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Inhibition of β-carotene-15,15′-dioxygenase activity by dietary flavonoids

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β-Carotene-15,15′-dioxygenase is an enzyme responsible for providing vertebrates with vitamin A by catalyzing oxidative cleavage of β-carotene at its central double bond to two molecules of retinal in intestinal cells. However, little data have been reported regarding regulation of the enzyme activity. We have evaluated the effects of antioxidants and dietary flavonoids on the β-carotene dioxygenase activity in vitro using a pig intestinal homogenate as the enzyme source. 2,6-Di-tert-butyl-4-methylphenol (BHT), a synthetic antioxidant, strongly inhibited the activity at the level of 10^{-6} M (a mixed-type inhibition), whereas butylated hydroxyanisole, nor-dihydroguaiaretic acid, n-propyl gallate, and curcumin were moderately inhibitory. Flavonoids such as luteolin, quercetin, rhamnetin, and phloretin remarkably inhibited the dioxygenase activity noncompetitively, whereas flavanones, isoflavones, catechins, and anthocyanidins were less inhibitory. The structure-activity relationship indicated that catechol structure of ring B and a planar flavone structure were essential for inhibition. The enzyme inhibition was also indicated in the cultured Caco-2 cells by the significantly reduced conversion of β-carotene to retinol when incubated with BHT and rhamnetin at 2 μM and 5 μM, respectively. The results suggest that some dietary antioxidants derived from food sources modulate conversion of β-carotene to vitamin A in intestinal cells. (J. Nutr. Biochem. 11:348–355, 2000)

Keywords: inhibition; β-carotene; dioxygenase; flavonoids; quercetin

Introduction

Dietary carotenoids play important roles as a major source of vitamin A and one of dietary antioxidants in human health. Most carotenoids are ingested from fruits and vegetables, but their bioavailability to the human body remains to be clarified to enhance absorption of carotenoids or conversion of β-carotene to vitamin A.[1] Part of β-carotene absorbed in intestinal cells is converted to vitamin A and incorporated as retinyl ester into chylomicron. The other part is directly incorporated into chylomicron without metabolic conversion. Thus vitamin A and intact β-carotene are delivered through lymph into tissues.[2] β-Carotene-15,15′-dioxygenase (CDOX) present in intestinal cells catalyzes cleavage reaction of β-carotene at the central double bond to two molecules of retinal.[3–5] Therefore, CDOX is a key enzyme to determine whether the provitamin A carotenoids that are absorbed are either metabolized to vitamin A or delivered to tissues as intact carotenoids. Consequently, CDOX activity can modulate biological function of dietary β-carotene: vitamin A or antioxidant. However, little is known about the regulation of CDOX activity. It was shown that CDOX activity is influenced by protein content in diet[6] and vitamin A status.[7,8] Recently we found that dietary polyunsaturated triacylglycerols enhanced both CDOX activity and the level of cellular retinol binding protein type II in rat intestine.[9] Dietary carotenoids have been found to inhibit CDOX activity. Canthaxanthin and zeaxanthin inhibited CDOX activity competitively in vivo as well as in

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vitro,[10] and α-carotene, β-cryptoxanthin, and lutein, but not lycopene inhibited CDOX in vitro.[11] Erchov and colleagues[12,13] found that lycopene, lutein, and astaxanthin competitively inhibited CDOX and that antioxidants such 2,6-Di-*tert*-butyl-4-methylphenol (BHT), butylated hydroxyanisole (BHA), and ascorbic acid inhibited the activity. These reports indicate that conversion of β-carotene to vitamin A can be modulated by inhibitory effects of various dietary components on CDOX activity. The purpose of this study was to investigate effects of dietary antioxidants, especially flavonoids, on CDOX activity.

Material and methods

Materials

All-*trans* β-carotene (type II), all-*trans* retinal, quercetin, rutin, and (−)-epicatechin were purchased from Sigma Chemical Co. (St. Louis, MO USA). All-*trans* retinol was prepared by reducing all-*trans* retinal with $NaBH_4$. *d*-α-Tocopherol was obtained from Eisai Co. (Tokyo, Japan). (+)-Catechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate were purchased from Kuritakogyo Co. (Tokyo, Japan). Flavone and flavanone were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan), respectively. Other flavonoids were purchased from Extrasynthese (Genay, France). High performance liquid chromatography (HPLC)-grade acetonitrile was purchased from Nacalai Tesque, Inc. Other chemicals and solvents were of reagent grade. All-*trans* β-carotene, all-*trans* retinal, and all-*trans* retinol were purified as described previously.[5] Purified all-*trans* β-carotene was stored at −80°C in *n*-hexane containing *d*-α-tocopherol at a molar ratio of 0.01 to the amount of β-carotene as antioxidant.

