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Iron dependency of beta-carotene 15, 15'-monooxygenase in Caco-2 TC7 cells

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ABSTRACT

Iron is an essential cofactor of β-carotene 15, 15'-monooxygenase (BCMO) which catalyzes the formation of vitamin A from β-carotene. In addition to being a cofactor, iron may play a role in the regulation of BCMO. In the present study, we investigated the effects of iron on the gene expression and enzyme activity of BCMO in the cultured TC7 clone of Caco-2 human intestinal cells, a human *in vitro* model. Cells on day 15 post-confluency were treated with ferrous sulfate ($FeSO_4$) at 0, 0.01 or 0.5 mM for 2, 4, 6 or 12 h for analysis of mRNA expression, or 2, 3, 4, 6, 24, or 48 h for determination of enzymatic activity. The results showed that compared with the 0 mM $FeSO_4$ controls, higher dose iron (0.5 mM) significantly increased BCMO mRNA expression in a time-dependent manner during 12 h of treatment with peak mRNA expression at 2 h (2.3-fold increase). The 0.01 mM $FeSO_4$ treatment enhanced BCMO mRNA expression at 2 h and 4 h incubation with iron. However, this lower concentration of FeSO4 had a lesser effect in stimulating BCMO mRNA expression than 0.5 mM $FeSO_4$, suggesting a dose-dependent enhancing effect of iron. Despite the increased mRNA expression, BCMO enzyme activities were not increased by 0.5 mM $FeSO_4$ treatment at any of the observed time points (2-48 h). These data suggest that iron up-regulates BCMO mRNA levels but not its catalytic activity under our cell culture conditions.

CHAPTER1. GENERAL INTRODUCTION

Introduction

β-Carotene 15, 15'-monooxygenase (BCMO) is a key enzyme involved in vitamin A biosynthesis from β-carotene. It cleaves β-carotene at the 15, 15' double bond to form two molecules of retinal. Retinal is further reduced to retinol in mucosal cells, then retinol will be esterified with a fatty acid to form a retinyl ester, incorporated into chylomicrons, and subsequently delivered to peripheral tissues via the lymphatic circulation (During and Harrison, 2007).

Due to the unique function of BCMO during β-carotene cleavage, its activity is especially important for people who are relying on β-carotene as their primary source of vitamin A. Therefore, there is an increasing interest in BCMO regulation. However, most studies of BCMO regulation were conducted in animal models and not in humans. Additionally, these studies concentrated on the regulatory effects of β-carotene and its metabolites. Studies of the potential significance of iron in BCMO regulation are limited.

In rats, intestinal BCMO activity was increased by vitamin A deficiency (Villard et al., 1986; van Vliet et al., 1996a), but decreased by supplements of β-carotene (van Vliet et al., 1996a) and retinoic acid (Bachmann et al., 2002; Takitani et al., 2006). These nutrients were suggested to down-regulate intestinal BCMO gene expression via interaction with retinoid acid receptors (RAR) and/or retinoid X receptors (RXR). These receptors may react with a direct repeat of retinoic acid response element (RARE) in the mouse BCMO promoter (Bachmann et al., 2002; Takitani et al., 2006). In addition, dietary polyunsaturated fatty acid was reported to enhance both BCMO activity and CRBP (II) levels in rat intestine (During et al., 1998a). Polyunsaturated fatty acid could activate peroxisome proliferator activated receptor gamma (PPAR γ) which then binds to peroxisome proliferator response element (PPRE) in the mouse BCMO promoter and CRBP (II) gene (Boulanger et al., 2003; Mangelsdorf et al., 1991; Suruga et al., 1999; Takase et al., 2000).

