

CAROTENOID METABOLISM IN MICE AND PROSTATE CANCER RISK

BY

NIKKI ANN FORD

DISSERTATION

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Doctoral Committee:

Associate Professor Manuba Nakamura, Chair
Professor John W. Erdman, Jr., Director of Research
Professor Elizabeth Jeffery
Professor James Drackley

ABSTRACT

Prostate cancer is the second most abundant cancer with a 32% mortality rate world-wide. Epidemiological studies suggest an inverse relationship between risk of prostate cancer and intake of tomato products or higher blood levels of lycopene.

β -carotene is centrally cleaved by carotene monooxygenase I (CMO-I) to form vitamin A and further metabolism results in formation of retinoic acid and other retinoids. The metabolism of β -carotene has been extensively studied, but very little is known about the metabolism of other carotenoids. We and others have hypothesized that other acyclic carotenoids, like lycopene, are eccentrically cleaved by carotene monooxygenase II (CMO-II). Like retinoids, we propose that carotenoid metabolites produced by CMO-II enzymatic cleavage are bioactive at small concentrations in tissues.

The primary aims of this proposal were to delineate tissue specific expression of CMO-I and CMO-II and evaluate resulting carotenoid bioaccumulation in CMO-I KO, CMO-II KO, and wild-type mice. Secondly, we investigated the effects of lycopene metabolites on human prostate cancer cells, *in vitro*. Thirdly, we investigated the effects of carotenoid metabolism in CMO-I KO, CMO-II KO, and WT mice on sex steroid hormone status.

Lycopene preferentially accumulated in CMO-II KO mice while β -carotene preferentially accumulated in CMO-I KO mice. Phytofluene and phytoene accumulation was not altered by genotype. Together these data suggest that lycopene is eccentrically metabolized by CMO-II and β -carotene is centrally metabolized to retinal by CMO-I. Phytoene and phytofluene may not be substrates for either CMO-I or CMO-II cleavage or did not accumulate in high enough concentrations in the liver to induce cleavage by these enzymes. We also report that the mRNA expression of CMO-I and CMO-II were not altered by the carotenoid-containing diets used in

our studies. Interestingly, serum and testes testosterone were reduced and related sex steroid metabolizing genes were altered in CMO-I KO mice. We hypothesize that due to induced expression of CMO-II in the testes of CMO-I KO mice that lycopenoids are at least in part responsible for these effects. Lastly, we demonstrate anti-proliferative effects of the lycopene-metabolite, apo-12'-lycopenal in androgen-dependent DU145 prostate cancer cells.

Overall, our findings provide support for previous *in vitro* data to suggest that lycopene is metabolized by the CMO-II enzyme, *in vivo*. Furthermore, we have evidence to suggest that lycopene metabolites reduce proliferation of prostate cancer cells *in vitro* and may beneficially alter sex steroid status in mice.

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CHAPTER 1

LITERATURE REVIEW

INTRODUCTION

Prostate cancer is the second most common cancer accounting for over 192,000 new cases in 2009 with approximately 27,000 deaths (1). One out of every six American men will experience this difficult and sometimes fatal disease in their lifetime. Many risk factors are associated with prostate cancer including family history, hormone status, race, age, diet, and environmental factors. Diet is one of the few risk factors that may be modified. Notably, consuming three to five servings of tomato products a week reduces the risk of prostate cancer in epidemiological studies (2).

The estimated cumulative cost of prostate cancer treatment over five years exceeds \$42,000 (3). Furthermore, treatment options often cause side effects with up to 43% of men experiencing sexual problems, 16% with urinary problems, and up to 11% of men experience bowel problems two years after treatment (4). With these points in mind and the fact that prostate cancer predominantly progresses slower than other types of cancer, dietary intervention should be explored as a way to prevent or reduce the progression of prostate cancer. Additionally, dietary modification may create a better quality of life for older men and an economic relief from the costs of healthcare associated with prostate cancer.

Lycopene, which is the red pigment of tomatoes (also in tomato products) and a major carotenoid in human plasma and tissues, is suggested to reduce prostate cancer risk. Therefore, it is common to find individuals consuming lycopene in supplement form. However, at this time, there is not enough evidence available to definitively determine whether lycopene is wholly responsible for the reduced risk of prostate cancer observed when consuming tomato products.

Since the *in vivo* metabolism of tomato carotenoids is poorly understood, it is not known whether it is lycopene, other carotenoids or components of tomatoes, or their metabolites that influence prostate cancer risk.

Tomato composition

Tomatoes contain significant amounts of provitamin A carotenoids, α -tocopherol, fiber, potassium, and folate. However, the nutrient content of tomato products varies widely depending upon variety, ripeness of the fruit, and the processing methods. Polyphenols, potentially beneficial anti-cancer compounds, are also found in tomato products, predominantly in the skin and seeds. Specifically, quercetin and kaempferol can be found in significant amounts (5).

Carotenoids in tomatoes

Tomatoes also contain a variety of carotenoids in addition to lycopene. While lycopene comprises approximately 60-64% of the total carotenoids found in tomato products, phytoene, phytofluene, neurosporene and γ -carotene are the next most abundant carotenoids (Figure 1.1).

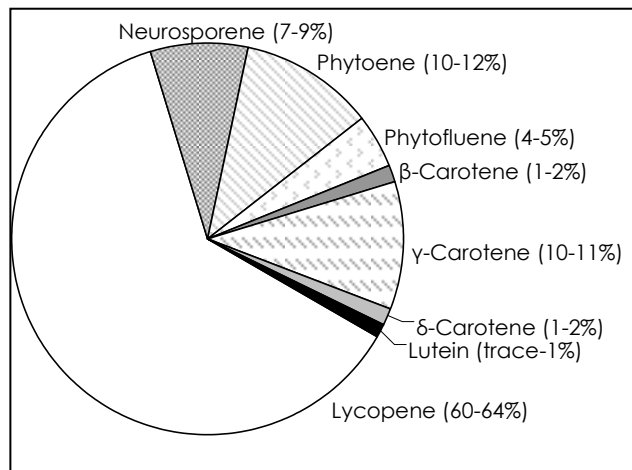


Figure 1.1: Carotenoid composition of a raw, ripe tomato (%). Adapted from (6).

The red pigment, lycopene, is also found in lesser amounts in guava, pink grapefruit, papaya, passionflower fruit, and watermelon (7). Upon consumption, it accumulates in human

and animal serum, predominantly in the liver, adrenals, testes, and prostate (8). Lycopene is a 40 carbon atom, acyclic, open chain polyisoprenoid with 11 conjugated double bonds (9).

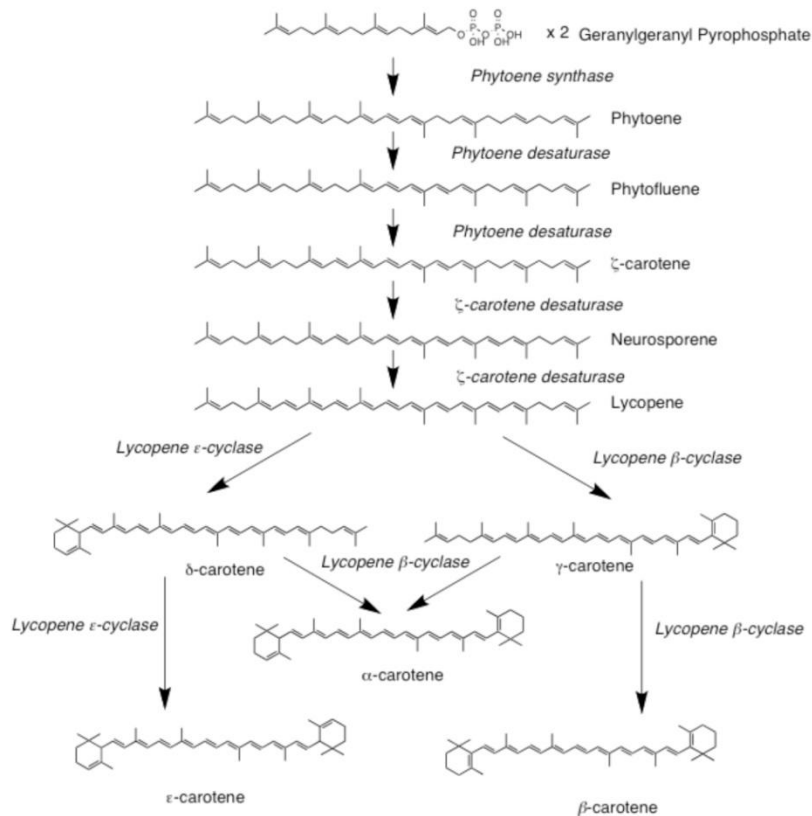


Figure 1.2: Plant carotenoid biosynthetic pathway

Many carotenoids are produced in plants through a number of desaturation and cyclase steps (Figure 1.2). Within the plant kingdom, the colorless carotenoid, phytoene is desaturated to form phytofluene which through further desaturation reactions results in accumulation of lycopene in tomatoes. Two different isoforms of lycopene cyclase ultimately produce α-carotene or β-carotene. Plant based dietary sources provide most of the serum and tissue carotenoids found in animals.

All-*trans* lycopene is the predominant isomer found in tomatoes, but *cis* isomers are the form most commonly found in human tissues suggesting preferential absorption of *cis* isomers or isomerization within the intestinal mucosa (10). Heating and processing of tomato products increases the amount of *cis* isomers of lycopene. *Cis* isomers of lycopene are more soluble in bile acid micelles and may be preferentially integrated into chylomicrons of the small intestinal mucosa, thus increasing the bioavailability of lycopene (10-12). Thermal processing also increases bioavailability by breaking down the tomato cell matrix and decreasing food particle size (13). Lastly, a tomato has a very high water weight while thermal processing of tomatoes causes water loss so lycopene becomes more concentrated.

Tomato carotenoids & prostate cancer

In a pivotal study published in 1995 using the Health Professional Follow-up Study cohort, lycopene and tomato product intake were associated with a decreased risk of prostate cancer (2). A follow-up study of this cohort revealed that the inverse association still existed 12 years after the original analysis (14). Another study investigated the association of the severity of prostate cancer and carotenoid intake and found a strong inverse association between the aggressive forms of cancer and lycopene intake (15). An association between plasma lycopene concentrations and the risk of non-familial prostate cancer was also reported (16). Lastly, a meta-analysis of epidemiological trials found marginal effects of tomato product consumption on prostate cancer and this relationship was restricted to high intakes of tomatoes (17). Together, these studies suggest that high intakes of tomatoes or lycopene reduce the risk of aggressive, sporadic prostate cancer in older men. The contention that tomato products and lycopene consumption can reduce the risk of prostate cancer prompts further research into this relationship. Moreover, we find that despite the evidence indicating a beneficial effect of lycopene against

cancer, the mechanism(s) of action have yet to be fully elucidated. It is unclear whether lycopene alone, its metabolites, or other bioactive components of tomato products reduce the risk of prostate cancer. Additionally, it is plausible that metabolites of lycopene may be more biologically active than the parent compound and may be responsible for lycopene's bioactivity.

Carotenoid cleavage enzymes

Carotenoid cleavage enzymes were first proposed in the 1930's as a mechanism for vitamin A formation from β -carotene cleavage at the central 15,15'-double bond (18). Three carotenoid cleavage oxygenases have been identified in mammals, carotene-15,15'-monooxygenase (CMO-I), carotene-9',10'-monooxygenase (CMO-II), and retinal pigment epithelium (RPE65). Many other members of this enzyme family are found in bacteria and plants with wide substrate specificity (19). These enzymes belong to a superfamily of iron-containing oxygenases which encompass a wide array of physiological roles. It has been suggested that RPE65, which is highly expressed in the eye of mammals, is a binding protein for *all-trans*-retinyl esters but is not responsible for apo-carotenal production (20).

CMO-I is also known in the literature as β,β -carotene 15,15'-dioxygenase, BCOI, and β CMOOX (21) while CMO-II is also known as β,β -carotene-9',10'-oxygenase, BCDO2 and BCOII (22, 23). Further characterization of these enzymes and their functions will help to clarify the appropriate nomenclature.

The central cleavage of β -carotene to form retinal was established in the rat liver and intestine in the separate laboratories of Goodman and Olson in 1965 (Figure 1.3) (24, 25). CMO-I is a cytoplasmic enzyme that was first cloned in 2000 by Wyss and colleagues (21). It is primarily found in the duodenal mucosa of mammals, but has also been found in numerous other

tissues (26-28). CMO-I symmetrically cleaves β -carotene to form two molecules of all-*trans* retinal. Lycopene was not cleaved by CMO-I in a model of transformed *E. coli* (23).

The second carotenoid cleavage enzyme, CMO-II, is proposed to be responsible for the eccentric cleavage of carotenoids (Figure 1.3) (22, 23). Although eccentric cleavage products of β -carotene have been identified *in vitro*, in a model of transformed *E. coli*, β -carotene was not metabolized by CMO-II (29). Further studies investigating eccentric cleavage of β -carotene are necessary.

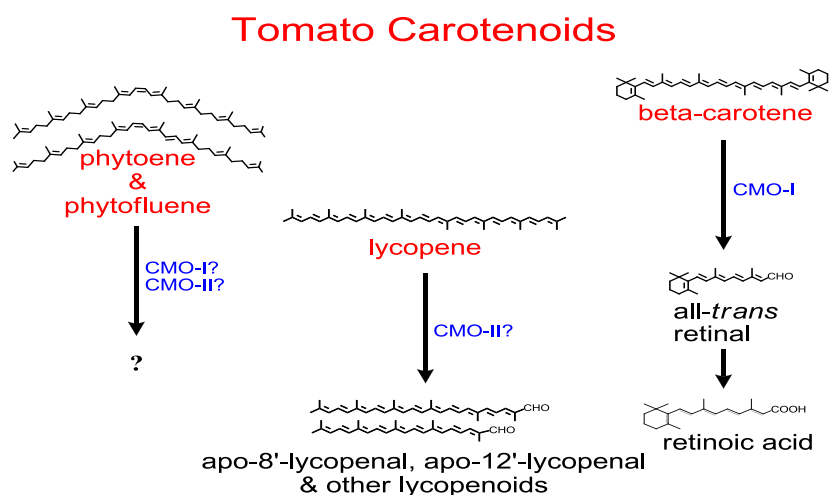


Figure 1.3: Proposed metabolic pathway of tomato carotenoids in animals.

CMO-II is expressed in many mammalian tissues including those not sensitive to vitamin A deficiency. Additionally, CMO-II mRNA expression can be found in tissues lacking expression of CMO-I. This suggests that CMO-II has a carotenoid cleavage function outside of vitamin A synthesis (30). Again, using a model of *E. coli* expressing CMO-II, the bright red lycopene pigment was lost suggesting metabolic cleavage by this enzyme (23). Therefore, *in vivo* investigation of eccentric cleavage of lycopene and other carotenoids is essential to confirm this observation.

CMO-I and CMO-II are similarly distributed and expressed in the human small intestine, liver, eye and adrenal glands (Table 1.1) (30). Both enzymes are also independently expressed in many other tissues. CMO-II appears to be less specific than CMO-I, accepting a wider range of substrates, including acyclic lycopene. Additionally, CMO-II has been reported to have a higher affinity for substrates of the *cis*-isomer conformation over *trans*-isomers (23, 31).

Tissue	CMO-I	CMO-II
Adrenals	++	++
Small Intestine	+++	+++
Liver	+++	+++
Skeletal Muscle	+	+++
Heart	-	+++
Eye	+++	+++
Prostate	+	++
Pancreas	+++	++

Table 1.1: Relative expression of CMO-I and CMO-II in human tissues. (-) zero, (+) low, (++) moderate, (+++) high expression levels (23).

The mRNA expression of carotenoid cleavage enzymes is altered with the consumption of lycopene in some rodent models. Recently, our laboratory measured the expression of CMO-I and CMO-II in F344 rats fed a lycopene-enriched diet. CMO-I expression was decreased in the kidney and adrenal with lycopene consumption while CMO-II was only significantly reduced in the kidney (32). This reduction may be a result of feedback inhibition by lycopene and/or its metabolites. In another study, the expression of CMO-II mRNA increased 4-fold in the lungs of ferrets fed lycopene (31). This apparent contradiction may be explained by the differential need

for lycopene metabolites as necessitated by the specie or tissue. Overall, the effect of carotenoid consumption on the expression of carotenoid cleavage enzymes warrants investigation.

Mutations in carotenoid cleavage enzymes

The relative conversion of β -carotene to vitamin A in healthy individuals and the resulting serum or lipoprotein concentrations of carotenoids varies greatly. This variation may be due to two recently reported single nucleotide polymorphisms (SNPs) existing in the human CMO-I gene. These SNPS are found in high frequency within the European, Chinese, and Japanese populations (33). A separate study in Italians found a common polymorphism near the CMO-I gene which lead to higher serum β -carotene and α -carotene levels (34). Lastly, in another independent study, a loss of function mutation in CMO-I lead to hypercarotenemia and hypovitaminosis A in a single patient (35).

Several SNPs have also been identified in the coding region of the CMO-II gene (36) Unfortunately, no human studies have investigated carotenoid tissue or serum levels with CMO-II gene SNPs. On the other hand, mutations in CMO-II resulted in altered carotenoid tissue deposition in the chicken, cow, and sheep (37-39).

Alterations in the function of CMO-I and CMO-II, due to SNPs, may explain the varied effect of diet on cancer risk owing to altered production of bioactive carotenoid metabolites.

Carotenoid metabolites

Theoretically, cleavage of lycopene by CMO-II produces lycopenoids, more specifically, apo-lycopenals. Apo-lycopenals are poly-isoprenoid compounds of less than 40 carbons in length containing at least one aldehyde end group and are derived from the parent compound, lycopene (Figure 1.4).

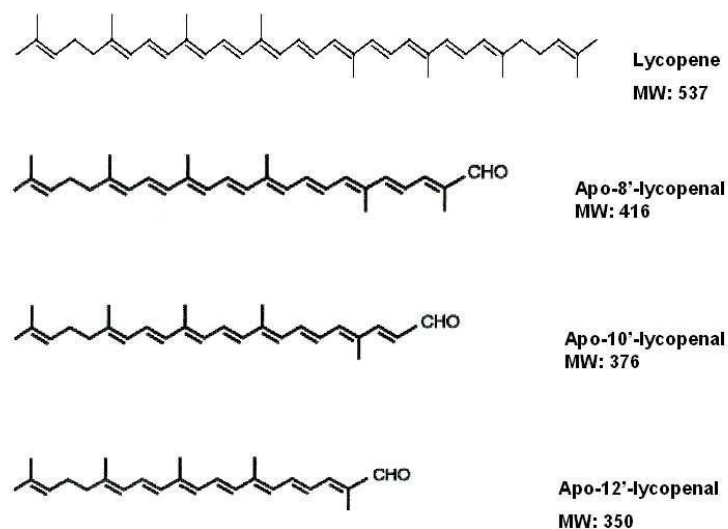


Figure 1.4: Structure and molecular weight of lycopene and 3 apo-lycopenals.

Recently, studies have identified and characterized lycopene metabolites and oxidative products generated *in vivo*. Some of these compounds demonstrate anti-cancer activity by inhibiting proliferation, inducing apoptosis, or enhancing cell to cell communication (40-42). These compounds appear to be physiologically bioactive and further identification and characterization of lycopene metabolites is warranted.

A series of apo-lycopenals and short-chain carbonyl compounds were produced by *in vitro* autoxidation of lycopene (43). Among other oxidation products formed, apo-14'-lycopenal, apo-12'-lycopenal, apo-10'-lycopenal and apo-6'-lycopenal were identified. The authors suggested that tissues exposed to oxidative stress may induce lycopene metabolite formation and these metabolites may be essential for the biological effects of fruit and vegetable consumption on chronic disease risk (43). Numerous other studies have investigated lycopene metabolite formation *in vitro* (44-46). Caris-Veyrat and colleagues studied metabolite formation by potassium permanganate oxidation and oxygen catalyzed by metalloporphyrin. Eleven apo-

lycopenals were found and characterized while a novel compound, apo-11-lycopenal, was identified. The authors proposed that apo-lycopenals were oxidatively cleaved from epoxides of lycopene.

There are some problems associated with the use of *in vitro* systems for the investigation of carotenoids. The partial pressure of oxygen during *in vitro* studies is often very high in comparison to most tissues in the body and supraphysiological concentrations of carotenoids are typically used in these studies. However, these *in vitro* models may be important representatives of carotenoid cleavage in the lungs of smokers, tumors in cancer patients, or other oxidatively stressed conditions.

Metabolites of lycopene have also been identified *in vivo*. Interestingly, *in vivo* concentrations of lycopene metabolites are of comparable biological concentrations to retinoids produced from β -carotene. Table 1.2 shows the relative concentrations of metabolites from β -carotene and lycopene reported in the literature.