Enzyme preparation

The pig intestinal homogenate was prepared as described previously and used as CDOX preparation.[5] Briefly, the intestinal mucosa of one female pig (11 months old and 128 kg in weight) was homogenized with a Potter Elvehjem homogenizer in 5 volumes of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES)-potassium hydroxide (KOH) buffer, pH 7.4, containing 0.154 M potassium chloride, 1 mM ethylenediamine-tetraacetic acid (EDTA), and 0.1 mM dithiothreitol (DTT). A supernatant solution of the homogenate, after centrifugation at 10,000 g for 30 min, was dialyzed against 10 mM HEPES-KOH buffer, pH 7.4, containing 0.1 mM EDTA, 0.05 M potassium chloride, and 0.1 mM DTT. The protein fraction after centrifugation was stored at −80°C until used as a cleavage enzyme preparation.

Assay of β-carotene dioxygenase activity

The reaction mixture contained 15 μM β-carotene, 0.1 M *N*-tris(hydroxymethyl)methylglycine-KOH buffer, pH 8.0, 0.5 mM DTT, 0.15% Tween 40, 0.1 mM α-tocopherol, and enzyme (<1.5 mg protein) in a total volume of 0.2 mL. The solubilization of β-carotene into reaction mixture was described previously.[5] The most compounds tested for inhibitory effect on the enzyme were dissolved in ethanol and the 10 μL was added to the reaction mixture to give a concentration less than 100 μM in the reaction mixture, whereas the polar compounds such as ascorbic acid, EDTA, diethylenetriamine-*N*,*N*′,*N*′,*N*′,*N*′-pentaacetic acid, and deferoxamine mesylate were dissolved in deionized water instead of ethanol. After preincubation at 37°C for 5 min, the cleavage reaction was started by adding the enzyme preparation. The mixture was then incubated at 37°C under atmospheric oxygen for 60 min. The reaction was terminated by adding 0.05 mL of 37% (w/w) formaldehyde and the mixture was incubated at 37°C for 10 min. Then 0.5 mL of acetonitrile was added to the mixture and mixed well and the supernatant after centrifugation was directly subjected to retinal analysis by HPLC as described previously.[14]

Conversion of β-carotene to retinol by Caco-2 cells

Caco-2 cells obtained from American Type Culture Collection (Rockville, MD USA) were cultivated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 4 mM L-glutamine, 40 U/mL penicillin, 40 μg/mL streptomycin, and 1% nonessential amino acids (Life Technologies Inc., Grand Island, NY USA). The cells were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide in air. The cells were grown in 5 mL of the medium on a 6-cm plastic dish at a seeding density of $1.5 \times 10^4/cm^2$, and the growth media were changed every 2 or 3 days. Three weeks after seeding, the growth media were changed to the fresh medium without supplementation of fetal calf serum. Thereafter, 50 μL of BHT or rhamnetin dissolved in dimethyl sulfoxide and 100 μL of 100 μM β-carotene solubilized in an aqueous 1% Tween 40 were added to the medium. After incubation for 24 hr, the cell monolayer was washed twice with 1 mL of Dulbecco's phosphate buffered saline (PBS) containing 5 mM sodium taurocholate. The cells were then scraped with a small spatula in 5 mL of PBS and pelleted by centrifugation at 1,000 rpm for 5 min (4°C). The cell pellet was suspended in 0.8 mL of PBS and was mixed well with 3 mL of dichloromethane/methanol (1:2, v/v) containing 0.01% pyrogallol and 0.003% BHT. Glacial acetic acid (0.05 mL) and *n*-hexane (1.5 mL) were mixed well with the above mixture and the resultant upper layer was withdrawn. The lower layer was similarly extracted with 1 mL of dichloromethane followed by 1.5 mL of *n*-hexane. The upper layer was combined with the initial extract. The combined extract was dried under a stream of argon gas and dissolved in 1 mL of dichloromethane/methanol (1:4, v/v). A 50 μL of the extract was subjected to β-carotene analysis by HPLC. The residual extract was again dried under a stream of argon gas and dissolved in 1 mL of acetonitrile/water (2:1, v/v). After filtration, 200 μL of the extract was subjected to retinol analysis by HPLC.

The HPLC system consisted of an LC-10AD pump (Shimadzu Co., Kyoto, Japan), an SPD-10A UV-VIS absorbance detector (Shimadzu Co.), an autosampler (Tosoh Co., Tokyo, Japan), and a personal computer with an EZChrome Chromatography Data System (Scientific Software Inc., Pleasanton, CA USA). Retinol was separated on a TSK gel ODS-80Ts, 4.6 × 150 mm, attached to a precolumn (2 × 20 mm) of Pelliguard LC-18 (Supelco Inc., Bellefonte, PA USA). Acetonitrile/methanol/water (75:15:10, v/v/v) containing 0.1% ammonium acetate was used as a mobile phase at a flow rate of 1.0 mL/min. β-Carotene was separated on the same column as in the case of retinol analysis, with ethyl acetate/methanol (25:75, v/v) containing 0.1% ammonium acetate as a mobile phase at a flow rate of 1.0 mL/min. Retinol and β-carotene were monitored at 325 nm and 450 nm, respectively, and were quantified from their peak area by use of standard curves with all-*trans* retinol and all-*trans* β-carotene. Incubation and extraction procedures were carried out under dim yellow light to minimize isomerization and degradation of β-carotene and retinoids by light irradiation.

