Naturally-occurring eccentric cleavage products of provitamin A β-carotene function as antagonists of retinoic acid receptors

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*Running title: β-apocarotenoids are retinoic acid receptor antagonists

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Background: Dietary beta-carotene can be cleaved centrally to vitamin A, an agonist of retinoic acid receptors, or eccentrically to yield beta-apocarotenoids.

Results: β-Apocarotenoids antagonize retinoic acid receptors by binding directly to the receptors.

Conclusion: β-Apocarotenoids function as naturally-occurring retinoid receptor antagonists.

Significance: The antagonism of retinoid signaling by these metabolites may explain the negative health effects of large doses of β-carotene.

SUMMARY

β-carotene (BC) is the major dietary source of provitamin A. Central cleavage of BC catalyzed by β-carotene oxygenase 1 yields two molecules of retinaldehyde. Subsequent oxidation produces all-trans retinoic acid (ATRA) which functions as a ligand for a family of nuclear transcription factors, the retinoic acid receptors (RARs). Eccentric cleavage of BC at non-central double bonds is catalyzed by other enzymes and can also occur non-enzymatically. The products of these reactions are β-apocarotenals and β-apocarotenones, whose biological functions in mammals are unknown. We used reporter gene assays to show that none of the β-apocarotenoids significantly activated RARs. Importantly however, β-apo-14'-carotenol, β-apo-14'-carotenoic acid, and β-apo-13-carotenone antagonized ATRA-induced transactivation of RARs. Competitive radioligand binding assays demonstrated that these putative RAR antagonists compete directly with retinoic acid for high affinity binding to purified receptors. Molecular modeling studies confirmed that β-apo-13-carotenone can directly interact with the ligand binding site of the retinoid receptors. β-Apo-13-carotenone and the β-apo-14'-carotenoids inhibited ATRA-induced expression of retinoid responsive genes in Hep G2 cells. Finally, we developed an LC/MS method and found 3-5 nM β-apo-13-carotenone was present in human plasma. These findings suggest that β-apocarotenoids function as naturally-occurring retinoid antagonists. The antagonism of retinoid signaling by these metabolites may have implications for the activities of dietary β-carotene as a provitamin A and as a modulator of risk for cardiovascular disease and cancer.

Capability for the synthesis of compounds with vitamin A activity is limited to plants and microorganisms (1). It has been known since the 1930’s that cleavage of the central double bond of β-carotene by vertebrates
gives rise to retinaldehyde (vitamin A aldehyde) (2-4) which can subsequently be reduced to retinol (vitamin A alcohol) or oxidized to all-trans retinoic acid (vitamin A acid) (5). In mammals, ATRA functions as a hormone agonist for the retinoic acid receptor family of nuclear transcription factors and directly activates several hundred genes which contain retinoic acid response elements (RAREs) in their promoters (6,7); it is this global effect on the regulation of gene transcription that renders vitamin A essential for embryonic development, growth, and differentiation in mammals, including humans. β-Carotene is cleaved eccentrically at double bonds other than the central one to yield β-apocarotenals and β-apocarotenones (8-10), molecules that have been detected in foods (11) and in the blood of both humans (12) and animals (13), but whose function in these is unknown. Here we show that some of these compounds (particularly β-apo-14′-carotenal, β-apo-14′-carotenoid acid, and β-apo-13-carotene) function as antagonists of retinoic acid receptors α, β, and γ and block the ATRA-induced activation of endogenous genes that contain RAREs in their promoters. Moreover, these molecules directly compete for ATRA binding to all receptor subtypes and in the case of β-apo-13-carotenone, the binding affinity is in the nanomolar range and comparable to ATRA itself. Thus, depending on the extent of oxidative cleavage at its various double bonds, dietary β-carotene can produce differing proportions of both agonists and antagonists of retinoic acid receptors. This Janus face may account for the unexpected and negative effects of large doses of β-carotene used in human clinical trials.

**EXPERIMENTAL PROCEDURES**

**Cell lines** – Cos-1 cells from ATCC (Rockville, MD) were cultured in DMEM supplemented with 10% FBS. Hep G2 cells were cultured in MEM supplemented with 10% FBS. Cells were maintained at 37°C with 5% CO₂ in air.

**Characterization of β-apocarotenoids** – Compounds were characterized by a mix of ¹H (400 MHz) and ¹³C (100 MHz unless noted) NMR spectroscopy, UV and mass (ESI) spectrometry and HPLC analysis (Polaris C18 analytical column with 1 ml/min methanol:water of appropriate ratio, all compounds were determined to be at least 94% pure). Essential compound data follows and procedures for their synthesis are provided below in Results and Discussion.

**β-Cyclocitrail:** HRMS calculated for C₁₀H₁₄O (M+Na) 175.1099, observed 175.1101.

**β-Cyclogeranic acid:** ¹H NMR (CDCl₃) δ 1.14 (6H, s), 1.45-1.49 (2H, m), 1.66-1.71 (2H, m), 2.01-2.04 (2H, m), 2.08 (3H, m); UV (ethanol) λ max 295 nm (ε 7,720); HRMS calculated for C₁₀H₁₄O₂ (M+Na) 191.1048, observed 191.1041.

