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Lycopene Biodistribution Is Altered in 15,15′-Carotenoid Monooxygenase Knockout Mice[1–3]

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Abstract

15,15′-carotenoid monooxygenase (CMO I) is generally recognized as the central carotenoid cleavage enzyme responsible for converting provitamin A carotenoids to vitamin A, while having little affinity for nonprovitamin A carotenoids, such as lycopene. To investigate the role of CMO I in carotenoid metabolism, ~90-d-old C57BL/6 × 129/SvJ [CMO I wild-type (WT)] and B6;129S6-Bcmo1tm1Dnp [CMO I knockout (KO)] mice were fed a high-fat, moderate vitamin A, cholesterol-containing diet supplemented with 150 mg/kg diet of β-carotene, lycopene, or placebo beadlets for 60 d ($n = 12–14$). CMO I KO mice fed lycopene (Lyc-KO) exhibited significant decreases in hepatic, spleen, and thymus lycopene concentrations and significant increases in prostate, seminal vesicles, testes, and brain lycopene concentrations compared with WT mice fed lycopene (Lyc-WT). Furthermore, in the serum and all tissues analyzed, excluding the testes, there was a significant increase in the percent lycopene *cis* isomers in Lyc-KO mice compared with Lyc-WT mice. CMO I KO mice fed β-carotene (βC-KO) had significantly lower hepatic vitamin A concentrations (17% of WT mice fed β-carotene [βC-WT]). Concordantly, βC-KO mice had higher serum and tissue β-carotene concentrations than βC-WT mice. In addition, phenotypically CMO I KO mice had significantly higher final body weights and CMO I KO female mice had significantly lower uterus weights than CMO I WT mice. In conclusion, CMO I KO mice fed low levels of vitamin A have altered lycopene biodistribution and isomer patterns and do not cleave β-carotene to vitamin A at appreciable levels. J. Nutr. 138: 2367–2371, 2008.

Introduction

Carotenoids are a large class of over 600 lipid-soluble compounds that are found throughout the plant kingdom. Among the 40 carbon carotenoids, β-carotene and lycopene are the most prevalent in human tissues (1). In 1929, Moore (2) showed that β-carotene could be converted to vitamin A. Not until 1965 was a putative enzyme identified that was responsible for this conversion (3,4). More recently, 2 animal carotenoid cleavage enzymes have been identified; the central cleavage enzyme, 15,15'-carotenoid monooxygenase (CMO I),[7] and the eccentric cleavage enzyme, carotenoid monooxygenase-II (CMO II) (1,5–11). CMO I is responsible for the central cleavage of the provitamin A carotenoids β-carotene, α-carotene, and β-cryptoxanthin to vitamin A. CMO II eccentrically cleaves β-carotene at the 9,10 (or 9′,10') position and the resulting β-apocarotenals undergo chain shortening and oxidation to form retinal or retinoic acid (12–14).

Hessel et al. (15) recently published the first paper characterizing CMO I knockout (KO) mice. Hepatic vitamin A concentrations were not altered in CMO I KO mice fed β-carotene and low vitamin A-containing diets. This suggests that CMO I is the primary enzyme that converts β-carotene to vitamin A in mice. This finding has recently been validated in another laboratory (16). In addition to the alterations in carotenoid metabolism, Hessel et al. (15) noted a surprising finding that CMO I KO mice had alterations in lipid metabolism, including liver steatosis, increased serum FFA, and increased body weights in older female mice fed a high-fat diet.

The primary purpose of this study was to determine whether tissue lycopene biodistribution was altered in CMO I KO mice compared CMO I wild-type (WT) mice. An additional aim was to validate that CMO I KO mice do not convert β-carotene to vitamin A at appreciable levels when fed β-carotene and low-vitamin A diets (15). Finally, because alterations in lipid metabolism have

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[3] Supplemental Figure 1 and Supplemental Table 1 are available with the online posting of this paper at jn.nutrition.org.
[7] Abbreviations used: βC-KO, knockout mice fed β-carotene; βC-WT, wild-type mice fed β-carotene; CMO I, 15,15′-carotenoid monooxygenase; CMO II, carotenoid monooxygenase-II; KO, knockout; Lyc-KO, knockout mice fed lycopene; Lyc-WT, wild-type mice fed lycopene; Pl-KO, placebo knockout group; Pl-WT, placebo wild-type group; WT, wild-type.
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been found in CMO I KO mice (15), we investigated whether there are alterations in serum or hepatic lipids in CMO I KO and CMO I WT mice fed high-fat, cholesterol-containing diets containing β-carotene, lycopene, or placebo beadlets.