Statistical analysis

Results were analyzed by one-way analysis of variance and Tukey's test, after examining for normal distribution and homogeneity of variances by Bartlett test.[15] *P*-values of less than 0.01 were considered significant. All analyses except for Tukey's test

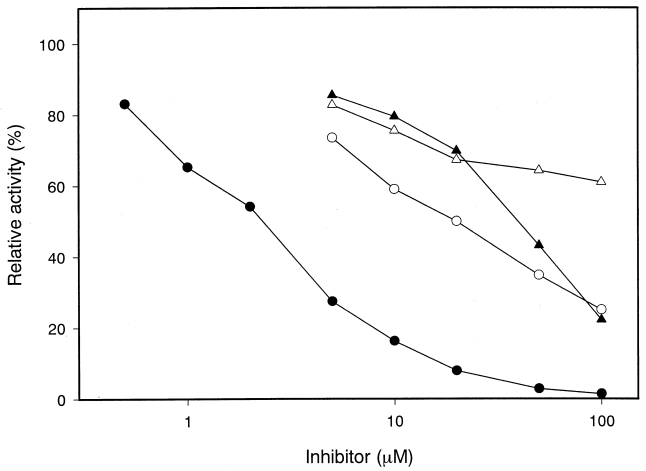

Figure 1 Effects of antioxidants on β-carotene dioxygenase activity. β-Carotene dioxygenase activity was determined in the presence of antioxidants: 2,6-di-*tert*-butyl-4-methylphenol, ●; nor-dihydroguaiaretic acid, ○; curcumin, ▲; pyrogallol, △. Values are expressed as the means of triplicate incubations.

were performed using the StatView software (Version 4.51, Abacus Concepts Inc., Berkeley, CA USA). Tukey's test was performed using a calculation table created with Excel 97 software (Microsoft Co., Redmond, WA USA).

Results

BHT, permitted as food additive for prevention of oxidative deterioration, was found to inhibit CDOX activity at a low concentration. The compounds analogous to BHT, the other synthetic antioxidants, and the natural antioxidants present in foods such as phenylpropanoids (caffeic, chlorogenic, and ferulic acids), capsaicin, and curcumin were evaluated for inhibition of CDOX activity of pig intestinal homogenate. Several antioxidants had inhibitory effects on CDOX activity in a concentration-dependent manner (*Figure 1*). Half-inhibition concentration (IC_{50}) was calculated from a curve plotting residual activity against inhibitor concentration. BHT showed the lowest IC_{50} value among the tested compounds (*Table 1*). The phenolic antioxidants analogous to BHT, such as BHA, tert-butylhydroquinone (TBHQ), and probucol, also had inhibitory effects on CDOX activity, but much less inhibitory than BHT. Although 3,5-di-*tert*-butyltoluene lacked a hydroxyl group of BHT, it showed weak but significant inhibition of CDOX. Among the antioxidants with catechol structure, nordihydroguaiaretic acid (NDGA), protocatechualdehyde, and *n*-propyl gallate significantly inhibited CDOX activity, whereas catechol, caffeic acid, and pyrogallol were less inhibitory. The chlorogenic acid, a sugar conjugate of caffeic acid showed no inhibition. On the other hand, vanillin and ferulic acid, in which one of the hyroxyl group of catechol was substituted by a methoxyl group, showed no effect on CDOX. Capsaicin and curcumin significantly inhibited CDOX activity, although they have no catechol structure, but had a phenolic hydroxyl group. Ascorbic acid and ascorbyl palmitate had no effect on CDOX activity up to 100 μM. α-Tocopherol increased the activity with a maximum (1.7-fold the activity in the absence of α-tocopherol) at 100 μM, but no inhibitory

Table 1 Inhibition of β-carotene dioxygenase activity by antioxidants

Antioxidants	IC_{50} (μM)	Ki (μM)
2,6-Di-*tert*-butyl-4-methylphenol (BHT)	2.5	0.79
Butylated hyroxyanisole (BHA)	59.9	37.3
3,5-Di-*tert*-butyltoluene	>100	118.7
tert-Butylhydroquinone (TBHQ)	>100	
Probucol	>100	
Protocatechualdehyde	31.3	
Nor-dihydroguaiaretic acid (NDGA)	19.9	
n-Propyl gallate	13.3	
Pyrogallol	>100	
Catechol	>100	
Caffeic acid	>100	
Chlorogenic acid	No inhibition	
Vanillin	No inhibition	
Ferulic acid	No inhibition	
Capsaicin	>100	
Curcumin	42.3	16.9
Ascorbyl palmitate	No inhibition	
Ascorbic acid	No inhibition	

effect was found up to 500 μM. Protection of β-carotene and retinal from oxidative degradation by α-tocopherol might cause the apparent enhancement of the activity. Therefore, 100 μM α-tocopherol was added to the standard reaction mixture in this study.