**β-Ionone:** HRMS calculated for C₁₀H₁₄O (M+H) 193.1592, observed 193.1579.

**β-Ionylideneacetaldehyde:** ¹H NMR (CDCl₃) δ 1.05 (6H, s), 1.46-1.55 (2H, m), 1.60-1.67 (2H, m), 1.74 (3H, s), 2.03-2.10 (2H, m), 2.39 (3H, s), 5.96 (1H, d, J = 8 Hz), 6.23 (1H, d, J = 16 Hz), 6.74 (1H, d, J = 16 Hz), 10.14 (1H, d, J = 8 Hz); UV (ethanol) λ max 272 nm (ε 14,800); HRMS calculated for C₁₂H₂₅O (M+H) 219.1749, observed 219.1737.

**β-Ionylideneacetaldehyde:** ¹H NMR (CDCl₃) δ 1.07 (6H, s), 1.50-1.53 (2H, m), 1.63-1.69 (2H, m), 1.75 (3H, s), 2.06-2.09 (2H, m), 2.36 (3H, s), 5.85 (1H, s), 6.23 (1H, d, J = 16.2 Hz), 6.67 (1H, d, J = 16.2 Hz), 10.14 (1H, d, J = 8 Hz); UV (ethanol) λ max 296 nm (ε 20,000); HRMS calculated for C₁₂H₂₅O (M+H) 235.1698, observed 235.1688.

**β-Apo-13-Carotenone:** ¹H NMR (CDCl₃) δ 0.98 (6H, s), 1.41-1.44 (2H, m), 1.54-1.60 (2H, m), 1.67 (3H, s), 1.99-2.00 (2H, m), 2.01 (3H, s), 2.25 (3H, s), 6.08-6.15 (3H, m), 6.37 (1H, d, J = 16 Hz), 7.53 (1H, dd, J = 15, 11.9 Hz); ¹³C NMR (CDCl₃; 75 MHz) δ 13.10, 19.14, 21.72, 27.63, 28.93, 33.14, 34.25, 39.59, 127.67, 129.27, 130.98, 131.27, 136.68, 137.48, 139.30, 145.55, 198.48; UV (ethanol) λ max 341 nm (ε 25,300); HRMS calculated for C₁₈H₃₀O (M+Na) 281.1881, observed 281.1859.

**β-Apo-14′-Carotenal:** ¹H NMR (CDCl₃) δ 1.00 (6H, s), 1.40-1.44 (2H, m), 1.54-1.60 (2H, m), 1.70 (3H, s), 1.93-2.04 (2H, m), 2.00 (3H, s), 2.07 (3H, s), 6.10-6.44 (6H, m), 6.90 (1H, dd, J = 14.9, 11.5 Hz), 7.50 (1H, dd, J = 14.9, 11.5 Hz), 9.58 (1H, d, J = 7.9 Hz); ¹³C NMR (CDCl₃)
δ 13.42, 19.26, 21.61, 27.57, 29.19, 33.55, 34.46, 40.10, 127.59, 128.06, 130.12, 130.95, 133.89, 136.31, 136.63, 137.43, 137.63, 137.99, 143.71, 190.39; UV (ethanol) λ_{max} 402 nm (ε 55,000); HRMS calculated for C_{22}H_{30}O (M+Na) 333.2194, observed 333.2190.

β-apo-14'-'Carotenoic acid: \(^1\)H NMR (CDCl\(_3\)) δ 1.05 (6H, s), 1.46-1.54 (2H, m), 1.61-1.71 (2H, m), 1.77 (3H, s), 2.06 (3H, s), 2.06-2.10 (2H, m), 2.13 (3H, s), 5.92 (1H, d, J = 15.0 Hz), 6.13-6.32 (4H, m), 6.39 (1H, d, J = 15.1 Hz), 6.90 (1H, dd, J = 15.0, 11.5 Hz), 7.88 (1H, dd, J = 15.1, 11.5 Hz); \(^13\)C NMR (CDCl\(_3\)) δ 13.65, 19.63, 22.19, 29.38, 33.53, 34.68, 40.01, 119.46, 128.58, 129.57, 130.37, 136.13, 137.81, 138.16, 139.23, 143.19, 146.14, 173.21 UV (ethanol) λ_{max} 378 nm (ε 52,200); HRMS calculated for C_{22}H_{30}O (M+Na) 349.2143, Observed 349.2136.

