high levels of lipophilic carotenoids (i.e. α- and β-carotene, α- and β-cryptoxanthin, lutein and lycopene) in the plasma of humans and other primates, while the poor plasma response to the less lipophilic epoxyxanthophylls may also be due to their lower affinity for the transporters. However, further investigations of these transporter proteins are needed to unravel the whole absorption mechanism and to assess the transport efficiency for each carotenoid.

It is also possible that the low concentration of the epoxyxanthophylls and their metabolites in plasma was due to a rapid excretion or unknown metabolic transformations such as hydrolysis of epoxide and formation of conjugates by detoxification enzymes after the intestinal uptake. The plasma concentration after the interventions was determined in the plasma samples obtained from overnight-fasting participants. Therefore, a rapid clearance of the epoxyxanthophylls was assumed to occur during the fasting. However, the plasma concentrations of the epoxyxanthophylls and their metabolites were very low (<1 nmol/l) even at 5 h after the first test meal intake on the first day of week 2 and the first day of week 4 (data not shown). More rapid excretion or metabolic transformation of epoxyxanthophylls might exist in humans.

In conclusion, the present results indicate that bioavailability of epoxyxanthophylls from spinach and wakame is very low. The mechanism underlying the poor incorporation of epoxyxanthophylls from diets into human plasma remains to be clarified. The enhancement of their bioavailability should be considered for their application as nutraceuticals for prevention of cancer and obesity.

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