Flavonoids present in fruits and vegetables are also natural antioxidants frequently ingested together with carotenoids. Flavonoids are basically composed of three rings (A, B, and C rings) (*Figure 2*) and more than 4,000 varieties with different substitutions are distributed in plants. Twenty-six flavonoids, which were present in foods and medicinal plants, were evaluated for inhibition of CDOX activity of pig intestinal homogenate. Some of them inhibited CDOX activity in a concentration-dependent manner (*Fig-*

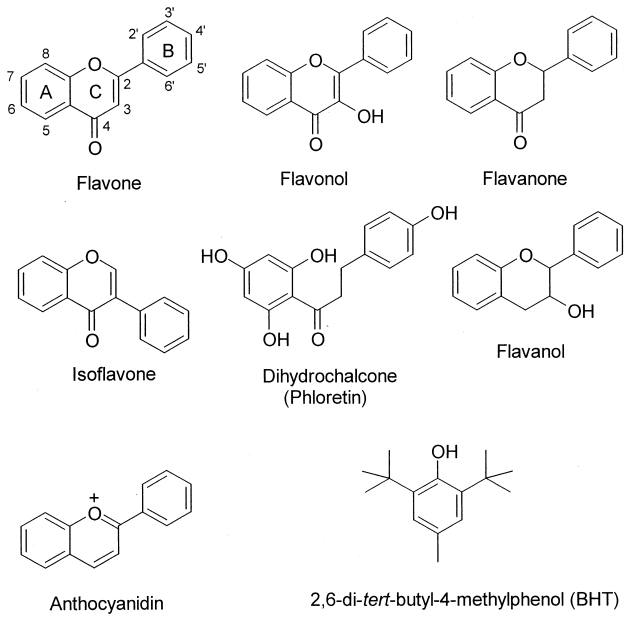

Figure 2 Basic structure of flavonoids and 2,6-di-*tert*-butyl-4-methylphenol (BHT) evaluated for inhibition of β-carotene dioxygenase.

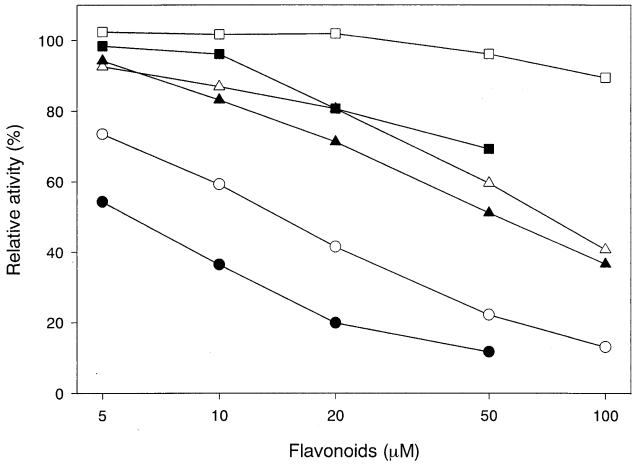

Figure 3 Effects of flavonols on β-carotene dioxygenase activity. β-Carotene dioxygenase activity was determined in the presence of flavonols: rhamnetin, ●; quercetin, ○; myricetin, ▲; kaempferol, △; isorhamnetin, ■; rutin, □. Values are expressed as the means of triplicate incubations.

ure 3). Chrysin showed little inhibition ($IC_{50} > 100$ μM), whereas luteolin, 3′,4′-dihyroxy derivatives of chrysin showed a strong inhibition ($IC_{50} = 9.2$ μM) for CDOX activity (*Table 2*). Quercetin (3-hydroxy luteolin) and rhamnetin (7-methoxy quercetin) also inhibited CDOX activity at low concentration. The modification of catechol structure in ring B of quercetin reduced inhibitory effect on CDOX activity as found in kampferol, myricetin, and isorhamnetin. Taxifolin (2,3-dihydroquercetin), (+)-catechin, and (−)-epicatechin with catechol structure in ring B was far less inhibitory than was quercetin. The anthocyanidins tested were far less inhibitory, although cyanidin had catechol structure in ring B. BHT, BHA, 3,5-di-tert-butyltoluene, and curcumin showed a mixed-type inhibition, whereas flavonoids such as luteolin, quercetin, rhamnetin, and phloretin showed a noncompetitive inhibition. The typical Lineweaver-Burk plots for BHT and rhamnetin are shown in *Figure 4*.

