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Antioxidant effect of lutein towards phospholipid hydroperoxidation in human erythrocytes

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Peroxidised phospholipid-mediated cytotoxity is involved in the pathophysiology of many diseases; for example, phospholipid hydroperoxides (PLOOH) are abnormally increased in erythrocytes of dementia patients. Dietary carotenoids (especially xanthophylls, polar carotenoids such as lutein) have gained attention as potent inhibitors against erythrocyte phospholipid hydroperoxidation, thereby making them plausible candidates for preventing diseases (i.e. dementia). To evaluate these points, we investigated whether orally administered lutein is distributed to human erythrocytes, and inhibits erythrocyte PLOOH formation. Six healthy subjects took one capsule of food-grade lutein (9·67 mg lutein per capsule) once per d for 4 weeks. Before and during the supplementation period, carotenoids and PLOOH in erythrocytes and plasma were determined by our developed HPLC technique. The administered lutein was incorporated into human erythrocytes, and erythrocyte PLOOH level decreased after the ingestion for 2 and 4 weeks. The antioxidative effect of lutein was confirmed on erythrocyte membranes, but not in plasma. These results suggest that lutein has the potential to act as an important antioxidant molecule in erythrocytes, and it thereby may contribute to the prevention of dementia. Therefore future biological and clinical studies will be required to evaluate the efficacy as well as safety of lutein in models of dementia with a realistic prospect of its use in human therapy.

Lutein: Phospholipid hydroperoxides: Erythrocytes: Dementia

Peroxidation of membrane phospholipids (PL) has received attention in relation to oxidative stress occurring during pathophysiological changes such as atherogenesis, ageing and others[1,2]. We previously confirmed that significantly higher levels of peroxidised PL (i.e. PL hydroperoxides; PLOOH) are accumulated in the erythrocytes of dementia patients[3]. Any compounds or food components that can minimise this accumulation could be used therapeutically as effective drugs or as functional foods to prevent the diseases. We therefore carried out animal studies and found that carotenoids were potential compounds to inhibit erythrocyte PL hydroperoxidation[4,5].

From these circumstances, we are particularly interested in the effects of carotenoids on human erythrocytes, but information is very limited. In contrast, plasma carotenoids have been investigated thoroughly for analytical methods as well as biological metabolism and their significance. To the best of our knowledge, occurrence and roles of carotenoids in human erythrocytes have not been fully understood, and this has been due, in part, to a lack of the suitable analytical method.

To address this need, we recently developed a method for determining human erythrocyte carotenoids by using HPLC[6]. By using the method, evidence is accumulating that erythrocyte carotenoids (especially xanthophylls, polar carotenoids such as lutein) decrease, and erythrocyte PLOOH increase, in correlation with the severity of dementia. Erythrocytes with high levels of lipid hydroperoxides have been postulated to have a decreased ability to transport oxygen to the brain, and they may impair blood rheology[7,8], thus facilitating dementia. On the basis of these points, it was hypothesised that xanthophylls would have potential to act as important antioxidant molecules in erythrocytes, and they thereby might contribute to the prevention of dementia.

To evaluate the hypothesis, we investigated whether administered lutein is distributed to human erythrocytes, and inhibits erythrocyte PL hydroperoxidation. To date, although there have been many reports about the health benefits of xanthophylls[9], they have never provided any information about the distribution and the effect of xanthophylls in erythrocytes. Therefore, our findings (the inhibitory effect of lutein on erythrocyte PLOOH) would provide a new insight into the application of lutein possibly as an anti-dementia agent.

Abbreviations: CL, chemiluminescence; DAD, diode array detection; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; PL, phospholipid; PLOOH, phospholipid hydroperoxide.