β-apo-12'-'Carotenal: \(^1\)H NMR (CDCl\(_3\)) δ 1.00 (6H, s), 1.45-1.43 (2H, m), 1.65-1.57 (4H, m), 1.69 (3H, s), 1.85 (3H, s), 1.99 (3H, s), 2.02 (3H, s), 6.14 (3H, m), 6.19 (1H, dd, J = 13.8, 14.2 Hz), 6.36 (1H, d, J = 11.9 Hz), 6.68 (1H, d, J = 11.8 Hz), 6.77 (1H, dd, J = 14.2, 13.8 Hz), 6.92 (1H, dd, J = 13.8, 11.6 Hz), 7.00 (1H, d, J = 11.6 Hz), 9.42 (1H, s); \(^13\)C NMR (CDCl\(_3\)) δ 10.12, 12.74,13.27, 20.24, 22.18, 25.49, 29.38, 33.53,34.68, 40.02, 127.31, 127.63, 127.66, 130.47, 130.81, 136.31, 136.62, 137.53, 137.67, 137.89, 138.22, 141.38,149.47,194.38; UV (methanol) λ_{max} 426 nm (ε 75,600); HRMS calculated for C_{22}H_{30}O (M+H) 351.2682, observed 351.2689.

β-apo-12'-'Carotenoic acid: \(^1\)H NMR (CDCl\(_3\)) δ 1.05 (6H, s), 1.47-1.50 (2H, m), 1.60-1.65 (2H, m), 1.74 (3H, s), 2.00-2.03 (1H, m), 6.14-6.22 (3H, m), 6.27 (1H, d, J = 12.2 Hz), 6.36 (1H, d, J = 14.9 Hz), 6.52 (1H, dd, J = 13.7, 12.4 Hz), 6.74 (1H, dd, J = 14.9, 11.9 Hz), 6.93 (1H, d, J = 12.2 Hz), 7.43 (1H, d, J = 11.5 Hz); \(^13\)C NMR (CDCl\(_3\)) δ 12.84, 13.25, 19.65, 22.19, 29.38, 33.53, 34.68, 40.03, 125.53, 127.51, 127.94, 128.04, 130.11, 130.86, 131.39, 136.92, 137.38, 137.98, 138.24, 141.09, 141.37, 173.92; UV (ethanol) λ_{max} 407 nm (ε 67,000); HRMS calculated for C_{22}H_{30}O (M+Na) 415.2613, observed 415.2608.

β-apo-10'-'Carotenoic acid: \(^1\)H NMR (CDCl\(_3\)) δ 1.07 (6H, s), 1.49-1.52 (2H, m), 1.60-1.69 (2H, m), 1.76 (3H, s), 1.94 (3H, s), 2.02-2.07 (2H, m), 2.02 (3H, s), 2.04 (3H, s), 6.15-6.23 (3H, m), 6.31 (1H, d, J = 11.6 Hz), 6.40 (1H, dd, J = 14.8 Hz), 6.49 (1H, d, J = 11.6 Hz) 6.64-6.84 (5H, m), 6.98 (1H, dd, J = 10.7 Hz); UV (ethanol) λ_{max} 441 nm (ε 108,700); HRMS calculated for C_{30}H_{40}O (M+Na) 455.2926, observed 455.2944.

Retinoids and other materials – All-trans retinoic acid, retinal, retinyl acetate, and 13-cis-retinoic acid were from Sigma (St. Louis, MO). 9-cis-RA was obtained from Enzo Life Sciences. RARβ/γ selective antagonist CD 2665 was from Tocris Bioscience (Ellisville, MO). All-trans-[\(^3\)H]RA (50.8 Ci/mmol), and 9-cis-[\(^3\)H]RA (52.9 Ci/mmol) were purchased from American Radiolabeled Chemicals (St. Louis, MO), and Perkin-Elmer Corp (Boston, MA) respectively. Recombinant proteins including
RARα, RARβ, RARγ, and RXRα were from Active Motif (Carlsbad, CA). Probes and primers for TaqMan assays were from Applied Biosystems (Carlsbad, CA).

Transactivation assays – Cos-1 cells were transfected with plasmids in serum-free medium using Lipofectamine 2000 reagent (Invitrogen). Reporter vectors used were renilla luciferase under the control of the thymidine kinase promoter and firefly luciferase under the control of a retinoic acid response element (RARE). Plasmids with cDNAs for retinoic acid receptors α, β, and γ were cotransfected in individual experiments. Four hours after transfection, cells were treated with test compounds that were dissolved in ethanol or with 0.1% ethanol alone for an additional 24 h. Cell lysates were then assayed for luciferase activities using a GloMax 96 Microplate Luminometer (Promega) and the Dual Luciferase Reporter (DLR) assay system (Promega). For each experiment, the firefly luciferase activity (experimental reporter) was normalized to renilla luciferase (control reporter) activity. The change in normalized firefly luciferase activity was calculated relative to that for cells that were transfected with vehicle (ethanol), which was set as 1. In some experiments we used the human RARγ stably-transfected reporter cell system from Indigo Biosciences, Inc. (State College, PA) according to the supplier’s directions. These cells show a much more robust response of ATRA-induced reporter gene expression than the transiently transfected cells.