A chelating activity for metal ions of flavonoids might cause inhibition of CDOX activity, because the enzyme was thought to require iron for its activity. Therefore, several chelators for iron were evaluated for inhibitory effects on the enzyme activity using the same assay conditions that were used for flavonoids (*Table 3*). Of the five chelators tested, only 1,10-phenanthroline showed a significant inhibition at 100 μM, but lower inhibition than quercetin and rhamnetin. Phenanthrene, an analogue of 1,10-phenanthroline without chelating activity, was also inhibitory. α,α′-Dipyridyl was not inhibitory, but its analogous hydrocarbon of 1,1′-biphenyl was slightly inhibitory.

Table 2 Inhibition of β-carotene dioxygenase activity by flavonoids

Class	Compound	Substitutes[1]	IC_{50} (μM)	Ki (μM)
Flavone	Flavone		>100	
	Chrysin	5,7,-OH	>100	
	Luteolin	5,7,3′,4′-OH	9.2	13.3
Flavonol	Kaempferol	3,5,7,4′-OH	75.3	
	Quercetin	3,5,7,3′,4′-OH	15.2	16.2
	Myricetin	3,5,7,3′,4′,5′-OH	53.8	
	Rhamnetin	3,5,3′,4′-OH; 7-OMe	6.0	5.8
	Isorhamnetin	3,5,7,3′-OH; 4′-OMe	>50	
	Tangeretin	3,5-OH; 6,7,8-OMe	99.7	
	Rutin	5,7,3′,4′-OH; 3-*O*-rutinosyl	>100	
Flavanone	Flavanone		>100	
	Taxifolin	3,5,7,3′,4′-OH	>100	
	Hesperetin	5,7,3′-OH; 4′-OMe	>100	
Isoflavone	Daizein	7,4′-OH	>100	
	Genistein	5,7,4′-OH	89.5	
Dihydrochalcone	Phloretin	[2]	9.1	9.9
Flavanol	(+)-Catechin	3,5,7,3′,4′-OH	>100	
	(−)-Epicatechin	3,5,7,3′,4′-OH	>100	
	(−)-Epicatechin gallate	5,7,3′,4′-OH; 3-*O*-galloyl	>100	
	(−)-Epigallocatechin	3,5,7,3′,4′,5′-OH	>100	
	(−)-Epigallocatechin gallate	5,7,3′,4′,5′-OH; 3-*O*-galloyl	>100	
Anthocyanidin	Cyanidin	3,5,7,3′,4′-OH	>100	
	Delphinidin	3,5,7,3′,4′,5′-OH	>100	
	Apigenidin	5,7,4′-OH	>100	
	Peonidin	3,5,7,4′-OH; 3′-OMe	>100	
	Pelargonidin	3,5,7,4′-OH	>100	

[1] The basic structure and specific carbon number are shown in *Figure 2*.
[2] The substituents of phloretin are shown in *Figure 2*.