Metabolite	Animal	Tissue	Concentration
Apo-6'-lycopenal	Humans	Plasma	0.076 ng/ml
Apo-8'-lycopenal	Rats	Liver	250 ng/g
Apo-8'-lycopenal	Humans	Plasma	0.142 ng/ml
Apo-10'-lycopenal	Humans	Plasma	0.076 ng/ml
Apo-12'-lycopenal	Rats	Liver	≥ 250 ng/g
Apo-12'-lycopenal	Humans	Plasma	0.137 ng/ml
Apo-10'-lycopenol	Ferrets	Lung	2-4 ng/g
Retinal	Rats	Intestine	8 µg/g
All-trans-Retinoic Acid	Humans	Plasma	3.5 ng/ml
All-trans-Retinoic Acid	Rats	Plasma	1.2 ng/ml
β-apo-8'-carotenal	Humans	Plasma	245 ng/ml

Table 1.2: Tissue & serum concentrations of lycopene and β-carotene metabolites, *in vivo* (31, 47-49)

Our lab has identified two apo-lycopenals in the liver of rats fed lycopene-enriched diets (48). For 30-d, male F344 rats consumed a diet of 8 mg / d of lycopene, an amount equivalent to humans consuming 2-3 tomatoes daily (50). A single oral dose of 0.152 mg (6,7,6'7')- ¹⁴C labeled lycopene was administered 24 hours before sacrifice. Apo-8'-lycopenal and putatively apo-12'-lycopenal were identified while many other polar metabolites or oxidative products were observed, but were not identified. Lycopenoic acids may have been present in the more polar fractions which were not further purified. It was estimated that apo-8'-lycopenal was present at levels of approximately two percent of total lycopene in the liver (48). Further analysis of the polar lycopene metabolites may have revealed additional short-chain apo-lycopenals produced in rat liver.

More recently, apo-6'-lycopenal, apo-8'-lycopenal, apo-10'-lycopenal, and apo-12'-lycopenal were identified in human plasma after the consumption of tomato juice for 8 wk (47). Additionally, all of these metabolites were also found in low concentrations in extracts of raw tomato, processed tomato products, and other lycopene containing foods.

Although a number of carotenoid cleavage products have been identified and described, we are only beginning to understand the biological importance of these compounds. It is critical to elucidate the metabolic pathways for the production of carotenoid metabolites and to further clarify both their biodistribution and tissue concentration achieved following tomato carotenoid consumption. Ultimately, it is of great importance to determine the roles of carotenoid metabolites in modifying chronic disease risks.

Lycopene's mechanisms of action

Many mechanisms of action have been suggested for lycopene's reduction of prostate cancer risk. Fourteen *in vitro* studies have reported that lycopene induces apoptosis in various cell lines (51-64). Two human (65, 66) and 6 animal studies (67-72) have also reported apoptosis induction with lycopene treatment. Numerous *in vitro* studies also suggest that lycopene induces normal cell cycle arrest while 2 animal studies report a reduction in cell cycle progression in both oral and prostate cancer models (70, 73).

Gap junction communication (GJC) is essential for cells and is often lost with cancer. GJC is achieved by small molecular signals passing through channels that are formed by gap junction proteins such as connexin 43. One *in vivo* study found a positive association between gap junction communication and connexin 43 expression with lycopene consumption (74). Additionally, a randomized clinical trial of lycopene supplementation reported a near significant increase in connexin 43 protein levels, which suggests an increased gap junction communication

(75). Lycopene or its metabolites may be responsible for this effect. More studies are necessary to elucidate lycopene's mechanisms of action in preventing or inhibiting chronic diseases.

Sex steroids and risk of prostate cancer

The risk of prostate cancer is strongly associated with androgen status (76, 77). Moreover a causal relationship is suggested in a few case reports of prostate cancer patients that previously used androgens as anabolic agents or medical treatment (78, 79). Additionally, administration of testosterone is commonly used to induce prostate cancer in rodent models (80-83). Huggins and Hodges first reported in 1941 that reducing testosterone levels was a potent therapeutic agent for patients with advanced prostate cancer (84).

Normal growth and function of the prostate requires androgens although higher serum levels of androgens, testosterone and dihydrotestosterone (DHT), are associated with increased prostate cancer risk (15, 77). The testes are the primary source of androgens and the major serum androgen is testosterone. Within the prostate, testosterone is converted to the more potent androgen DHT, primarily by 5α -reductase type II (Figure 1.5). DHT is also found in plasma at about 10% of the total plasma testosterone level (85). In androgen synthesis, 17β -HSDs catalyze the conversions between the active 17β -hydroxysteroids and less-active 17β -ketosteroids. 17β -HSD isozymes 1, 3, 5, and 7 convert substrates to more potent androgens, whereas isozymes 2, 4, 8, 10, and 11 create less active substrates and more estrogenic compounds (86). Although 17β -HSD's are also involved in other pathways unrelated to sex steroid metabolism, types 1, 3, and 5 have the greatest impact on sex steroid concentrations (87). Lastly, CYP3A is involved in oxidizing testosterone to some of its less active metabolites in the liver (88).

Some additional factors influence serum testosterone levels. Serum testosterone levels have circadian rhythms with highest levels occurring just after waking and the lowest levels

before going to sleep (89). Testosterone circulates in the blood bound to plasma proteins. Approximately 60-70% of circulating testosterone is bound to steroid hormone binding globulin (SHBG), 30% bound to albumin, and transcortin with 1-2% as free or unbound (90). Free testosterone is the most bioavailable and is commonly what is most commonly measured clinically with a radioimmunoassay. Androgens bind the androgen receptor and induce transcription of genes. DHT has a much higher binding affinity with the androgen receptor compared to testosterone thus making DHT much more efficient at inducing transcription genes (90). Lastly, serum testosterone concentrations may not indicate tissue testosterone concentrations; therefore this discrepancy needs to be considered in future studies (91, 92).

Dietary intervention studies have recently evaluated a possible diet-endocrine interaction in prostate cancer prevention (8, 93-95). In a phase II randomized-controlled trial, it was found that serum free testosterone decreased with lycopene supplementation in men with clinically localized prostate cancer (96).

Previous studies with rats from our laboratory suggest androgen ablation through castration results in altered lycopene metabolism whereby castrated rats accumulated twice as much hepatic lycopene than intact controls even though castrated rats consumed 20% less lycopene (97, 98). We also reported that serum testosterone concentrations were significantly decreased in rats fed lycopene for 4-d (99) although longer term lycopene feeding did not alter serum testosterone or DHT levels (67). Feeding lycopene for 4-d did not alter mRNA expression of prostatic 17 β -HSD or 5 α -reductase II. The mRNA expression of hepatic CYP3A1 was decreased but there was no effect on its activity. Other studies reported reduced mRNA expression of 5 α -reductase in the prostate and prostate tumor when rats were fed lycopene for four or eight wk (93, 94). Additionally, another study found that Japanese males, who are less

likely to develop prostate cancer than Westerners, have reduced circulating levels of 5 α -reductase metabolites (100).

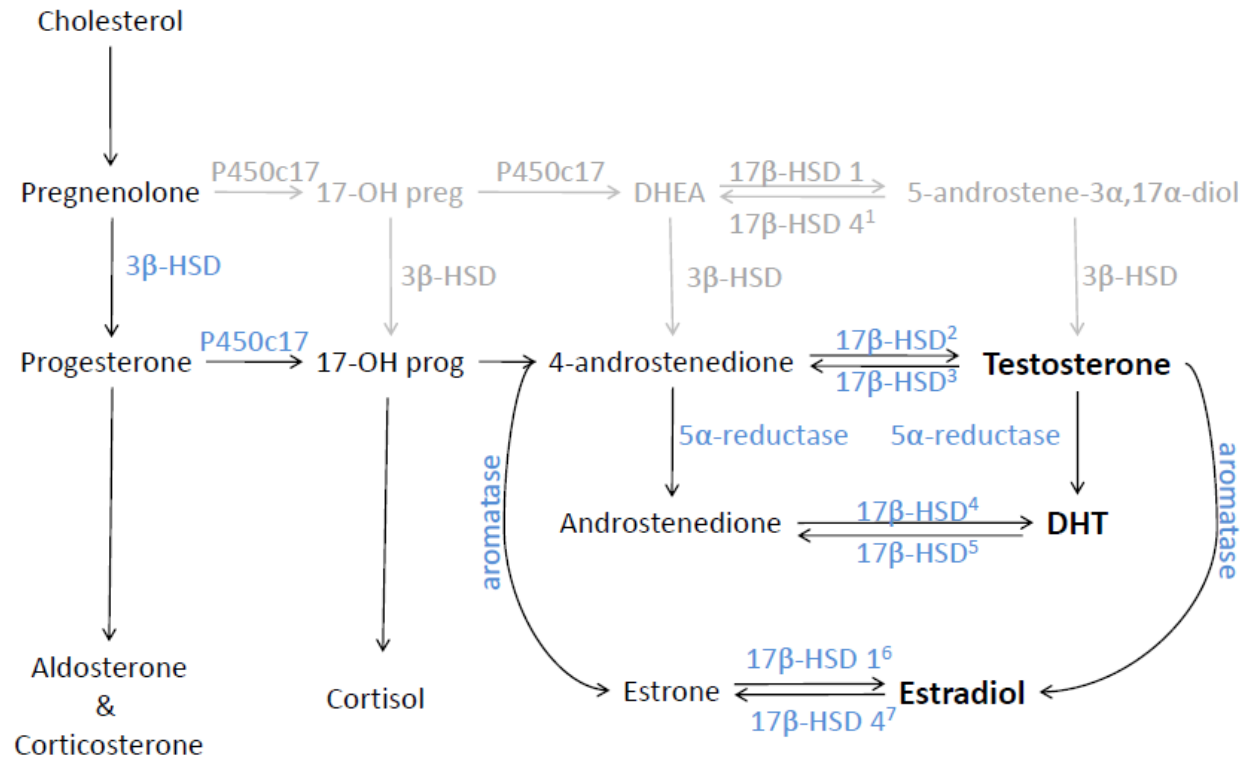


Figure 1.5: Schematic of steroidogenic pathway. The grey text represents enzymes and products that are produced in humans but are minimally produced in rodents. Other isomers of 17 β -hydroxysteroid dehydrogenase acting upon these substrates: ¹isomer 2; ²isomers 3,5; ³isomers 2,10; ⁴isomers 3,5,15; ⁵isomers 2,9,10; ⁶isomers 1,12; ⁷isomers 2,8,14. Adapted from Luu-The, et al (90).

It is important to note that the androgen synthesis pathways in the rodent and human differ. The rodent 17 α -hydroxylase enzyme is more active on 4-ene steroids while the human enzyme specificity prefers 5-ene steroids (Figure 1.5) (101-103). Therefore, rodents do not produce dehydroepiandrosterone (DHEA) but instead produce 4-androstenedione. Additionally, rodents lack expression of the 17 α -hydroxylase enzyme in the adrenal gland, therefore adrenal sources of steroid precursors are not present in rodent serum. These differences must be considered when interpreting results from rodent studies for human application.

Overall, *in vivo* evidence suggests that short-term consumption of lycopene may decrease androgen concentrations or signaling. This may be related to lycopene itself or to lycopene metabolites, but these relationships have not been investigated in depth.

Androgens can be converted to estrogens by the enzyme, aromatase. Although androgen modulation has been the conventional method of prostate cancer treatment, it is apparent in recent literature that estrogens also play a role in prostate cancer development and treatment. Estrogens were classically used to suppress serum testosterone through biofeedback to the hypothalamus (104). Estrogen treatment has proven useful in androgen-resistant tissues suggesting that estrogens must work through a second mechanism (92, 105). This mechanism is most likely regulated by estrogen receptors. ER β is the primary isoform found in the prostate although expression is lost with development of prostate cancer which parallels induced expression of ER α in prostate cancer cells (106). The binding of estrogens to ER α induces proliferation, inflammation, and development of premalignant lesions while binding to ER β reduces proliferation and in general acts as a tumor suppressor (107). Therefore, it has been hypothesized that tissue expression levels of estrogen receptors radically affects growth, differentiation, malignancy and treatment of prostate cancer.

Testosterone levels decrease with age in men while serum estrogens remain relatively constant; therefore it has been hypothesized that the ratio of testosterone to estrogen may be a key regulator of prostate cancer development (92, 104, 105, 108). Moreover, African-American men, who have an increased risk of developing prostate cancer, are exposed to higher circulating estrogen levels in utero compared to Caucasian men. This estrogen exposure may be correlated to increased prostate cancer risk later in life (109-111). Overall, the effects of estrogens in the prostate are complex and warrant further investigation.

To our knowledge, no studies have investigated the effects of lycopene on estrogens in prostate cancer cells, *in vivo* or *in vitro*. However, a couple studies have investigated this relationship with breast cancer incidence. Within the Women's Health Initiative Observational Study, women had a reduced risk of developing ER- and progesterone-receptor positive breast cancers with higher intakes of lycopene or β -carotene (112). This inverse association was not found with other types of breast cancers suggesting that lycopene and β -carotene may inhibit breast cancer development through the estrogen and progesterone receptors. An *in vitro* study found that lycopene, phytoene, phytofluene, and β -carotene inhibited estrogen signaling and reduced proliferation of hormone-dependent MCF-7 and T47D breast cancer and ECC-1 endometrial cancer cells (113).

A number of anti-androgen drugs (including finasteride, dutasteride) or anti-estrogenic drugs (tamoxifen) have been utilized to reduce steroid-induced cancers but unfortunately adverse events are often seen with these regimens. Preliminary evidence suggests that lycopene influences androgen and estrogen signaling in cancer cells. Whether through intact lycopene or its metabolites, dietary intervention could provide a more sustainable, cost effective treatment with fewer side effects.

Mouse models

The CMO-I KO mouse was created by researchers at DSM Nutritional Products (Switzerland) and at the University of Freiburg (Germany) through a targeted deletion of the CMO-I gene in mice. We confirmed this knock-out in the liver of CMO-I KO mice. Only two papers have been published using CMO-I KO mice and therefore limited information is known about this model (114, 115).

It is known that CMO-I KO mice have altered lipid metabolism. CMO-I KO mice were reported to develop hepatic liver droplets at 3 months of age while much larger and wide spread lipid droplets were identified at 12 months of age (114). Collagen deposition was unaltered and therefore eliminated the possibility of hepatic fibrosis. At 25 wk of age, CMO-I KO mice had significantly increased hepatic triglycerides. The mRNA expression of hepatic fatty acid oxidase I (ACOX1) and stearoyl-CoA-deaturase (SCD1) was significantly increased while there was no effect on the expression of hepatic peroxisome proliferator-activated receptor (PPAR- α), Sterol Regulatory Element Binding Protein (SREBP-1c), fatty acid synthase (FAS) or retinol binding protein (RBP4). Within visceral adipose, the expression of PPAR- γ target genes, fatty acid binding protein (FABP4) and scavenger receptor (CD36), were significantly increased. Additionally, serum non-esterified fatty acids were significantly increased. Further analysis reported no alterations in serum glucose concentrations or tolerance in CMO-I KO mice. Lastly, glutamate pyruvate transaminase expression was significantly increased, which suggests liver injury (114).

Hessel, et al. reported that CMO-I mice are prone to obesity (114). Twenty wk old female CMO-I KO mice were fed an obesogenic diet (30% soybean oil) which resulted in significantly increased serum cholesterol esters compared to control mice. Additionally, increased hepatic lipid droplets were reported with an increase in serum free fatty acids.

We reported an alteration in body and organ weight and alterations in lycopene bioaccumulation in CMO-I KO mice. Overall, at 22 wk of age, body weight was significantly increased in CMO-I KO mice compared to age-matched wild-type mice. Additionally, females had a significantly increased uterus weight (as a percent of body weight) compared to control mice (115). Furthermore, lycopene concentration was reduced in the liver, spleen, and thymus of

CMO-I KO mice fed lycopene-containing diets compared to wild-type mice. Interestingly, lycopene concentration was increased in the prostate, seminal vesicles, testes, and brain of CMO-I KO mice compared to wild-type control mice. Additionally, the percent of *cis* lycopene isomers was greatly increased in most tissues (excluding serum and testes) of CMO-I KO mice compared to wild-type mice.

The CMO-II KO mouse was created by DSM and the University of Freiburg (Germany) through a targeted deletion of the CMO-II gene in mice (Amengual, et al., submitted). We confirmed this knock-out in the liver of CMO-II KO mice. A recently submitted paper by Dr. von Lintig's group at Case Western Reserve University suggests that 3-hydroxylated carotenoids impair mitochondrial function and induce oxidative stress in CMO-II KO mice (Amengual, et al., submitted). With consumption of lutein or zeaxanthin, CMO-II KO mice developed liver steatosis with increased triacylglycerides, induction of PPAR- γ , and reduction in the expression of PPAR- α . Mitochondrial respiration was reduced in CMO-II KO mice consuming these carotenoids and manganese superoxide dismutase, an indicator of mitochondrial dysfunction, was induced in hepatic mitochondria. Additionally, carotenoid consumption induced stress pathways (HIF-1 α , p-AKT, p-MAPK) through radical oxygen species production. Impaired mitochondria and induction of oxidative stress in CMO-II KO mice was caused by 3-dehydro lutein or zeaxanthin, but not from the parent compounds alone. This suggests that CMO-II may play a role in protecting the mitochondria from carotenoid oxidation products.

The transgenic adenocarcinoma of the mouse prostate (TRAMP) mouse model has been used extensively in prostate cancer and dietary intervention or drug therapy studies (116-121). The TRAMP model does not require external carcinogens as it relies on an androgen-driven and prostate specific expression of the large and small T antigen PB-SV40 genes (122). The large T

antigen abolishes p53 and Rb function while the small T antigen inhibits PP2A function (123). Although this system is driven by a viral oncogene, typical prostate cancer expression of IGF-1 is observed (124, 125). The TRAMP model is initially androgen-sensitive and then develops androgen-insensitivity which is commonly seen in human prostate cancer (126, 127). The pathobiology of the TRAMP model has also been reviewed in depth (Figure 1.6) (128).

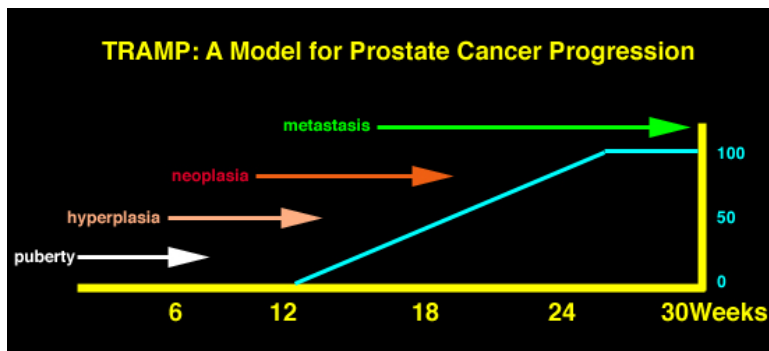


Figure 1.6: TRAMP model prostate cancer progression. (<http://thegreenberglab.fhrc.org/index.html>)

Aims of dissertation

The metabolism of β -carotene is widely studied, but very little is known about the metabolism of other tomato carotenoids including lycopene, phytofluene, and phytoene. Through the use of novel mouse knock-out models, we investigated carotenoid metabolism, *in vivo* and its effects on a risk factor for prostate cancer, androgen status. The effects of lycopene metabolites on prostate cancer proliferation were investigated, *in vitro*.

The specific aims of this thesis were to:

1. Evaluate the expression of carotenoid cleavage enzymes and resulting carotenoid accumulation in CMO-I KO, CMO-II KO, and wild-type mice.
2. Evaluate the effects of lycopene and its metabolites, apo-8'-lycopenal and apo-12'-lycopenal on the proliferation of DU145 prostate cancer cells.

3. Evaluate the effects of feeding lycopene or tomato powder on androgen status in mice lacking of the carotenoid cleavage enzymes.

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CHAPTER II

TOMATO CAROTENOID METABOLISM IN GENETICALLY DEFINED MICE

DEVOID OF CMO-I OR CMO-II

ABSTRACT

Two enzymes have been implicated in oxidative metabolism of carotenoids. Carotene-15,15'-monooxygenase (CMO-I) centrally cleaves β,β -carotene to form vitamin A. We hypothesize that carotene-9',10'-monooxygenase (CMO-II) plays a key role in metabolism of non-provitamin A carotenoids, such as lycopene. We investigated carotenoid bioaccumulation in young adult, male wild-type (WT) mice or mice lacking either CMO-I or CMO-II. In study 1, mice consumed an AIN-93G diet or identical diets supplemented with 10% tomato powder, 130 mg lycopene / kg diet (DSM, 10% lycopene beadlets) or placebo beadlets for 30-d. Lycopene preferentially accumulated in CMO-II KO mouse tissues while β -carotene primarily accumulated in CMO-I KO mouse tissues. In this study, phytofluene and phytoene did not preferentially accumulate in tissues of the genotypes investigated. Relative tissue mRNA expression of CMO-I and CMO-II in response to diet and/or genotype was evaluated. CMO-II, but not CMO-I, was expressed in the prostate. The expression of testicular CMO-II was significantly greater in CMO-I KO mice compared to WT. A longer (60-d) feeding study evaluating relative carotenoid uptake in CMO-II KO and WT mice confirmed enhanced lycopene, but not phytofluene or phytoene, bioaccumulation in most CMO-II KO mouse tissues. In conclusion, CMO-II, but not CMO-I, is expressed in the prostate and CMO-II appears to specifically metabolize lycopene, but not the more saturated carotenes, phytofluene and phytoene.

INTRODUCTION

Recent landmark discoveries have established that the carotenoid, β -carotene, is metabolized by carotene-15,15'-monooxygenase (CMO-I) through central chain cleavage to form vitamin A with subsequent metabolism resulting in the formation of retinoic acid and other retinoids (1). The metabolism of β -carotene is widely studied, yet very little is known about the metabolism of many other carotenoids and the biological significance of carotenoid metabolites. Lycopene is the red pigment and predominant carotenoid in tomato products followed by phytoene, phytofluene, and β -carotene. Phytofluene and phytoene are precursors to lycopene in the plant biosynthetic pathway and are desaturated to form lycopene. Accumulating studies from human epidemiology (2, 3), clinical studies (4-6), rodent studies (7, 8), and cells in culture have suggested that tomato carotenoids may have health promoting properties for a variety of acute and chronic disease processes.

