Strong and weak plasma response to dietary carotenoids identified by cluster analysis and linked to beta-carotene 15,15'-monooxygenase 1 single nucleotide polymorphisms ☆

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Abstract

The mechanisms as well the genetics underlying the bioavailability and metabolism of carotenoids in humans remain unclear. To begin to address these questions, we used cluster analysis to examine individual temporal responses of plasma carotenoids from a controlled-diet study of subjects who consumed carotenoid-rich beverages. Treatments, given daily for 3 weeks, were watermelon juice at two levels (20-mg lycopene, 2.5-mg β-carotene, $n=23$ and 40-mg lycopene, 5-mg β-carotene, $n=12$) and tomato juice (18-mg lycopene, 0.6-mg β-carotene, $n=10$). Cluster analysis revealed distinct groups of subjects differing in the temporal response of plasma carotenoids and provided the basis for classifying subjects as strong responders or weak responders for β-carotene, lycopene, phytoene and phytofluene. Individuals who were strong or weak responders for one carotenoid were not necessarily strong or weak responders for another carotenoid. Furthermore, individual responsiveness was associated with genetic variants of the carotenoid metabolizing enzyme β-carotene 15,15'-monooxygenase 1. These results support the concept that individuals absorb or metabolize carotenoids differently across time and suggest that bioavailability of carotenoids may involve specific genetic variants of β-carotene 15,15'-monooxygenase 1.
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1. Introduction

Carotenoids, fat-soluble orange, yellow, or red pigments commonly found in fruits and vegetables, are purported to provide protection against a number of chronic diseases of aging [1–7]. The capacity of carotenoids to modulate disease initiation or progression is expected to depend, in large part, on the bioavailability of these compounds, a factor that varies among individuals. However, the mechanisms as well the genetics underlying bioavailability and metabolism of carotenoids in humans remain unclear. The most common carotenoids found in human blood and tissues are α-carotene, β-carotene and β-cryptoxanthin, which have provitamin A activity, and lycopene (LYCO), lutein and zeaxanthin, which have no vitamin A activity [7]. Phytoene (PE) and phytofluene (PF), non-pigmented precursors of LYCO in plants, occur at lower concentrations in blood and in plant-based foods. Tomato juice, for example, contains ~10-fold less PE and PF than LYCO [8,9].

To date, provitamin A activity is the only physiologic function of carotenoids clearly demonstrated in humans. However, epidemiological studies have linked decreased risk for cardiovascular disease with consumption of β-carotene (b-CAR) as well as LYCO [10,11]. The red-pigmented carotenoid, LYCO, alone or in concert with PE and PF, may provide protection against heart disease and prostate cancer [11–13], one of the most common cancers among US men (United States Cancer Statistics: 2007 http://apps.nccd.cdc.gov/uscs). Although b-CAR is found widely in plant-based foods, LYCO as well as PE and PF are found in relatively few foods, with the dominant share in the US diet coming from tomato products. Watermelon [14] and red grapefruit [15] are other major sources of LYCO and, to a lesser extent, PF.

The intestinal absorption and metabolism of carotenoids involves several crucial steps [16,17]. That fraction of a carotenoid dose that is

absorbed and reaches systemic circulation is said to be bioavailable. In humans, bioavailability is typically measured as a surrogate, plasma response. The proportion of a bioavailable carotenoid that is converted to retinol is said to be bioconverted. Two enzymes are thought to be involved in the initial cleavage of carotenoids. One enzyme, b-CAR 15,15'-monooxygenase 1 (BCMO1), the key enzyme for retinoid production in mammals, has limited substrate specificity. It splits b-CAR at the central double bond (15,15') to yield retinal in intestinal cells and liver cytosol [18]. The cleavage product, all-*trans* retinal, can be reversibly reduced to retinol (vitamin A) or irreversibly oxidized to retinoic acid [19]. Recently, Kiefer et al. [20] cloned and characterized a second carotenoid-cleaving enzyme termed b-CAR 9,10-monooxygenase (BCO2) that catalyzes asymmetric cleavage of b-CAR to yield one molecule of β-apo-10-carotenal and one molecule of β-ionone. Interestingly, compared to BCMO1, BCO2 appears to accept a variety of xanthophyll and carotene substrates, including LYCO [20]. This implies that BCO2 may have physiological roles beyond providing precursors for vitamin A synthesis. In addition, carotenoids [21–23] including LYCO induce Phase 1 and 2 xenobiotic metabolizing enzymes, suggesting involvement of these enzymes in the metabolism of certain carotenoids.