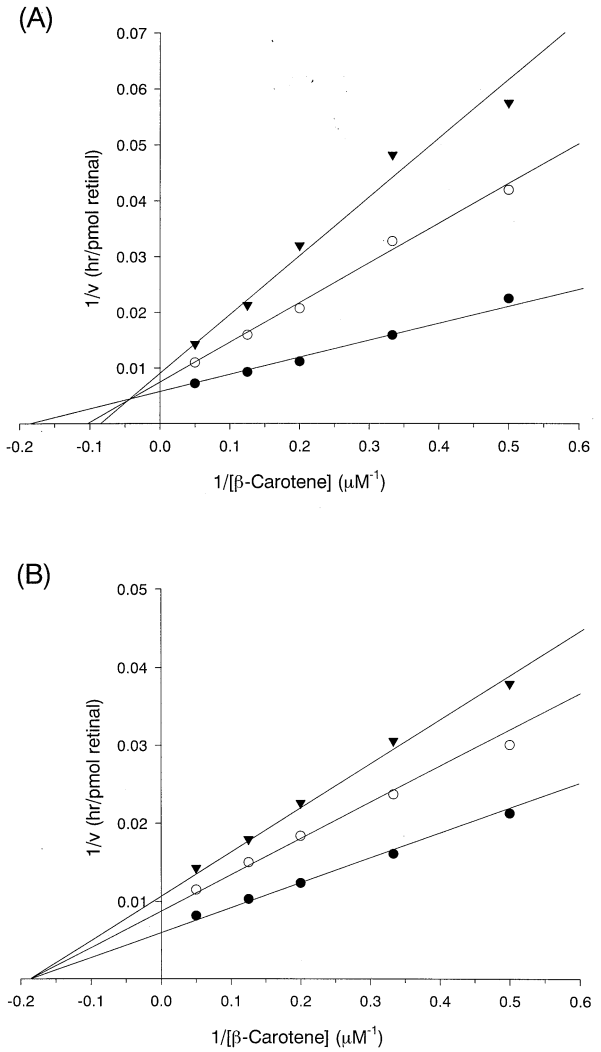

Figure 4 Lineweaver-Burk plots for the inhibition of β-carotene dioxygenase by 2,6-di-*tert*-butyl-4-methylphenol (BHT) and rhamnetin. (A) Inhibition by BHT: ●, control; ○, 1 μM; ▼, 2 μM. (B) Inhibition by rhamnetin: ●, control; ○, 2.5 μM; ▼, 5 μM. Values are expressed as the means of triplicate incubations.

To elucidate whether BHT and rhamnetin are absorbed in intestinal cells and inhibit CDOX activity, these compounds, which showed a strong inhibition for CDOX activity of pig intestinal homogenate at lower concentrations in vitro, were evaluated for their effects on conversion of β-carotene to vitamin A by Caco-2 cells. β-Carotene was effectively converted to retinol for 24 hr by the cells. In the control incubation, β-carotene accumulation, retinol, and retinyl ester formed in the cells were about 2.4 nmol/dish, 69 pmol/dish, and 6.3 pmol/dish, respectively. Retinol and retinyl ester in the medium were 30 pmol/dish and 6 pmol/dish, respectively. The major product of β-carotene was retinol, whereas retinoic acid and retinal were not detected either in the medium or in the cells. The ratio of retinol in medium to intracellular retinol was nearly constant at 40% irrespective of the culture conditions. The total amount of retinol formed from β-carotene was approximately 100 pmol/24 hr/60-mm culture dish, which was

Table 3 Effects of chelators and related compounds on β-carotene dioxygenase activity

Compounds	Relative activity (%)
None	100
α,α′-Dipyridyl	100.9
1,1′-Biphenyl (Diphenyl)	78.2
1,10-Phenanthroline	28.4
Phenanthrene	71.0
EDTA	102.7
Diethylenetriamine-*N*,*N*′,*N*′,*N*′,*N*′-pentaacetic acid	99.5
Deferoxamine mesylate	88.2

Values are expressed as the means of triplicate incubations.

comparable to the value of approximately 12 to 20 pmol retinyl ester/hr/100-mm culture dish (approximately 104–172 pmol/24 hr/60-mm culture dish) in cultured Caco-2 cells estimated by Quick and Ong[16] Retinol accumulation in the cells from β-carotene was significantly reduced to 63.5% of the control by BHT added to the culture medium at 2 μM, which was 2.5-fold the K_i value for the dioxygenase (*Figure 5*). Rhamnetin also significantly repressed to 70.4% at 20 μM, which was 3.3-fold the K_i value. Accumulation of β-carotene was slightly reduced at high concentrations of BHT and rhamnetin. Rhamnetin at 20 μM decreased β-carotene accumulation by 14.4% of the control. When the β-carotene accumulation was reduced by incubating Caco-2 cells at lower concentrations of β-carotene (<2 μM in the absence of inhibitors), retinol formation was repressed by 4.4% per 10% decrease of β-carotene accumulation in the cells (data not shown). On the basis of this ratio, the decrease of β-carotene accumulation (14.4%)

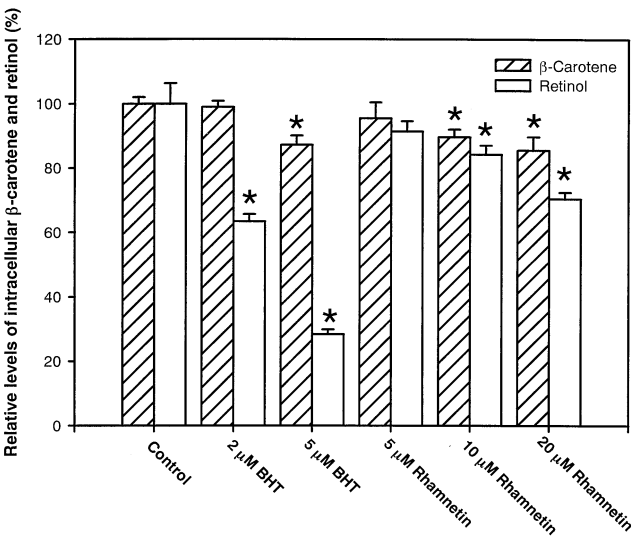

Figure 5 Effects of 2,6-di-*tert*-butyl-4-methylphenol (BHT) and quercetin on conversion of β-carotene to retinol by Caco-2 cells. β-Carotene was incubated with Caco-2 cells for 24 hr and the amount of intracellular β-carotene and retinol were quantified. Control incubations were done without β-carotene. The values obtained were normalized against those of the control incubation without inhibitors. Bar indicates standard deviation of four incubations. The asterisks indicate a significant difference from the control incubation ($P < 001$).

observed at 20 μM rhamnetin could cause 6.3% reduction of retinol formation, whereas actual reduction was 29.6%. Thus, reduction of retinol formation by BHT and rhamnetin was largely dependent on their direct effect on conversion of β-carotene to retinol rather than decreased availability of β-carotene.