Ligand-Binding Assays – Purified human recombinant retinoid receptors were used at 50 fmol with a non-retinoid-binding protein (keyhole limpet hemocyanin) to maintain ~0.1 mg protein/mL to prevent loss of receptor protein and ligand during the course of binding experiments. For binding analyses, proteins and ligands were incubated at 4°C for 16h. For equilibrium saturation binding assays, proteins were incubated with various concentrations of all-trans-[3H]RA for RARs and 9-cis-[3H]RA for RXRα and in the absence or presence of a 100-fold excess of unlabeled compound to determine nonspecific binding. Specific binding was determined by a hydroxyapatite-binding assay (14). The quantity of labeled compound bound was determined by liquid scintillation counting. For competitive binding assays, a constant amount of all-trans-[3H]RA (5 nM) for RARs or 9-cis-[3H]RA (10 nM) for RXRα was incubated with test compounds (from 10^-6 M to 10^-10 M). Apparent dissociation constants (Kd) and inhibition constants (Ki) were determined using GraphPad Prism Version 5.0 with non-linear regression.

Gene expression Assays - Hep G2 cells were treated with test compounds for 4 h and total RNA was isolated from each well and subjected to reverse transcription. Quantitative PCR was carried out using TaqMan chemistry for human CYP26A1 (Hs01084852_g1), and human RARβ (Hs00977137_m1)) as target genes and human GAPDH as a housekeeping gene. The comparative Ct method (ΔΔCt) was used to quantify the results obtained by real-time RT-PCR.

Molecular modeling – Structures were displayed and modeled on a Silicon Graphics O2 running Sybyl v.7.1 (Tripos). Structures were minimized using Sybyl’s Maximin method and docked to the RARβ ligand binding domain crystal structure (PDB entry: 1xap) using the available FlexX routine (15).

Quantitative HPLC/MS analysis of β-apo-13-carotenone in human plasma – Blood plasma was purchased from Innovative Research (Novi, MI). Fresh blood plasma was prepared from six individuals over the course of two weeks, shipped on dry ice to Ohio State University, and stored at -80°C before extraction. For each individual, 1 mL of plasma was split among 5 glass tubes (200 µL per tube). Ethanol (1 mL) was added to each tube, and the samples were sonicated using an Ultrasonic Dismembrator (Model 150E, Fisher Scientific). Hexanes (10 mL) were added to each tube and again the mixture was sonicated. The tubes were centrifuged for 5 min at 300 g to facilitate phase separation. The upper organic layers from each vial were pooled into a single vial, and the extraction with hexanes repeated a second time. The pooled extracts were dried under nitrogen gas. The samples were redissolved in 200 µL 1:1 methyl t-butyl ether/methanol and analyzed using HPLC-MS/MS.
HPLC was conducted on a C30 column (4.6x150mm, s5, YMC) in reversed phase with (A) methanol/0.1% formic acid (80/20 v/v) and (B) methanol/0.1% formic acid/methyl t-butyl ether (20/2/78 v/v/v) as mobile phase solvents at 1.8 mL/min and 35 °C (Agilent 1200 SL, Agilent Technologies, Santa Clara, CA). Eluent was introduced to a triple quadrupole mass spectrometer (QTrap 5500, AB Sciex, Concord, Canada) via an atmospheric pressure chemical ionization probe operated in positive ion mode. Three MS/MS transitions monitored for \( \beta \)-apo-13-carotenone were m/z 259>175, 119 and 69 at collision energies of 21, 31, and 27 eV respectively with 150ms dwell times and using nitrogen as CAD gas. Other MS parameters included: curtain gas 10, heated nebulizer temperature 425 °C, nebulizer current 5 µA, declustering potential 60, entrance potential 10, and exit potential 11. Calibrating solutions of \( \beta \)-apo-13-carotenone were prepared in 1:1 methyl t-butyl ether/methanol with concentrations based on an extinction of 25,300 at 368 nm in ethanol. The dried residue of serum extract was redissolved in 100 µL of methyl t-butyl ether then 100 µL of methanol added with mixing before centrifuging prior to injection (10 µL). For quantitation, the m/z 259>175 transition was used as it had superior signal:noise and the other two transitions were used for qualitative purposes to confirm peak identity. The limit of detection for \( \beta \)-apo-13-carotenone was 280 pM. We have found that stock solutions of \( \beta \)-apo-13-carotenone in ethanol are stable for 2.5 years at 5°C. The compound is stable in plasmas frozen at -80°C for periods of at least two weeks.

RESULTS & DISCUSSION

Synthesis of \( \beta \)-Apocarotenoids – Although the occurrence and biological activity of selected \( \beta \)-apocarotenoids in mammals has been reported in various systems, progress has been hampered because many of the compounds are not available commercially. In order to comprehensively assess the activity of \( \beta \)-apocarotenoids we first undertook to purify or synthesize all of the possible eccentric cleavage products of \( \beta \)-carotene (Fig. 1). Retinal, retinoic acid, and \( \beta \)-apo-8'-carotenal (Fluka) were used as obtained. \( \beta \)-Cyclocitral and \( \beta \)-ionone (both Sigma-Aldrich) were purified by preparative TLC prior to use. Eleven of the \( \beta \)-apocarotenoids were synthesized by standard organic chemical transformations including oxidation of aldehydes to acids (16), Wadsworth–Emmons and Wittig homologation, and reduction of esters to acids followed by oxidation to aldehydes (Fig. 2).