Our work (9) and that of others (10) has identified several metabolites and oxidative products of lycopene in rodent and human plasma. Interestingly, levels of lycopene metabolites identified *in vivo* are of comparable biological concentrations to retinoids suggesting that they may exert physiological functions (11, 12). Studies with CMO-I knockout mice show an alteration in lycopene metabolism and tissue accumulation (11) and, *in vitro* evidence suggests that lycopene is metabolized primarily by carotene-9',10'-monooxygenase (CMO-II), the enzyme resulting in eccentric chain cleavage of carotenoids (13). However, at this stage, the metabolic production of apo-carotenals and their biological impact is poorly understood.

We investigated the metabolism of lycopene and other tomato carotenoids in mice with normal genotype (wild type) (WT) and those devoid of CMO-I or CMO-II derived by knock-out (KO) technology. In our first study, we evaluated tissue carotenoid accumulation and relative

tissue expression of CMO-I and CMO-II genes in male WT and CMO-I KO, and CMO-II KO mice fed diets containing tomato powder or lycopene beadlets for 30-d. In a second 60-d study, we compared tissue carotenoid bioaccumulation in male and female CMO-II KO and WT mice fed tomato powder.

MATERIALS & METHODS

Mice and Diet.

In study 1, C57BL/6 x 129/SvJ (F1) mice (WT, n=40) were purchased from Jackson Laboratory (Bar Harbor, ME). Breeder pairs of *Bcmo1tm1Dnp* (CMO-I KO, n=4) and *B6;129S6-Bcdo2tm1Dnp* (CMO-II KO, n=4) mice were shipped to the University of Illinois courtesy of collaborators, Drs. Adrian Wyss at DSM Nutritional Products (Basel, Switzerland) and Johannes von Lintig at Case Western Reserve University. The generation of CMO-I KO mice was previously described (1). The generation of CMO-II KO mice is described by Amengual, et al. (submitted). For this study, CMO-I KO and CMO-II KO mice were bred at the University of Illinois animal facilities. We confirmed the genotype of these models using genomic DNA from mouse tail biopsies and the Sigma Extract-N-Amp Tissue PCR Kit (St. Louis, MO). Mice were housed in shoebox cages with free access to water and weighed every other day. Six to nine wk old male mice consumed an AIN-93G diet for 3 wk prior to randomization onto experimental diets. This study was conducted in 2 cohorts, one month apart.

The tomato powder diet contained 204 nmol lycopene/g diet while the lycopene beadlet diet provided 248 nmol lycopene/g diet. The tomato powder diet also contained 10.1 nmol phytoene, 2.6 nmol phytofluene, 1.3 nmol β -cryptoxanthin, and 0.8 nmol β -carotene/g diet. All diets were balanced for total fiber, nitrogen, and calories (Table 2.1). Additionally, vitamin A levels were reduced in all diets to 1500 IU retinyl palmitate per kg diet to ensure adequate

absorption of carotenoids without resulting in a vitamin A deficiency (11). Fresh diet was provided every 48 hours and new diet was made monthly. The diet was stored in the dark at 4°C.

Nine- to twelve-wk old male mice were randomly assigned to one of four experimental diets; AIN-93G (AIN), 10% tomato powder (Futureceuticals) (TP), lycopene (DSM, 10% lycopene beadlet) (LYC), or placebo (DSM, 10% beadlet) (PB); n=10 per genotype/diet. Mice consumed the experimental diets for 30-d, ad libitum. At the conclusion of the study, mice were asphyxiated by CO₂ and blood was collected by cardiac puncture. Brain, thymus, lungs, liver, spleen, duodenum mucosa, kidneys, adrenals, seminal vesicles, testes, and prostate were removed and snap-frozen in liquid nitrogen and then stored at -80°C.

In study 2, 23-27 wk old male CMO-II KO (n= 11) and C57BL/6 (WT) mice (Harlan, Indianapolis, IN) (n=14) and female CMO-II KO (n= 8) and C57BL/6 (WT) (n=13) were fed a 10% tomato powder diet (Gilroy Foods, Gilroy, CA) for 60-d, ad libitum (Table 2.1). Vitamin A levels were again reduced in all diets to 1500 IU retinyl palmitate per kg diet. In contrast to study 1, the tomato powder diet was supplemented with β-carotene (beadlets, DSM) and the AIN-93G diet was supplemented with placebo beadlets (DSM). A different source of tomato powder was used in study 2 resulting in the tomato powder diet containing different amounts and proportions of carotenoids (17.5 nmol lycopene, 29.3 nmol phytofluene, 33.5 nmol phytoene, and 28.7 nmol β-carotene). All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

Carotenoid extraction and HPLC analysis.

All chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Tissue and serum samples were extracted with hexanes as described previously with minor modifications (14). Briefly, 0.08g tissue (0.15g liver was used for β-carotene analysis in study 1) was added to 5 ml

of ethanol containing 0.01% butylated hydroxytoluene (BHT). Saturated KOH was added and the samples were vortexed. The samples were saponified in a waterbath at 60°C for 30 minutes with vortexing every 10 minutes. Distilled water (2 ml) was added and carotenoids were extracted three times with 6 ml of hexanes. Serum carotenoids were extracted from 0.15 ml serum in 0.15 ml ethanol/BHT solution with 0.15 ml distilled water and 1.5 ml hexanes.

All extracts were kept on ice under yellow lights and flushed with argon to prevent degradation. For analysis, samples were dissolved in methyl-*tert*-butyl ether and injected onto a C30 HPLC column (4.6 x 150 mm, 3µm; YMC, Wilmington, NC) maintained at 18°C. A reverse-phase HPLC-PDA method was utilized (9, 15). Individual carotenoids were identified by comparison of elution times to standards and absorption spectral analysis.

Real-time PCR analysis.

In study 1, relative tissue expression of CMO-I and CMO-II mRNA was evaluated using a Trizol (Invitrogen, Carlsbad, CA) extraction as per manufacturer's instructions followed by Dnase I treatment with the following modifications (New England Biolab, Ipswich, MA). Prostate tissue mRNA was extracted with 2 ml Trizol and testes tissue mRNA was extracted using 1.5 ml Trizol. mRNA concentration and quality were determined by spectrophotometry and agarose gel electrophoresis before synthesis of complimentary DNA by Superscript II Reverse Transcriptase (Invitrogen) using random hexamers (Applied Biosystems, Foster City, CA). Primer pairs were selected to measure CMO-I (NM_021486): Forward-5'-ATGGAGATAATATTTGGCCAG and Reverse-5'-AACTCAGACACCACGATTC; and CMO-II (NM_1332217): Forward-5'-GTTATCTACTTCGAGTTGGACCTGG and Reverse-5'-AAGCAACGCCATTCCATCA. Real-time PCR was performed using a 7900HT Fast Real-Time

PCR detection system (Applied Biosystems) with the SYBR green fluorescence dye. Threshold cycle (C_t) values for the 18S gene were used as a control.

Statistical analysis.

In study 1, a 3 x 4 factorial design was used with three genotypes (CMO-I KO, CMO-II KO, or WT) and four diets (AIN, TP, LYC, or PB). Interactions between genotype and diet were investigated. All parameters were analyzed by analysis of variance (2-way ANOVA) followed by Tukey's post hoc analysis using SAS 9.1 (Cary, NC) with $\alpha = 0.05$. The body weight analysis model blocked for age. When assumptions for ANOVA were violated, data were transformed by natural log. In study 2, a 2 x 2 factorial design was used with two genotypes (CMO-II KO or WT) and two sexes. Body and organ weights and carotenoid accumulation were analyzed using multiple linear regression. Dummy coding was used for variables and violations of model assumptions were corrected for using natural log transformation. Results were expressed as means \pm SEM, unless otherwise specified.

RESULTS

Body and organ weight: Study 1.

Upon enrollment, WT mice were approximately 1 wk older than CMO-I KO and CMO-II KO mice; therefore, age was blocked in the final ANOVA model analysis. Diet had no effect on body weight, growth, or organ weight (data not shown). Body weight and change in body weight over the study duration was not significantly impacted by genotype (Table 2.2). However, CMO-II KO mice had significantly heavier spleens as a percent of body weight. CMO-II KO mice also had significantly smaller testes and seminal vesicles while CMO-I KO mice had smaller seminal vesicles compared to wild-type animals. No other changes in tissue weight or body weight were noted (data not shown).

Body and organ weight: Study 2.

Final body weights of CMO-II KO mice were non-significantly reduced compared to WT mice. Additionally, some differences in organ weights of CMO-II KO mice were noted (data not shown). The weights of the lungs ($p=0.035$) and testes ($p=0.0003$) were increased in male CMO-II KO mice; however the seminal vesicle ($p=0.034$) weight was reduced in CMO-II KO mice compared to WT mice. Male and female CMO-II KO mice had reduced liver weight as a percent of total body weight ($p=0.003$). Other tissue weights, as a percent of total body weight, did not differ by sex or genotype.

Tissue and serum carotenoids: Study 1.

Carotenoids were not detected in tissues of mice consuming diets not supplemented with carotenoids (data not shown). CMO-II KO mice accumulated substantially higher amounts of lycopene in the liver (5-fold in tomato fed and 3-fold in lycopene fed) compared to WT mice. Higher levels of lycopene were found in the testes of both CMO-I KO and CMO-II KO mice (Table 2.3). Serum lycopene concentrations were higher for CMO-I KO and CMO-II KO mice compared to WT (Table 2.3). Overall, there was a tendency of WT and CMO-II KO mice that consumed the TP diet to have significantly higher serum and testicular lycopene concentrations compared to mice that consumed the LYC diet.

Levels of β -carotene in TP were quite low and resulted in marginally detectable levels in WT mouse tissues and serum (Table 2.4). CMO-I KO mice accumulated greater concentrations of β -carotene in tissues and serum compared to WT mice. CMO-II KO mice also accumulated more β -carotene in the liver and testes compared to WT mice, but less than CMO-I KO mice.

After 30-d of feeding tomato powder, phytofluene and phytoene were not detected in serum or most tissues; although the liver accumulated detectable levels, concentrations did not differ between genotypes (Table 2.5).

Tissue and serum carotenoids: Study 2.

Carotenoid biodistribution did not differ by sex (data not shown). CMO-II KO mouse tissues accumulated higher amounts of lycopene compared to WT mice, with the exception of the liver, spleen, and seminal vesicles (Table 2.6). Interestingly, compared to WT mice, CMO-II KO mice accumulated higher amounts of β -carotene in the liver, adrenal, kidney, and prostate but reduced accumulation in the lungs (Table 2.7). CMO-II KO mice in study 2 accumulated 5 times more hepatic β -carotene, 10 times more testes β -carotene, and 90 times more serum β -carotene compared to study 1 due primarily to the addition of β -carotene to the TP diet in study 2. Like study 1, phytofluene and phytoene were quantified in the liver but did not accumulate in detectable concentrations in other tissues (Table 2.5). Hepatic accumulation of phytofluene and phytoene did not differ by genotype.

Lycopene isomers: Study 1.

The percentage of lycopene as *cis* isomers was altered in tissues of CMO-I KO and CMO-II KO mice fed lycopene containing diets (TP and LYC) for 30-d. CMO-II KO mice had significantly less hepatic 13-*cis* lycopene and 9-*cis* lycopene compared to WT mice as a percent of total lycopene (Table 2.8). Additionally, CMO-II KO mice accumulated less 5-*cis* lycopene and more all-*trans* lycopene in the testes compared to WT mice.

mRNA Expression of CMO-I and CMO-II.

Of the four tissues analyzed from mice in study1, the liver had the highest relative mRNA expression of CMO-I while the prostate had no detectable expression of CMO-I (Figure 2.1).

CMO-II was most highly expressed in the mouse duodenum followed by the liver, prostate, and testes. There was no substantial impact of the diet on the expression of CMO-I or CMO-II in these tissues. A significant interaction was identified between genotype and diet (AIN, TP) for CMO-I and CMO-II expression in the liver. The expression of testicular CMO-II was significantly greater in CMO-I KO mice compared to WT mice consuming any of the four diets while the expression of CMO-I in the testes was significantly elevated only in CMO-II KO mice consuming the PB diet. In contrast, CMO-II expression was significantly reduced in the prostate of CMO-I KO mice compared to WT mice (LYC, TP, AIN).

DISCUSSION

Two separate feeding studies were designed to investigate carotenoid tissue bioaccumulation and the expression of carotenoid cleavage enzymes in transgenic mice lacking the enzymes CMO-I or CMO-II. Wild-type and transgenic mice consumed carotenoid containing diets or their respective controls for 30 or 60-d. We hypothesized that CMO-I and CMO-II are responsible for production of tomato carotenoid metabolites in animals.

In study 1, the lycopene content in the two experimental diets was comparable (204 nmol for the TP vs 247 nmol for the LYC / g) although the tomato powder diet also contained other carotenoids, especially phytofluene and phytoene. In study 2, but not study 1, β -carotene was added to the tomato powder diet for a final concentration of 28.7 nmol. The tomato powder diet in study 2 contained much less lycopene (17.5 nmol). The level of tomato powder for these studies was selected to result in hepatic lycopene concentrations similar to that found in humans (14, 16).

Given that these mouse models are new, we measured body weight, change in body weight, and organ weights in young, adult male mice (Table 2.2). We noted no differences in

body weight or change in body weight by genotype or diet; however, as a percentage of body weight, CMO-II KO mice had significantly enlarged spleens but smaller testes and seminal vesicles. CMO-I KO mice had smaller seminal vesicles as a percentage of total body weight compared to wild-type mice. The weights of the brain, lungs, liver, and kidneys did not differ (as a percentage of body weight). We previously reported CMO-I KO mice are significantly heavier than WT mice with resulting alterations in hepatic and serum lipids (1, 11). The mice in our study were young, sexually mature males while the earlier studies were conducted in 22-30 wk old mice. The age of the mice may be a critical factor distinguishing alterations in total body weight between these two studies. In study 2, we again found that seminal vesicle weight was reduced in CMO-II KO mice compared to WT mice. In contrast to study 1, CMO-II KO mouse testes were significantly larger than WT mouse testes in study 2. Furthermore, in study 2 we did not find enlarged spleens in CMO-II KO mice although we report liver weight, as a percentage of body weight, was reduced compared to WT mice. Further phenotypic characterization is necessary to clarify the impact of age and sex on these phenotypes.

CMO-II KO mice had significantly higher tissue and serum concentrations of lycopene compared to WT mice in the 30-d study (Table 2.3). The liver, which accumulates substantial quantities of lycopene compared to other tissues (7, 14, 16-18), had 3-5 times more lycopene in CMO-II KO mice compared to WT mice. Interestingly, CMO-I KO mice also accumulated somewhat greater concentrations of lycopene in the serum and testes compared to WT. Increased lycopene bioaccumulation in CMO-I KO mice is not explained by alterations in the expression of CMO-II in these mice (Figure 2.1). Instead, other enzymes may be partially responsible for lycopene biodistribution.

In study 1, WT and CMO-II KO mice that consumed the TP diet had higher circulating lycopene levels than mice that consumed the LYC diet (Table 2.3). Moreover, WT mice that consumed the TP diet also accumulated more lycopene in the testes compared to mice that consumed the LYC diet. We have reported similar findings previously in rats fed lycopene-containing diets (7).

In our second study, CMO-II KO mice fed tomato powder for 60-d also preferentially accumulated lycopene in tissues compared to WT mice, with exceptions of the liver, spleen, and seminal vesicles (Table 2.6). The apparent inconsistency between lycopene accumulation in some organs in the 30-d feeding study and the 60-d feeding study may be explained by the choice of mouse genotype for comparison. In study 1, a mixed background WT mouse was used for comparison but in study 2, inbred C57BL/6 mice were used as WT mice. The apparent inconsistencies between these studies may also be explained by variation in lycopene content or total carotenoid content in the diet. Regulation of intestinal absorption may also play a role. In the 60-d study, the diet was supplemented with additional β -carotene. Beta-carotene supplementation can result in retinoic acid production that down-regulates SR-B1 in the intestine (19). SR-B1 is also partially responsible for lycopene uptake and circulating lycopene has been shown to reduce the expression of SR-B1 (20, 21).

Data from study 1 confirmed increased accumulation of β -carotene in CMO-I KO mouse tissues and serum compared to other genotypes, supporting the supposition that CMO-I is the primary cleavage enzyme for β -carotene (1, 11). We previously reported vitamin A production from β -carotene was abolished in CMO-I KO mice (1, 11). Furthermore, Paik, et al. demonstrated recombinant murine CMO-I efficiently converted β -carotene to vitamin A (22). Although not as dramatic an increase in β -carotene accumulation observed in CMO-I KO mice,

CMO-II KO mice also preferentially accumulated β -carotene in tissues compared to WT mice (Table 2.4 & 2.7). Greater accumulation of β -carotene in CMO-II KO mice is not explained by alterations in the expression of CMO-I (Figure 2.1). These data suggest that under some conditions, β -carotene may be a substrate for CMO-II cleavage, but accumulating evidence suggests that the primary cleavage enzyme for β -carotene is CMO-I. Further research is necessary to clarify the differential carotenoid substrate specificity and binding affinities for CMO-II.

CMO-II is hypothesized to cleave carotenoids at the 9',10' bond. Lycopene, phytofluene, and phytoene all have the requisite 9',10' double bond. In these studies, phytofluene and phytoene only accumulated in detectable quantities in the liver, but hepatic concentrations did not differ between genotypes in either the 30-d or 60-d feeding studies (Table 2.5). These data suggest that lycopene is a substrate for CMO-II while apparently phytofluene and phytoene are not. This important finding suggests that despite almost identical structures, the reduced number of conjugated double bonds of phytofluene (5) and phytoene (3) compared to lycopene (11) may affect the affinity of the carotenoid for the CMO-II enzyme. Furthermore, carotenoid cleavage enzymes are highly evolutionarily conserved. Carotenoid cleavage dioxygenase (CCD1) and CMO-II are eccentric cleavage enzymes for plants and animals, respectively, and apparently, phytoene is also not a substrate for CCD1 in plants (23). However, we speculate that other carotenoids with a 9',10' bond may be substrates for CMO-II.

We investigated the bioaccumulation of lycopene isomers in the tissues and serum of CMO-I KO, CMO-II KO, and WT mice. Although foods contain a relatively low percentage of *cis*-lycopene, previous studies demonstrated that human and ferret tissues preferentially accumulated *cis* lycopene rather than all-*trans* lycopene (24, 25). In studies 1 and 2, most of the

serum and tissue lycopene was found in the *cis* isomer form. Moreover, the percentage of *cis* lycopene isomers was significantly altered in CMO-I KO and CMO-II KO mice in study 1, but not study 2 (data not shown). It is reported that *cis* lycopene, rather than all-*trans* lycopene, is a preferred substrate for CMO-II cleavage, *in vitro* (10). Therefore, we might have expected to find more *cis* lycopene in CMO-II KO mice than in WT mice. Our data is contrary to this idea. In fact, we generally found that CMO-II KO mice have reduced *cis*-lycopene isomers in tissues compared to WT mice.

Previously, we reported CMO-I KO mice accumulate more *cis*-lycopene isomers in tissues (11). While inconsistent with the results presented here, the study design differed considerably. The previous study fed ~22 wk old mice a high fat (16.7%) diet for 60-d. Consuming a high fat diet would be expected to increase lipid oxidation which may have altered the *cis/trans* lycopene isomer profiles in these mice. Overall, these data suggest that lycopene isomerization is altered or preferential uptake of lycopene isomers occurs in carotenoid cleavage knock-out mice.

In study 1, we investigated the expression of CMO-I and CMO-II in response to altered genotypes (CMO-I KO and CMO-II KO mice) and consumption of carotenoid containing diets (LYC and TP). Of the tissues analyzed for carotenoid cleavage enzyme mRNA expression, the liver had the highest relative mRNA expression of CMO-I while CMO-II was most highly expressed in the duodenum (Figure 2.1). Previously, we reported in rats that the relative mRNA expression of CMO-I was greatest in the small intestine followed by the liver, testes, and prostate (26). Moreover, expression of CMO-II was greatest in ventral prostate followed by the liver, testes, and small intestine in rats (26). Our current relative mRNA expression data are in line with that of two previous studies in mice and one in humans (1, 27-29).

Notably, CMO-I expression was not detected in the prostate in these mouse models (any genotype). CMO-II was expressed in the prostate of CMO-I KO mice although its expression was reduced compared to WT mice. These data may imply that retinoids may not be locally produced in the prostate of young, adult, male mice, while lycopene metabolites may be produced through CMO-II cleavage. The expression of testicular CMO-II was significantly increased in CMO-I KO mice while the expression of CMO-I in the testes was significantly elevated in CMO-II KO mice compared to WT mice. This might suggest a compensatory regulation of expression for these enzymes in the testes. Clearly, further work regarding relative tissue expression, protein levels, and enzyme activity of carotenoid cleavage enzymes is warranted.

Although there was no major impact of the diets alone on the transcript of CMO-I or CMO-II in these tissues, a significant interaction was identified between genotype and diet (AIN, TP) for hepatic CMO-I and CMO-II expression. Specifically, the expression of CMO-I was decreased in CMO-II KO mice that consumed the TP diet (compared to the AIN diet) while the expression of CMO-II was slightly greater in WT mice that consumed the TP diet. Furthermore, the expression of CMO-II was increased in CMO-I KO mice that consumed the TP diet while the expression of CMO-II slightly decreased in WT mice that consumed the TP diet. These interactions may affect the efficacy of tomato product consumption for improving health outcomes.

