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β-Carotene regulates expression of β-carotene 15,15′-monoxygenase in human alveolar epithelial cells

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ABSTRACT

β-Carotene 15,15′-monooxygenase (CMO1, BCMO1) converts β-carotene to retinaldehyde (retinal) and is a key enzyme in vitamin A metabolism. CMO1 activity is robust in the intestine and liver, where *cmo1* gene transcription may be subject to negative feedback by accumulation of its metabolic products. Evidence from CMO1 null animals also indicates that non-gastrointestinal CMO1 may be required for tissue-specific conversion of β-carotene into vitamin A. The aim of this study was to investigate the effects of the enzymatic substrate, β-carotene, on regulation of CMO1 in a cell model of human alveolar pneumocytes. We demonstrate that CMO1 is expressed in human alveolar epithelial (A549) cells and converts β-carotene into retinal and biologically active retinoic acids (RA). Exposure to β-carotene suppresses CMO1 expression at both mRNA and protein levels. β-Carotene, but not *all-trans* RA, decreases CMO1 promoter activity in a time- and dosage-dependent manner. This β-carotene-mediated inhibition of CMO1 expression results from decreased binding of peroxisome proliferator-activated receptor γ (PPARγ) and retinoid X receptor α (RXRα) in the CMO1 promoter. β-Carotene treatment also antagonizes PPARγ activity in HEK293 cells that stably express CMO1 wild-type, but not in cells that express the CMO1 mutant or vector alone. These findings have implications for local vitamin A synthesis in the lung, especially during systemic vitamin A insufficiency and may also help to explain, in part, the mechanism underlying the increased lung cancer risk upon β-carotene supplementation in smokers.

Introduction

Vitamin A (retinol) is a fat-soluble micronutrient essential for normal morphogenesis and epithelial differentiation and maintenance [1,2]. The importance of vitamin A throughout the life cycle is well established [3] and begins in embryogenesis [3,4]. Vitamin A exerts its biological actions largely through its principal active metabolites, specific retinoic acid (RA) isomers. RA activity, in turn, is mediated by binding to RA receptors (RARα, RARβ, RARγ) and retinoid X receptors (RXRα, RXRβ, RXRγ), ligand-inducible transcription factors which are members of the superfamily of nuclear hormone receptors [5]. RARs and RXRs form ligand-dependent heterodimers that bind to regulatory regions in specific target genes and modulate gene transcription [6]. Circulating vitamin A concentrations must be maintained in narrow range in order to avoid either deficiency or toxicity. Consequently, vitamin A production is tightly controlled for normal physiological processes.

A major dietary source of vitamin A is the pro-vitamin A carotenoids, particularly β-carotene, which is ingested from many vegetables and fruits. In humans, increased β-carotene intake leads to increased plasma concentrations of β-carotene and its metabolites. β,β-carotene 15,15′-monooxygenase ([1]CMO1, BCMO1) symmetrically cleaves β-carotene to yield two molecules of *all-trans* retinal (retinaldehyde) [7–10]. Studies undertaken in CMO1 null mice and in human subjects further establish the fundamental role of this enzyme in producing vitamin A aldehyde (retinaldehyde, or retinal) from dietary β-carotene [11,12]. Retinal is converted to retinol, the transport and storage form of vitamin A, and is further oxidized to RA.

CMO1 expression and enzyme activity are robust in digestive sites in intestinal mucosa and liver [13] but the enzyme also is expressed in peripheral tissues [14,15]. In humans, unlike rodents, substantial amounts of absorbed β-carotene are not enzymatically cleaved in the intestine [16] and up to 15–30% of absorbed β-carotene remains intact and is delivered to peripheral tissues [17,18].

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[1] *Abbreviations used:* CMO1, β-carotene 15,15′-monooxygenase; ATRA, *all-trans* retinoic acid; RAR, retinoic acid receptor; RXR, retinoid X receptor; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-response element; EMSA, electrophoretic mobility shift assay.

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Local tissue-specific β-carotene to retinal metabolism may contribute to vitamin A production in extra-intestinal tissues. β-Carotene metabolism and CMO1 activity are present in human and rodent lung tissue [19–21]. However, the molecular details of how β-carotene metabolism in the lung is regulated are largely unknown.

Although CMO1 enzymatic biochemistry has been studied for several decades, regulation of CMO1 gene expression and enzyme activity has largely focused on the intestinal tract, where CMO1 is subject to transcriptional feedback inhibition by RA [22]. Intestinal cell CMO1 gene expression in rodents also is stimulated by PPARγ agonist and PPARγ/RXRα heterodimer binding to a PPAR regulatory element (PPRE) [23]. In human intestinal cells, PPARγ is essential but not sufficient to activate human CMO1 gene expression which is instead dependent on cooperation between PPARγ and MEF2 isoforms [24]. More recently, an intestine specific homeodomain transcription factor, ISX, has been found to regulate CMO1 and scavenger receptor class B type 1 (SR-BI) expression, also via metabolic feedback [25,26]. Mechanisms of CMO1 regulation in extra-intestinal sites remains less studied.