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Subjects and methods

Supplementation study

Six healthy subjects (aged 21–28 years) participated in the present study. The subjects gave written informed consent to the experimental protocol. Subjects took one capsule of Flora-Glo Lutein (containing 9·67 mg lutein, 0·73 mg zeaxanthin and 0·12 mg α-tocopherol; Kemin Foods, Des Moines, IA, USA) once per d (after breakfast) for 4 weeks. During the experimental period, the subjects were instructed to avoid foods rich in carotenoids (for example, spinach, broccoli, carrots and tomatoes). Before, 2 and 4 weeks after the ingestion, blood was collected into a tube containing EDTA-2Na as an anticoagulant. The blood was subjected to centrifugation at 1000 ***g*** for 10 min at 4°C. After the plasma and buffy coat were removed, erythrocytes were washed three times with PBS (pH 7·4) to prepare packed cells. The packed cells were immediately subjected to determination of carotenoids and PLOOH. Plasma samples were stored at −80°C until analysis.

Measurement of carotenoids in erythrocytes

For determination of erythrocyte carotenoids[6], packed cells (2·5 ml) were diluted with 2·5 ml of water, and were mixed with 5 ml of 80 mM-ethanolic pyrogallol, 1·0 ml of 1·8 M-aqueous KOH and 40 μl of 1 μM-ethanolic echinenone (internal standard). After addition of 1·25 ml of 0·1 M-aqueous sodium dodecyl sulfate, the sample was mixed with 15 ml of hexane–dichloromethane (5:1, v/v) for extraction of erythrocyte carotenoids. The extract was purified by Sep-Pak silica cartridge (Waters, Milford, MA, USA), and then subjected to HPLC coupled with UV diode array detection (DAD) and atmospheric pressure chemical ionisation MS. A C30 Carotenoid column (4·6 × 250 mm, 5 μm; YMC, Kyoto, Japan) was used. The column was eluted using a binary gradient consisting of the following HPLC solvents: A, methanol–*tert*-butyl ether (MTBE)–water (83:15:2, by vol.; containing 3·9 mM-ammonium acetate); B, methanol–MTBE–water (8:90:2, by vol.; containing 2·6 mM-ammonium acetate). The gradient profile was as follows: 0–12 min, 10–45 % B linear; 12–24 min, 45–100 % B linear; 24–30 min, 100 % B. The flow rate was adjusted to 1 ml/min, and the column temperature was maintained at 20°C. The column eluent was sent to a Shimadzu SPD-M10Avp DAD detector (Kyoto, Japan) for monitoring carotenoids at 463 nm. Concentrations of erythrocyte carotenoids were calculated using the equation corresponding to the external standard curve of each carotenoid and were adjusted by the percentage recovery of the added echinenone (internal standard).

Measurement of phospholipid hydroperoxides in erythrocytes

For determination of erythrocyte PLOOH[2–5], total lipids were extracted from packed cells with a mixture of 2-propanol and chloroform containing butylated hydroxytoluene. PLOOH (i.e. phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH)) in the total lipids were measured by HPLC with chemiluminescence (CL) detection. The column was a 4·6 × 250 mm, 5 μm Finepak SIL NH2-5 (Japan Spectroscopic Co., Tokyo, Japan), the eluent was 2-propanol–methanol–water (135:45:20, by vol.) and the flow rate was 1·0 ml/min. Post-column CL detection was carried out using a CLD-100 detector (Tohoku Electronic Industries Co., Sendai, Japan). A mixture of luminol and cytochrome c in 50 mM-borate buffer (pH 10·0) was used as a hydroperoxide-specific post-column CL reagent. Calibration was carried out using standard PCOOH or PEOOH.

Other analytical methods

For plasma samples, carotenoids and PLOOH were determined by HPLC-UV[4] and HPLC-CL[2–5], respectively. Tocopherols in erythrocytes and plasma were measured by HPLC with fluorescence detection[10]. Erythrocyte and plasma PL were quantified by Bartlett's method[11]. Erythrocyte Hb was measured by a commercial kits (Wako, Osaka, Japan).

Statistical analyses

Data were expressed as mean values and standard deviations. Statistical analysis was performed by ANOVA followed by Tukey's test. Differences were considered significant at $P<0.05$.