There is wide individual variation in LYCO bioavailability [24,25], as well as carotenoid bioavailability in general [26–28]. The degree to which carotenoids are absorbed depends on a number of dietary factors [29,30], including coconsumption of dietary fat, the amount of carotenoid consumed and the degree to which the plant matrix is disrupted. Nondietary factors including age and smoking status can also influence circulating carotenoid concentrations. These factors can be held constant in controlled diet studies, yet large intersubject variability is commonly reported even under these conditions [28], suggesting that absorption or metabolism of carotenoids is, at least to some degree, under genetic control. Provitamin A carotenoids in plasma, for example, would be expected to be lower in individuals who efficiently convert these carotenoids to vitamin A, a process more easily facilitated by certain genotypes of carotenoid cleavage enzymes.

Genetic variation, such as single nucleotide polymorphism (SNP), plays a role in the metabolism of various diet-derived compounds, including carotenoids [31–35]. For example, Ferrucci et al. [34] reported that the G allele at SNP rs6564851, near the BCMO1 gene, was associated with higher plasma concentrations of b-CAR and α-carotene and lower plasma concentrations of LYCO, zeaxanthin and lutein. However, whether rs6564851 is related to BCMO1 expression or activity remains unclear. Herron et al. [35] reported that plasma cholesterol and lutein responses to egg consumption differ among individuals as a function of polymorphism of ABC65, a transporter protein. In addition, several genetic polymorphisms of the Phase 1 and 2 enzymes exist and are known to affect their activities [36,37]. Overall, how genetic variation in the enzymes or proteins involved in carotenoid absorption and metabolism affects uptake, metabolism and efficacy of carotenoids remains largely unknown and warrants further investigation.

Cluster analysis has been used by us and others for global gene expression analysis [38–40]. It allows for grouping of genes that respond similarly to a treatment temporally or in a concentration-dependent fashion [40]. We reasoned that cluster analysis would effectively segregate subjects by temporal differences with regard to plasma carotenoid response to diet and, thus, be useful for identifying individuals with strong and weak plasma responses to various carotenoids. Furthermore, because specific genetic variants of BCMO1 or BCO2 may influence an individual's plasma response to b-CAR and LYCO, links between responsiveness to these carotenoids and genetic variants of these enzymes would be important to elucidate. Hence, the purposes of this study were (a) to assess temporal changes of circulating carotenoids using cluster analysis to identify strong and weak plasma responders; and (b) to determine whether individual differences in plasma carotenoid response are related to specific genetic variants of BCMO1 and BCO2 enzymes.