Materials and Methods

Mice. C57BL/6 × 129/SvJ (CMO I WT, $n = 41$) and B6;129S6-Bcmo1tm1Dnp (CMO I KO, $n = 37$) were provided courtesy of DSM Nutritional Products. Generation of these mice has been described previously (15). Mice were bred at RCC Ltd Laboratory Animal Services and fed a low-vitamin A (450 μg retinol/kg diet in the form of retinol palmitate) diet prior to shipment to the University of Illinois where they were individually housed in shoebox cages with free access to water. For 3 wk after receipt, mice consumed ad libitum a standard AIN-93G diet (17) that was adjusted to contain 825 μg retinol palmitate/kg.

After 3 wk, mice were assigned to 1 of 3 experimental diets supplemented with placebo, β-carotene, or lycopene beadlets (gift from DSM) to provide 150 mg/kg diet of β-carotene or lycopene using a randomized complete block design (blocked variables; strain, sex, and shipment date). The study groups were as follows: placebo WT (Pl-WT), placebo KO (Pl-KO), β-carotene wild-type (βC-WT), β-carotene KO (βC-KO), lycopene wild-type (Lyc-WT), and lycopene KO (Lyc-KO). The carotenoid level consumed is roughly equivalent to humans consuming 230 mg/d of one of these carotenoids. Placebo beadlets were added at equivalent concentrations to placebo diet. **Table 1** shows the number of males and females assigned to the study diet groups. Diets were adapted from Kirk et al. (18) and were high fat (16.7%), contained 0.1% cholesterol, and lower vitamin E and A levels (22.5 mg of α-tocopherol and 825 μg retinol palmitate/kg of diet; TD.04070 Harlan Tekland; **Table 2**). New batches of experimental diets were made every 3 wk by adding new beadlets to the basal diet and stored in the dark at 4°C. Individual feed intake was monitored when fresh feed was provided every 48 h and mice were weighed weekly. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

After 60 d of receiving their experimental diets, mice were anesthetized with 200:10.5 mg/kg ketamine: xylazine. The thoracic cavity was opened and blood was taken via cardiac puncture, after which mice were killed by heart removal. Tissues were removed and snap-frozen in liquid nitrogen and stored at −80°C until analysis.

Carotenoid and retinoid extraction. Carotenoids and retinoids were extracted as previously described (19) with minor modifications described below. For hepatic tissue, 0.2 g was minced in a 50-mL test tube containing 6 mL of ethanol containing 0.1% butylated hydroxytoluene. Echinenone (a gift from DSM), the internal standard for carotenoids, and 3 mL of saturated potassium hydroxide was then added and vortexed before saponifying for 30 min at 60°C. The test tubes were removed, placed on ice, and 3 mL of deionized water was added. The samples were then extracted 3 times with 6 mL of hexane. For extrahepatic tissues, 2 mL ethanol with 0.1% butylated hydroxytoluene, 1 mL of saturated potassium hydroxide, and 1 mL of deionized water were utilized.

For diet analysis, ~10 mL of ethanol with 0.1% butylated hydroxyltoluene and 4 mL of saturated potassium hydroxide were added to 1 g of diet. The samples were then vortexed and saponified at 70°C for 30 min. The test tubes were removed, placed on ice, and 8 mL of deionized water was added. The samples were extracted initially with 14 mL of hexane and then twice with 7 mL of hexane. Echinenone was then added as an internal standard. All extracts were dried down in a Speedvac concentrator (model AES1010; Savant), flushed with argon and stored at −20°C for <48 h before HPLC analysis.