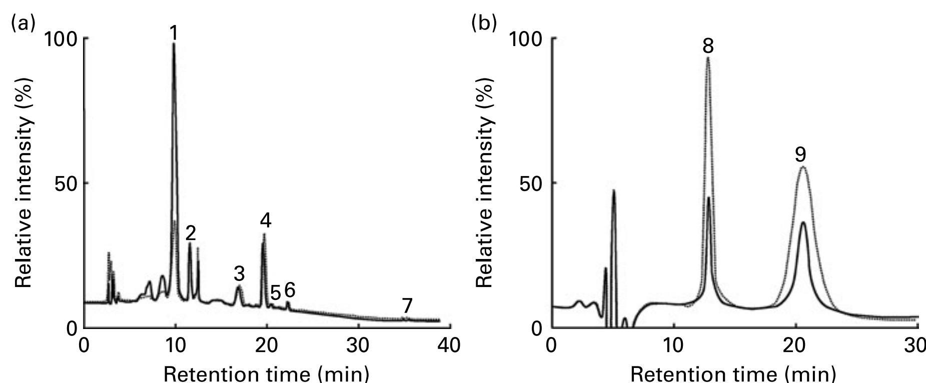

Fig. 1. Typical diode array detection (DAD) and chemiluminescence (CL) chromatograms of carotenoids (a) and phospholipid hydroperoxides (PLOOH) (b) in erythrocytes taken before (····) and 4 weeks after (—) ingestion of food-grade lutein (Flora-Glo Lutein; Kemin Foods, Des Moines, IA, USA). Erythrocyte carotenoids and PLOOH were determined by HPLC-DAD and HPLC-CL, respectively. Peak identifications are as follows: 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin; 4, echinenone (internal standard); 5, α-carotene; 6, β-carotene; 7, lycopene; 8, phosphatidylcholine hydroperoxide; 9, phosphatidylethanolamine hydroperoxide.

Results

In a typical DAD chromatogram of erythrocyte extract taken before the ingestion of Flora-Glo Lutein, six endogenous carotenoids (lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene) were separated, detected by DAD (Fig. 1 (a)), and concurrently identified based on atmospheric pressure chemical ionisation MS and UV spectra profiles (data not shown). The relative amounts of each carotenoid were consistently as follows: lutein > β-cryptoxanthin > zeaxanthin > β-carotene > lycopene > α-carotene (Table 1). These data support our previous finding that, in human erythrocytes, xanthophylls (especially lutein) are the most prevalent carotenoids[6]. In contrast, non-polar carotenoids (for example, β-carotene) were more abundant in plasma (Table 1). In a typical CL chromatogram of erythrocyte total lipids taken before the ingestion, PCOOH and PEOOH were the predominant forms of PLOOH (Fig. 1 (b)). On the other hand, only PCOOH was detected in plasma (Table 1).

After ingestion of Flora-Glo Lutein for 4 weeks, erythrocyte lutein concentration increased from baseline (160 (SD 42) pmol/g Hb) to 449 (SD 120) pmol/g Hb, whereas erythrocyte PLOOH (sum of PCOOH and PEOOH) decreased from 4·9 (SD 2·5) μmmol/mol PL to 1·5 (SD 1·2) μmmol/mol PL (Table 1). Flora-Glo Lutein ingestion did not affect the levels of zeaxanthin, β-carotene, lycopene and α-carotene in erythrocytes and plasma, PLOOH in plasma, and tocopherols in erythrocytes and plasma (Table 1). Also, erythrocyte Hb, erythrocyte PL as well as plasma PL were not affected by Flora-Glo Lutein ingestion (data not shown). Based on these results, it is suggested that when humans ingest lutein, lutein is absorbed, distributed and accumulated in erythrocytes, where it acts as an antioxidant molecule, thereby reducing PLOOH as an index of oxidative stress.