\( \beta \)-Apocarotenoids antagonize retinoic acid-induced expression of reporter and endogenous retinoic acid-responsive genes – We first screened all of the compounds for their potential to activate RAR\( \alpha \), RAR\( \beta \), and RAR\( \gamma \), using Cos-1 cells transiently transfected with cDNAs for the individual RARs and with an RARE-luciferase reporter. None of the compounds was as effective as the pan-agonist, ATRA, in activating the RARs and indeed most showed no agonist activity at all (Fig. 3). The slight activity of some of the longer chain \( \beta \)-apocarotenoids at high concentration is consistent with previous reports (17-19). We then used the same transactivation assay to screen all of the compounds for their potential to antagonize the ATRA-induced activation of the individual RARs by treating the cells with maximally effective doses of ATRA and equimolar concentrations of the \( \beta \)-apocarotenoids (Fig. 4). While the shorter products of the eccentric cleavage of \( \beta \)-carotene had little or no effect on ATRA-induced transactivation, \( \beta \)-apo-10'-carotenonic acid and \( \beta \)-apo-12'-carotenonic acid both led to 40-50% inhibition of ATRA-induced activation of all three RAR isoforms. Even more striking inhibition was observed for the products of the “d” cleavage (Fig. 1) of \( \beta \)-carotene (viz., \( \beta \)-apo-14'-carotenal, \( \beta \)-apo-14'-carotenonic acid, and \( \beta \)-apo-13-carotenone), with the greatest inhibitory activity being displayed by \( \beta \)-apo-13-carotenone. Thus, it appeared that these five \( \beta \)-apocarotenoids could be functioning as RAR antagonists.

In order to characterize the antagonist function \( \beta \)-apo-13-carotenone more quantitatively we used stably-transfected RAR\( \gamma \) reporter cells. Cells were treated with ATRA in a concentration range of 0.5 nM to 3 µM in the absence or presence of fixed concentrations (1, 10 or 100 nM) of \( \beta \)-apo-13-carotenone (Fig.
We observed a progressive shift in the ATRA dose-response curve with increasing concentrations of β-apo-13-carotenone in the nanomolar range. Higher concentrations of ATRA were able to overcome inhibition by β-apo-13-carotenone, suggesting direct competition between the two compounds for binding. This suggestion is supported by the results of competitive radioligand binding assays and molecular modeling discussed below.

We then asked whether β-apo-13-carotenone and the β-apo-14′-carotenoids would antagonize the ATRA-induced transcription of endogenous genes. For these experiments, we treated Hep G2 cells in culture with ATRA for 4 hours and measured the mRNA levels for RARβ and cytochrome P450-26A1 (CYP26A1). Both of these genes have canonical RAREs in their promoters and their transcription is directly upregulated by ATRA treatment (19,20). As shown in Fig. 5B, treatment with 10 nM ATRA led to 9-fold induction in RARβ mRNA levels and ~20-fold increases on CYP26A1 mRNA levels. Treatment with 10 nM BC, β-apo-13-carotenone, or other β-apocarotenoids expectedly did not markedly induce expression of either gene (data not shown). However, co-treatment with ATRA and β-apo-13-carotenone or the β-apo-14′-carotenoids led to marked inhibition of the ATRA-induced gene expression (Fig. 5B). Importantly, the inhibition by β-apo-13-carotenone was greater than that for β-apo-14′-carotenal or β-apo-14′-carotenoic acid. This is in keeping with the greater affinity of β-apo-13-carotenone for the RARs than that of the β-apo-14′-carotenoids. Co-treatment of the cells with ATRA and the parent compound (BC) or with a β-apocarotenoid that does not antagonize ATRA-induced transactivation (viz. β-ionylideneacetic acid) had no effect on ATRA-induced gene expression in HepG2 cells.

**β-Apocarotenoids bind to the ligand binding site of RARs with high affinity** – Retinoic acid receptors (like other type II nuclear receptors) function in the regulation of endogenous gene expression by binding as heterodimers with retinoid X receptors (7,22). The heterodimers bind to specific response elements (RAREs and RXREs) in the promoter regions of genes via their respective DNA-binding domains. In the unliganded state the transcription factor complex binds to nuclear co-repressors and transcription is repressed. Binding of ATRA (or other agonist) to the ligand-binding domain of RAR induces a conformational change in the RAR (at helix 12) and this leads to co-repressors dissociating from the receptor and the unmasking of a co-activator binding site (22). In the case of RAR-RXR heterodimer the binding of an agonist to RXR alone does not lead to activation, but the binding of an RXR agonist in concert with an RAR agonist leads to supraactivation of transcription (22). We wanted to know whether the β-apocarotenoids that demonstrated an antagonist “activity” in the cell-based assays did so by directly binding to the RAR ligand-binding domain and competing for ATRA binding. Thus, we conducted radioligand binding assays using purified recombinant RARα, RARβ, and RARγ and tritium-labeled ATRA in the presence of increasing concentrations of unlabeled ATRA (as a positive control), CD 2665 (a synthetic antagonist of RARβ/γ known to bind to the ligand binding site), retinyl acetate (a retinoid that does not bind and used as a negative control), and the selected β-apocarotenoids (Fig. 6A). For all three receptors the three β-apocarotenoids with the highest antagonist activity competed for ATRA binding. In fact, β-apo-13-carotenone displayed the same affinity for the RARs as ATRA itself (i.e., 2-6 nM) (Fig 6B). The affinity of binding for β-apo-14′-carotenal and β-apo-14′-carotenoic acid to RARs was in the 15-60 nM range, while those of β-apo-10′- and β-apo-12′-carotenoic acids were greater than 300 nM.