TABLE 1 Gender distribution of CMO I WT and CMO I KO mouse groups

Group	Pl-WT	Pl-KO	βC-WT	βC-KO	Lyc-WT	Lyc-KO
				n		
Male	6	5	6	6	6	6
Female	7	7	8	7	8	6
Total	13	12	14	13	14	12

TABLE 2 Basal diet composition[1]

Ingredient	*g/kg diet*
Casein	200
Sucrose	414.2
Maltodextrin	100
Cellulose	50
Cocoa butter	150
Corn oil	17
DL-Methionine	3
AIN-93 vitamin mix[a]	12
Mineral mix, Ca-P deficient (TD 79055)	16
Calcium phosphate, dibasic $CaHPO_4$	11.4
Calcium carbonate $CaCO_3$	7.9
Cholesterol	10
Sodium cholate	5
Choline bitartrate	3.5

[1] Adjusted to provided 22.5 mg of RRR-α-tocopherol (as α-tocopherol acetate) and 450 μg retinol (as retinol palmitate)/kg diet.

HPLC analysis. Retinol (20) and carotenoids (21,22) were analyzed using previously described methods. The HPLC system consisted of Rainin SD-200 Dynamax pumps, a C30 column (4.5 × 150 mm, 3 μm; YMC) or C18 column (4.6 × 250 mm, 5 μm; Supelco), a UV-1 or UV-DII Detector, and a Dynamax HPLC Methods Manager integrator (Rainin Instrument). Our laboratory routinely participates in the National Institutes of Standards in Technology micronutrient proficiency testing program for carotenoids and retinoids and our carotenoid values are normally within 1 SD of the median.

Cholesterol and lipid analysis. Serum total cholesterol was measured in triplicate using kit instructions and an enzymatic colorimetric assay (Thermo DMA catalogue no. 2350–500). Serum triglycerides were measured per kit instructions using an enzymatic assay (Wako catalogue no. 998–40391, 998–40491). Liver lipids were extracted using a modification of the Folch method (23). The sample (0.5 g) was placed in chloroform:methanol (1:1), homogenized, and filtered by gravity. The interface was washed twice with 0.29% sodium chloride solution, centrifuged at 183 × *g* for 4 min at 25°C, and the top layer was discarded. The remaining solution was evaporated, placed to dry in a desiccator for at least 48 h, and weighed to determine total lipids. PBS was then added to the dried lipid fraction of the liver, heated to 37°C, and vortexed. Dissolved liver fractions were then quantified for total cholesterol content using the same method as described above for serum.

Statistics. Initial body weights, final body weights, serum cholesterol, serum triglycerides, liver weights, liver lipids, and liver cholesterol concentrations were analyzed using dummy coded multiple linear regression (24,25). Sex was used as a covariate (dummy coded) along with the 3 main variables (genotype, β-carotene, and lycopene). Hierarchical analysis was used to determine whether covariate interaction terms and treatment interaction terms accounted for a significant amount of the variance; if not, these terms were removed from the model. To correct for violations of model assumptions the natural logs of initial body weights, final body weights, serum triglycerides and liver weight as percent body weight were used for statistical analysis. Lycopene concentrations, percent all-*trans* lycopene, and β-carotene concentrations were analyzed by 2-sample *t* tests. The natural logs of most of these measures were analyzed to correct for assumption violations. When this transformation did not correct assumption violations, we used the Wilcoxon's Rank Exact Sum test (26)

to analyze the data. Serum and hepatic vitamin A concentrations were analyzed using ANOVA and Tukey's test to compare group means. Values in the text are means ± SEM, statistics were performed using SAS 9.1, and $P < 0.05$ was considered significant.

Results

βC-KO mice had significantly higher β-carotene serum and tissue concentrations (3.5- to 22-fold of βC-WT; **Table 3**). Concordantly, βC-KO mice had lower (17% of βC-WT) hepatic vitamin A concentrations (**Table 4**; $P < 0.05$). However, there was no difference in serum vitamin A concentrations between βC-KO and βC-WT mice (Table 4).

