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Carotenoid Oxidative Degradation Products Inhibit Na^+–K^+-ATPase

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This study investigates the biological significance of carotenoid oxidation products using inhibition of Na^+–K^+-ATPase activity as an index. β-Carotene was completely oxidized by hypochlorous acid and the oxidation products were analyzed by capillary gas–liquid chromatography and high performance liquid chromatography. The Na^+–K^+-ATPase activity was assayed in the presence of these oxidized carotenoids and was rapidly and potently inhibited. This was demonstrated for a mixture of β-carotene oxidative breakdown products, β-Apo-10′-carotenal and retinal. Most of the β-carotene oxidation products were identified as aldehydic. The concentration of the oxidized carotenoid mixture that inhibited Na^+–K^+-ATPase activity by 50% (IC_{50}) was equivalent to 10 μM non-degraded β-carotene, whereas the IC_{50} for 4-hydroxy-2-nonenal, a major lipid peroxidation product, was 120 μM. Carotenoid oxidation products are more potent inhibitors of Na^+–K^+-ATPase than 4-hydroxy-2-nonenal. Enzyme activity was only partially restored with hydroxylamine and/or β-mercaptoethanol. Thus, *in vitro* binding of carotenoid oxidation products results in strong enzyme inhibition. These data indicate the potential toxicity of oxidative carotenoid metabolites and their activity on key enzyme regulators and signal modulators.

Keywords: Carotenoids, carotenoid breakdown products, aldehydes, 4-hydroxynonenal, Na^+–K^+-ATPase

Abbreviations: Apo-10, β-Apo-10′-carotenal; ARMD, age related macular degeneration; BME, β-mercaptoethanol; CBP, carotenoid breakdown products; GC-MS, gas liquid chromatography-mass spectrometry; HA, hydroxylamine; HPLC, high performance liquid chromatography; HNE, 4-hydroxy-2,3-*trans*-nonenal, 4-hydroxy-2-nonenal; NE, *trans*-2,3-nonenal; NA, nonanal (nonyl aldehyde); PUFA, polyunsaturated fatty acids; TCA, trichloroacetic acid

INTRODUCTION

Carotenoids have many biological functions, including the quenching of singlet oxygen, and preventing the peroxidative degradation of polyunsaturated fatty acids (PUFA).[1,2] Antioxidant functions are associated with mitigating DNA damage, malignant transformation, and other

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parameters of *in vitro* cell damage as well as epidemiologically with decreased incidence of cancers (primarily epithelial), and other degenerative diseases, such as Alzheimer's disease, atherosclerosis, cardiac ischemia, cataracts, and age related macular degeneration (ARMD).[3–5] These associations originated from excellent observational epidemiology, that has consistently indicated that diets containing fruits and vegetables enriched in carotenoids have a significant risk reduction for chronic diseases. However, in some studies, high dose supplementation of β-carotene to men who were heavy smokers increased the incidence of lung cancer.[6–8] The conclusion that major public health benefits could be achieved by increasing consumption of carotenoid-rich fruits and vegetables still appears to stand. However, the pharmacological supplementation of β-carotene to prevent cardiovascular disease and lung cancer, particularly in smokers, can no longer be recommended.[7]

In an attempt to understand the phenomenon of partial toxicity seen during supplementation of β-carotene in smokers, Paolini *et al.* have shown that β-carotene induces cytochrome P450 in rat lung,[9] whilst Gradelet *et al.* showed that β-apo-8′-carotenal but not β-carotene increased rat hepatic cytochrome P450 levels.[10] In humans elevated levels of these carcinogen-metabolizing enzymes would translate into increased cancer risk from tobacco carcinogens. In addition Krinsky has exposed ferrets, fed high dose β-carotene, to cigarette smoke and has observed unexpected changes in the lung nuclear receptors and histology. These changes were attributed to oxidative metabolites of β-carotene,[11] rather than to the intact carotenoid precursor. This could perhaps explain how these compounds, lacking specific binding proteins or nuclear receptors, can exert such profound biological effects. Handelman *et al.*[12] identified and characterized β-breakdown products of autoxidized β-carotene. Krinsky suggested that these oxidative metabolites modulated the activity of protein kinase C, a major player in cell signaling.[11]