The high affinity binding of β-apo-13-carotenone to the ligand binding site of RARβ was also demonstrated by the results of molecular modeling studies. We displayed the crystal structure of RARβ with the retinoid agonist TTNPB in the binding site (PDB entry:1xap), “extracted” the ligand computationally, and then attempted to dock both ATRA and β-apo-13-carotenone into the binding site using FlexX (15). Both molecules docked smoothly (Fig. 6C left); indeed the RMSD for β-apo-13-carotenone was very slightly better than for ATRA. Moreover, the
crystal conformation of TTNPB and the energy-minimized and then docked conformations of ATRA and β-apo-13-carotenone had nearly identical RMSD values (Fig. 6C right).

The studies reported here demonstrate that the products of the oxidative eccentric cleavage of β-carotene at the C13-C14 double bond yields products that are antagonists of RARs and that the most active molecule in this regard is β-apo-13-carotenone. We previously showed that this compound was also the most effective β-apocarotenoid in inhibiting the transactivation of RXRα by its agonist, 9-cis-retinoic acid, and molecular modeling studies demonstrated that it could potentially bind to the transcriptionally silent tetramer of RXRα (23). Therefore, we tested the three β-apocarotenoids resulting from cleavage of the C13-C14 double bond of β-carotene for their binding to purified recombinant RXRα (Fig. 7). The β-apo-13-carotenone competed for 9-cis-retinoic acid binding with an affinity (7-8 nM) identical to 9-cis-retinoic acid itself. The affinities of β-apo-14'-carotenal and β-apo-14'-carotenoic acid for RXRα were greater than 250 nM in keeping with their lack of effect on inhibiting RXR transactivation. Given that a number of nuclear receptors form heterodimers with RXR and that ligand binding (either agonist or antagonist) to the RXR leads to modulation of the transcriptional activity of the heterodimers (22), these eccentric cleavage products of β-carotene could have complex global effects on gene expression.

The most potent β-apocarotenoid antagonist of RARs, β-apo-13-carotenone, is found in human plasma at concentrations that are biologically significant – Although the mechanisms responsible for the formation of the eccentric cleavage products of β-carotene in mammals are not fully known, it is clear that some of the long-chain β-apocarotenals (e.g., 8', 10', 12', 14') are found in the plasma of humans (12) and experimental animals (13) and that these are increased under conditions of oxidative stress and high dietary doses of β-carotene (24). We have also found that all of these β-apocarotenals and, specifically, β-apo-13-carotenone are present in fresh cantaloupe and orange-fleshed melons (11); thus these compounds may be absorbed directly from the diet.

In order to further establish the relevance of β-apo-13-carotenone’s potent antagonist activity on retinoid receptors, we developed sensitive HPLC/MS procedures for its detection in human plasma. Figure 8 shows the analysis of human plasma and authentic standards by HPLC/MS. We used multiple reaction monitoring to insure the specificity and sensitivity of the assay. We then analyzed the plasmas of six free-living individuals and found the plasma concentration of β-apo-13-carotenone to be 3.8 ± 0.6 nM. Importantly, this is in the range of normal concentrations of retinoic acid in plasma and approximately the same as the binding constant of the compound for the retinoid receptors. This would suggest that β-apo-13-carotenone can function at physiological concentrations as an endogenous modulator of retinoid signaling in humans.