Lyc-KO mice exhibited significant decreases in hepatic, spleen, and thymus lycopene concentrations (Table 3) compared with Lyc-WT. At the same time, there were significant increases in prostate, seminal vesicles, testes, and brain lycopene concentrations in Lyc-KO compared with Lyc-WT mice. In addition, serum, liver, spleen, adrenals, kidney, lungs, prostate, and seminal vesicles of Lyc-KO mice exhibited significant decreases in the percent of lycopene that was in the all-*trans* isomer form compared with Lyc-WT mice. Instead, the tissues contained a higher percentage of *cis* lycopene isomers (**Supplemental Fig. 1**).

CMO I KO and WT mice did not differ in initial body weights; however, CMO I KO mice weighed more at the end of the study (**Supplemental Table 1**; $P = 0.04$). Experimental diets did not affect final body weights. We also found that CMO I KO female mice had lower uterus weights (0.10 ± 0.05 g) than the CMO I WT female mice (0.23 ± 0.08; $P < 0.0001$). Other organ weights as a percent of body weight did not differ (data not shown).

Given that we fed an atherogenic diet and reported alterations in CMO I KO mice (15), we also evaluated whether carotenoid feeding or genotype altered various lipid parameters (Supplemental Table 1). Neither diet nor genotype significantly affected liver lipids or liver cholesterol concentrations; however, there was a significant gender × genotype interaction, where male KO mice had smaller livers as a percent of body weight and females had larger livers as a percent of body weight ($P = 0.009$).

Lycopene consumption led to a significant increase in serum cholesterol ($P = 0.008$) and there was a significant interaction between lycopene and genotype ($P = 0.0003$). Serum cholesterol concentrations were decreased in Lyc-KO mice and increased in Lyc-WT mice compared with the placebo groups. Diet did not affect serum triglycerides, but CMO I KO mice had higher ($P = 0.03$) serum triglyceride concentrations than CMO I WT mice.

Discussion

In this study, we fed CMO I KO and CMO I WT mice diets containing placebo, β-carotene, or lycopene beadlets and discovered some important outcomes. First, we confirmed the work of others (15,16) showing that CMO I is the primary enzyme responsible for converting β-carotene to vitamin A in mice. This was clear from the substantial increases in tissue β-carotene concentrations in βC-KO mice (3.5–22-fold of βC-WT) and the much lower hepatic vitamin A concentrations (17% of βC-WT). Apparently, CMO II or other murine enzymes failed to cleave β-carotene to vitamin A to any great extent when mice are fed low-vitamin A diets as administered in this study. Although relatively low dietary vitamin A was fed, the mice were not vitamin A deficient, as indicated by hepatic vitamin A concentrations in excess of 100 nmol/g in all groups and normal serum vitamin A concentrations, which are homeostatically controlled and are reduced only when mice become deficient.

One novel finding was the substantially altered lycopene biodistribution in Lyc-KO mice. Lycopene concentrations were significantly lower in liver, spleen, and thymus but significantly higher in the prostate, seminal vesicles, testes, and brain in Lyc-KO mice than in Lyc-WT mice. We hypothesize that the difference between the genotypes is partly due to the relative tissue-specific expression levels of CMO II (27). Previous evidence suggests that CMO I has little cleaving activity toward lycopene (9,10),

TABLE 3 Serum and tissue β-carotene and lycopene concentrations and percent all-trans lycopene isomer in CMO I WT and CMO I KO mice fed β-carotene or lycopene diets for 60 d[1]