Interactions of endogenous aldehydic compounds such as 4-hydroxynonenal (HNE), the end product of lipid peroxidation, with enzymes and other proteins are well known.[13–19] In this study we examined the effect of oxidative carotenoid breakdown products on Na^+–K^+-ATPase activity, which we previously reported to be irreversibly inhibited by HNE with a IC_{50} value = 120 μM.[13] The activities of carotenoid metabolites, HNE and related aldehydes were compared. Additionally restoration of enzyme activity was evaluated in the presence of hydroxylamine (HA) and β-mercaptoethanol (BME).

MATERIALS AND METHODS

Materials

Na^+–K^+-ATPase preparation (adenosine 5′-triphosphatase; ATPase; ATP phosphohydrolase; sodium- and potassium-activated; sensitive to ouabain and to inhibition by vanadate; EC 3.6.1.3) from porcine cerebral cortex, 0.3 units/mg protein at pH 7.8, 1 unit liberates 1.0 μmol inorganic phosphorus of ATP/min at pH 7.4 at 37°C in the presence of Na^+, K^+, and Mg^{2+}, BME and trichloroacetic acid (TCA) were obtained from Sigma Chemical Co (St. Louis, MO). Ouabain, ATP, and ammonium molybdate were from Fluka Chemie AG (Buchs, Switzerland). Nonanal (nonyl aldehyde, NA) *trans*-2,3-nonenal (NE), ethyl hydroxylamine and HA were from Aldrich Chem., Co. (Milwaukee, WI). HNE was synthesized as previously described.[20] The purity of the HNE preparation was monitored by thin-layer and gas–liquid chromatography (GC) as previously described[20] and was > 97%. Carotenoid standards were generous gifts from Hoffman–La Roche (Nutley, NJ).

Carotenoid Breakdown Conditions

Samples of 0.2 ml methanolic β-carotene stock solution of 0.04 mM (20 mg/L) were 'bleached' whilst mixing with 1 mM hypochlorous acid at

room temperature for 15 min. Because β-carotene is completely destroyed, the initial undissolved fraction goes into solution, and the breakdown products are well solubilized.[21] The extent of β-carotene oxidative breakdown was monitored by high performance liquid chromatography (HPLC).[22] In all samples which were used as carotenoid breakdown products (CBP) there was no detectable intact β-carotene. After the 15 min of 'bleaching' the CBP were extracted twice with 2 ml hexane. The hexane phases were combined, and evaporated to dryness with argon. The residue was reconstituted in 0.2 ml ethanol. These solutions were used for Na^+–K^+-ATPase inhibition experiments.

Gas–Liquid Chromatography of CBP and CBP-Oxime Derivatives

CBP and their oxime derivatives were analyzed using a 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA) with a split–splitless injector and a flame ionization detector that was interfaced to a computer with Chem-Station software (Hewlett-Packard, Palo Alto, CA). A DB-23 capillary column (15 m × 0.2 mm ID, 0.15 µm film thickness) was used. Dry helium at 35 kPa pressure was the carrier gas. Air and hydrogen were used as fuel gases chosen to give maximum sensitivity at flow rates of 350 and 42 kPa, respectively. The injector port and the interphase were kept at 260°C. The oven temperature was kept at 50°C for 2 min followed by an increase to 110°C at a rate of 5°C/min, held at 110°C for 1 min followed by a 5°C/min increase to a final temperature of 240°C and held for 10 min. Ethyl-oxime derivatives of CBP were prepared as previously described.[23]