Recently, two common single nucleotide polymorphisms (SNPs) in the human CMO-I gene were identified and found to reduce β -carotene metabolism by 32-69% (30). Importantly, these variant allele frequencies are found at up to 42% of the study population. Additionally, one case study noted a loss of function mutation in the human CMO-I gene (31). SNPs for the CMO-

II gene have not yet been investigated although mutations in the ovine, bovine and chicken CMO-II gene have been identified (32-34). Therefore, our results in CMO KO mice may parallel humans with SNPs or mutations in CMO-I and CMO-II whereby each species might respond to the reduced activity of one carotenoid cleavage enzyme with alterations in other protein/enzymes.

The inverse relationship between prostate cancer incidence and tomato product consumption (2, 35-37) has often been attributed to lycopene. It is unknown whether lycopene, and/or other carotenoids or tomato components, or their metabolites primarily affect prostate cancer risk. We propose that lycopene metabolites, produced by carotenoid cleavage enzymes, are partially responsible for this effect (37).

The findings in these studies support the hypothesis that lycopene, but not phytofluene or phytoene, is metabolized by the CMO-II enzyme. Both studies further demonstrate that the primary metabolism of β -carotene is by central cleavage via CMO-I. We also provide evidence that CMO-I KO and CMO-II KO mice have unique phenotypes. Interestingly we showed that the prostate lacks expression of CMO-I in all mouse genotypes studied. We also found differential tissue expression of CMO-I and CMO-II in mouse tissues. A separate publication will present additional information regarding the generation of CMO-II KO mice and the impact of CMO-II on the metabolism of other carotenoids (Amengual et al., submitted). Further characterization of these models is essential and could provide valuable insight into carotenoid metabolism and the impact of carotenoids on health outcomes.

Table 2.1. Composition of diets (g/kg diet) employed in studies of lycopene metabolism in CMO-I and CMO-II mutant mice.

	AIN-93G ¹	10% Tomato Powder ^{2,3}	Placebo Beadlet ⁴	Lycopene Beadlet ⁵
Ingredient	(AIN)	(TP)	(PB)	(LYC)
Cornstarch	398	331	398	398
Casein	200	188	200	200
Maltodextrin	102	132	102	102
Sucrose	100	100	100	100
Non-nutritive cellulose	50	34	50	50
Mineral Mix ⁶	35	35	35	35
Vitamin mix ⁷	10	10	10	10
L-cystine	3	3	3	3
Choline bitartrate	2.5	2.5	2.5	2.5
Soybean oil	100	65	100	100
Tomato powder	0	100	0	0
10% lycopene beadlets	0	0	0	0.1
Placebo beadlets	0	0	0.1	0

¹In study 2, the AIN diet was supplemented with (DSM) water-soluble placebo beadlets.

²In study 2, the tomato powder was supplemented with (DSM) water-soluble beadlets to a final dietary concentration of 28.7 ± 2.7 nmol β -carotene.

³As measured by HPLC; the tomato powder (FutureCeuticals, Momence, IL) diet from study 1 contained 10.1 ± 0.8 nmol phytoene, 2.6 ± 0.5 nmol phytofluene, 1.5 ± 0.3 nmol β -cryptoxanthin, 0.8 ± 0.05 nmol β -carotene, and 204 ± 30 nmol lycopene per gram diet. The tomato powder (Gilroy, CA) diet from study 2 contained 33.5 ± 5.6 nmol phytoene, 29.3 ± 3.4 nmol phytofluene, and 175 ± 13 nmol lycopene.

⁴As measured by HPLC; the placebo beadlet (DSM) diet provided 0 nmol carotenoids per gram diet.

⁵As measured by HPLC; the lycopene beadlet (10% DSM) diet provided 248 ± 58 nmol lycopene per gram diet.

⁶AIN93M-MX formulation

⁷AIN93-VX formulation with reduced vitamin A content (1500 IU retinyl palmitate / kg)

Table 2.2. Body (g) and organ weight (% of total body weight) in CMO-I and CMO-II mutant mice after 30-d of feeding (study 1)¹

Weight	Genotype		
	CMO-I KO	CMO-II KO	Wild-type
Final body weight ²	30.9 ± 0.62	29.9 ± 0.51	33.3 ± 0.68
Liver	3.72 ± 0.10	3.71 ± 0.15	3.58 ± 0.03
Spleen	0.24 ± 0.01	0.31 ± 0.01*	0.23 ± 0.00
		(p<0.0001)	
Testes	0.70 ± 0.02	0.63 ± 0.01*	0.72 ± 0.01
		(p<0.0001)	
Seminal vesicles	0.76 ± 0.02*	0.75 ± 0.02*	0.86 ± 0.01
	(p=0.0007)	(p=0.0001)	

¹Means ± SEM; n=40

²Mice were 12-15 wk of age at termination.

* Asterisks indicate means within a row differ from WT.

Table 2.3. Tissue lycopene concentrations (nmol / gram wet weight) by dietary intervention in CMO-I KO, CMO-II KO, and WT mice (study 1)¹

Genotype	10% tomato powder diet			Lycopene diet		
	CMO-I KO	CMO-II KO	Wild-type	CMO-I KO	CMO-II KO	Wild-type
Liver	6.28 ± 2.46	38.1 ± 12.5*	7.64 ± 2.84	4.13 ± 0.82	37.0 ± 6.12*	12.2 ± 2.90
		(p<0.0001)			(p<0.0001)	
Testes	1.29 ± 0.18*	1.76 ± 0.41*	0.74 ± 0.16	1.60 ± 0.31*	1.06 ± 0.13*	0.51 ± 0.05#
	(p=0.0004)	(p<0.0001)		(p<0.0001)	(p=0.005)	
Serum	0.90 ± 0.13*	0.89 ± 0.12*	0.63 ± 0.10	1.17 ± 0.24*	0.46 ± 0.05*,#	0.22 ± 0.04#
	(p=0.03)	(p=0.04)		(p<0.0001)	(p=0.003)	

¹Values are means ± SEM; n=6

* Asterisks indicate means within a row differ from WT.

Pound sign indicates significantly different from 10% tomato powder diet of corresponding genotype, p<0.05.

Table 2.4. Tissue β -carotene concentrations (nmol / gram wet weight) of CMO-I KO, CMO-II KO, and WT mice that consumed a 10% tomato powder diet for 30-d (study 1)¹

Tissue	Genotype		
	CMO-I KO	CMO-II KO	Wild-type
Liver	0.170 \pm 0.061 ^c (p<0.0001)	0.087 \pm 0.019 ^b (p=0.02)	0.002 \pm 0.001 ^a
Testes	0.092 \pm 0.018 ^c (p<0.0001)	0.015 \pm 0.005 ^b (p=0.003)	0.003 \pm 0.002 ^a
Serum	0.006 \pm 0.001 ^b (p<0.0001)	0.001 \pm 0.000 ^a	² 0.000 \pm 0.000 ^a

¹Data are presented as means \pm SEM, n=8. Means in a row with superscripts without a common letter differ, p < 0.05.

²Below limit of detection (0.001 nmol).

Table 2.5. Hepatic phytofluene and phytoene concentrations (nmol / gram wet weight) of CMO-I KO, CMO-II KO, WT and WT mice that consumed a 10% tomato powder diet for 30- or 60-d (study 1 & 2)¹

Carotenoid	² 30-d feeding			³ 60-d feeding	
	CMO-I KO	CMO-II KO	Wild-type	CMO-II KO	WT
Phytofluene	1.57 \pm 0.58	1.99 \pm 0.45	1.35 \pm 0.27	9.08 \pm 2.12	8.98 \pm 1.47
Phytoene	4.19 \pm 0.36	7.23 \pm 1.26	4.82 \pm 0.84	3.87 \pm 0.90	3.73 \pm 1.05

¹Data are presented as means \pm SEM, n=8.

² FutureCeutical tomato powder contains 2.6 nmol phytofluene and 10.1 nmol phytoene; 217 nmol total carotenoids.

³ Gilroy tomato powder contains 29.3 nmol phytofluene and 33.5 nmol phytoene, 525 nmol total carotenoids.

Table 2.6. Tissue lycopene concentrations (nmol / gram wet weight) in CMO-II KO and WT mice fed tomato powder for 60-d (study 2)^{1,2}

Tissue	Genotype	
	CMO-II KO	WT
Liver	58.3 ± 6.90	67.5 ± 6.08
Adrenals	9.62 ± 0.83*	5.97 ± 0.34
	(p=0.005)	
Spleen	5.75 ± 1.37	5.88 ± 0.58
Testes	1.44 ± 0.14*	0.74 ± 0.15
	(p=0.002)	
Kidney	1.17 ± 0.14*	0.73 ± 0.09
	(p=0.02)	
Lung	1.25 ± 0.05*	0.61 ± 0.11
	(p=0.0005)	
Uterus	3.52 ± 0.68*	1.01 ± 0.13
	(p=0.02)	
Serum	0.42 ± 0.05*	0.21 ± 0.02
	(p=0.005)	
Prostate	1.11 ± 0.10*	0.32 ± 0.06
	(p=0.004)	
Brain	0.13 ± 0.01*	0.04 ± 0.01
	(p<0.0001)	
Seminal vesicles	0.04 ± 0.01	0.05 ± 0.02

¹ Data are presented as means ± SEM; n=3-11(prostate, adrenals, and seminal vesicles were pooled for analysis).

² Gilroy tomato powder contains 175 nmol lycopene, 525 nmol total carotenoids.

* Asterisks indicate means within a row differ from WT.

Table 2.7. Tissue β -carotene concentrations (nmol / gram wet weight) in CMO-II KO and WT mice fed tomato powder for 60-d (study 2)^{1,2}

Tissue	Genotype	
	CMO-II KO	WT
Liver	0.49 \pm 0.04*	0.25 \pm 0.02
	(p=0.005)	
Adrenals	0.83 \pm 0.10*	0.46 \pm 0.04
	(p=0.007)	
Spleen	0.25 \pm 0.05	0.23 \pm 0.02
Testes	0.15 \pm 0.01	0.15 \pm 0.00
Kidney	0.13 \pm 0.01*	0.11 \pm 0.00
	(p=0.009)	
Lung	0.17 \pm 0.02*	0.29 \pm 0.04
	(p=0.001)	
Uterus	0.25 \pm 0.02	0.20 \pm 0.01
Serum	0.09 \pm 0.01	0.07 \pm 0.01
Prostate	0.09 \pm 0.01*	0.05 \pm 0.00
	(p=0.0007)	
Brain	0.12 \pm 0.02	0.15 \pm 0.02
Seminal vesicles	0.08 \pm 0.02	0.06 \pm 0.01

¹ Data are presented as means \pm SEM; n=3-11(prostate, adrenals, and seminal vesicles were pooled for analysis).

²Gilroy tomato powder diet contained 28 nmol β -carotene as DSM water-soluble beadlets, 109 nmol total carotenoids.

* Asterisks indicate means within a row differ from WT.

Table 2.8. Tissue lycopene isomer concentration (% of total lycopene) from mice fed lycopene containing diets for 30-d (study 1)¹

Tissue	Isomer	Genotype		
		CMO-I KO	CMO-II KO	Wild-type
Liver	13- <i>cis</i>	24.9 ± 3.72	24.0 ± 0.78*	27.9 ± 1.93
			(p=0.04)	
	9- <i>cis</i>	15.1 ± 1.36	9.11 ± 0.75*	16.2 ± 1.45
			(p<0.0001)	
	5- <i>cis</i>	27.3 ± 2.46	26.1 ± 3.16	22.6 ± 6.54
	All- <i>trans</i>	32.6 ± 6.28	38.9 ± 5.66	32.3 ± 4.61
Testes	13- <i>cis</i>	20.4 ± 3.23	21.1 ± 3.64	17.8 ± 1.22
	9- <i>cis</i>	13.9 ± 0.96	15.0 ± 2.32	15.7 ± 1.32
	5- <i>cis</i>	43.1 ± 0.88	31.6 ± 0.98*	42.2 ± 0.85
			(p<0.0001)	
	All- <i>trans</i>	22.7 ± 1.38	31.5 ± 3.77*	22.0 ± 0.75
			(p=0.0001)	
Serum	13- <i>cis</i>	8.92 ± 0.64	13.0 ± 1.06*	8.40 ± 1.39
			(p=0.009)	
	9- <i>cis</i>	10.3 ± 0.64*	18.1 ± 1.39	17.2 ± 1.48
		(p=0.004)		
	5- <i>cis</i>	56.0 ± 2.1*	39.8 ± 1.62*	49.0 ± 1.2
		(p=0.005)	(p<0.0001)	
	All- <i>trans</i>	25.1 ± 1.09	29.3 ± 2.78*	24.2 ± 1.36
			(p=0.01)	

¹ Data are presented as means ± SEM; n=8

Table 2.8 (cont)

²The lycopene containing diets contained approximately 9% 13-*cis*, 6% 9-*cis*, 17% 5-*cis*, and 68% all-*trans* lycopene.

* Within a row, asterisks indicate different from WT.

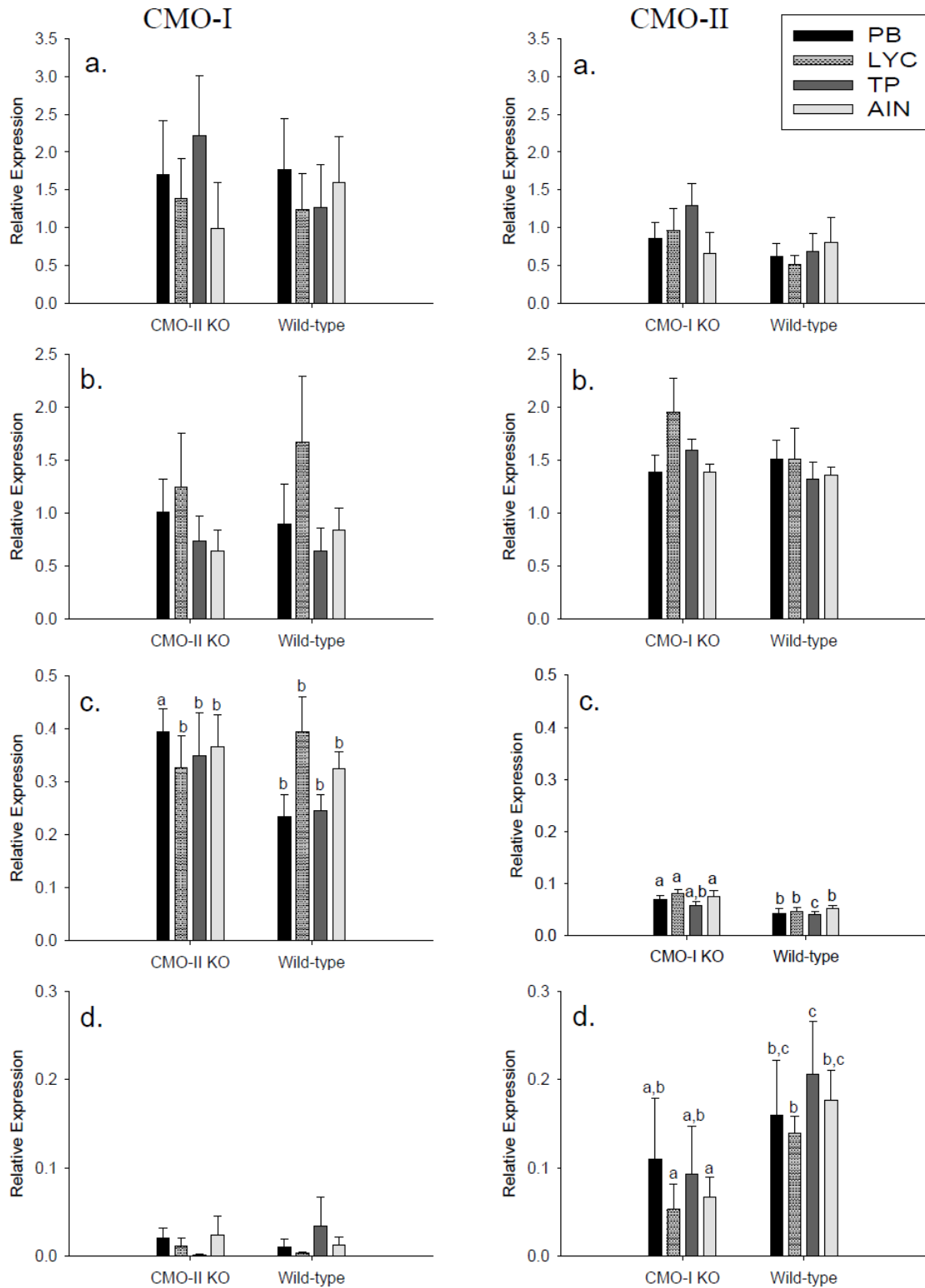


Figure 2.1. Relative mRNA expression of CMO-I and CMO-II in mice fed experimental diets for 30-d (study 1). Relative mRNA expression of CMO-I and CMO-II in (a) liver, (b) duodenum, (c) testes, (d) prostate. Different letters signify statistically significant. Expression was analyzed by ANOVA, $p < 0.05$; $n = 9-11$.

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CHAPTER III

LYCOPENE AND ITS METABOLITE, APO-12'-LYCOPENAL, REDUCE CELL PROLIFERATION AND ALTER CELL CYCLE PROGRESSION IN HUMAN PROSTATE CANCER CELLS

ABSTRACT

Lycopene is associated with a reduced risk of prostate cancer. Due to difficulties with stability and solubility of lycopene in cell culture systems, it is difficult to elucidate the mechanisms of action. Moreover, lycopene may not be wholly responsible for the effects seen *in vivo* or in cell culture systems. Apo-lycopenals, whether produced by cleavage enzymes within the body or consumed from tomato products, can be found in human blood plasma at concentrations equivalent to physiological retinoid concentrations. Therefore, it is plausible that lycopeneoids, like retinoids, are bioactive within tissues. Androgen-independent DU145 prostate cancer cells were treated with lycopene, apo-8'-lycopenal, or apo-12'-lycopenal. DU145 cell proliferation was significantly reduced by supraphysiological levels of lycopene and apo-12'-lycopenal, in part, through significant alteration of the normal cell cycle. Levels of the gap junction protein, connexin 43, were unaltered by treatments while cell apoptosis rates significantly decreased. The present data indicates that lycopene and apo-12'-lycopenal reduce the proliferation of prostate cancer cells, in part, by inhibiting cells from progressing through the normal cell cycle.

INTRODUCTION

Prostate cancer is the second most abundant male cancer with over 192,000 newly diagnosed cases annually (1). Males born today have a 16% risk of developing prostate cancer in their lifetime according to the 2002-2006 SEER data (1). Diet is one of the few risk factors that

may be modified. Notably, consuming three to five servings of tomato products a week reduces the risk of prostate cancer according to epidemiological studies (2, 3). Additionally, blood serum levels of lycopene, the red pigment of tomatoes, is inversely associated with prostate cancer risk (4).

Lycopene is the predominant carotenoid in tomato products followed by phytoene, phytofluene, and β -carotene. β -carotene is metabolized by carotenoid monooxygenase I (CMO-I) through central chain cleavage to form vitamin A and further metabolism results in formation of retinoic acid and other retinoids (5). *In vitro* evidence suggests that lycopene is metabolized by carotenoid monooxygenase II (CMO-II) (6). Enzymatic cleavage by CMO-II results in eccentric chain cleavage of carotenoids to form aldehyde metabolites.

In vivo studies identified and characterized tomato carotenoid metabolites and oxidative products (6, 7). Levels of lycopene metabolites identified *in vivo* are of comparable biological concentrations to retinoids. Kopec, et al. identified apo-6'-lycopenal, apo-8'-lycopenal, apo-10'-lycopenal, and apo-12'-lycopenal in plasma of individuals consuming tomato juice for 8 wk (8). These lycopene metabolites were also identified in tomato products although at much lower concentrations therefore dietary consumption may contribute to blood plasma apo-lycopenal concentrations.

Epidemiological trials established an inverse association between the risk of prostate cancer and both the intake of tomato products and higher serum levels of the lycopene (2, 9). The inverse association is likely attributed to the proposed mechanisms of lycopene, including: antioxidant function, alteration in growth factors, modifying androgen status, induction of apoptosis, modulation of normal cell cycle progression, and enhancing gap junction communication. The interpretation of current literature is confused by variation of *in vitro*

techniques, culture conditions, cell types, etc. Therefore, at this time, lycopene's mechanism of action is still ambiguous. We investigated proliferation, apoptosis, cell cycle progression, and gap junction communication of androgen-independent DU145 cells treated with lycopene or apo-lycopenals.

MATERIALS AND METHODS

Cell lines and culture conditions.

DU145 cells were purchased from ATCC (Manassas, VA) and maintained in RPMI 1640 (Sigma-Aldrich, St. Louis, MO) without phenol red media (10% per volume FBS (fetal bovine serum), 2.0g/L sodium bicarbonate, 2.5g/L glucose, 2.38g/L HEPES, and 1% sodium pyruvate) with 5.6mg/ml amphotericin B. Fresh media was made monthly and cells were subcultured 2-3 times per week. The cells were maintained at 37°C with 5% CO₂.

Preparation of carotenoids for cell culture.