	β-Carotene[2]		Total lycopene[3]		All-*trans* lycopene[3]	
Group	βC-WT	βC-KO	Lyc-WT	Lyc-KO	Lyc-WT	Lyc-KO
	μmol/L				*%*	
Serum	0.76 ± 0.20 (6)	10.31 ± 0.99 (6)**	0.59 ± 0.11 (4)	0.61 ± 0.078 (6)	19.0 ± 2.1 (4)	8.5 ± 1.3 (6)*
	nmol/g					
Liver	18.0 ± 2.2 (6)	246 ± 61 (6)*	469 ± 107 (6)	100 ± 18 (6)**	51.3 ± 2.8 (5)	27.6 ± 1.1 (5)**
Spleen	25.0 ± 6.6 (7)	89.4 ± 15.9 (6)**	270 ± 41 (7)	29.8 ± 6.5 (6)**	58.9 ± 1.0 (5)	45.9 ± 1.6 (5)**
Adrenals[4]	20.2 ± 1.5 (7)	266.7 ± 8.7 (7)**	48.8 ± 15.3 (7)	37.9 ± 14.4 (4)	32.0 ± 2.3 (7)	17.9 ± 1.6 (4)**
Kidney	1.53 ± 0.16 (6)	22.5 ± 1.5 (6)**	2.60 ± 0.21 (12)	3.75 ± 0.51 (12)	47.5 ± 2.8 (5)	30.2 ± 2.1 (5)**
Lungs	2.05 ± 0.17 (8)	22.7 ± 2.4 (8)**	1.94 ± 0.38 (7)	1.38 ± 0.21 (6)	41.1 ± 2.7 (7)	18.7 ± 1.2 (6)**
Brain	0.072 ± 0.019 (6)	1.44 ± 0.18 (5)*	0.019 ± 0.005 (6)	0.048 ± 0.006 (5)*	ND[5]	ND
Thymus[4]	3.74 ± 0.39 (4)	37.4 ± 3.6 (4)**	4.71 ± 1.03 (5)	2.24 ± 0.47 (4)*	ND	ND
Adipose	2.08 ± 0.99 (6)	45.6 ± 10.8 (6)**	3.06 ± 0.79 (5)	4.90 ± 1.16 (5)	ND	ND
Uterus[4]	3.67 ± 0.58 (4)	31.3 ± 4.6 (3)**	4.43 ± 0.81 (4)	3.29 ± 1.85 (2)	ND	ND
Prostate[4]	1.32 ± 0.07 (3)	22.7 ± 5.6 (3)**	0.81 ± 0.08 (3)	3.24 ± 0.79 (3)*	33.1 ± 1.3 (3)	20.5 ± 1.3 (3)*
Testes	3.56 ± 0.37 (6)	37.4 ± 3.8 (6)**	2.12 ± 0.14 (6)	5.11 ± 0.80 (6)*	25.6 ± 2.9 (6)	20.3 ± 1.5 (6)
Seminal vesicles	0.28 ± 0.10 (6)	3.56 ± 0.50 (5)*	0.04 ± 0.01 (6)	0.15 ± 0.05 (5)*	31.2 ± 3.3 (6)	18.4 ± 0.9 (5)*

[1] Values are means ± SEM, (*n*). Asterisks indicate different from corresponding WT, *$P < 0.05$, **$P < 0.001$.
[2] β-Carotene was not detected in mice fed placebo or lycopene beadlet-containing diets.
[3] Lycopene was not detected in mice fed placebo or β-carotene beadlet-containing diets.
[4] Pooled from 2–3 mice.
[5] ND, Not determined.

TABLE 4 Serum and liver vitamin A concentrations in CMO I WT and CMO I KO mice following consumption of placebo, β-carotene, or lycopene diets for 60 d[1]

Group	Pl-WT	Pl-KO	βC-WT	βC-KO	Lyc-WT	Lyc-KO
Serum, *μmol/L*	1.35 ± 0.13 (11)	1.56 ± 0.08 (11)	1.37 ± 0.09 (10)	1.38 ± 0.06 (10)	1.33 ± 0.15 (11)	1.46 ± 0.09 (11)
Liver, *nmol/g*	144 ± 24 (6)[a]	130 ± 14 (6)[a]	876 ± 94 (6)[b]	148 ± 14 (6)[a]	190 ± 36 (6)[a]	166 ± 16 (6)[a]

[1] Values are means ± SEM, (*n*). Labeled means in a row with superscripts without a common letter differ, $P < 0.05$.

but work with *Escherichia coli* suggests that CMO II may cleave this compound (8). It has been reported that *cis* lycopene isomers of lycopene are preferential substrates for CMO II in vitro compared with all-*trans* lycopene (28). In food, lycopene is primarily found in the all-*trans* form; however, tissues such as the prostate accumulate higher concentrations of *cis* isomers (29). We and others have shown previously that *cis* isomers are more bioavailable than the all-*trans* parent (30,31). It is not clear if there is a difference in antioxidant or other biological activity of *cis* isomers as compared with all-*trans* lycopene in vivo.