Measurement of Na^+–K^+-ATPase Activity

Na^+–K^+-ATPase activity was determined as the difference between the total activity and the Mg^{2+}-stimulated activity (ouabain-insensitive component) in medium B according to Schoot *et al.*[24] For most measurements 20 µl diluted enzyme preparation (1.85 nmol/µl) were added to 400 µl chilled medium, which was freshly prepared for each experiment. Medium A contained 110 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 30 mM imidazole–HCl, pH 7.4, and medium B contained 110 mM NaCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ouabain, and 30 mM imidazole–HCl, pH 7.4. ATPase activity measurement was started by the addition of ATP (final concentration 5 mM) and reagents were incubated at 37°C for 30 min. After incubation the samples were chilled on ice and 3 ml stop-color reagent was added. This comprised 1.32 N H_2SO_4 with 1.15% (w/v) ammonium heptamolybdate, which was supplemented before use with 9.2% (w/v) $FeSO_4$. Stop reagent was 8.6% (v/v) TCA, which was mixed with an equal volume of color reagent before use, to constitute the stop-color reagent. The samples were stirred, incubated at room temperature for 30 min, and the inorganic phosphorus concentration was measured spectrophotometrically at 700 nm, using KH_2PO_4 solutions as standards. To correct for partial hydrolysis of ATP solutions, reference measurements of the incubation media, were taken with and without the addition of the same volume of ATP, used for starting ATPase activity measurements. Although this hydrolysis is known to be minimal, it has to be taken into account, especially when the ATPase activity of the sample is low.

Inhibition of ATPase Activity by CBP, HNE and Related Aldehydes

Na^+–K^+-ATPase was preincubated for 20 min with varying concentrations of CBP, or Apo-10′-carotinal (Apo-10), or retinal (vitamin A aldehyde), or β-carotene (0.1, 0.25, 1, 2.5, and 10 µM), respectively before assaying its activity. Comparative parallel assays were performed with HNE and other C9-aldehydes, such as NE and NA, as their inhibition of ATPase is well-established. HNE, NE, and NA were added at 1, 10, 50, and 100 µM concentrations. All reagents i.e. 0.4 ml

medium A or B, supplemented with the equivalent of 0.4 mM non-degraded β-carotene of CBP, and similar amounts of Apo-10, retinal, β-carotene, HNE, NE, or NA, respectively, and the enzyme solution (0.4 mg enzyme/ml, 1.2 nmol ATPase) were kept on ice. Samples were then transferred to the water bath at 37°C and the enzyme was preincubated for 20 min. To determine the time-dependent inhibition rate, the incubation time for the binding of the CBP was varied from 5 to 60 min. It was found that the inhibition was achieved within 10 min of incubation (data not shown). For HNE, NE, and NA that was already established.[13] Therefore, a preincubation time of 20 min was chosen for all following experiments.

Restoration of ATPase Activity after Incubation with BME and HA

Na^+–K^+-ATPase was reacted with CBP, Apo-10, or retinal as described above, and restoration of enzyme activity was attempted with HA and/or BME, as described in detail previously.[13] The optimal concentrations of BME and HA for the incubation conditions were previously established and shown to be 0.5 and 1 mM, respectively.[13] HA is known to reverse Schiff's base bonds, to form oxime derivatives with aldehydes, which are simultaneously released from the protein lysine or histidine residues.[23] In the presence of BME, free sulfhydryl groups of the enzyme can be regenerated.

Measurements were repeated for each aldehyde, and the average and standard deviation were calculated. Statistical analysis of data in Table II was done using ANOVA with Bonferroni's method for comparison of HA and or BME treated samples with control (CBP, no HA or BME added) samples.