Stock solutions of 15-25µM lycopene water-soluble beadlets (5.7% pure lycopene; DSM Nutritional Products, Basel, Switzerland), water-soluble placebo beadlets (DSM), apo-8'-lycopenal (BASF, Ludwigshafen, Germany), or apo-12'-lycopenal (BASF) were dissolved in dimethyl sulfoxide (DMSO) by sonication under yellow lights. Solubilization was confirmed through quick observation under a light microscope.

Cell proliferation.

DU145 cells were plated in 96-well plates (Sigma Aldrich) at 10,000 cells per well and were treated with 1, 15, or 25µM lycopene, apo-8'-lycopenal, or apo-12'-lycopenal 24 hours after plating. Control wells were treated with DMSO or placebo beadlets solubilized in DMSO. The number of viable cells was measured 72 hours after treatment using a Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit (Madison, WI). In separate experiments,

fresh media and lycopene were provided daily to DU145 cells (changed). For comparison, 48 wells on the plate were incubated with lycopene for 72 hrs without replacement of media or lycopene treatment (unchanged). The number of viable cells was also measured using a Promega CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit. Values are the average of eight treated sample wells in triplicate.

Flow cytometric analysis of apoptosis and cell cycle distribution.

The Annexin V-FITC apoptosis Detection Kit I (BD Bioscience, San Jose, California) was used to the manufacturer's instructions. Briefly, cells were split and washed with PBS two times before the cells were resuspended in 1x Binding Buffer. Annexin V-FITC and propidium iodide (PI) stains were added to the suspension and then analyzed with flow cytometry. The protocol for cell cycle analysis was modified from Ueno et. al (6). Briefly, treated DU145 cells were trypsinized, pelleted, and resuspended in 300 μ L of PBS. Subsequently, 700 μ L of ice cold 100% ethanol was added a drop at a time while vortexing the cells. The cells were kept at -20°C for less than 96 hours. Cells were then centrifuged for 15 minutes at 747 x *g* and the ethanol supernatant was removed. Cells were resuspended in 500 μ L of ice cold PBS and transferred to a 5mL polystyrene round-bottom tube from BD Falcon (Franklin Lakes, NJ). Next, 12.5 μ L RNase (20mg/ml) and 12.5 μ L of PI were added to the cells. Cells were incubated at 37°C in the dark for 30 minutes. Cells were analyzed by flow cytometry with the BD Sciences LSRII System (San Jose, CA) and FCS Express software from DeNovo (Los Angeles, CA) with the Multicycle AV add-on.

Western blotting analysis.

Media was aspirated off of the cells and the cells were removed from the plate with a cell scraper in 5mL of ice cold PBS. Cells were pelleted by centrifugation at 4°C for 20min at 269 x *g*. Cells

were resuspended with 500 μ L cold CellLytic (Sigma-Aldrich) + 5 μ L Protease Inhibitor Cocktail (Sigma-Aldrich) and lysed on ice for 15min. Cells were pelleted again by centrifugation at 4°C for 20 min at 12,000 x g, and the lysate was stored at -80°C. The Bio-Rad Protein Assay Kit (Hercules, CA) was used per manufacturer's instructions to create a standard protein curve for protein concentration. Samples were prepared for gel electrophoresis by combining sample Buffer (Sigma-Aldrich) and 2-Mercaptoethanol (9:1) with the cell lysate (1:1). Samples were then incubated at room temperature for 15min. Samples and a 5 μ l protein marker were loaded into a 12% Tris-HCl Ready Gel (Bio-Rad). Gel electrophoresis was run for 1 hour at 105V. The protein sample was transferred from the gel onto an Immobilon-P Transfer Membrane from Millipore (Bedford, MA) at 95V for 1hr. The membrane was allowed to dry for at least one hour. The membrane was incubated overnight while on ice and rocking with GAPDH 1^o antibody (1:20,000) (Millipore) and Cx43 (1:125) 1^o antibody (Sigma) in 1% casein solution. The membrane was washed with TBS-T 3x's and incubated with a mouse anti-GAPDH monoclonal antibody (1:20,000) (Amersham Biosciences, Sunnyvale, CA) and rabbit anti-connexin 43 affinity isolated antibody (Invitrogen, Carlsbad, CA) for 2hrs at room temperature. The membrane was visualized using ECL plus (Amersham) with a Storm Image Analyzer (Amersham) and a photo multiplier at 650V and an emission of 520LP. Densitometry was analyzed using ImageQuant 5.2 (Amersham).

Statistics.

DU145 cell proliferation, cytotoxicity, apoptosis, and cell cycle analysis were analyzed by one-way ANOVA followed by Tukey's post hoc test (SAS 9.1, Carey, NC). A p-value of ≤ 0.05 was considered significant. Proliferation data were log transformed to meet statistical test assumptions.

RESULTS

Effects of lycopene treatment on proliferation.

DU145 cells were treated with 1, 15, or 25 μ M lycopene or vehicle alone for 72 hours. DU145 cell proliferation was significantly reduced by 10% and 19% with 15 μ M and 25 μ M lycopene, respectively (ANOVA, $p < 0.05$, $n = 3$) (Fig 3.1a).

Lycopene treatment reduces apoptosis and inhibits cell-cycle progression.

Flow cytometry was used to assess apoptosis and cell cycle distribution after DU145 cells were treated with various doses of lycopene. Incubating cells with lycopene for 72 hours significantly reduced apoptosis of cells at all concentrations. Figure 3.1b shows all treatments resulted in a 92-93% reduction in apoptosis rates compared to control cells (ANOVA, $p < 0.05$, $n = 9$). Lycopene at 1, 15, and 25 μ M caused a significant reduction in the total number of cells in the S phase (ANOVA, $p < 0.05$, $n = 9$) (Fig 3.1c). Neither the G1 nor G2/M phases were significantly altered with lycopene treatment.

Lycopene treatment does not affect the levels of gap junction protein, Cx43.

DU145 cells were treated with 0-25 μ M lycopene for 72 hours and the cell lysates were analyzed by Western blot for protein levels of Cx43, normalized to GAPDH. Lycopene treatment did not affect the protein level of Cx43 ($n = 4$) (Fig 3.2a).

Not replacing media and lycopene treatment daily most greatly affects proliferation.

Cells were treated with lycopene (10 μ M) for 72 hours. Fresh media and lycopene were provided daily to half of the cells and compared to cells treated with unchanged media and lycopene treatment. Figure 3.3 shows a 23% inhibition of DU145 cells when media was unchanged during the 72 hr incubation period while only a 4% inhibition was found when media and treatment were replaced daily (ANOVA, $p < 0.05$, $n = 3$).

Apo-12'-lycopenal, but not apo-8'-lycopenal reduces proliferation.

Apo-12'-lycopenal (1-25 μ M) reduced proliferation of androgen-independent DU145 cells in a dose-dependent manner (ANOVA, $p < 0.05$, $n = 3$). Conversely, apo-8'-lycopenal did not affect proliferation of this prostate cancer cell line (Fig 3.4a). Apo-12'-lycopenal (25 μ M) significantly inhibited proliferation by 7%.

Apo-12'-lycopenal treatment reduces apoptosis and inhibits cell-cycle progression.

Apoptosis and cell cycle distribution of DU145 cells were analyzed by flow cytometry after treatment with 0-25 μ M of apo-12'-lycopenal for 72 hours. Incubating cells with 1, 15, or 25 μ M apo-12'-lycopenal significantly reduced apoptosis of cells (ANOVA, $p < 0.05$, $n = 9$). All 3 treatments resulted in an 87-96% reduction in apoptosis rates compared to control cells (Fig 3.4b). Apo-12'-lycopenal treatment also resulted in a significant reduction in the total number of cells in the S phase at all concentrations (ANOVA, $p < 0.05$, $n = 9$) (Fig 3.4c). No significant changes in the number of cells accumulating in the G1 or G2/M phases were found.

Apo-12'-lycopenal treatment does not affect gap junction protein, Cx43, levels.

DU145 cells were treated with varying concentrations of apo-12'-lycopenal and the cell lysate was analyzed by Western blot for protein level of Cx43 and normalized to GAPDH. Apo-12'-lycopenal treatment did not affect the levels of the Cx43 protein at any concentration (Fig. 3.2b).

DISCUSSION

Numerous studies report lycopene reduces proliferation of prostate cancer cells, *in vitro* (10-18). Due to variability in cell lines, culture conditions, solvents used to deliver lycopene, and the source of lycopene, the mechanisms of reduced proliferation are still open to further investigation. We investigated several of these mechanisms *in vitro*. This study provides

evidence that at least one lycopene metabolite, apo-12'-lycopenal, is bioactive and may contribute to reduced prostate cancer risk as previously observed with consuming lycopene-containing diets.

The investigation of carotenoids in cell culture conditions has proved difficult and standard methods have not been developed. Lycopene is an excellent antioxidant; therefore it suffers considerable degradation and isomerization under cell culture conditions (19). Vitamins C and E have been used to protect lycopene in these conditions, but inclusion of these antioxidants complicates outcomes (20-22). Another issue faced in biological and artificial systems is carotenoid aggregation or stacking (19). All-*trans* lycopene is a linear hydrocarbon and in hydrophobic media lycopene can quickly form aggregates of closely stacked lycopene monomers in parallel sheets that result in reduced contact with the cells and the culture environment compared to monomers. Because lycopene is not soluble in cell culture media it must be solubilized. Many solvents including tetrahydrofuran, Tween, and dimethylsulfoxide have been used in addition to mammalian serum and micelles. Each lycopene delivery method has its advantages and limitations related to crystallization, commercial availability, solubilization ability, toxicity, and labor time. For example, THF readily solubilizes lycopene but possesses a moderate Materials Safety Data Sheet hazard rating and can form peroxides at greater than 1% v/v ratio. Lastly, duration of lycopene treatment varies widely within the literature. Cells treated with lycopene from 12 hours to 7-d with increased time significantly reducing proliferation. Overall, *in vitro* results investigating lycopene vary considerably most likely due to variation in culturing techniques and care taken to avoid isomerization, oxidations, and stacking.

Our study shows that lycopene significantly reduces proliferation of DU145 cells at supra-physiological concentrations (15 and 25 μ M). However, when the physiological concentration of lycopene (>2 μ M) (23) was investigated, we do not see reduced proliferation. To our knowledge, the effects of lycopene on the proliferation of DU145 cells have been investigated in 3 previous studies. Two of those studies demonstrate that supra-physiological doses reduced proliferation while one found no changes in proliferation with physiological concentrations (15, 18, 24). Therefore, the proliferation results of this study are in alignment with previous *in vitro* findings.

Results from *in vivo* and *in vitro* studies suggest that lycopene induces apoptosis of cancer cells (12, 13, 15, 23, 25). We found no evidence to support these findings in the current study but rather surprisingly found that lycopene, at all concentrations investigated, significantly reduced apoptosis compared to control cells. Our data matches previous findings in lymphocytes and macrophages but disagrees with previous findings in prostate cancer cells (26-28). Disagreement between our findings and previous studies may be due to pro-oxidant effects caused by supra-physiological lycopene concentrations in cell culture conditions.

We also investigated cell-cycle regulation, another commonly-reported mechanism of action of lycopene. The cell cycle of DU145 cells was altered with 1-25 μ M lycopene resulting in cells accumulating in the G₁ and G₂/M phase. Moreover, significantly fewer DU145 cells accumulating in the S phase suggests that the cancer cells were not progressing through the cell cycle normally. Therefore, in these experiments, cell cycle arrest contributes, in part, to reduced proliferation of DU145 cells by lycopene treatment. Our findings corroborate other reports which indicate lycopene inhibits normal cell cycle progression of prostate cancer cells (12, 15, 25). Tang, et al. reported DU145 cells treated with 16 μ M and 32 μ M lycopene reduced the

accumulation of cells in the S phase by approximately 50% (15), which is similar to our current findings. Additionally, as shown in our study using similar concentrations, increasing lycopene concentration from 16 μ M to 32 μ M did not further reduce cells accumulating in the S phase. Moreover, Tang, et al. report significant increases in DU145 cells accumulating in the G₂/M phase which supports the non-significant trend we demonstrate in this study. Altogether, we and others report that lycopene alters cell cycle progression in prostate cancer cells.

Lastly, one of the earliest suggested mechanisms of action of lycopene is enhancement of GJC through increased concentrations of the connexin 43 protein. Research consistently demonstrates the importance of GJC in maintaining normal cell function (29). We did not find an increase in the protein levels of Cx43 with lycopene treatment in DU145 cells. Cancer cells often express very low levels of Cx43 protein; therefore, very little protein was detected in the control cells. Overall, we found no change in the protein levels of Cx43 *in vitro* with lycopene. The apparent contradiction in our DU145 cell results and those of others may be due to the malignancy of the cells. King and Bertram suggest that GJC may not be restored by carotenoids in cell lines containing multiple mutations (29). They further state that only forced expression of connexins, gene transfer, or hypomethylation may overcome loss of expression of the connexin genes. Further research in this area is warranted. On the other hand, due to the short half-life of Cx43 (1.5-5 hours), functional assays may not be appropriate and may further complicate results in the literature (30).

Unfortunately, due to high temperatures and presence of oxygen, lycopene rapidly oxidizes and degrades in cell culture systems making it difficult to interpret results of *in vitro* studies with lycopene. For this reason, we compared proliferation of DU145 cells treated with fresh cell culture medium and treatment daily (changed) to that of cells treated with lycopene

without replacing medium or treatment (unchanged) during the 72 hour incubation. Interestingly, proliferation of DU145 cells was most significantly reduced when culture media and treatment were unchanged for 72 hours. We interpret this to suggest that oxidative products of lycopene produced *in vitro* and/or metabolic products of lycopene produced in DU145 cells reduce the proliferation of prostate cancer cells. Subsequently, to rule out cytotoxicity as a factor, we performed CytoTox 96 Non-Radioactive Cytotoxicity Assays (Promega, Madison, WI). No significant increase in cytotoxicity resulted with up to 25 μ M lycopene treatment compared to the control cells. Given our findings, we propose that oxidative products or lycopene metabolites are bioactive and reduce DU145 cell proliferation of prostate cancer cells, alone, or in combination, with lycopene.

We investigated the effects of two known lycopene metabolites, apo-8'-lycopenal and apo-12'-lycopenal, on the proliferation of DU145 cells (7). Both metabolites have been identified as metabolic products of lycopene that was exposed to cell culture conditions for 72 hours (31). Moreover, our lab previously identified apo-8'-lycopenal and potentially apo-12'-lycopenal in the liver of rats fed lycopene containing diets (7). Apo-12'-lycopenal significantly reduces proliferation of DU145 cells at 15 μ M and 25 μ M, while apo-8'-lycopenal has no effect on proliferation. Similarly, others report that lycopene metabolites reduced proliferation in several cell types (32-36). For instance, an apo-lycopenoid, apo-10'-lycopenoic acid (3-10 μ M), reduced proliferation of non-small-cell lung cancer cells and 2 bronchial epithelial cell types (36).

We further investigated the mechanisms of reduced DU145 proliferation by apo-12'-lycopenal. Results of other studies suggest lycopene metabolites have antioxidant properties, mediate retinoid signaling through RAR- β , induce phase II enzymes, and interfere with insulin-

like growth factors (32, 36-39). We analyzed apoptosis and cell cycle progression by flow cytometry and GJC as indicated by protein levels of Cx43.

As similarly seen with lycopene, apo-12'-lycopenal significantly reduced apoptosis and inhibited normal cell cycle progression. Cells treated with apo-12'-lycopenal accumulated in the G₁ and G₂/M phase while significantly fewer DU145 cells accumulated in the S phase. Therefore, apo-12'-lycopenal appears to reduce proliferation of DU145 cells, in part, through cell cycle arrest. Few other studies have investigated the effects of lycopene metabolites on apoptosis or cell cycle progression. Zhang, et al. indicate the oxidative lycopene metabolite, (E,E,E)-4—methyl-8-oxo-2,4,6-nonatrienal, induced apoptosis and inhibited normal cell cycle progression in human promyelocytic leukemia cells (HL-60) (35). Other studies suggest that lycopene metabolites have no effect on apoptosis. Lian, et al. reported apo-10'-lycopenoic acid reduced proliferation of NHBE normal human bronchial epithelial cells, BEAS-2B-immortalized normal bronchial epithelial cells and A549 non-small cell lung cancer cells, altered cell cycle progression, but did not alter apoptosis (36). Our study adds to the *in vitro* literature by demonstrating that apo-12'-lycopenal reduces apoptosis of DU145 cells. Overall, apo-12'-lycopenal, in part, reduces proliferation of DU145 cells through disruption of normal cell cycle progression and our results are supported by other *in vitro* lycopene metabolite studies.

Lastly, we measured Cx43 protein levels by Western blot as an indicator of GJC. Two papers report that longer incubation with carotenoids (3-7 d) is required to induce the expression of Cx43 compared to retinoids. The authors hypothesize that carotenoids must first be oxidatively or metabolically cleaved before significant increases in GJC can be observed (40, 41). Therefore, we proposed that carotenoid metabolites would have a greater capacity to increase GJC, via expression of Cx43, than does the parent carotenoid compound. Other studies showed

that lycopene metabolites effectively increased Cx43 protein levels in rat liver epithelial WB-F344 cells, human keratinocytes, and human fetal skin fibroblasts (HFFF2) (42-44). However, apo-12'-lycopenal treatment did not alter the protein levels of Cx43 in DU145 cells. Apparent conflict in results between our study and the previously reported study may be due to species, tissue, or lycopene metabolite. However, we are the first to report that the lycopene metabolite, apo-12'-lycopenal, does not affect protein levels of Cx43 in DU145 cells.

In summary, we found that lycopene, at supra-physiological concentrations, reduces proliferation of DU145 cells, in part, through alteration in normal cell cycle progression. Lycopene did not alter Cx43 protein levels in DU145 cells. This suggests that lycopene does not reduce prostate cancer risk by enhancing GJC through Cx43. Our results suggest that unknown lycopene oxidative or metabolic products reduce proliferation of DU145 cells more than the parent compound lycopene. Lastly, we are the first to report that the lycopene metabolite, apo-12'-lycopenal, reduces proliferation of DU145 cells through modulation of cell cycle progression. Altogether, our results suggest that lycopene metabolites produced by cell metabolism or by oxidation may be bioactive and play a role in reducing prostate cancer risk.

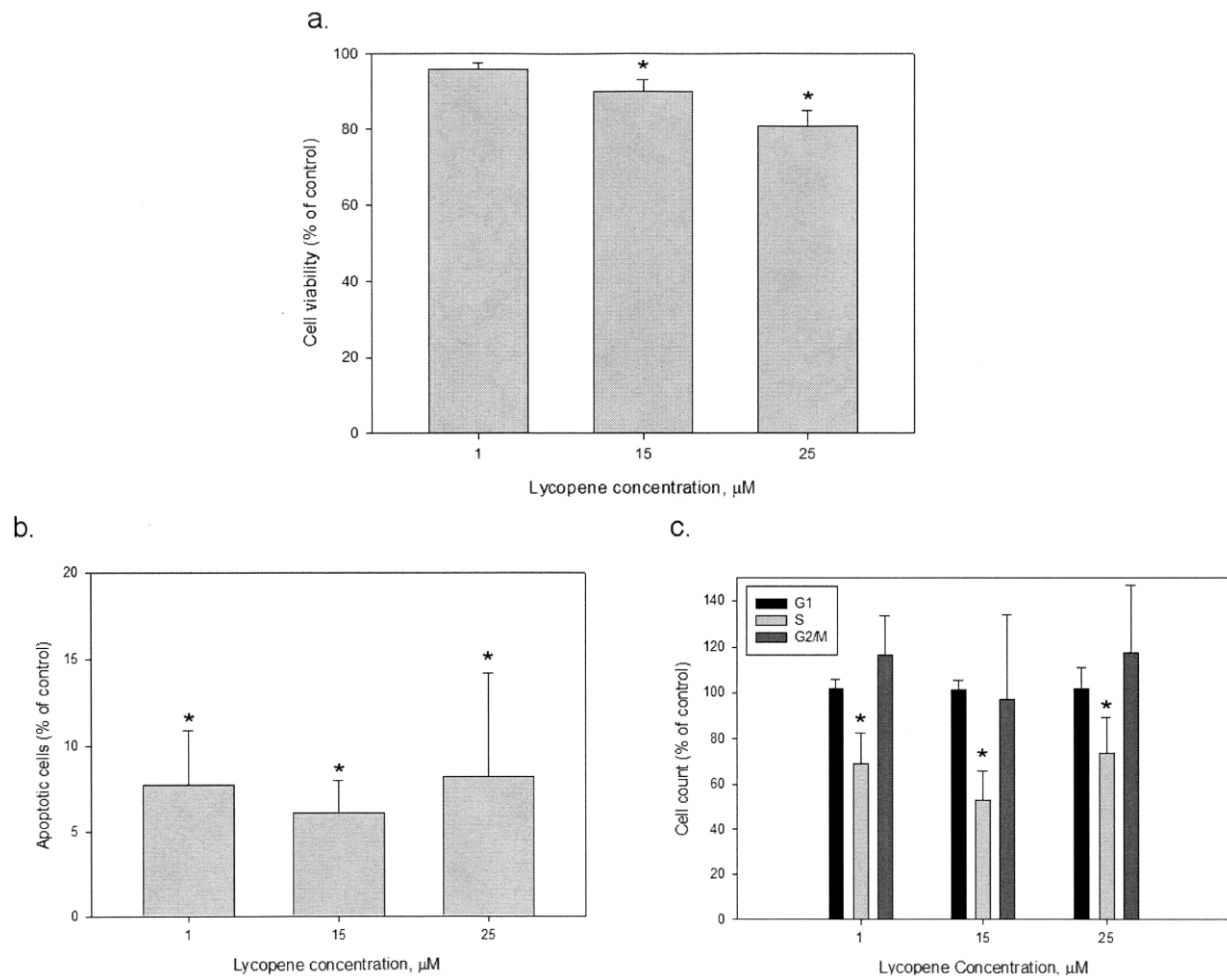


Figure 3.1. a: Effect of varying concentrations of lycopene on the 72 hour proliferation of DU145 cells. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. b: DU145 cells were treated with lycopene and analyzed by flow cytometry using Annexin V-FITC and Propidium Iodide dyes c: Propidium Iodide was used for detection of cell cycle distribution by flow cytometry in DU145 cells after treatment with varying concentrations of lycopene for 72 hours. Data are presented as a percentage of the control cells treated with vehicle only plus placebo beadlets. Bars are means of three independent experiments \pm SEM. Asterisk indicates significantly different from control cells. Lycopene significantly reduced DU145 cell proliferation (n=3), apoptosis (n=9), and accumulation of cells in the S phase (n=9) (ANOVA, $p < 0.05$).