The relationship between iron and BCMO activity was also recognized. For example, iron concentration in the rat small intestine showed a positive association with the intestinal

BCMO activity *in vivo* (During et al., 1999 & 2000), but in this phenomenon iron was considered to be a cofactor of BCMO. A recent study (During et al., 2001) suggested that iron may have multiple roles in BCMO metabolism. TC7 cells which are derived from the Caco-2 cell line were used successfully as a human *in vitro* model since apparent substrate affinity in TC7 subclones of Caco-2 cells was found to be similar to that in human intestinal preparations. Additionally, elevated or decreased BCMO activities were observed depending upon addition of iron or desferrioxamine (DFO), respectively, into the cell culture medium. Moreover, the inhibitory effect of DFO could be reversed by iron addition to the medium. Also, a linear increase in the BCMO activity was shown with increasing incubation time up to 48 h in the presence of 0.5 mM of ferrous sulfate ($FeSO_4$), but no change in the enzyme activity was found during the first 12 h of incubation with iron. Although these data provided more evidence for iron as a cofactor of BCMO, the delayed effect (the latency of 12 h) of iron on BCMO activity strongly suggested that there is (are) mechanism(s) involving the regulation of BCMO by iron.

Based on the accumulated data, our objective is to investigate the effects of iron on mRNA expression and enzyme activity of human BCMO using TC7 cells as a human *in vitro* model. The results derived from this study will help understand the underlying mechanism of the complicated relationship between iron and BCMO.

Thesis organization

This dissertation was written in the following order: general introduction, manuscript, general conclusions, and appendices. References are listed at the end of each chapter.

Chapter 1, the general introduction containing a literature review, provides background information for our hypothesis and objectives regarding the iron effect on β-carotene 15, 15'-monooxygenase.

Chapter 2 is the manuscript describing our study of the iron dependency of β-carotene 15, 15'-monooxygenase written in journal format.

Chapter 3 presents the general conclusions from our research project with recommendations for future experiments.

The appendices are found after Chapter 3 and provide supplemental information with regard to supporting experiments used to refine and interpret our final study.

Literature Review

Vitamin A (retinol) and its derivatives (retinal and retinoic acid) are fat-soluble organic components. They are essential for mammals to maintain many normal physiological functions, such as vision, growth, cell differentiation, immunity and embryonic development (Napoli, 1996; Biesalski, 1997). Despite the nutritional importance and biological significance of vitamin A, mammals generally lack the ability to synthesize vitamin A *de novo.* Animals satisfy their own vitamin A requirement directly by consuming retinyl esters and/or indirectly by cleaving plant-derived provitamin A (mainly β-carotene, α-carotene and β-cryptoxanthin) (During and Harrison, 2007). In humans, plant-derived provitamin A carotenoids are estimated to contribute to 40-80% of vitamin A stores, and are, of course, the sole vitamin A source for vegetarians (Gong et al., 2006). Among the dietary provitamin A compounds, β-carotene is the major contributor of vitamin A due to its more widespread distribution in fruits and vegetables and higher bioefficacy when compared with other provitamin A compounds (During and Harrison, 2004).

Oxidative cleavage of β-carotene

β-Carotene is an organic pigment in plants. It contains 40 carbons and 2 unsubstituted β-ionone rings. This specific chemical structure equips the molecule to be the most potent to be converted into retinal. Retinal is the intermediate product of the conversion of retinol to retinoic acid. It can be either reduced to retinol or oxidized to retinoic acid (RA) *in vivo*. Absorbed β-carotene is first converted into retinal via an enzymatic cleavage reaction in the intestinal mucosa. Retinal is then quickly reduced to retinol by retinal reductase. Retinol will be esterified with a fatty acid to form a retinyl ester, incorporated into chylomicrons, and subsequently delivered to peripheral tissues via the lymphatic circulation (During and Harrison, 2007). Due to the pivotal role in retinal formation from β-carotene, the enzymatic cleavage mechanisms have been subjected to intense study. It has been

suggested that in mammals two enzymatic cleavage pathways are involved in converting β-carotene into retinal, referred to as central cleavage and eccentric cleavage.

The formation of vitamin A from all-trans β-carotene was first reported in 1930 (Moore, 1930). Moore found that all-trans β-carotene could be converted into vitamin A *in vivo*, and thus served as a precursor of vitamin A to meet the animal's requirement. However, the mechanism underlying the conversion of β-carotene to vitamin A remained unclear until three decades later. Glover (Glover, 1960) proposed two possible mechanisms in which β-carotene undergoes oxidative cleavage to form retinal, a direct precursor to retinol and retinoic acid. The central cleavage pathway postulated that all-trans β-carotene is cleaved at central double bond (15, 15' position) and as a result yields two molecules of all-trans retinal. Eccentric cleavage was suggested as a stepwise cleavage activity. In the eccentric cleavage pathway, β-carotene is first converted to β-apocarotenals (cleavage position is an eccentric double bond rather than the 15, 15' double bond). The β-apocarotenal is subject to subsequent cleavage and eventually produces one molecule of retinal.