However, all tissues examined, except the testes, exhibited significantly lower percentages of all-*trans* lycopene and thus a greater proportion of *cis* isomers in CMO I KO mice than in CMO I WT mice. If *cis* isomers were indeed a preferred substrate for CMO II, we would have expected to find the opposite outcome. It is also possible that other unidentified enzyme(s) may cleave lycopene in CMO I KO mice. If so, it would seem likely that this uncharacterized enzyme either prefers all-*trans* lycopene as a substrate or that there is a lycopene isomerase present, which would explain the high *cis* concentrations in CMO I KO tissues. Another possibility is a binding protein that preferentially binds to *cis* forms of lycopene and facilitates tissue uptake and/or prevents their degradation and excretion.

Recently, Vogel et al. (32) evaluated eccentric carotenoid cleavage of lycopene and other carotenoids in maize, Arabidopsis, and tomatoes. Interestingly, the plant eccentric cleavage enzyme (carotenoid cleavage diooxygenase 1) cleaved lycopene but not its more saturated precursor phytoene. The authors note "carotenoid cleavage enzymes are an ancient and highly conserved family, with members present in plants, animals, and bacteria (32)." Further work is needed to determine whether the carotenoid specificity of CMO II is similar to carotenoid cleavage diooxygenase 1.

Additional studies are needed to characterize tissue CMO II mRNA expression and protein levels and determine whether CMO II levels are responsible for the altered lycopene biodistribution. However, given what is known about the relative tissue expression levels of CMO II, it does not seem that CMO II alone is entirely responsible for the altered lycopene biodistribution or altered isomer distribution (1,11). It may be that CMO I KO mice exhibit alterations in CMO II protein or enzyme activity levels that would further explain this relationship. Furthermore, CMO I KO mice may have differences in lipid transporters such as scavenge receptor class B, type 1, which is involved in carotenoid transport (33,34). The mechanism(s) responsible for the altered lycopene biodistribution is currently under investigation.

In addition to alterations in carotenoid metabolism, Hessel et al. (15) also reported a significant increase in weight gain in 28-wk-old female CMO I KO mice fed a high-fat diet. We found a significant increase in final body weights of CMO I KO mice compared with CMO I WT mice. Hessel et al. (15) also reported increases in serum FFA, hepatic total lipids, cholesterol esters, and FFA. In contrast, we found that CMO I KO mice had significantly higher serum triglyceride concentrations but no differences in serum cholesterol, hepatic lipids, or hepatic cholesterol concentrations. These different outcomes may be due to the age of the mice (21.5 wk vs. 28 wk at the end of the respective studies) and/or the fact that we fed high-fat, cholesterol-containing diets.

We did not find evidence to suggest that either β-carotene or lycopene positively altered lipid homeostasis. However, the serum cholesterol concentrations led to an interesting result. We found a significant diet × genotype interaction; serum cholesterol concentrations were increased in Lyc-WT mice but decreased in Lyc-KO mice. More research is needed to clarify the relationship between lycopene, serum cholesterol, and CMO I expression. We were surprised to find that uterus weights in CMO I KO mice were less than one-half the size of CMO I WT mice. It is possible that there may be an alteration in estrogen status in CMO I KO mice. We did not collect other estrogen-responsive tissues nor have remaining tissue for analysis. We are pursuing this currently with additional mice.

Alterations in CMO I have been reported in humans. For instance, a CMO I mutation resulted in drastically reduced enzyme activity and led to hypovitaminosis A and hypercarotenemia (35). Thus, it is clear that CMO I function is critical to the production of vitamin A, but its function in lycopene metabolism may or may not be important for understanding the reported health benefits of tomatoes and lycopene (27).

In conclusion, our results in CMO I KO mice support the belief that CMO I is the primary central cleavage enzyme responsible for conversion of β-carotene to vitamin A. Furthermore, CMO I KO mice had altered lycopene biodistributions, greater final body weights, higher serum triglyceride concentrations, and smaller uterus sizes. It is possible that CMO II and/or another carotenoid cleavage enzyme is/are responsible for differential lycopene metabolism. Additional work is currently underway to further clarify these novel findings in these mice.

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