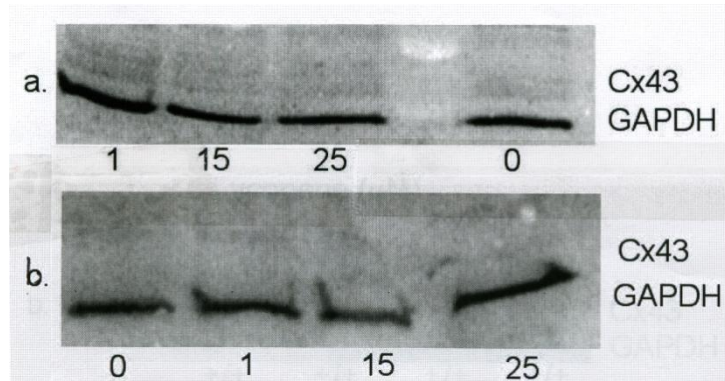


Figure 3.2. Gap junction protein, Connexin 43 (Cx43), levels in DU145 with: a. lycopene or b. apo-12'-lycopenal treatment. GAPDH was included as a control for loading. All panels denote a representative Western blot of three independent experiments.

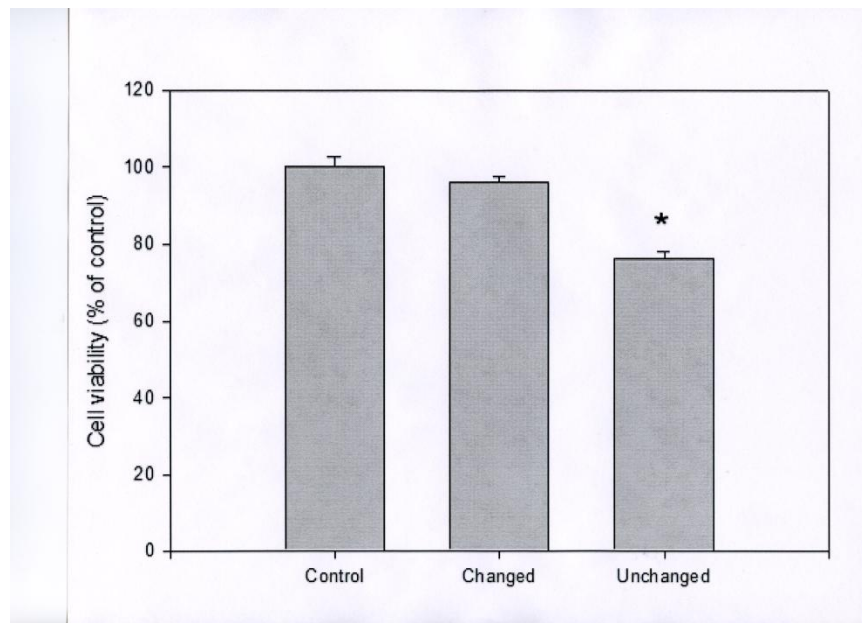


Figure 3.3. Effect of replacing media and lycopene treatment daily (changed) or not changing media and lycopene treatment daily (unchanged) on the proliferation of DU145 cells. Cells were treated with 10 μ M lycopene for 72 hours. Cell numbers are expressed as the percentage of the control cells and bars are means of three independent experiments \pm SEM. Asterisk indicates significantly different from control cells. Unchanged media and lycopene treatment significantly reduced proliferation of DU145 cells (ANOVA, $p < 0.05$, $n = 3$).

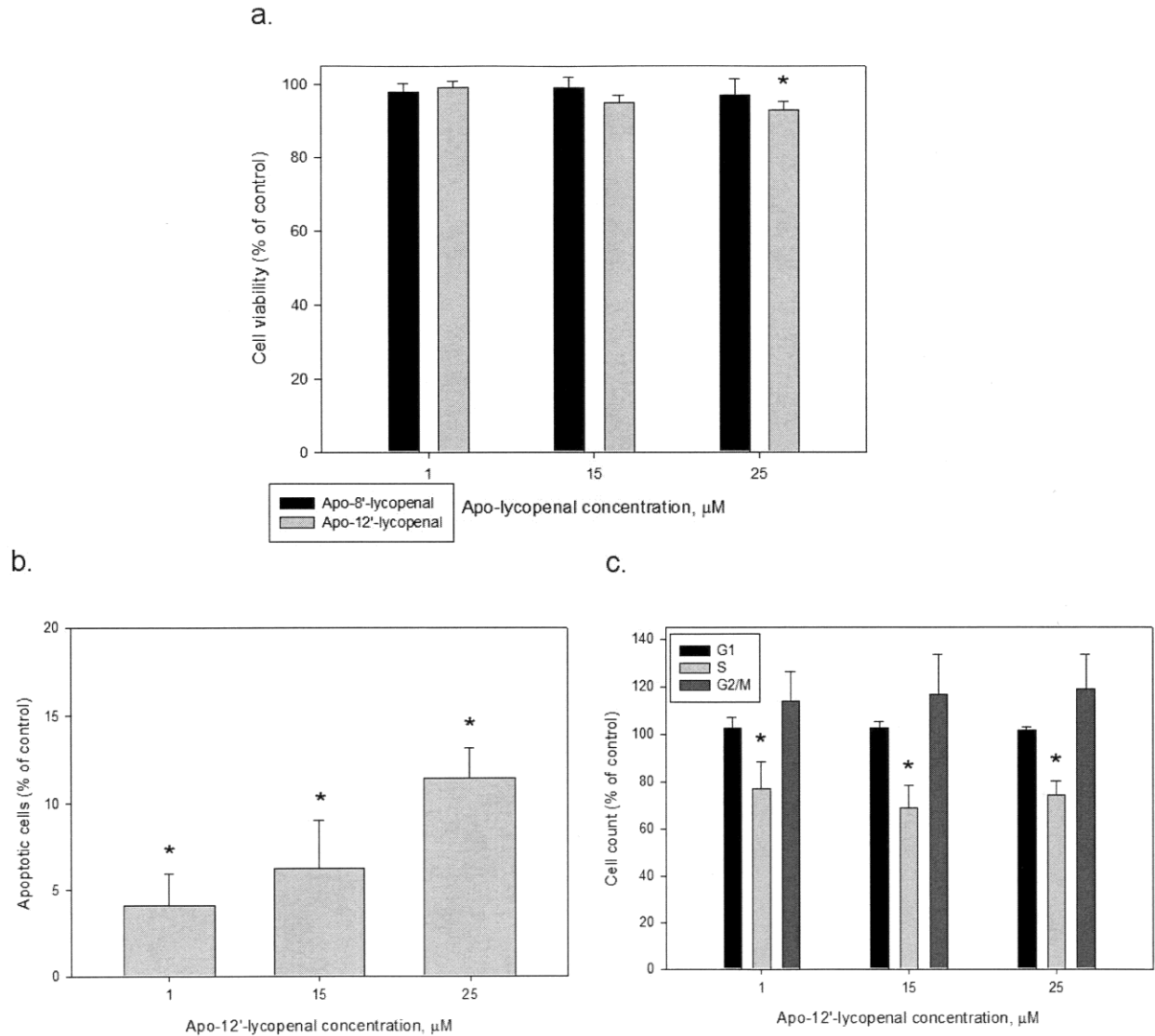


Figure 3.4. a: Effect of varying concentrations of apo-12'-lycopenal on the 72 hour proliferation of DU145 cells. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. b: DU145 cells were treated with apo-12'-lycopenal and analyzed by flow cytometry using Annexin V-FITC and Propidium Iodide dyes c: Propidium Iodide was used for detection of cell cycle distribution by flow cytometry in DU145 cells after treatment with varying concentrations of apo-12'-lycopenal for 72 hours. Data are presented as a percentage of the control cells treated with vehicle only. Bars are means of three independent experiments \pm SEM. Asterisk indicates significantly different from control cells. Apo-12'-lycopenal significantly reduced proliferation of DU145 cells, apoptosis, and accumulation of cells in the S phase (ANOVA, $p < 0.05$, $n = 3$).

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CHAPTER IV

TOMATO POWDER OR LYCOPENE REDUCES SERUM AND TESTES TESTOSTERONE AND ENZYMES CONTROLLING ANDROGEN AND ESTROGEN METABOLISM IN MICE LACKING CAROTENE MONOOXYGENASE I

ABSTRACT

Serum levels of lycopene, the red pigment of tomatoes, are inversely associated with prostate cancer incidence while serum sex steroids, testosterone, dihydrotestosterone, and possibly estrogen, are associated with increased risk. Previously, we reported that serum testosterone was reduced in rats fed lycopene-containing diets. In this study, transgenic mice lacking the expression of carotene-monooxygenase II (CMO-II KO), wild-type mice, or mice with increased expression of CMO-II in the testes (CMO-I KO) were used to investigate the effects of lycopene metabolism on sex steroid status. The lycopene ($p=0.015$) and tomato powder diets ($p=0.0062$) significantly reduced testicular testosterone in CMO-I KO mice after 4-d of feeding. Serum testosterone was also reduced in CMO-I KO mice by 4-d of consuming the lycopene ($p=0.012$) and the tomato powder ($p=0.017$) diets. The genes for the sex steroid metabolizing enzymes, 5α -reductase, and 17β -hydroxysteroid dehydrogenase, were also altered in response to dietary lycopene in the testes of CMO-I KO mice ($p=0.001$). The mRNA expression of the testicular androgen ($p=0.0008$) and estrogen ($p<0.0001$) receptors was down-regulated by dietary lycopene in CMO-I KO mice. We hypothesize that lycopene metabolites, produced through oxidative cleavage by CMO-II, reduced serum testosterone alone, or in combination with the parent compound, lycopene. A small reduction in androgen and estrogen status by tomato powder and lycopene following short term feeding may reduce the risk of prostate cancer over a lifetime.

INTRODUCTION

Prostate cancer is the second most common cancer accounting for over 192,000 new cases in 2009 with approximately 27,000 deaths (1). The cumulative cost of prostate cancer over five years is estimated to exceed \$42,000 (2). Moreover, African-American men are more likely to develop prostate cancer and more likely to die from this disease. According to the 2004 US census, the average African-American household income was only \$30,134. Therefore, the group of men with the greatest risk of developing prostate cancer cannot afford the current treatment options. Furthermore, current treatment options have unwanted side-effects with up to 43% of men experiencing sexual problems, 16% with urinary problems, and up to 11% of men experience bowel problems two years after treatment (3). For these reasons and because prostate cancer normally progresses slowly, dietary intervention is a plausible alternative to reduce prostate cancer risk.

The risk of prostate cancer is strongly associated with androgen status (4, 5). Additionally, a causal relationship is suggested in a few case reports of prostate cancer patients that previously used androgens for medical treatment or as anabolic agents (6, 7). Additionally, administration of testosterone is commonly used to induce prostate cancer in rodent models (8-11). Androgens can be converted to estrogens by the enzyme aromatase. Although androgen modulation has been the conventional method of prostate cancer treatment, it is apparent in recent literature that estrogens also play a role in prostate cancer development and treatment (12-18).

In a pivotal study published in 1995 using the Health Professional Follow-up Study cohort, intake of tomato products and their red pigment, lycopene, was associated with a decreased risk of prostate cancer (19). Notably, consuming three to five servings of tomato products a week reduced the risk of prostate cancer (19). Dietary intervention studies have

recently evaluated a possible diet-endocrine interaction in prostate cancer prevention (20-23). In a phase II randomized-controlled trial, serum free testosterone was decreased with lycopene supplementation in men with clinically localized prostate cancer (24). Previously we reported that serum testosterone concentrations were significantly decreased in rats fed lycopene or tomato powder for 4-d (25) although longer term lycopene feeding did not alter serum testosterone or DHT levels (26). Furthermore, we reported that lycopene accumulation was enhanced in castrated rats (27, 28). Other studies reported reduced mRNA expression of the androgen metabolizing gene 5 α -reductase in the prostate and in prostate tumors when rats were fed lycopene for four or eight wk (20, 21).

To our knowledge, no studies have investigated the effects of lycopene on estrogens in testes or prostate. However, a couple of studies have investigated the effects of lycopene on estrogen status in other tissues. Within the Women's Health Initiative Observational Study, women had a reduced risk of developing estrogen receptor-positive and progesterone receptor-positive breast cancers with higher intakes of lycopene or β -carotene (29). This inverse association was not found with other types of breast cancer suggesting that lycopene and β -carotene may alter breast cancer risk through the estrogen and progesterone receptors. An *in vitro* study found that the carotenoids lycopene, phytoene, phytofluene and β -carotene, inhibited estrogen signaling and reduced proliferation of hormone-dependent MCF-7 and T47D breast cancer and ECC-1 endometrial cancer cells (30). Together, these data suggest that lycopene alters estrogen status within the endometrial and breast tissues.

Lycopene is the predominant carotenoid in tomato products. Tomatoes also contain other carotenoids, including β -carotene which is metabolized by carotene monooxygenase I (CMO-I) through central chain cleavage to form vitamin A and further metabolism results in formation of

retinoic acid and other retinoids (31). Carotene monooxygenase II (CMO-II) is responsible for eccentric cleavage of carotenoids, including lycopene to form aldehyde metabolites (Chapter 2 (32)).

Recently, studies have identified and characterized lycopene metabolites and oxidative products generated *in vivo*. Some of these compounds demonstrate anti-cancer activity by inhibiting proliferation, inducing apoptosis, or enhancing cell to cell communication (33-35). Interestingly, *in vivo* concentrations of lycopene metabolites, also known as lycopenoids (36), are of comparable biological concentrations to retinoids produced from β -carotene. Like retinoids, we propose that carotenoid metabolites, produced through eccentric cleavage by CMO-II, are bioactive in small concentrations within tissues (36). Additionally, we hypothesize that lycopenoids, in combination with lycopene or other tomato components, reduce prostate cancer risk.

We and others have reported that lycopene alters androgen and estrogen status in rats, cell-culture, and clinical studies (20-25). The current study investigates the effects of lycopene-containing diets on testosterone and estrogen status and further investigates possible mechanisms. Specifically, we investigated how the metabolism of lycopene affects sex steroid status using transgenic mice that lack the production of oxidative lycopene metabolites (CMO-II KO), wild-type mice, or mice with increased expression of CMO-II in the testes (CMO-I KO). Whether through intact lycopene or its metabolites, consuming lycopene or tomato products may provide a cost-effective way to manage prostate cancer risk over a man's lifetime through a moderate reduction in testosterone and estrogen.

MATERIALS AND METHODS

Mouse husbandry and diets.

We received breeder pairs of *Bcmo1tm1Dnp* (CMO-I KO, n=4) and *B6;129S6-Bcdo2tm1Dnp* (CMO-II KO, n=4) courtesy of collaborators, Drs. Adrian Wyss at DSM Nutritional Products (Basel, Switzerland) and Johannes von Lintig at Case Western Reserve University. The generation of CMO-I KO mice was previously described (37). The generation of CMO-II KO mice is described by (Amenguel, submitted). C57BL/6 x 129/SvJ (F1) mice (WT, n=40) were purchased from Jackson Laboratory (Bar Harbor, ME). For this study, CMO-I KO and CMO-II KO mice were bred at the University of Illinois animal facilities. Genomic DNA from mouse tail biopsies and the Sigma Extract-N-Amp Tissue PCR Kit (St. Louis, MO) were used to confirm the genotype of the mice. Mice were housed in shoebox cages with free access to water and weighed and handled daily. Six to nine wk old male mice consumed an AIN-93G diet for 3 wk prior to randomization onto experimental diets. Fresh diet was provided every 48 hr and new diet was made monthly. The diet was stored in the dark at 4°C.

Nine- to 12-wk old male mice were randomly assigned to one of two experimental lycopene-containing diets; 10% tomato powder (Futureceuticals) (TP), or lycopene (DSM, 10% water-soluble lycopene beadlet) (LYC). The TP group was compared to an AIN-93G dietary group while the LYC group was compared to a placebo beadlet (PB) dietary group; n=25 per genotype/diet. Because previously we demonstrated that 4-d of feeding or orally gavaging lycopene, tomato powder, and phytofluene significantly reduced serum testosterone in rats, the mice in this study also consumed the experimental diets for 4-d, *ad libitum*. At the conclusion of the study, mice were asphyxiated by CO₂ and blood was collected by cardiac puncture. Cohorts of mice were sacrificed daily in the afternoon within a three hr time frame to avoid diurnal

alterations in serum testosterone. Brain, thymus, lungs, liver, spleen, duodenum mucosa, kidneys, adrenals, seminal vesicles, testes, and prostate were removed and snap-frozen in liquid nitrogen and then stored at -80°C. All animal procedures were approved by the University of Illinois Institutional Animal Care and Use Committee.

The TP diet contained 204 nmol lycopene/g diet while the LYC diet provided 248 nmol lycopene/g diet. The TP diet also contained additional carotenoids: 10.1 nmol phytoene, 2.6 nmol phytofluene, 1.3 nmol β -cryptoxanthin, and 0.8 nmol β -carotene/g diet. All diets were balanced for total fiber, nitrogen, and calories (Chapter 2). Additionally, vitamin A levels were reduced in all diets to 1500 IU retinyl palmitate per kg diet to ensure adequate absorption of carotenoids without resulting in a vitamin A deficiency (38).

HPLC analysis of carotenoids.

All chemicals were purchased from Fisher Scientific (Pittsburgh, PA). Tissue and serum samples were extracted with hexanes as described previously with minor modifications (Chapter 2) (27). Briefly, 0.08g tissue (0.15g liver was used for β -carotene analysis in study 1) was added to 5 ml of ethanol containing 0.01% butylated hydroxytoluene (BHT). Saturated KOH was added and the samples were vortexed. The samples were saponified in a waterbath at 60°C for 30 minutes with vortexing every 10 minutes. Distilled water (2 ml) was added and carotenoids were extracted three times with hexanes (6 ml). Serum carotenoids were extracted from 0.15 ml serum in 0.15 ml ethanol/BHT solution with 0.15 ml distilled water and 1.5 ml hexanes.

All extracts were kept on ice under yellow lights and flushed with argon to prevent degradation. For analysis, samples were dissolved in methyl-*tert*-butyl ether and injected onto a C30 HPLC column (4.6 x 150 mm, 3 μ m; YMC, Wilmington, NC) maintained at 18°C. A

reverse-phase HPLC-PDA method was utilized (39, 40). Individual carotenoids were identified by comparison of elution times to standards and absorption spectral analysis.

Serum and testes testosterone.

Testicular testosterone was extracted as previously described with minor modifications (41). Briefly, half of a testis was homogenized in PBS (125 μ l) at room temperature for 20 s. Diethyl ether (2.5 ml) was added, the sample was vortexed for 2 min, and then centrifuged at 183 x g for 1 min. The upper phase was removed and saved. Another 1.4 ml diethyl ether was added, the sample was vortexed and placed on dry ice for 10 min. The upper phase was again removed and combined with the previous extract. Samples were allowed to evaporate at room temperature and stored at -80°C until use. Samples were diluted 1:10 in PBS before quantification by radioimmunoassay. Serum and testicular testosterone were quantified with a radioimmunoassay kit (DSL-4000 ACTIVE Testosterone Coated-Tube Radioimmunoassay Kits; Diagnostic Systems Laboratories) as per manufacturer's instructions.

Real-Time PCR analysis.

Relative tissue expression of genes were evaluated using a Trizol (Invitrogen, Carlsbad, CA) extraction as per manufacturer's instructions followed by Dnase I treatment with the following modifications (New England Biolab, Ipswich, MA) (Table 4.1). Prostate tissue mRNA was extracted with 2 ml Trizol and testicular tissue mRNA was extracted using 1.5 ml Trizol. Concentration and quantity of mRNA were determined by spectrophotometry and agarose gel electrophoresis before synthesis of complimentary DNA by Superscript II Reverse Transcriptase (Invitrogen) using random hexamers (Applied Biosystems, Foster City, CA). CMO-I and CMO-II primers have been published previously (Chapter 2) while all other primers are listed in Table 1. Real-time PCR was performed using a 7900HT Fast Real-Time PCR detection system

(Applied Biosystems) with the SYBR green fluorescence dye. Threshold cycle (C_T) values for the 18S, β -actin, and L7a gene were used as control genes.

Statistical analysis.

