Free Radical Lipid Peroxidation Inhibits Enzymatic Conversion of β-Carotene into Vitamin A

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Free radical oxidation of arachidonic acid with soybean lipoxygenase was accompanied by inhibition of retinal synthesis from β-carotene catalyzed by enzyme preparation from rabbit intestinal mucosa. Lipoxygenase inhibitor salicylhydroxamic acid and antioxidants suppressing free radical reactions (ethyl gallate, α-tocopherol, astaxanthine, and quercetin) promoted conversion of β-carotene into retinal catalyzed by β-carotene-15,15'-dioxygenase. These results indicate that lipoperoxides and/or products of their homolysis attenuate enzymatic conversion of β-carotene and confirm the important role of natural antioxidants in the maintenance of stable vitamin A content in mammals.

Key Words: *β-carotene; retinal; β-carotene-15,15'-dioxygenase; soybean lipoxygenase; antioxidants*

β-Carotene, an important component of plant-derived food products, is converted in mammals into retinal and other forms of vitamin A essential for the maintenance of growth, development, and vision. More than 60% retinoids in the body are synthesized from β-carotene [5]. However, under physiological conditions only small amounts of β-carotene are converted into retinal. The coefficient of molar conversion of β-carotene into retinal varies from 3 to 20; for humans, the mean value of this parameter is 6 [14]. The formation of vitamin A from β-carotene decreases during vitamin deficiency, infectious or parasitic diseases [7].

β-Carotene-15,15'-dioxygenase (CDO, EC 1.13.11.21) is a key enzyme catalyzing conversion of β-carotene into retinal. It cleaves the central double bond in the substrate molecule. Our previous studies demonstrated sharp inhibition of β-carotene conversion into retinal in the presence of potent oxidant sodium hypochlorite, which is primarily related to degradation of the enzyme molecule (not the substrate) [2]. These data suggest that lipid hydroperoxides, free radical intermediates, and other lipid peroxidation (LPO) products can modulate biotransformation of β-carotene into vitamin A. It can not be excluded that under physiological conditions lipid radicals formed after homolysis of hydroperoxides of polyunsaturated fatty acids (PUFA), which are present in food or synthesized under the effect of exogenous or endogenous lipoxygenases (LO), attack CDO in mammalian intestine. LO and their substrates are abundant in plants [8] and various organs of birds and mammals, including the gastrointestinal tract [15].

Here we studied the mechanism of free radical inactivation of CDO and the possibility of protecting this enzyme with natural antioxidants.

MATERIALS AND METHODS

CDO was isolated from rabbit intestinal mucosa by ammonium sulfate precipitation (60% saturation) [3]. CDO activity was measured in a medium (1 ml) containing 0.2 M potassium-phosphate buffer (pH 8.0),

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2 mM reduced glutathione, 20 mM sodium dodecyl sulfate, 30 μM β-carotene (emulsion), and 2-3 mg CDO preparation. Incubation was performed in the dark at 37°C for 2 h. The reaction was stopped by protein precipitation with acetone (3-fold volume). The products were extracted with hexane (3 portions), pooled extracts were vacuum evaporated, and the residue was dissolved in hexane and fractionated by thin-layer chromatography on aluminum oxide-coated plates in a hexane-acetone system (85:15). Retinal (R_f=0.5) was extracted with diethyl ether, vacuum evaporated, and dissolved in ethanol. The absorption spectrum was recorded at 320-450 nm. Soybean LO activity was measured spectrophotometrically in 0.2 M potassium-phosphate buffer (pH 8.0) in the presence of 10 μM arachidonic acid (micelles) at 20°C. Accumulation of arachidonate 15-hydroperoxide was estimated by the formation of diene conjugates at 233 nm. The concentration of conjugated dienes was calculated at ε=25,000 M^{-1} cm^{-1} [6]. In our experiments arachidonate oxidation catalyzed by soybean LO was completed within 3 min (kinetic curve reached the plateau) due to autoinactivation of the enzyme. The concentrations of arachidonate 15-hydroperoxyde after 3-min incubation with 0.04, 0.1, 0.15, and 0.2 U/ml LO were 4, 10, 15, and 20 μM, respectively (±10%).

When CDO was incubated with LO, 10 μM arachidonic acid and 0.15 U soybean LO were added to an incubation medium containing reduced glutathione, sodium dodecyl sulfate, and enzyme preparation and after 3 min β-carotene emulsion was added. LO inhibitors and antioxidants were added to the incubation medium before addition of LO in ethanol (final ethanol concentration 0.5%). The antioxidants were added in concentrations providing maximum CDO activity (previous data).

We used 4% emulsion of β-carotene (BASF AG), astaxanthine, α-tocopherol (Fluka), soybean lipoxygenase-1, salicylhydroxamic acid, quercetin (Sigma), reduced glutathione (Calbiochem), aluminum oxide (Merck), ethyl gallate, ammonium sulfate, acetone, hexane, and diethyl ether purified by fractional distillation with sodium metabisulfite.