RESULTS

Table I shows the inhibition of Na^+–K^+-ATPase activity by carotenoid oxidation products (CBP, Apo-10, and retinal), β-carotene itself, and HNE at 10 μM concentration. CBP were the most potent inhibitors of Na^+–K^+-ATPase activity. 4-HNE and non-degraded β-carotene were equipotent inhibitors, and were less active than retinal. Figure 1A shows the inhibition of Na^+–K^+-ATPase activity by CBP and related compounds. For comparison the inhibition of HNE and related compounds NA, and NE on the enzyme activity is shown in Figure 1B. Figure 1A shows that the inhibition of Na^+–K^+-ATPase activity by CBP, Apo-10, and retinal (0.1–10 μM) is concentration-dependent. Similarly a concentration-responsive inhibition for HNE, NE, and NA (1–100 μM) is shown in Figure 1B. The slopes for both curves are similar except for the concentration range at which equivalent inhibition is obtained. The IC_{50} values for the ATPase inhibition were obtained from these curves. The IC_{50} for CBP is 11 μM, which is one tenth of the value for equivalent inhibition by HNE. Thus, CBP are more potent than HNE as inhibitors of Na^+–K^+-ATPase. Figure 2 shows the chromatograms of CBP separated by GC. Figure 2A shows the CBP without derivatization, whereas Figure 2B shows the ethyl-oxime derivatives of these same samples. By comparing these chromatograms it is apparent that some of the peaks in Figure 2A are not present in Figure 2B and vice versa. This implies that several of the CBP are aldehydes which were converted to ethyl-oxime derivatives. In Table II the restoration

TABLE I Inhibition of Na^+–K^+-ATPase by carotenoid oxidation products, β-Carotene, and HNE

Compound	ATP splitting	% of control	*n*
CBP	291 ± 27	52.4 ± 4.8	20
β-Carotene	474 ± 73	85.3 ± 13.1	36
Apo-10	461 ± 44	83.0 ± 7.9	20
Retinal	403 ± 7	72.5 ± 1.2	12
4-HNE	445 ± 18	89.0 ± 3.2	8

Values expressed as nmol/mg protein/min for ATP splitting and as % of control. The data are given as mean ± standard deviation. The rate of ATP splitting in the control was 556 ± 33 (49 measurements). The concentration used was 10 μM; for all compounds. For CBP this represented the mixture of products from 10 μM of completely oxidized β-carotene as monitored by HPLC.

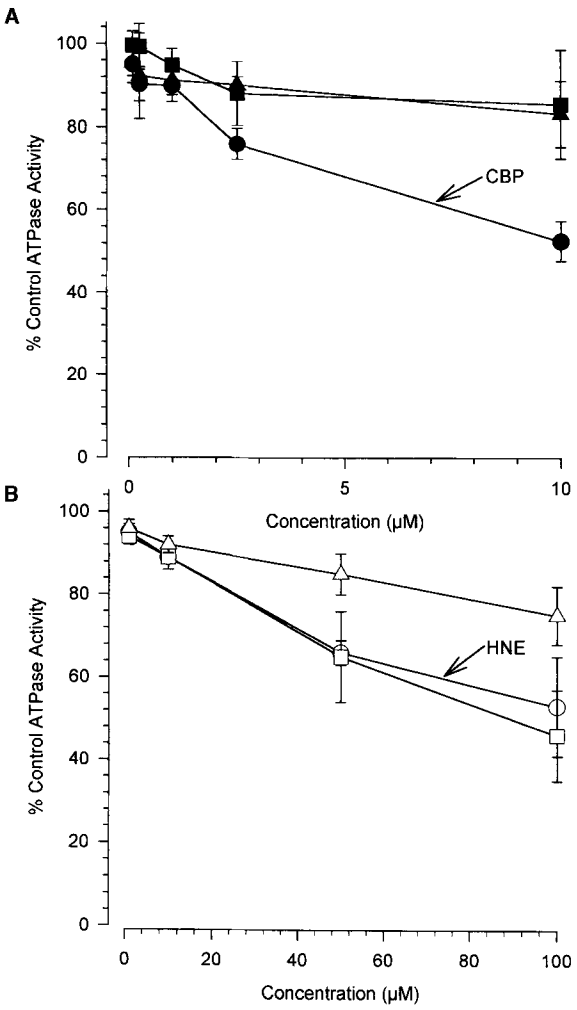

FIGURE 1 Concentration dependent inhibition of Na^+–K^+-ATPase activity by aldehydes. The top portion (A) compares the inhibition of oxidized β-carotene (CBP, shown as filled circles) with non-oxidized β-carotene (shown as filled squares), and apo-10-carotenal (shown as filled triangles). The bottom portion (B) compares HNE (shown as open circles) with NE (shown as open squares), and NA (shown as open triangles). CBP are more effective inhibitors (A) of Na^+–K^+-ATPase than HNE (B).