A 3 x 4 factorial design was used with three genotypes (CMO-I KO, wild-type, or CMO-II KO) and four diets (AIN, TP, LYC, or PB). Interactions between genotype and diet were investigated. All parameters were analyzed by analysis of variance (ANOVA) followed by Tukey's post hoc analysis using SAS 9.2 (Cary, NC) with $\alpha = 0.05$. When assumptions for ANOVA were violated, data were transformed by natural log. For analysis of serum and testicular testosterone, all data points outside two standard deviations were considered extreme outliers and were removed from the dataset. Results were expressed as means \pm SEM, unless otherwise specified.

RESULTS

Tomato powder and lycopene reduced serum and testes testosterone.

Consumption of the LYC and the TP diet significantly reduced serum and testicular testosterone concentrations in CMO-I KO mice (Figure 4.1). Testosterone was also significantly reduced in the testis of CMO-II KO mice consuming the TP diet. The experimental diets did not alter serum or testes testosterone in wild-type mice or serum testosterone in CMO-II KO mice.

Lycopene-containing diets altered testicular mRNA expression of sex steroid metabolizing genes and receptors.

The mRNA expression of the androgen receptor, estrogen receptor α , estrogen receptor β , steroid hormone binding globulin, aromatase, 5 α -reductase I, 5 α -reductase II, 17 β -hydroxysteroid dehydrogenase I, and 17 β -hydroxysteroid dehydrogenase IV were measured in the testis. The LYC and TP diets affected the expression of steroid metabolizing genes in CMO-I KO mice

(Table 4.2). The lycopene diet significantly decreased the mRNA expression of the androgen receptor and 5 α -reductase I in the testes of CMO-I KO mice (p=0.01). Consumption of the TP diet significantly up-regulated mRNA expression of 17 β -hydroxysteroid dehydrogenase I (17 β -HSD I) (p=0.01) and 17 β -hydroxysteroid dehydrogenase IV (17 β -HSD IV) (p=0.0004) in the testes of CMO-I KO mice. The expression of the estrogen receptor alpha was significantly decreased in CMO-I KO mice compared to CMO-II KO and wild-type mice consuming the lycopene diet (p<0.0001). The expression of steroid metabolizing genes was also altered in CMO-II KO mice consuming the lycopene-containing diets. The mRNA expression of the androgen receptor in the testis was significantly increased in CMO-II KO mice that consumed the lycopene diet (p=0.008) and but not by the TP diet (Table 4.2). Consumption of the lycopene diet also resulted in a slight decrease in the expression of 5 α -reductase I. The expression of estrogen receptor alpha was significantly up-regulated in CMO-II KO and wild-type mice that consumed the lycopene diet. The experimental diets did not affect the mRNA expression of other genes investigated.

CMO-I KO mice and CMO-II KO mice have altered mRNA expression of steroid metabolizing genes.

The mRNA expression of steroid metabolizing genes was altered in testis and prostate of CMO-I KO and CMO-II KO mice compared to wild-type mice (Figure 4.2 and Table 4.4). The mRNA expression of 5 α -reductase I was reduced in the testes of CMO-I KO and CMO-II KO mice while the mRNA expression of 5 α -reductase II was also reduced in the testes of CMO-II KO. The mRNA expression of testicular aromatase and estrogen receptor beta and prostatic androgen receptor was elevated in CMO-II KO mice. The mRNA expression of the 17 β -HSD

isoforms 1 and 4 were upregulated in the testes and prostate of CMO-I KO mice and testes of CMO-II KO mice.

Dietary lycopene and tomato powder altered the expression of hepatic PPAR- γ but not CYP26a1.

The TP and LYC diets increased the hepatic mRNA expression of PPAR- γ in CMO-I KO mice (Figure 4.3). The LYC diet increased expression of hepatic PPAR- γ and the TP diet reduced expression of PPAR- γ in wild-type mice. The dietary interventions in this study did not affect hepatic CYP26a1 mRNA expression (Table 4.3). On the other hand, overall, CMO-II KO mice had reduced mRNA expression of hepatic CYP26a1 ($p < 0.0001$).

Short term feeding of lycopene or tomato powder results in preferential accumulation of carotenoids in CMO-I KO mice and CMO-II KO mice and alterations in CMO gene expression.

CMO-II KO mice preferentially accumulated tissue and serum lycopene after 4 d of consuming a LYC or TP diet ($p < 0.0001$) (data not shown). CMO-I KO mice accumulated significantly less hepatic lycopene compared to WT mice ($p < 0.0001$). CMO-I KO mice preferentially accumulated tissue and serum β -carotene compared to wild-type and CMO-II KO mice ($p < 0.0001$). The mRNA expression of CMO-I and CMO-II was investigated in the liver, duodenum, prostate, and testes of mice. The mRNA expression of CMO-II was significantly down-regulated in the prostate ($p < 0.0001$) and up-regulated in the testes ($p = 0.004$) of CMO-I KO mice compared to wild-type mice. The mRNA expression of CMO-I was not altered in CMO-II KO mice. The lycopene-containing diets did not affect CMO-I or CMO-II mRNA expression (data not shown).

DISCUSSION

Normal growth and function of the prostate requires sex steroids although higher serum levels of androgens, testosterone and dihydrotestosterone (DHT), and higher estrogens are reported to be associated with increased prostate cancer risk (5, 12, 13, 16, 42-44). Previously, we reported that serum testosterone was reduced in rats that consumed lycopene or tomato powder for 4-d (25). In contrast, longer term feeding of lycopene-containing diets (22 wk) did not alter serum testosterone (26, 45). Here, we show in a second animal model that consumption of lycopene and tomato powder has modest effects on testosterone levels and metabolism of sex steroids which may translate to a reduced risk of prostate cancer over a man's lifetime. Because these effects were primarily found in CMO-I KO mice, the current study further suggests that metabolites of lycopene reduce serum and testes testosterone in mice (Figure 4.1).

The testis is the primary site of testosterone production in the human and rodent. Therefore, we investigated the effects of tomato powder and lycopene on testosterone production the testes and in circulation. The TP and LYC diets significantly reduced serum and testes testosterone in CMO-I KO mice by 30-40% (Figure 4.1). Additionally, the mRNA expression of 5 α -reductase I was significantly reduced in the testes of CMO-I KO mice that consumed the lycopene diet. Testosterone is converted to the more active androgen, DHT, by 5 α -reductase. The enzyme, 5 α -reductase I, is predominantly expressed in the testes (Table 4.2) while isoform II is more highly expressed in the prostate. Current androgen ablation therapies, Dutasteride and Finasteride, target 5 α -reductase to reduce serum DHT levels by 65-98% (13, 46). In contrast, serum testosterone levels significantly increase with these treatments (47-49). Although DHT has a much higher binding affinity for the androgen receptor, testosterone is also a ligand for the androgen receptor and may induce transcription of target genes. The lycopene-containing diets

used in this study significantly reduced serum testosterone and possibly DHT synthesis through reduced expression of 5 α -reductase in the testes. Therefore, lycopene appears to moderately reduce both serum androgens which may result in a reduced risk of prostate cancer.

Not only is the ligand testosterone (and possibly DHT) reduced in CMO-I KO mice, but the expression of the androgen receptor is also reduced in the testes by lycopene-containing diets. CMO-II KO mice have the highest mRNA expression of the androgen receptor, followed by wild-type, and CMO-I KO mice (Table 4.2). These data would suggest reduced transcription of androgen-related genes in the testes of CMO-I KO mice by lycopene-containing diets.

The role of estrogens in prostate cancer treatment and development is not clear at this time. Estrogen can inhibit synthesis of testosterone and has more traditionally been used as a prostate cancer therapeutic (12, 13, 15). In contrast, other studies have reported that estradiol is integral for prostate cancer development (13, 14, 16, 18). In this study, the lycopene-containing tomato powder diet up-regulated the expression of 17 β -hydroxysteroid dehydrogenase (17 β -HSD). It is important to note that the androgen pathways in the rodent and human differ. The rodent 17 α -hydroxylase enzyme is more active on 4-ene steroids while the human enzyme specificity prefers 5-ene steroids (50-52). As a result, rodents do not produce dehydroepiandrosterone (DHEA) but instead 4-androstenedione. Therefore, the 17 β -HSD enzyme isoforms I and IV are primarily responsible for activation and inactivation of estradiol, respectively. Consumption of the TP diet significantly increased the expression of 17 β -HSD I and IV in CMO-I KO mice (Table 4.2). Isoform I is responsible for conversion of estrone to the more potent steroid, estradiol, whereas, isoform IV inactivates estradiol to estrone. The type IV isoform is much more highly expressed (>80x) in testis. Therefore, we hypothesize that up-

regulation of both isoforms of 17 β -HSD would result in a net inactivation of estradiol to estrone by lycopeneoids in CMO-I KO mice.

The lycopene diet also down-regulated the expression of estrogen receptor (ER) alpha in the testes of CMO-I KO mice compared to wild-type and CMO-II KO mice. The primary isoform found in the prostate is ER β , although expression is lost with development of prostate cancer which parallels induced expression of ER α in prostate cancer cells (43). The binding of estrogens to ER α induces proliferation, inflammation, and development of premalignant lesions while ER β reduces proliferation and in general acts as a tumor suppressor (44). Therefore, it has been hypothesized that tissue expression levels of estrogen receptors radically affects growth, differentiation, malignancy and treatment of prostate cancer. The lycopene-containing diets may reduce prostate cancer risk by inducing 17 β -HSD IV and reducing estrogen receptor alpha expression in CMO-I KO mice.

Overall, the lycopene-containing diets (LYC and TP) reduced serum and testes testosterone, expression of testicular androgen receptor, testicular estrogen receptor, and altered other sex steroid metabolizing genes in CMO-I KO mice. CMO-I KO mice have significantly induced expression of the eccentric carotenoid cleavage enzyme, CMO-II, in the testes and reduced tissue lycopene accumulation compared to wild-type mice (Chapter 2). Therefore, we hypothesize that testosterone is significantly reduced in CMO-I KO mice due to increased production of lycopeneoids.

Evidence suggests the positive effects of the lycopene-containing diets on sex steroid status is due to lycopeneoids rather than decreased production of retinal by CMO-I cleavage. Although CMO-I KO mice have a substantially reduced ability to convert pro-vitamin A carotenoids to retinal, vitamin A deficiency has not been noted in these mice (37, 38). The diet

provided a reduced level of retinyl palmitate relative to NRC requirements for rodents, but this level is adequate enough to avoid vitamin A deficiency symptoms in the current and previous longer term feeding studies (37, 38). Interestingly, we have determined that wild-type mice lack the mRNA expression of CMO-I in the prostate (Chapter 2). This might suggest that retinoids do not have a functional role in the prostate of adult male mice. Therefore, lycopeneoids, but not retinoids, may have reduced serum and testes testosterone levels in mice consuming LYC or TP diets.

Peroxisome proliferator-activated receptor gamma (PPAR- γ) agonists have been shown to inhibit the production of estradiol and testosterone, decrease prostate tumor growth, and decrease a signaling target of DHT, prostate specific androgen (PSA) (53-56). We previously showed that lycopene diets decrease the expression of PPAR- γ in the kidney and adrenal of rats (57). In contrast, we also reported that a lycopene-containing diet increased the expression of PPAR- γ in the prostate of castrated rats (25). In the current study, lycopene may have reduced testosterone production by targeting PPAR- γ . The mRNA expression of hepatic PPAR- γ in CMO-I KO mice was significantly up-regulated when mice consumed the LYC or TP diets (Figure 4.3). This study suggests that activation of PPAR- γ by lycopene or its metabolites may inhibit testosterone synthesis.

Surprisingly, some alterations in sex steroid status were also found in CMO-II KO mice. The lycopene-containing diets increased the mRNA expression of 17 β -HSD IV and estrogen receptor alpha in the testes of CMO-II KO mice (Table 4.2). These data may suggest that estradiol synthesis is down-regulated and negative feedback is causing increased expression of the estrogen receptor in the testes of CMO-II KO mice. Testosterone levels in the testis were also

decreased in CMO-II KO mice consuming the lycopene diet. These data suggest that intact lycopene may have some moderate effects on estrogen and testosterone concentrations or activity.

The basal expression of steroid metabolizing genes was altered in the transgenic mice and may predict prostate cancer susceptibility in these models. Overall, significant changes in the mRNA expression of 5 α -reductase I and 17 β -HSD IV were identified in the testes and alterations in expression were found in both isoforms of 17 β -HSD within the prostate of CMO-I KO mice (Figure 4.2 and Table 4.4). CMO-II KO mice had significant alterations in the mRNA expression of 5 α -reductase I and II and aromatase in the testis, and alterations in the expression of the androgen receptor in the prostate of CMO-II KO mice.

Consuming lycopene-containing foods may prove a cost-effective, dietary approach to reduce prostate cancer risk with few side effects. In this short term, 4-d feeding study, the lycopene-containing diets reduced serum and testes testosterone and decreased expression of 5 α -reductase I and the androgen receptor in the testes of CMO-I KO mice. The lycopene-containing diets also reduced expression of 17 β -HSD IV and the estrogen receptor in the testes of CMO-I KO mice. Our previous work with rats demonstrates that 4-d of feeding lycopene or tomato powder reduces serum testosterone (25) but this effect was not seen with chronic feeding for months (26, 45). This may suggest that intermittent consumption of tomato powder or lycopene may have anti-androgenic effects in the testes which matches human feeding patterns whereby man may eat 3-5 servings of tomatoes a week to reduce prostate cancer risk.. Clearly, more work needs to be done to follow-up on this speculation.

Evidence suggests CMO-I KO mice may produce more bioactive lycopene metabolites in the testes compared to wild-type mice, therefore, we hypothesize that the positive effects of the lycopene-containing diets found in this study can be attributed at least in part to lycopeneoids.

This is the first report of the effects of dietary lycopene on estrogen status in the mouse testes and prostate. Furthermore, these data suggest that consuming tomato products or lycopene a few days a week over a lifetime may reduce prostate cancer risk by moderately reducing androgens and estrogens.

Table 4.1. Primers used for Real-Time PCR analysis.

Gene	Accession number	Forward/reverse primers
Androgen receptor	NM_013476.3	5'-GGCCCCATCCAAGACCTATC-3' 5'-CAGCTGCCTCCCCGAGCC-3'
SHBG	NM_011367.2	5'-ACATTGACCCTGCCCTGAGAC-3' 5'-CGCTCGTGCCTGCCCTG-3'
Aromatase	NM_007810.2	5'-CCC GGA AAC TGT GAC TGT CA -3' 5'-GAG AAG GAG GCC CAT GAT CA -3'
5 α -reductase I	NM_175283.3	5'-CTGGAGGGTTTCCTGGCTTTC-3' 5'-AAGCCCACCCTGCTGTTCAC-3'
5 α -reductase II	NM_053188.2	5'-GTGCCATCAGGTCCCGGT-3' 5'-GCTGGGTCTCTTCTCCGCACA-3'
17 β -HSD I	NM_010475.1	5'-GCT CCT CTG GAA TCG GCA TGC-3' 5'-CCA CAC GAC CCT CAG TCA CG-3'
17 β -HSD IV	NM_008292.2	5'-CCA TCG TCA GAA AGC GGA AT-3' 5'-CTT CTC CCA GTT GTC CCT CAC T -3'
Cyp26a1	NM_007811.1	5'- CAG TGC TGC GAC ATC ACT GA-3' 5'-CGA ACT TTC TGG AGG ACA TGT G-3'
PPAR- γ	NM_011146.2	5'-GCC CAC CAA CTT CGG AAT C-3'

Table 4.1 (cont.)

Gene	Accession number	Forward/reverse primers
		5'-TGC GAG TGG TCT TCC ATC AC-3'
18S	NR_003278.1	5'-GAT CCA TTG GAG GGC AAG TCT-3'
		5'-AAC TGC AGC AAC TTT AAT ATA CGC-3'
β -actin	NM_007393.2	5'-CGT GAA AAG ATG ACC CAG ATC A-3'
		5'-AGC CTG GAT GGC TAC GTA CAT G-3'
L7a	NM_013721.3	5'-CCT CTA CAA GCG GCT CAA AGT C-3'
		5'-CCA GGG CCT GGG TGA AC-3'
ER α	NM_01193.4	5'-GGA GTA CTG CCT GCT GAA AGC T-3'
		5'-CAC AGC CTC AGC ATC TTC AAT G-3'
ER β	NM_007953.2	5'-CTC AGC CAG CCG GAA CTC-3'
		5'-CAA AAA GAG CAG TGG GTA CAG AAC-3'

Table 4.2. Testicular steroid metabolizing gene expression in mice fed lycopene-containing diets.

Gene	Dietary intervention			
	PB	LYC	AIN	TP
Androgen Receptor ¹				
Wild-type	37.1 ± 5.31 ^b	36.9 ± 7.66 ^b	39.5 ± 8.38 ^b	29.5 ± 8.32 ^{ab}
CMO-I KO	34.9 ± 6.23 ^b	22.7 ± 1.94 ^a	49.1 ± 11.1 ^{bc}	39.3 ± 9.32 ^b
CMO-II KO	42.1 ± 7.23 ^{bc}	56.4 ± 7.03 ^c	38.8 ± 7.40 ^b	46.4 ± 11.0 ^{bc}
5 α -reductase I ²				
Wild-type	79.2 ± 7.74 ^c	83.1 ± 6.49 ^{cd}	70.9 ± 7.83 ^{bc}	76.4 ± 6.26 ^{bc}
CMO-I KO	61.3 ± 2.86 ^b	49.7 ± 7.14 ^a	56.6 ± 7.28 ^{ab}	59.5 ± 11.1 ^{ab}
CMO-II KO	96.6 ± 9.43 ^d	85.3 ± 6.38 ^{cd}	102 ± 10.09 ^d	82.2 ± 7.80 ^{cd}
17 β -HSD I ³				
Wild-type	1.19 ± 0.15 ^{bc}	1.25 ± 0.17 ^{bc}	1.47 ± 0.21 ^c	1.26 ± 0.29 ^{bc}
CMO-I KO	0.71 ± 0.12 ^b	0.67 ± 0.39 ^b	0.25 ± 0.04 ^a	1.20 ± 0.33 ^{bc}
CMO-II KO	1.10 ± 0.26 ^{bc}	2.05 ± 0.59 ^c	1.13 ± 0.20 ^{bc}	2.23 ± 0.76 ^c
17 β -HSD IV ³				
Wild-type	58.1 ± 6.06 ^d	66.6 ± 12.14 ^{de}	68.1 ± 12.97 ^{de}	64.6 ± 10.52 ^d
CMO-I KO	34.6 ± 5.83 ^{bc}	24.5 ± 4.67 ^b	16.1 ± 2.09 ^a	40.4 ± 4.30 ^c
CMO-II KO	60.4 ± 12.0 ^d	93.5 ± 16.40 ^e	86.4 ± 9.08 ^e	153 ± 44.4 ^e
ER- α ¹				
Wild-type	244 ± 63.6 ^b	445 ± 130 ^c	187 ± 70.0 ^{ab}	385 ± 84.3 ^c
CMO-I KO	253 ± 84.8 ^b	137 ± 9.77 ^a	366 ± 95.4 ^b	224 ± 78.8 ^{ab}
CMO-II KO	232 ± 63.2 ^b	461 ± 97.1 ^c	226 ± 66.4 ^b	380 ± 83.0 ^c

Table 4.2 (cont.)¹Compared to L7a.²Compared to beta-actin.³Compared to 18s.Letters signify significantly different; $p < 0.05$. Means \pm SEM. $n = 9-11$.**Table 4.3.** Hepatic cyp26a1 mRNA expression is altered in CMO-II KO mice, but not affected by dietary lycopene or tomato powder.

Diet	Wild-type	CMO-I KO	CMO-II KO
PB	1.16 ± 0.24^a	0.95 ± 0.35^a	0.24 ± 0.08^b
LYC	1.15 ± 0.33^a	0.92 ± 0.46^a	0.36 ± 0.11^b
AIN	1.13 ± 0.33^a	0.34 ± 0.17^{ab}	0.37 ± 0.09^b
TP	0.63 ± 0.13^a	0.46 ± 0.14^{ab}	0.31 ± 0.06^b

Letters signify significantly different; $p < 0.05$. Means \pm SEM. $n = 9-11$.