In the present report, we examined the effects of β-carotene on the expression of CMO1 in pulmonary alveolar epithelial cells. Lung terminal air sacs, or alveoli, are an important target for retinoid action, especially in development and during repair. We present evidence that β-carotene, but not ATRA, decreases CMO1 expression and inhibits CMO1 promoter activity through suppression of PPARγ/RXRα binding to the CMO1 promoter. Moreover, a CMO1-dependent decrease in PPARγ activity by β-carotene appears to be a key determinant in regulation of CMO1 expression.

Materials and methods

Plasmids and chemicals

The plasmid pGL3-basic (Promega, Madison WI) and constructs for wild type, truncated and mutated pGL3-BCO1-Luciferase (pGL3-BCO1-Luc) reporter genes, pPPRE-tk-Luc reporter gene, pRARE-200Luc reporter gene, the expression vectors for PPARα, β, and γ, RARβ, RXRα, pCMV-β-Gal and pcDNA3.1 were described previously [24]. The pCMV-BCO1 expression vector was the gift of Dr. Stefan Andersson (Center for Nuclear Receptors and Cell Signaling, University of Houston, Houston, Texas). Dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), β-carotene, retinaldehyde and *all-trans* retinoic acid (ATRA) were from Sigma–Aldrich (St. Louis, MO). The PPARγ agonist, GW1929, was obtained from Alexis Biochemicals (San Diego, CA).

Antibodies

The synthetic peptide [Ac-C]TKKQAASEEQRDRASD-Amide, corresponding to the C-terminal 15 amino acid residues 525–540 in human CMO1, was coupled to keyhole limpet hemocyanin and used for immunization of rabbits as described previously [27]. Polyclonal antibodies to CMO1 were characterized by an enzyme-linked immunosorbent assay using the synthetic peptide as antigen and by Western blot analysis.

Cell culture and treatments

Human pulmonary alveolar epithelial A549 cells and HEK293T cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal bovine serum (HyClone, Logan, UT) at 37 °C in a humidified atmosphere of 95% air/5% CO_2. All the above-mentioned reagents, with the exception of fetal bovine serum, were purchased from Invitrogen (Carlsbad, CA). Equal numbers of A549 cells (1×10^6/well) were plated into six-well cell culture plates for 24 h before addition of various treatments. β-carotene (stock solution, 1 mM) was dissolved in DMSO:THF (4:1). ATRA and retinaldehyde (in DMSO) at a concentration of 1 mM were prepared freshly before each experiment. Control cells were incubated with vehicle alone.

Site-directed mutagenesis

A panel of mutant CMO1 was made using a pcDNA3-CMO1 construct as template. The 5 residues of human CMO1 under study (His172, His237, His308, Glu450, His514) as described previously [28] were mutated to alanine using QuikChange™ multiple site-directed mutagenesis kit (Stratagene). The validity of all point mutations and integrity of the open reading frame were verified by DNA sequencing.

Stable expression of wild-type and mutated CMO1 in HEK293 cells

Stable CMO1 wild type (CMO1-wt), mutated (CMO1-mt) and pcDNA3.1 cell lines were established as previously described [29]. Briefly, the pcDNA3.1 vector and pcDNA3.1 plasmids containing the full-length wild type or [H172, H308, E450, H514] CMO1 mutants were transfected into HEK293 cells using Lipofectamin 2000 reagent (Invitrogen) and cells were selected in the presence of geneticin (G418, Gibco-BRL) (400 μg/ml). Isolated clones were selected by limited dilution and then expanded. Stable transfected clones were selected in the presence of G418 (500 μg/ml) and verified with PCR using primers for CMO1 and western blot using CMO1 antibody.

Extraction of carotenoids and retinoids

β-Carotene and its metabolites and retinoids were extracted as described previously, with minor modifications [30]. After incubation of cells with known amounts of β-carotene for the indicated times, culture plates were placed on ice, medium removed and monolayers washed with 0.5 ml of 10 mM sodium taurocholate in phosphate-buffered saline (PBS) to remove surface-bound carotenoids. After PBS washes 2×, cells were harvested by brief trypsinization, cell pellets were homogenized in 0.5 ml ice-cold PBS and transferred it to glass test tubes. An aliquot (0.1 ml from 0.2 mM stock) of butylated hydroxytoluene (BHT) was added to the homogenate and, when required, a known amount of internal standard (echinenone) was added. Homogenates were extracted with 1.5 ml dichloromethane/methanol (1:2, v/v) and hexane. Following centrifugation, the resulting upper hexane-dichloromethane layer was collected. The lower layer was extracted two more times and the hexane-dichloromethane layer was combined with the initial extract. The combined extract was dried, re-dissolved in 0.1 mL dichloromethane/methanol (1:4, v/v) and subjected to HPLC analysis. We also analyzed the concentration of β-carotene in the medium before and after incubations. Sample handling, homogenization and extraction were carried out under cold conditions and dim yellow light to minimize carotenoid isomerization and oxidation.

HPLC analysis of β-carotene and retinoids

β-Carotene and retinoids were analyzed and quantified as described previously, with minor modifications [31]. A Shimadzu HPLC system (Model: UFLC, Kyoto, Japan) equipped with PDA detector, SPD-M20A monitoring from 210 to 670 nm, comprising of a gradient pump system, LC-20AT and a personal computer equipped with LC Solution (Shimadzu) software was used for the