Table 1. Carotenoid, phospholipid hydroperoxide (PLOOH) and tocopherol contents in erythrocytes and plasma taken before and after ingestion of food-grade lutein (Flora-Glo Lutein*) (*n* 6)

(Mean values and standard deviations)

	Before ingestion		2 weeks after ingestion		4 weeks after ingestion	
	Mean	SD	Mean	SD	Mean	SD
Erythrocytes						
Carotenoids (pmol/g Hb)						
Lutein	160[a]	42	364[b]	113	449[b]	120
Zeaxanthin	52	18	66	21	71	18
β-Cryptoxanthin	66	19	64	17	68	21
α-Carotene	8	5	9	6	5	4
β-Carotene	20	9	22	14	11	4
Lycopene	12	6	14	10	5	2
Xanthophylls†	278[a]	47	495[b]	116	589[b]	118
Non-polar carotenoids‡	40	18	46	28	21	10
Total carotenoids	318[a]	57	540[b]	110	610[b]	110
PLOOH (μmol/mol phospholipids)						
PCOOH	2·7[a]	1·4	1·7[a]	0·6	0·7[b]	0·5
PEOOH	2·1[a]	1·1	0·7[b]	0·2	0·8[b]	0·8
Total PLOOH (PCOOH + PEOOH)	4·9[a]	2·5	2·4[a]	0·8	1·5[b]	1·2
Tocopherols (nmol/g Hb)						
α-Tocopherol	20·2	3·1	21·2	2·9	20·7	0·8
γ-Tocopherol	3·0	0·6	3·8	1·1	3·2	0·5
Total tocopherols	23·2	3·0	25·0	3·2	23·9	0·9
Plasma						
Carotenoids (pmol/ml)						
Lutein	341[a]	119	682[a,b]	280	768[b]	295
Zeaxanthin	115	33	122	46	120	38
β-Cryptoxanthin	411[b]	111	284[a]	77	224[a]	31
α-Carotene	184	94	147	56	135	48
β-Carotene	475	189	409	168	337	175
Lycopene	303	140	249	119	301	112
Xanthophylls†	868	151	1083	361	1112	317
Non-polar carotenoids‡	961	377	805	303	772	255
Total carotenoids	1829	452	1894	445	1885	279
PLOOH (μmol/mol phospholipids)						
PCOOH	9·5	5·1	7·7	2·2	11·3	3·9
Tocopherols (nmol/ml)						
α-Tocopherol	24·4	4·3	22·7	4·0	23·7	3·6
γ-Tocopherol	2·7	0·6	3·1	0·5	3·0	0·4
Total tocopherols	27·1	4·6	25·7	3·6	26·6	3·8

PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide.
[a,b,c] Mean values within a column with unlike superscript letters were significantly different ($P < 0·05$).
* Kemin Foods (Des Moines, IA, USA).
† Xanthophylls are the sum of lutein, zeaxanthin and β-cryptoxanthin.
‡ Non-polar carotenoids are the sum of α-carotene, β-carotene and lycopene.

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Discussion

In recent years, the antioxidative property of food constituents has been seriously noted by medical and nutritional experts, since the reactive oxygen species-mediated peroxidation of biological molecules (for example, lipids) has been postulated to induce a variety of pathological events such as atherogenesis, ageing and dementia. Although many *in vitro* studies on the antioxidative property of food constituents have been reported, little has been known about the biological functions of dietary antioxidants *in vivo* (especially in humans), except for major antioxidants (for example, tocopherols and ascorbic acid). Since the bioavailability of food constituents is limited by their digestibility and metabolic fate, oral administration trials are favoured to evaluate their biological function.

The present study demonstrates that when human subjects ingest food-grade lutein (Flora-Glo Lutein), lutein is absorbed, distributed and accumulated in erythrocytes, where it exhibits antioxidative effects *in vivo* (inhibition of erythrocyte PL hydroperoxidation). It is interesting that the antioxidative effect observed in the present study was brought about by relatively short-term supplementation with lutein (2–4 weeks). In the present study, each volunteer received 9·67 mg lutein per d. The dosage was about 2-fold compared with that of human daily intake[12].