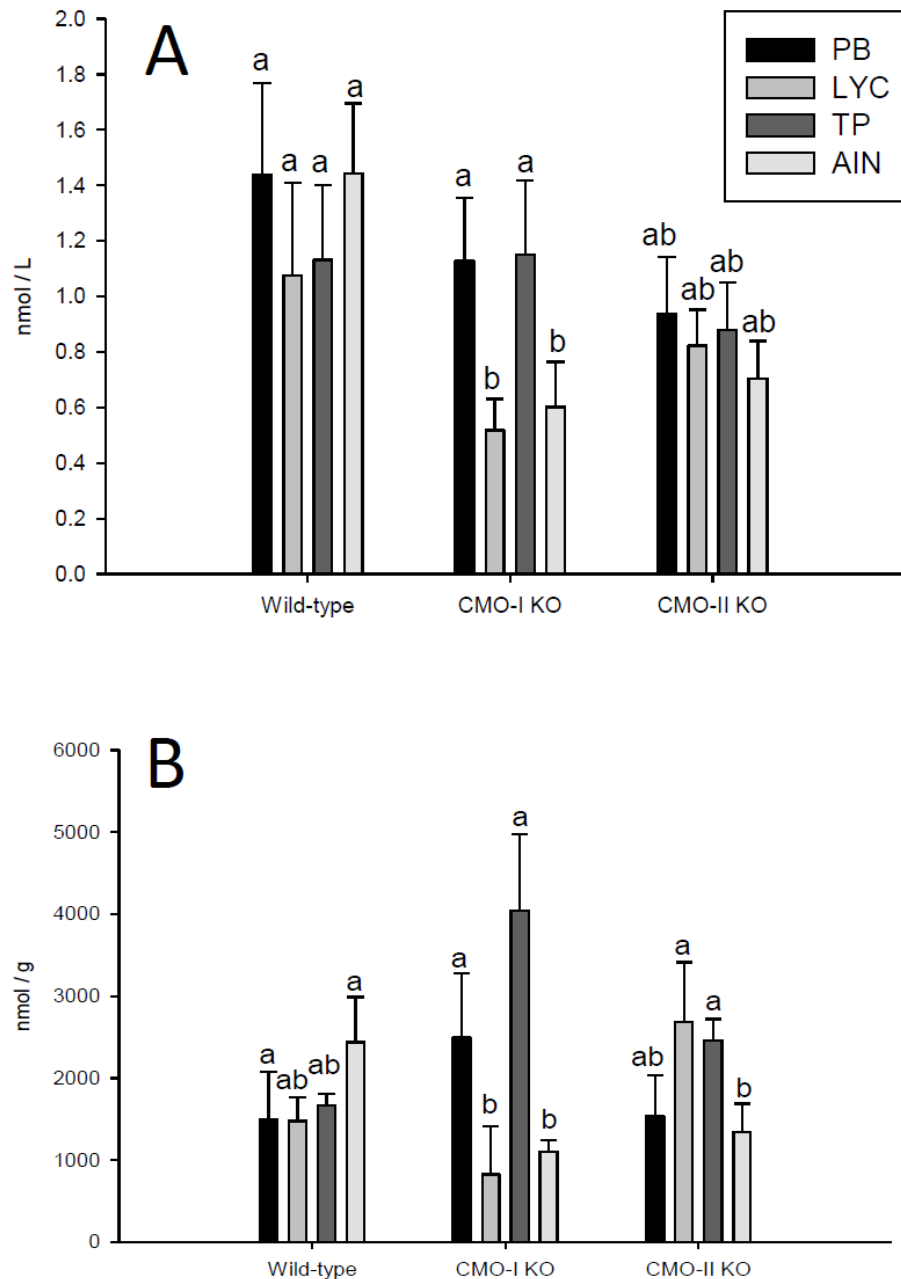


Figure 4.1. CMO-I KO mice, CMO-II KO mice, and wild-type mice were fed AIN-93G diet (AIN) or diets supplemented with tomato powder (TP), lycopene water-soluble beadlets (LYC), or placebo beadlets (PB) for 4-d and then serum and testes testosterone were measured. Serum (A) and testes (B) testosterone were quantified with a radioimmunoassay kit (DSL-4000 ACTIVE Testosterone Coated-Tube Radioimmunoassay Kits; Diagnostic Systems Laboratories). Bars represent means \pm SEM. For each variable, labeled means without a common letter differ, $p < 0.05$; serum $n = 10-15$, testes $n = 8-10$.

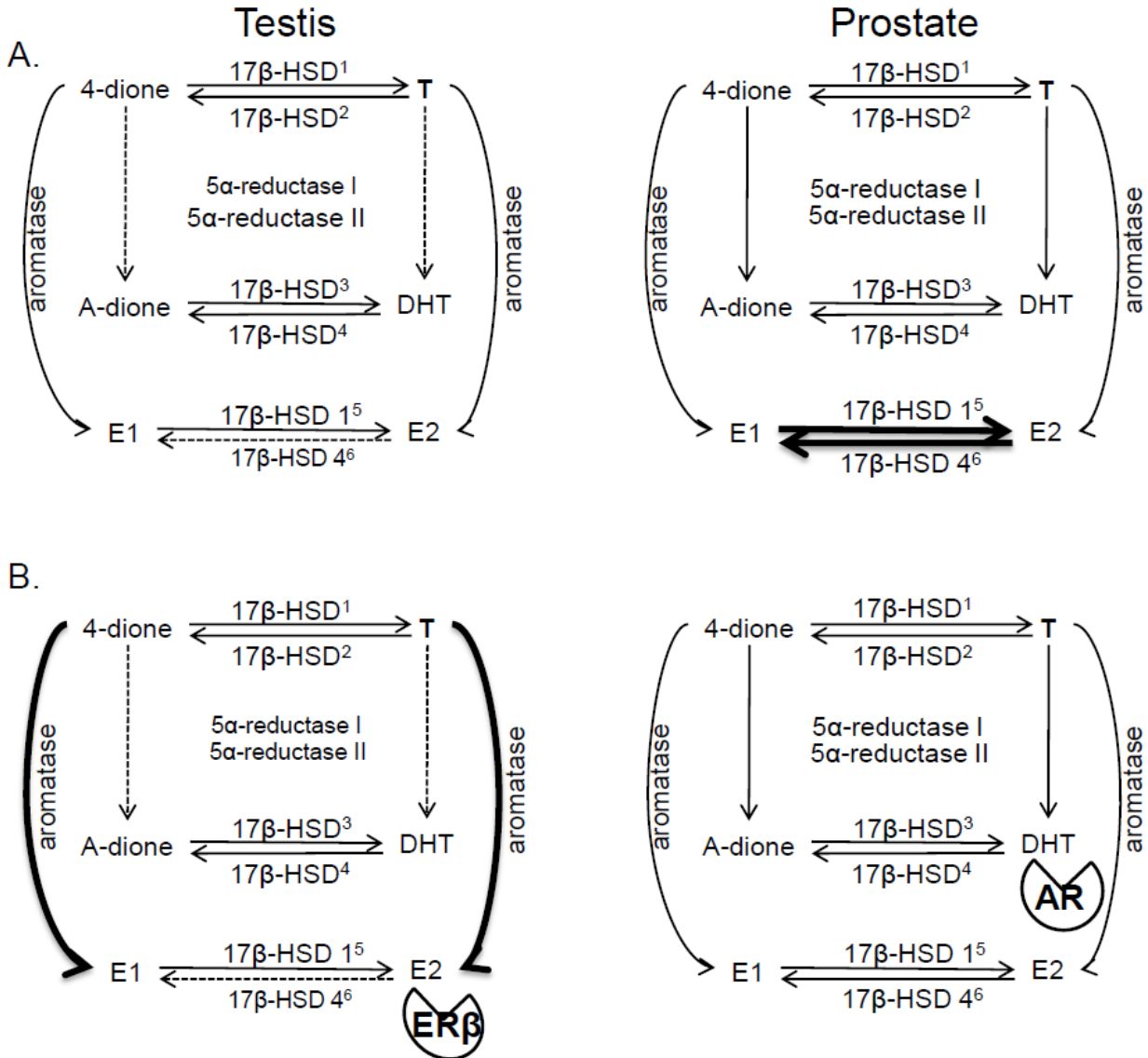


Figure 4.2. Steroid metabolizing genes were altered in the testis and prostate of CMO-I KO mice (A), and testis and prostate of CMO-II KO mice (B). mRNA expression was quantified by Real Time PCR and are expressed as $2^{-\Delta\Delta C_t} * 1000$. The mRNA expression of aromatase, androgen receptor (AR), and estrogen receptor (ER β) were compared to L7a, 5 α reductase I and 5 α reductase II were compared to β -actin, and 17 β -hydroxysteroid dehydrogenase I (17 β -HSD I) and 17 β -hydroxysteroid dehydrogenase IV (17 β -HSD IV) were compared to 18S ct values. Additional isomers of 17 β -HSD acting on these substrates include: ¹isomers 3,5; ²isomers 2,10; ³isomers 3,5,15; ⁴isomers 2,9,10; ⁵isomers 1,12; ⁶isomers 2,8,14. Adapted from Luu-The, et al (58). 4-dione, 4-androstenedione; A-dione, androstenedione; T, testosterone; DHT, dihydrotestosterone; E2, estradiol; E1, estrone. Significant increases in mRNA expression are noted with bolded line or text and reductions in mRNA expression are noted with dashed line and reduced text size.

Testis			Prostate		
Gene	Genotype	<i>P</i> -value	Gene	Genotype	<i>P</i> -value
5 α -reductase I	↓ CMO-I KO	<0.0001	17 β -HSD I	↑ CMO-I KO	<0.0001
5 α -reductase I	↓ CMO-II KO	<0.0001	17 β -HSD IV	↑ CMO-I KO	<0.0001
5 α -reductase II	↓ CMO-II KO	0.0085	AR	↑ CMO-II KO	<0.0001
Aromatase	↑ CMO-II KO	0.0071			
17 β -HSD IV	↓ CMO-I KO	0.0231			
17 β -HSD IV	↓ CMO-II KO	<0.0001			
ER β	↑ CMO-II KO	0.0232			

Table 4.4. The p-value for genes with significant alterations in basal mRNA expression for each genotype, n=9-11.

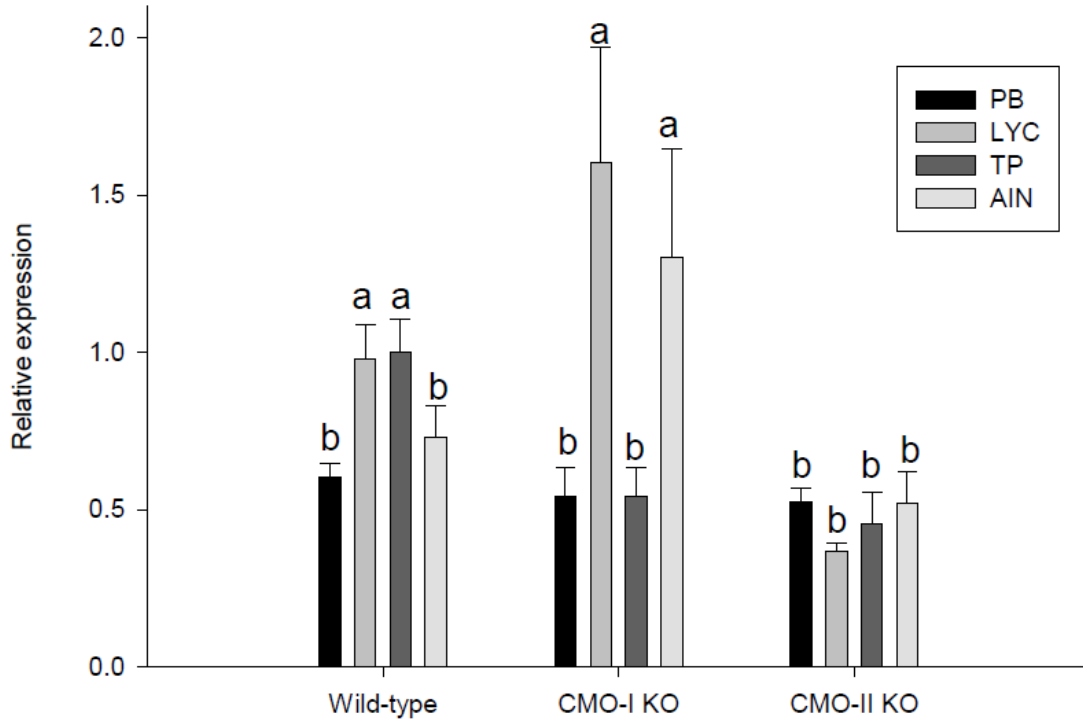


Figure 4.3. Hepatic PPAR- γ mRNA expression is upregulated in CMO-I KO mice. Results are expressed as $2^{-\Delta Ct} * 1000$ with β -actin as the control gene. Bars represent means \pm SEM. For each variable, labeled means without a common letter differ, $p < 0.05$; $n = 9-11$.

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CHAPTER V

DISCUSSION AND FUTURE DIRECTIONS

INTRODUCTION

Lycopene is the predominant carotenoid in tomato products followed by phytoene, phytofluene, and β -carotene. Beta-carotene is metabolized by carotene monooxygenase I (CMO-I) through central chain cleavage to form vitamin A and further metabolism results in formation of retinoic acid and other retinoids. Metabolism of β -carotene is widely studied, but very little is known about the metabolism of other carotenoids. *In vitro* evidence suggests that lycopene is metabolized by carotenoid monooxygenase II (CMO-II). Enzymatic cleavage by CMO-II results in eccentric chain cleavage of carotenoids. Like retinoids, we propose that carotenoid metabolites produced through eccentric cleavage by CMO-II are bioactive in small concentrations within tissues. Additionally, we hypothesize that the metabolic products of lycopene and possibly other tomato carotenoids, alone and in combination with the parent compounds, reduce prostate cancer risk. *In vivo* studies identified and characterized tomato carotenoid metabolites and oxidative products. Levels of lycopene metabolites identified *in vivo* are of comparable biological concentrations to retinoids. Recent data suggests that lycopene metabolites are biologically active and affect important molecular targets and pathways. However, at this stage, the metabolic production of non-provitamin A carotenoids is poorly understood. Since we are one of only three labs breeding CMO-I KO and CMO-II KO mice, we have the unique opportunity to investigate the metabolism of non-provitamin A carotenoids in these models. Therefore, the overall objective of this thesis is to investigate the metabolism of carotenoids from tomatoes, *in vivo*, and its impact upon prostate cancer risk factors.

Summary of study 1

In the first study, we investigated the metabolism of tomato carotenoids, lycopene, phytoene, phytofluene, and β -carotene in novel transgenic mice lacking the carotenoid cleavage enzymes CMO-I or CMO-II. Lycopene preferentially accumulated in tissues of CMO-II KO mice compared to wild-type mice thereby demonstrating that lycopene is metabolized by CMO-II *in vivo*. Interestingly, although phytoene and phytofluene are structurally similar to lycopene, neither CMO-I KO nor CMO-II KO mice preferentially accumulated these carotenoids suggesting they are not substrates for either enzyme. The differential expression of CMO-I and CMO-II in tissues suggests functional roles of lycopene or retinoids in those tissues. Of further interest, the prostate lacks CMO-I expression which suggests that retinoids do not have a function in the adult male prostate. We were surprised to find that the lycopene-containing diets did not affect the expression of either CMO-I or CMO-II in the duodenum, liver, testes, or prostate. Lastly, we again confirmed through bioaccumulation and mRNA expression data that β -carotene is primarily metabolized by CMO-I.

Summary of study 2

The second study investigated the effects of lycopene and two of its oxidative metabolites, apo-12'-lycopenal, and apo-8'-lycopenal on prostate cancer cells, *in vitro*. Androgen-independent DU145 cells were treated with physiological and supraphysiological concentrations of lycopene and its metabolites. Lycopene and apo-12'-lycopenal, but not apo-8'-lycopenal reduced proliferation of the DU145 cells only at supraphysiological concentrations. Further investigation of the mechanism of reduced proliferation suggests that lycopene and apo-12'-lycopenal inhibit normal cell cycle progression but do not alter gap junction communication of DU145 cells. This study demonstrated bioactivity of oxidative lycopene metabolites, *in vitro*.

Summary of study 3

A previous study in our lab showed that short term feeding of lycopene or tomato powder significantly reduced serum testosterone. In the third study, CMO-I KO, CMO-II KO, and wild-type mice were fed lycopene-containing diets for 4-d to investigate whether these diets influenced androgen or estrogen metabolism in mice. Serum and testicular testosterone were significantly reduced in CMO-I KO mice. CMO-I KO mice that consumed the lycopene-containing diets had decreased expression of 5 α -reductase I and the androgen receptor in the testes. Additionally, the experimental diets up-regulated expression of 17 β -hydroxysteroid dehydrogenase and decreased expression of the estrogen receptor in the testes of CMO-I KO mice. Because CMO-I KO mice have induced expression of CMO-II in the testes and reduced accumulation of lycopene, this would suggest that CMO-I KO mice have an increased production of lycopene metabolites in this tissue. Therefore, results from the 3rd study suggest that the expression of CMO-II and the production of lycopene metabolites significantly impacts sex steroid status in mice. In conclusion, a small reduction in androgen and estrogen status by tomato powder and lycopene following short term feeding might reduce the risk of prostate cancer over a lifetime.

FUTURE DIRECTIONS

Based upon the results of this dissertation, four follow-up studies are proposed:

1. TRAMP x CMO-II KO prostate cancer study
2. Identification of novel lycopene, phytoene, and phytofluene metabolites
3. Alterations in the expression of CMO-I and CMO-II in mice
4. Phenotype characterization of new transgenic mouse models

The first future study is actually supported by an NIH grant that is ongoing with collaborators at The Ohio State University. CMO-II KO mice are being crossed with a mouse model of prostate cancer, TRAMP, to investigate the effects of carotenoid metabolism on prostate cancer risk. The TRAMP x CMO-II KO and TRAMP x WT mice are being fed lycopene, tomato powder, or a control diet starting before sexual maturity or the induction of pre-cancerous changes in the prostate. We hypothesize that lycopene metabolites produced through CMO-II cleavage will be effective in reducing prostate cancer risk. Therefore, we hypothesize that wild-type mice will have significantly reduced prostate cancer progression compared to CMO-II KO mice that will produce fewer lycopene metabolites.

The second study will strengthen our current studies by identifying and quantifying carotenoid metabolites, *in vivo*. The metabolism of lycopene by CMO-II produces apolycopenals. These aldehydes are quite unstable and highly reactive therefore make identification and quantification very difficult. Additionally, because the metabolites are produced in small concentrations and mouse tissues are quite small, future studies will necessitate pooling of tissues to try to quantify metabolites. We will use an HPLC-MS tandem system to optimize use of tissue. Additionally, ¹⁴C labeled lycopene, phytoene, and phytofluene can be used to trace the metabolism of carotenoids and synthesis of new metabolites.

The third proposed study investigates the effects of castration, age, sex, and progression of prostate cancer on the tissue expression of CMO-I and CMO-II in our mouse models. Previously, TRAMP and wild-type mice were castrated or sham-castrated at 9 wk of age and sacrificed 1 wk later. The expression of CMO-I and CMO-II will be measured in the liver, prostate, duodenum, and adrenals. We also have sacrificed mice from our aging CMO-I KO, CMO-II KO, and wild-type mice colonies to investigate the effects of age and sex on the mRNA

expression of CMO-I and CMO-II in selected tissues. Male and female mice were sacrificed at 5, 9, 31, 51, and 71 wk of age. Preliminary analysis suggests that CMO-I and CMO-II are most highly expressed before sexual maturity. Preliminary analysis also suggests there is no sex difference in the expression of CMO-I or CMO-II. Further analysis of this data is required. Lastly, we have sacrificed male TRAMP mice at 9, 15, and 20 wk of age to determine the effects of the progression of prostate carcinogenesis on the mRNA expression of the carotenoid cleavage enzymes. At nine wk of age, most mice had normal prostate tissue, but by 15 wk of age, neoplasia was apparent in the prostate of these mice. The 20 wk time point has not yet been evaluated, but we expect that some 20 wk old TRAMP mice will have developed prostate tumors. The expression data has not yet been completed. All of the tissues for this proposed study have been collected. Further analysis is necessary to investigate mediating factors on the tissue expression of CMO-I and CMO-II.

Lastly, we propose further investigation of the phenotypical characteristics of CMO-I KO and CMO-II KO mice. Because these knock-out mouse models are new, very few papers have been published using these models. Preliminary evidence suggests alterations in lipid metabolism in both mouse models and alterations in organ weights needs to be further investigated. We will measure liver lipids using the Folch method, serum lipids by colorimetric assay, and genes for lipid metabolizing enzymes by RT-PCR. We have reported that female CMO-I KO mice have reduced uterus weights. This might suggest alterations in estrogen production in these mice. Serum estrogen levels and estrogen metabolizing genes should be investigated. Lastly, we have reported that CMO-II KO mice have enlarged spleens. We have histology slides prepared of a portion of each spleen from our CMO-II KO mice from previous studies. Additionally, we collected tail snip blood smears to look at erythrocyte morphology all of our mouse models.

Overall, the phenotype of CMO-I KO and CMO-II KO mice may impact study outcomes, therefore further characterization of these models is critical.

AUTHOR'S BIOGRAPHY

Nikki A. Ford was born in Slatington, Pennsylvania. She graduated from Northern Lehigh High School in May of 2000 and then attending The Pennsylvania State University from 2001 - 2005. During her tenure at Penn State, Nikki won an NSF fellowship to participate in a Biology Summer Research Program at Towson University, Maryland, working with Dr. Gerald Robinson. While at Towson, Nikki investigated hormonal control of skin hydration electrical properties in amphibians earning her first publication. Upon returning to Penn State for the fall semester, Nikki joined a lab investigating bovine and ovine reproductive physiology with Dr. Alan Ealy and then later a veterinary pathology laboratory with Dr. Biao He. Nikki decided to apply to pursue a graduate level nutrition degree when in her final semester at Penn State, she took a 400 level human nutritional biochemistry course with Dr. Thompson. Nikki graduated from Penn State in May of 2005 and joined the Division of Nutritional Sciences at UIUC in August of 2005.

Nikki completed her dissertation work relating to carotenoid metabolism and prostate cancer in the lab of Dr. John W. Erdman, Jr in 2010. She was actively involved in professional development serving as co-treasurer and treasurer of the Nutritional Sciences Graduate Student Association, student representative for the Carotenoid Research Interest Group and Gordon Carotenoids Conference. Nikki earned several awards including the ASN/Kraft Fellowship, numerous poster and oral competition awards, the Frank Kari Memorial Award, the Baker Award, and was an ASN/NSC Graduate Student Research abstract competition finalist. Nikki will be joining the University of Texas at Austin as a post-doctoral student in November of 2010 to investigate the effects of diet on breast cancer incidence.