Conclusions and Implications

Our results demonstrate that β-carotene can generate both RAR agonists (ATRA) and RAR antagonists (β-apo-14'-carotenal and β-apo-13-carotenone) depending on the extent of cleavage at the central C15-C15' double bond or the C13-14 double bond, respectively. These findings may have implications for the unexpected and negative effects of high doses of β-carotene in human clinical trials of cancer prevention (25). An example is the now-famous CARET trial which, based on observational epidemiology, explored whether supplemental β-carotene would decrease incidence of lung cancer in a highly susceptible population, namely smokers and asbestos workers (26,27). Surprisingly, the supplemented subjects had a higher incidence of disease and the trial had to be halted early. It was apparent that the doses of β-carotene used in the trial (30 mg/day) were much higher than the range of normal dietary intakes associated with a decreased risk of disease in the observational studies (25). The possible mechanisms involved were explored in elegant studies employing a novel animal model, the smoking ferret (24,28). These studies revealed that under conditions of high dietary β-
carotene and the oxidative stress of smoking there was a clear increase in preneoplastic lung cancer lesions in the animals. The authors concluded that oxidative stress led to increased eccentric cleavage of β-carotene and that the mixture of cleavage products led to disruption of retinoid-signaling via indirect mechanisms. The present work demonstrates that specific β-apocarotenoids exert an anti-vitamin A activity by directly interacting with RARs as high-affinity antagonists. Our analyses of both β-carotene-containing animal diets and fruits containing β-carotene suggest that any dietary source of β-carotene also contains β-apocarotenoids. It may also be useful to consider these findings in attempts to alleviate vitamin A deficiency in humans through the biofortification of crops with high levels of β-carotene.
References:


FOOTNOTES

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The abbreviations used are: BC, β-carotene; BCO1, β-carotene oxygenase 1; ATRA, all-trans retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; RARE, retinoic acid response element; K_d, dissociation constant; K_i, inhibition constant; C13 ketone, β-apo-13-Carotenone; 14'-AL, β-apo-14'-Carotenal; 14'-CA, β-apo-14'-Carotenoid acid.
FIGURE LEGENDS

FIGURE 1. β-Apocarotenoids. Structures of the β-apocarotenoids synthesized (indicated by [S]), purified and characterized for this study. R = CHO in the carotenals and R = COOH in the carotenoic acids.

FIGURE 2. Chemical synthesis of all possible β-apocarotenoids. Reagents and conditions used in the synthesis of the various compounds are shown in lower case roman numerals and yields are shown in parentheses. i) 5N KOH/EtOH (+/- benzene), rt, 12 h (99%, BIAA; 96%, 12'-CA; 92%, 10'-CA; 94%, 14'-CA); ii) LAH, THF, rt, 45 min (for compound 2, 9); DIBAL-H, CH₂Cl₂, rt, 30 min (for compound 6); iii) MnO₂, Celite, CH₂Cl₂, rt, 4 h (73%, BIA; 36%, 10'-AL; 6%, 14'-AL); iv) (triphenylphosphoranylidene)-2-propanone, toluene, reflux, 12 h (61%); v) NaH, dialdehyde shown, CH₂Cl₂, 0 °C to rt, 48 h (59%); vi) KCN, CH₃COOH, MnO₂, MeOH, rt, 90 h (21%, 4; 2%; 10); vii) NaH, triethylphosphonoacetate, THF, 0 °C to rt, 48 h (83%, 5; 94%, 1; 74%, 8); viii) O₂, CH₂Cl₂, 48 h (quant.).

FIGURE 3. β-Apocarotenoids do not transactivate retinoic acid receptors. Histograms of activation of RARE-reporter genes in cells transfected with retinoic acid receptors α (left), β (middle) and γ (right). Normalized fold-activation relative to vehicle-treated cells is shown for all-trans retinoic acid (left most bar in each histogram) or the β-apocarotenoids resulting from cleavage at the “a”, “b”, “c”, or “d” sites from top to bottom, respectively. Compounds were tested individually at 10⁻⁵ M. n = 3 to 6; mean +/- s.d. Compound abbreviations are given in Fig. 1.

FIGURE 4. β-Apocarotenoids antagonize ATRA-induced transactivation of retinoic acid receptors. Histograms of activation of RARE-reporter genes in cells transfected with retinoic acid receptors α (left), β (middle) and γ (right). Percent of maximal activation of cells treated with 10⁻⁵ M ATRA alone (left most bar in each histogram) or co-treated with 10⁻⁵ M ATRA and 10⁻⁵ M of the β-apocarotenoids resulting from cleavage at the “a”, “b”, “c”, or “d” sites are shown in a, b, c, and d, respectively. n = 3 to 6; mean +/- s.d. Compound abbreviations are given in Fig. 1.