Discussion

The conversion of β-carotene in intestinal cells plays an essential role in providing vertebrates with vitamin A. However, knowledge about regulation of the responsible enzyme, CDOX, is limited. The ingestion of fruits and vegetables rich in carotenoids is accompanied by other phytochemicals. The intestinal cells are exposed to these substances, and thereby physiologic function of intestinal cells would be directly modified by food components in comparison with other tissues. The low bioavailability of β-carotene in dark green leafy vegetables as vitamin A source is considered as the results of poor absorption of β-carotene.[17–19] Moreover, some phytochemicals present in the vegetables might reduce the conversion of β-carotene to vitamin A. In this study, we found that some flavonoids and antioxidants present in foods strongly inhibited β-carotene dioxygenase activity in vitro as well as in cultured cells.

Among the tested compounds, BHT showed the most potent inhibition for CDOX activity. The four BHT analogues with or without a phenolic hydroxyl group also showed a significant inhibition of CDOX activity, but far less inhibition than BHT. The phenolic hydroxyl group as well as the carbon skeleton of BHT is suggested to be essential for a strong inhibition. Ershov et al.[12] also reported the inhibitory effects of BHT and BHA, although IC_{50} values (130 μM and 320 μM, respectively) were much higher than those obtained in this study. This discrepancy might be due to the difference of the conditions for the dioxygenase reaction. Mixed-type inhibition observed in BHT, its analogues (BHA and 3,5-di-*tert*-butyltoluene), and curcumin suggests that these hydrophobic substances bind with the dioxygenase both at the substrate-binding site competitively and at the other site to inactivate the enzyme. BHT may be an analogue of β-carotene for CDOX, because the carbon chain consisted of the two *tert*-butyl groups and the three carbons (C-2, C-1, and C-6) of the benzene ring in BHT are similar to the central part (C-12 to C-12′) or to the end part (C-2, C-1, and C-6 to C-10) of β-carotene molecule. Grolier et al.[10] found that CDOX activity was inhibited by canthaxanthin (K_i = 1.6 μM) in a mixed-type mode and by zeaxanthin (K_i = 7.8 μM) in a noncompetitive mode. These results indicate that carotenoid and its analogous compounds inhibit CDOX activity by binding at the active site competitively and/or at the other site noncompetitively. The results on the rest of the antioxidants shown in *Table 1*, except for capsaicin and curcumin, suggested that the antioxidants with catechol structure inhibited CDOX activity, although the degree of inhibition was largely varied. The inhibition mode of these antioxidants might be different from that of BHT and its analogues without catechol structure.

The structure-activity relationships observed in inhibition of CDOX activity by flavonoids clearly indicate that catechol structure of ring B in flavonol is essential for inhibition. Moreover, the lower inhibition by taxifolin (2,3-dihydroquercetin) than by quercetin suggest that the nonplanar structure due to saturated ring C of quercetin might reduce the inhibitory effect. This is also suggested by low inhibitory effects of (+)-catechin and (−)-epicatechin with catechol structure in ring B. These different inhibitory effects between planar and nonplanar flavonoids were also observed in cyclooxygenase and xanthine oxidase.[20,21] Noncompetitive inhibitions observed in flavonoids suggest their exclusive binding to a site other than the substrate-binding site. The planar flavonols with catechol structure in ring B might have higher affinity for the binding site to inactivate CDOX than the other flavonoids. In rutin, a conjugate of rutinose with quercetin may loosen binding to the enzyme of its bulky sugar moiety. Nonplanar flavonoids may not be able to bind the enzyme because of the molecular shape does not fit the binding pocket. Anthocyanidins may not be able to access the enzyme, because of its presence as flavylium cation.