of Na^+–K^+-ATPase activity by HA and BME is shown. With CBP the enzyme activity could only be partially recovered. The BME was more effective than HA. An almost complete restoration of enzyme activity resulted after addition of HA to the samples which were incubated with Apo-10 or retinal, but for these compounds, the addition of BME did not increase enzyme activity.

DISCUSSION

Na^+–K^+-ATPase Inhibition by CBP, HNE and Related Aldehydes

The regulation of Na^+–K^+-ATPase activity including its phosphorylation by protein kinases[25–27] and subunit interactions[28,29] has been extensively studied. Furthermore, pharmacological effects especially those related to the activity of cardiac glycosides such as digoxin (see list of exogenous inhibitors of Na^+/K^+-transporting ATPase 3.6.1.37[30] have been described. There are also endogenous inhibitors of Na^+–K^+-ATPase and endogenous digitalis-like factors have been partially isolated and characterized from mammalian organs.[31–37] Catecholamines and endogenous brain factors such as endobains II-A, and II-E modulate neuronal Na^+–K^+-ATPase activity.[38–40]

Several studies have reported inactivation of Na^+–K^+-ATPase by agents that react with either lysine or cysteine residues.[24,41–46] Kim and Akera reported that oxygen free radicals caused ischemia-reperfusion injury to cardiac Na^+–K^+-ATPase.[47] They showed a partial inactivation of ATPase during lipid peroxidation following ischemia reperfusion injury, which could be reduced by antioxidants.[47] The kinetic parameters of Na^+–K^+-ATPase are modified by free radicals *in vitro* and *in vivo*.[48–51] Oxygen free radicals directly attack the ATP binding site of the cardiac Na^+–K^+-ATPase.[52] The inhibition of Na^+–K^+-ATPase by endogenous HNE, the end product of lipid peroxidation, was previously reported.[13] Here we report that this enzyme is even more potently inhibited by another group of endogenous aldehydes, those derived from oxidative degradation of carotenoids.

Carotenoid Toxicity Mediated by their Degradation Products

Aldehydes derived from either the oxidation of PUFA and carotenoids, unlike reactive oxygen