Until now, there have been about ten reports concerning human erythrocyte carotenoids. Some studies successfully detected erythrocyte carotenoids (mainly β-carotene)[13], while the others were unable to detect them[14]. Incorporation of carotenoid (β-carotene) into erythrocytes after oral supplementation was described in some reports[15]. However, there has been no study evaluating whether administered carotenoids other than β-carotene are distributed to the erythrocytes. In the present study, incorporation of lutein into erythrocytes after oral supplementation was well documented (Fig. 1 (a)). Because erythrocyte lutein concentration increased along with the elevation of plasma lutein (Table 1), it seems likely that lutein in plasma lipoprotein particles is partly transferred into erythrocytes. By this hypothesis, lutein would be located on the outer region of plasma lipoprotein, which facilitates its transfer to erythrocytes. On the other hand, the concentrations of endogenous antioxidants (i.e. carotenoids and tocopherols) other than erythrocyte and plasma lutein and plasma β-cryptoxanthin showed no changes before and after Flora-Glo Lutein ingestion (Table 1). This is advantageous for elucidation of the antioxidative contribution of lutein. According to the decrease of plasma β-cryptoxanthin (Table 1), this seems to be because the volunteers did not ingest foods enriched in β-cryptoxanthin throughout the supplementation period.

In the present study, to evaluate the peroxidisability, we measured PLOOH. Because PLOOH are the primary oxidation products of PL, an increase in PLOOH directly reflects *in vivo* oxidative stress[16]. As evidenced here, lutein supplementation clearly reduced erythrocyte PLOOH concentration (Fig. 1 (b)), indicating that lutein incorporated into erythrocytes attenuated PL peroxidation of erythrocyte membranes. On the other hand, the antioxidative effect of lutein was confirmed on the erythrocyte membrane, but not in the plasma (Table 1). Erythrocytes are rich in PUFA in the PL bilayer, and contain high concentrations of molecular oxygen and ferrous ions as constituents of oxyhaemoglobin Hb. The oxidation of Hb accompanies the formation of superoxides, the source of more reactive oxygen species. Therefore, erythrocyte membrane PL would be more susceptible to peroxidation than other organelle membranes, although being protected by several antioxidative systems such as superoxide dismutase, catalase and glutathione peroxidase.

Recently, polar carotenoids (xanthophylls) have gained increasing scientific interest due to their antioxidative, anti-obesity and anti-inflammatory activities[17], which differ somewhat from those of non-polar carotenoids (for example, β-carotene). As mentioned in the Introduction, we previously found that the higher accumulation of PLOOH in the erythrocytes of dementia patients[3] and the inhibitory effect on PLOOH formation in erythrocyte membranes was confirmed in mice by dietary supplementation with carotenoids[4,5]. In another study, a pronounced distribution of xanthophylls (for example, lutein) to erythrocytes was confirmed[6]. In the present study, orally administered lutein was incorporated into erythrocytes, and erythrocyte PLOOH levels decreased. This was supported by our ongoing study, in which human volunteers daily received chlorella rich in lutein. According to another ongoing study, erythrocyte PLOOH increased, and erythrocyte xanthophylls (for example, lutein) decreased, in correlation with the severity of dementia. Erythrocytes high in lipid hydroperoxides have been suggested to have a decreased ability to transport oxygen to brain, and they may impair blood rheology[7,8], thus facilitating dementia. On the basis of these points, it seems that xanthophylls, especially lutein, have the potential to act as important antioxidant molecules in erythrocytes, and they thereby may contribute to the prevention of dementia. This possibility warrants their testing in other models of dementia with a realistic prospect of their use in human therapy.

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K. N. was involved in data collection, data analysis, data interpretation, literature search and manuscript preparation. T. K., K. H. and P. S. were involved in data collection, data analysis and data interpretation. H. A. and T. M. were involved in study design, data interpretation and review of the manuscript.

None of the authors has conflicts of interest with respect to the present study.

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