Flavonoids are known to show antioxidant activity through their oxygen-radical trapping and heavy metal ion-chelating activity. The three possible sites of flavonol (C-5 hydroxyl and C-4 carbonyl groups, C-3 hydroxyl and C-4 carbonyl groups, and C-3′, C-4′ catechol structure in ring B) are thought to be responsible for chelating activity.[22] Thus, the flavonoids might inhibit the β-carotene dioxygenase activity by chelating iron, which has been suggested to be required for the catalysis.[23] However, the result that the chelators tested did not show a significant inhibition except for 1,10-phenanthroline suggests that the inhibition observed by the flavonoids in this assay condition is not due to their chelating activity for iron. Lipoxygenase with nonheme iron as an essential component for the activity was reported to be more inhibited by hydroxypyridione iron chelators as their lipophilicity was higher, and it was suggested that the hydrophobic chelators accessible to substrate binding pocket can interact with iron at the active center of the enzyme.[24] If it is the case with β-carotene dioxygenase, it is reasonable that bulky and polar flavonols relative to β-carotene cannot inhibit the enzyme activity because of the lack of accessibility to iron in the enzyme. In fact, the hydrophilic chelators such as EDTA, diethylenetriamine-*N*,*N*′,*N*′,*N*′,*N*′-pentaacetic acid, and deferoxamine mesylate did not inhibit the CDOX activity, but hydrophobic *O*-phenanthroline did. Several antioxidants with catechol structure are well known to inhibit lipoxygenases. Reduction of fatty acid radical intermediate or reduction of catalytically active ferric ion of the enzyme to inactive ferrous ion was suggested to account for the inhibition.[25] Whether such antioxidant nature of phenolic hydroxyl group is also involved in inhibition of CDOX activity by BHT and the flavonoids is not clear from the results obtained in this study. However, direct effect of phenolic hydroxyl group of flavonoid with reaction intermediate or iron may not occur, because flavonoids are suggested to interact with the enzyme at the site other than the active center.

These inhibitions found in the enzyme reaction with the pig intestinal homogenate were confirmed in the cultured cells of Caco-2, which was derived from a human colon

adenocarcinoma and has been used as a model system of human intestinal cells.[26] The result that retinol was formed as a major product from β-carotene during incubation with Caco-2 cells certainly indicates the presence of CDOX in the cells, although CDOX activity might be too small to be detected in the cell homogenate as reported by During et al.[27] Effect of BHT and rhamnetin on conversion of β-carotene to retinol could be evaluated by measuring intracellular retinol level. The result that the two compounds clearly reduced retinol formation from β-carotene suggests that β-carotene dioxygenase was directly inhibited by these compounds absorbed into the cells, although the possible effects of the compounds on the reduction of retinal to retinol was not excluded. Some flavonoids were reported to be absorbed and metabolized in the Caco-2,[28,29] and recent studies have shown that most quercetin present in human plasma was found in the form of metabolites.[30] Therefore, it was not certain whether rhamnetin and/or its metabolites inhibited the conversion of β-carotene to vitamin A in the Caco-2 cells. Nonetheless, the intact rhamnetin present in cells before metabolic conversion could inhibit the CDOX activity.

The flavonoids that showed low K_i value for CDOX activity were rhamnetin, phloretin, luteolin, and quercetin. Rhamnetin and phloretin are present in some spices and an apples, respectively, whereas quercetin and luteolin are distributed widely in plants. Quercetin is most abundant in diets among the above four flavonoids. The average intake of quercetin was reported to range from 2.6 to 38.2 mg/d in various populations of seven countries.[31] Based on these values, quercetin was estimated to be present at 0.96 to 14.0 μM in a digestive fluid (9 L/day) of intestinal tract. In the population with the highest intake of quercetin, its level in an intestinal fluid would reach the value close to IC_{50} value of quercetin (15.2 μM) against β-carotene dioxygenase activity. However, dietary flavonoids are mostly present as glycosides in plant foods. Recent studies have suggested that flavonoid glycosides as well as aglycons are absorbed to intestinal cells, where they are hydrolyzed to aglycons by a cytosolic β-glycosidase and then converted to glucuronides by a microsomal enzyme.[32–35] Thus, free quercetin may interact with a cytosolic enzyme, β-carotene dioxygenase, in the intestinal cells. However, quercetin level attained in the intestinal cells still remains to be quantitatively estimated to elucidate a possible in vivo effect of quercetin diet on β-carotene dioxygenase reaction. BHT is currently approved for use in food as an antioxidant. The average intake of BHA and/or BHT in the Netherlands was estimated to be 0.075 mg/kg body weight/day, based on their maximum permitted levels in foods.[36] Based on this value, the concentration of BHT was calculated to be 2.3 μM in a digestive fluid of intestine. As 2 μM BHT in the Caco-2 cell cultures reduced significantly the conversion of β-carotene to vitamin A, the dietary intake of BHT would affect vitamin A formation in a certain condition. Thus, BHT would not be appropriate as food preservative for the population at risk of vitamin A deficiency.

In this study, a wide range of antioxidants and dietary flavonoids was evaluated for the inhibition of CDOX activity. The planar flavonols with a catechol structure in ring B and BHT had a highly inhibitory effect on CDOX. The detailed mechanism for inhibition and the in vivo effect of these inhibitors on conversion of β-carotene to vitamin A remain to be studied to clarify the bioavailability of dietary provitamin A carotenoids in various foods.

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