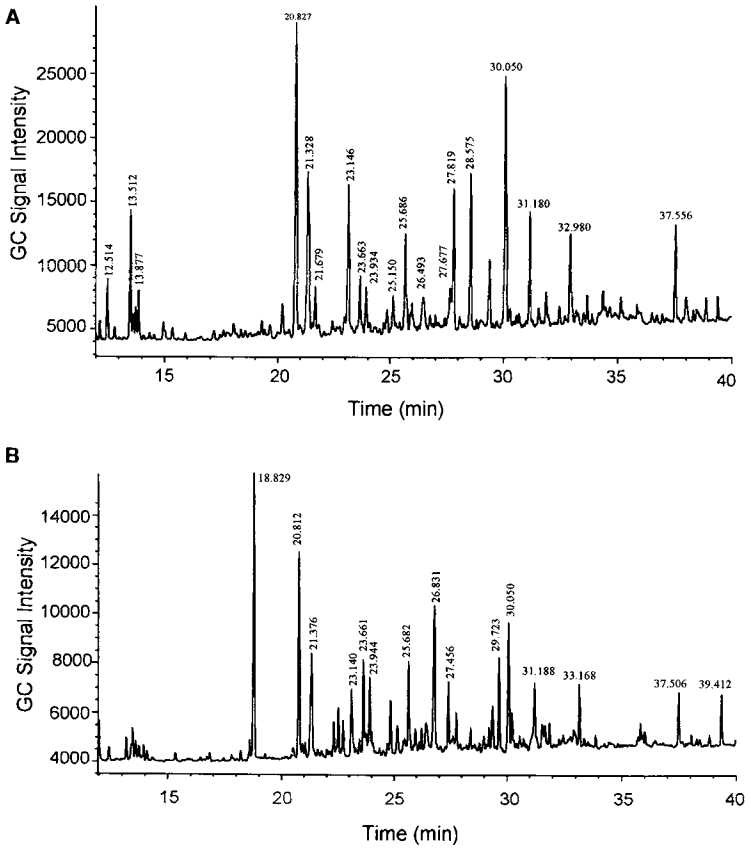

FIGURE 2 Gas chromatography of completely oxidized β-carotene before (A) and after formation of O-ethyl-oxime derivatives (B). Note that several peaks present in A are absent in B and vice versa. This is due to the fact that ethyl-oxime derivatives of aldehydes have longer retention times. β-Carotene is not shown since it does not volatilize under conditions employed.

TABLE II Restoration of ATPase activity following incubation with HA and BME

	CBP[1]	Apo-10[1]	Retinal[1]	4-HNE[1]
None added[2]	52.1 ± 5.4	84.5 ± 8.3	72.2 ± 1.2	62 ± 2
HA[2]	58.3 ± 3.7	95.3 ± 6.3	92.2 ± 5.6	72 ± 1
BME[2]	62.8 ± 6.3	80.1 ± 4.7	76.6 ± 6.8	76 ± 1
BME + HA[2]	65.0 ± 3.4	94.9 ± 3.9	90.8 ± 6.6	83 ± 5

[1]Values are presented as % control and given as mean ± standard deviation. Data points for CBP and Apo-10 is based on 12 measurements, for retinal on 8 measurements, and for 4-HNE on 3 measurements. Concentrations used were 10 μM except for 4-HNE which was 100 μM.

[2]Statistics was done using ANOVA with Bonferroni's adjustment. The differences observed for CBP and 4-HNE without and with HA and or BME are significant. For Apo-10 and retinal the differences were significant, except when BME was added alone. $P < 0.05$ in all cases of significance.

species, are generally stable and can act as both autocrine and paracrine agents at the intra- and extracellular levels, attacking targets near and remote from the site of generation. Therefore, they are not only end products of oxidative breakdown processes but may also act as 'second messengers' for the primary reactive species, free radicals and/or singlet oxygen that initiated their formation. We propose that aldehydic breakdown products of carotenoids and lipid peroxidation should be classified together within the same group. These endogenous aldehydes can bind to and interact with proteins, which was extensively demonstrated for HNE.[13,15,16,18,53] In our studies

the inhibition of Na^+–K^+-ATPase by CBP was shown *in vitro*. Furthermore, CBP demonstrated higher *in vitro* toxicity than HNE. However, the toxicity of both CBP and HNE *in vivo* will ultimately depend on the capacity of the metabolic pathways for both products. The metabolic pathways for HNE including their capacity in different tissues has been previously elucidated.[54–56] The metabolic pathways of some carotenoid metabolites are known, such as for β-apo-8′-carotenal, and 4,4′-dimethoxy-β-carotene.[57] However very little is known about the capacity of CBP metabolic pathways in animal and human tissues.

In this study we show that there is an inhibition of ATPase activity at CBP concentrations between 0.25 and 1 μM. At 2.5 μM an inhibition of 25% was observed. We suggest that the inhibitory effect of CBP may be cumulative because of the irreversible binding of these metabolites to the enzyme. Carotenoid levels, especially β-carotene, in blood plasma and in various animal and human tissues are dependent on the carotenoid content of food and may contain higher CBP concentrations than used in our Na^+–K^+-ATPase inhibition studies. We propose that under conditions of oxidative stress tissues with high carotenoid levels accumulate aldehydic metabolites that could activate stress signaling pathways similar to those postulated by Uchida *et al.* for HNE.[58]

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