2. Materials and methods

2.1. Study design

A controlled diet study with repeated measures and a crossover design was conducted at the Beltsville Human Nutrition Research Center. The 23 adult subjects were healthy, nonsmokers who agreed to eat all of the foods and only the foods provided by the study. The Committee on Human Research of the Johns Hopkins School of Public Health and Hygiene approved the study procedures, and subjects gave written informed consent. Details of study design and procedures, diet composition, subject characteristics, subject recruitment criteria and diet composition were previously reported [8]. Diets provided ample dietary fat for carotenoid absorption and were designed to meet the recommended dietary allowances of required nutrients. Diets provided a stable, daily intake of non-LYCO carotenoids, which were held constant as a function of energy required to maintain body weight. For example, at the 10,040 kJ level (2400 kcal), background diets contained 2.2-mg carotenoid/d including 0.8-mg b-CAR and 1.3-mg lutein. All 23 subjects consumed a watermelon juice treatment (W-20) containing 20.1-mg/d LYCO, 2.5-mg/d b-CAR, 0.90-mg/d PE and 0.45-mg/d PF. In addition, subjects received a second treatment of either an increased level of watermelon juice (W-40) (n=12) containing 40.2-mg/d LYCO, 5.0-mg/d b-CAR, 1.8-mg/d PE and 0.9-mg/d PF or tomato juice (T-20) (n=10) containing 18.4-mg/d LYCO, 0.6-mg/d b-CAR, 2.1-mg/d PE and 1.1-mg/d PF. Each treatment was provided daily for 3 weeks with duplicate blood samples taken at each treatment baseline (Week 0) and at the end of Weeks 1, 2 and 3 of each treatment. Treatment periods were separated by 4-week washout periods during which subjects consumed free-choice diets with the exception that LYCO-containing foods were restricted as documented by food records.

2.2. Plasma and food analysis

Extraction and high performance liquid chromatography analysis of b-CAR, LYCO, PE, PF, lutein and retinol from plasma and food were conducted as previously described [8].

2.3. Cluster analysis

Weekly plasma carotenoid concentrations were normalized to treatment period baseline (Week 0) and expressed as fold changes in plasma carotenoid concentrations. The data for temporally normalized fold changes were then analyzed using cluster analysis (Cluster 3.0, k-mean method) [39] to group individuals' temporal carotenoid responsiveness to watermelon juice or tomato juice interventions. We used five clusters for the analysis. The results from cluster analyses were then visualized using TreeView (http://jtreeview.sourceforge.net) and illustrated as a heat map.

2.4. BCMO1 and BCO2 SNP analysis

BCMO1 and BCO2, enzymes proposed to catalyze the first committed step of carotenoid metabolism [18,20], were selected for SNP analysis. Because the number of study subjects was small (n=23), we narrowed the candidate SNPs to be analyzed. We first queried the db SNP database of the National Center for Biotechnology Information (NCBI) (www.ncbi.nim.nih.gov/projects/SNP/) to obtain a list for cSNPs, SNPs located in the coding region of candidate genes. This list was then reduced to those cSNPs corresponding to nonsynonymous changes because they represent changes in amino acids and hence potential alterations in enzyme activity. The list was further limited by selecting cSNPs with >0.1 heterozygosity within the population.

Genomic deoxyribonucleic acid (DNA) for SNP analysis was collected from blood lymphocytes. Whole blood, 10 ml from each subject, was diluted with 10 ml of RPMI-1640+10% fetal bovine serum, mixed by gentle inversion and divided between two 50-ml screw cap tubes containing 10 ml of Ficoll-Paque Plus (GE Healthcare, Piscataway, NJ, USA). Samples were centrifuged at 700× g for 30 min at 20°C. After removal of the top 5 ml of supernatant, cells floating as a white band at the Ficoll/blood interface were harvested in a volume of 5 ml from each of the two tubes and pooled in a fresh 50-ml tube. Then, 15 ml of RPMI-1640+10% FBS were added to each tube of pooled cells. Cells were centrifuged at 700× g for 15 min at 20°C, and the pelleted lymphocytes were stored at −80°C until processed for genomic DNA isolation. The Trizol method (Invitrogen, Carlsbad, CA, USA) was used to isolate genomic DNA according to the manufacturer's protocol. Purified genomic DNA was resuspended in Tris-EDTA (pH 8.0) buffer and stored at −20°C for SNP analysis. Individual SNPs were determined using the Taqman real-time polymerase chain reaction method according to the manufacturer's (Applied Biosystems, Carlsbad, CA, USA) protocol. Primer and probes for cSNPs rs12934922 and rs7501331 were purchased from Applied Biosystems. The PCR were performed with 10–20-ng genomic DNA/tube as determined from our preliminary experiments. Triplicate PCR were performed per gene per sample. SNPs with homo/heterozygous