FIGURE 5. β-Apo-13-Carotenone is a potent antagonist of retinoic acid receptor-mediated induction of reporter gene expression and blocks all-trans retinoic acid (ATRA) induction of endogenous gene expression. a, Dose response curves for transactivation of RARγ (left-upper panel) by ATRA in the absence (●) or presence of 1 nM (green ▲), 10 nM (▲), or 100 nM (blue ▲) C13 ketone. Points shown are the means of 6 determinations for ATRA alone or 3 determinations for each of the curves with C13 ketone. Variations about the means were generally less than 10% except at very low concentrations of ATRA. b, Induction of expression of mRNAs for RARβ (left-lower panel) or cytochrome P₄₅₀, 26A1 (CYP26A1) (right-lower panel) by 10 nM ATRA treatment alone or by co-treatment with ATRA and the test compounds at 10 nM including β-carotene (BC), β-ionylideneacetic acid (BIAA), β-apo-14'-carotenal (14'-AL), β-apo-14'-carotenoic acid (14'-CA), and β-apo-13-carotenone (C13 ketone). mRNA levels were quantified by RT-PCR and are shown as the fold induction compared to vehicle treated cells. n = 3; mean +/- s.d.
FIGURE 6. β-Apo-13-Carotenone is a high affinity ligand for purified retinoic acid receptors and fits into the ligand binding site. a, Competitive displacement of 5 nM tritiated ATRA from purified RAR proteins by unlabeled ATRA (●) as a positive control, C13 ketone (▲), 14'-CA (●), 14'-AL (●), and 13-cis-retinoic acid (●) as a negative control for RARα (left) experiment, CD 2665 (●), retinyl acetate (●) as a negative control for RARβ (middle) and RARγ (right) experiments. Points shown are means of n=3 with a variance of less than 10%. b, Binding affinities (in nM) of β-apocarotenoids to RARs calculated from the data shown in Fig. 6a and additional experiments with β-apo-12'- and β-apo-10'-carotenoic acids. For ATRA and the C13 ketone variance shown is for three independent experiments. c, Molecular modeling of the docking of ATRA (red) and β-apo-13-carotenone (purple) into the ligand binding site (protein backbone in green) of RARβ (PDB entry:1xap)(left). On the right is shown the energy minimized then docked conformations of ATRA (red) and β-apo-13-carotenone (purple) overlaid onto the conformation of the agonist TTNPB (white) as observed in the X-ray structure.

FIGURE 7. β-Apo-13-Carotenone is a high affinity ligand for purified retinoid X receptor alpha. a, Competitive displacement of 10 nM tritiated 9-cis RA from purified RXRα protein by unlabeled 9-cis RA (●) as a positive control, C13 ketone (▲), 14'-AL (●), 14'-CA (+), and retinyl acetate (●) as a negative control. Points shown are means of n=3 with a variance of less than 10%. b, Binding affinities of β-apocarotenoids to RXRα calculated from the data shown in Fig. 7a.

FIGURE 8. Analysis of β-apo-13-carotenone in human plasma by HPLC/MS. Multiple reaction monitoring (MRM) chromatogram of β-apo-13-carotenone in blood plasma (top) and a standard (bottom) as analyzed by atmospheric pressure chemical ionization in positive mode after C30 HPLC. The MRM was composed of three transitions – m/z 259.2>175.1 (blue), 119.1 (red) and 69.0 (green) and the matching elution time and relative intensities of the transitions confirm the peak identity.
Figure 1

\[
\beta\text{-CAROTENE (BC)}
\]

\[
\begin{align*}
\beta\text{-apo-8'}\text{-CAROTENAL (8'}\text{-AL}) & \quad \beta\text{-Cyclocitrinal (BCL)} \\
\beta\text{-apo-8'}\text{-CAROTENOIC ACID (8'}\text{-CA}) [S] & \quad \beta\text{-Cyclogeranic Acid (BCA) [S]} \\
\beta\text{-apo-10'}\text{-CAROTENAL (10'}\text{-AL}) [S] & \quad \beta\text{-Ionone (BI)} \\
\beta\text{-apo-10'}\text{-CAROTENOIC ACID (10'}\text{-CA}) [S] & \\
\beta\text{-apo-12'}\text{-CAROTENAL (12'}\text{-AL}) [S] & \quad \beta\text{-Ionylideneacetaldehyde (BIA) [S]} \\
\beta\text{-apo-12'}\text{-CAROTENOIC ACID (12'}\text{-CA}) [S] & \quad \beta\text{-Ionylideneacetic Acid (BIAA) [S]} \\
\beta\text{-apo-14'}\text{-CAROTENAL (14'}\text{-AL}) [S] & \quad \beta\text{-apo-13-Carotenone (C13 ketone) [S]} \\
\beta\text{-apo-14'}\text{-CAROTENOIC ACID (14'}\text{-CA}) [S] & \\
\end{align*}
\]

\[
\text{RETINAL (RAL) and all-trans-RETINOIC ACID (ATRA)}
\]

\[R = \text{CHO, COOH}\]
Figure 3
**Figure 6a**

![Graphs showing binding affinity for RARα, RARβ, and RARγ](image)

**Figure 6b**

<table>
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<th>β-Apocarotenoids</th>
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<th>RARβ</th>
<th>RARγ</th>
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**Figure 6c**

![Molecular structures showing binding sites](image)
Figure 7

(a) Graph showing the binding of various apocarotenoids to RXRα.

(b) Table showing the Kᵢ values (nM) for different β-apocarotenoids to RXRα:

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<tr>
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Figure 8
Naturally-occurring eccentric cleavage products of provitamin A beta-carotene function as antagonists of retinoic acid receptors
Abdulkerim Eroglu, Damian P. Hruszkewycz, Carlo dela Sena, Sureshbabu Narayanasamy, Ken M. Riedl, Rachel E. Kopec, Steven J. Schwartz, Robert W. Curley, Jr. and Earl H. Harrison

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