RESULTS

Incubation of β-carotene in the presence of CDO yielded 1.21±0.06 nmol/mg protein/h retinal, which was taken as 100%. No conversion of β-carotene into retinal was observed in the presence of arachidonic acid and LO in the incubation medium. However, arachidonic acid or LO alone did not inhibit retinal synthesis (100±5 and 100.0±6.2%, respectively). CDO activity did not differ from the control (100.0±4.9%) after incubation with arachidonic acid and LO in the presence of the specific LO inhibitor salicylhydroxamic acid (0.2 mM), which attested to involvement of lipoxygenase products in CDO inactivation.

Antioxidants in various concentrations added to the incubation medium containing CDO and β-carotene had no effect on enzyme activity (Table 1). However, antioxidants added to the incubation medium containing LO alone decelerated enzymatic oxidation of arachidonate and reduced the concentration hydroperoxide. Previous studies demonstrated LO inhibition in the presence of ethyl gallate, quercetin, α-tocopherol, and carotenoids [9,11,12]. In our experiments the content of arachidonate hydroperoxides depended on the type and concentration of antioxidants (Table 1). Phenol antioxidant ethyl gallate and α-tocopherol in test concentrations inhibited accumulation of hydroperoxides in the incubation medium during lipoxygenase-catalyzed oxidation of arachidonate (Table 1). At the same time, polyphenol quercetin and carotenoid astaxanthine had no effect on lipid peroxide accumulation (Table 1). A negative correlation between CDO activity and the content of accumulated lipoperoxides was found both in the absence and presence of antioxidants (Fig. 1). Our findings do not allow us to conclude that CDO is inhibited by lipid peroxides, since

TABLE 1. Effects of Lipoxygenase Products and Antioxidants on β-Carotene Conversion into Retinal in Preparations from Rabbit Intestinal Mucosa (*M*±*m*, *n*=3)

Series	[LOOH]*, μM	CDO activity, %
Control	0	100±5
	4	79±4
	10	51±6
	15	0
Ethyl gallate, 0.2 mM	0	100±5
	0.3	81±6
α-Tocopherol, 0.35 mM	0	100±5
	1.1	100±4
	2.8	74±6
	4.2	53±5
Quercetin, 0.01 M	0	100±5
	7.2	100±6
	10.8	65±5
	14.5	0
Astaxanthine, 0.005 mM	0	100±5
	3	100±5
	7.4	91±6
	11.1	82±7
	15.8	0

Note. *[LOOH]: concentration of arachidonic acid hydroperoxides generated by soybean LO over 3 min in the incubation medium.

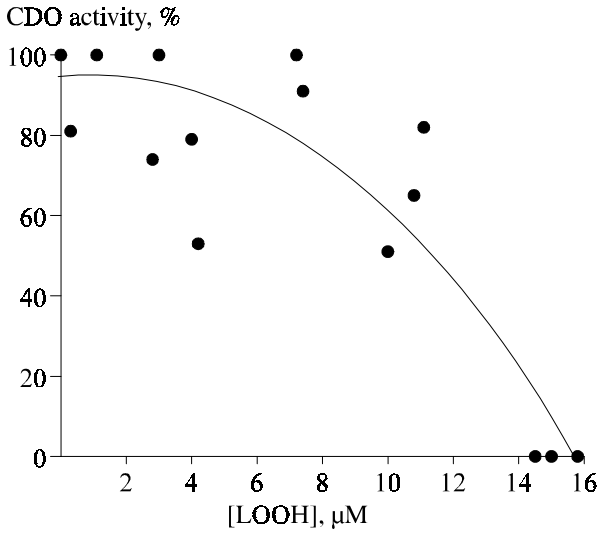

Fig. 1. β-Carotene-15,15'-dioxygenase (CDO) activity as a function of lipid hydroperoxide concentration (LOOH) in the incubation medium. r=0.81, p<0.001.

incubation can be accompanied by homolysis of these compounds with the formation of reactive alkoxyl radicals that damage lipids in biological membranes, lipoproteins, and biopolymers (protein enzymes and nucleic acids). Homolysis of hydroperoxides is activated in the presence of iron ions. In our experiments autoinactivation of Fe-containing LO accelerated this reaction. Our previous studies showed that alkoxyl radicals, but not soybean LO product 15-hydroperoxyarachidonate, initiate oxidative degradation of β-carotene in the model system [1]. The inhibitory effect of free radical oxidation on enzyme activities *in vitro* and *in vivo* was reported [4,10,13]. The data indicate that CDO is sensitive to free radical oxidation. Antioxidants hold much promise as CDO protectors during oxidative stress *in vitro* and, probably, *in vivo*.

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