Several years later, direct evidence for the central cleavage mechanism was provided by two separate research groups (Olson and Hayaishi, 1965; Goodman and Huang, 1965). Although performed under independent experimental conditions, both studies demonstrated an enzymatic oxidative reaction of β-carotene and showed that vitamin A was synthesized from β-carotene within cell-free homogenates of rat intestine or liver. Retinal was the first and sole cleavage product of β-carotene. Central cleavage (cleavage at 15, 15' double bond) was suggested to be involved in the formation of retinal. The soluble supernatant fraction was found to catalyze the reaction and oxygen was required. Bile salts, which might be essential for the uptake and cleavage of β-carotene by gut slices *in vitro* and *in vivo*, are not required for cleavage of β-carotene in intestinal homogenates. Due to these properties the enzyme was termed β-carotene 15, 15'-oxygenase (E.C. 1.13.11.21). Further observations suggested this oxygenase had dioxygenase activity. Molecular oxygen was suggested to react with the two central carbon atoms (C-15 and C-15') of β-carotene, followed by a central cleavage within β-carotene (Goodman et al., 1966a; Fidge et al., 1969). However, recent studies suggested that a monooxygenase mechanism may better explain this enzyme's oxidative cleavage (Leuenberger et al., 2001). This newly proposed mechanism suggested

that the oxygen molecules in the two cleavage products (retinal) of β-carotene are derived from different sources. It was demonstrated that the oxygen atoms from both $^{17}O_2$ and $H_2{}^{18}O$ were incorporated into the retinal products. Based on these findings, the enzyme responsible for central cleavage of β-carotene has been renamed as β-carotene 15, 15'-monooxygenase (BCMO, E.C. 1.14.99.36) (Wirtz et al., 2001).

In contrast to the widely accepted role of central cleavage in β-carotene metabolism, the eccentric cleavage pathway had been the subject of controversy and debate since Glover proposed this hypothesis in 1960. The debate was mainly focusing on the existence and importance of this alternative pathway. The existence of the pathway began to be accepted once there was clear *in vitro* and *in vivo* evidence that apo-carotenals and retinoic acid were detected in significant amounts as the cleavage products of β-carotene (Wang et al., 1991; 1992; 1993). The subsequent work of cloning and identification of a mammalian enzyme responsible for the asymmetric oxidative cleavage of provitamin A further supported the existence of the eccentric cleavage (Kiefer et al., 2001). This enzyme was reported to specifically cleave β-carotene at the 9', 10' double bond to yield β-apo-10'-carotenal and β-ionone.

A systematic comparison of the extent of central versus eccentric cleavage contributing to vitamin A formation in mammals was explored by several later studies (Nagao et al., 1996; Wolf, 2001). The central cleavage mechanism was seen to be the predominant mechanism in mammals whereas apo-carotenals were found in trace amounts as a minor product of the cleavage of β-carotene. Additional research suggested that eccentric cleavage more likely occurred under specific conditions, such as low concentrations of antioxidants and/or high levels of β-carotene. Otherwise, the central cleavage pathway predominates over its eccentric counterpart in the β-carotene metabolic process under normal physiological conditions (Yeum and Russell, 2002).

Physiological and biochemical properties and molecular characterization of BCMO

As a key enzyme catalyzing the central cleavage of β-carotene, BCMO has been intensely studied since 1960s. Due to the technical limitations in protein purification, prior to the 1990s, most of the cleavage activities of BCMO were analyzed using various tissue

homogenates or partially purified protein from these tissue homogenates. Although crude proteins were applied in these studies, the enzymatic activity and other biochemical properties of BCMO from different species and tissues were preliminarily identified. In 2000, the first BCMO was cloned from *Drosophila melanogaster* (von Lintig and Vogt, 2000). Thus, a new era was opened to better understand the activity of BCMO based on the purified protein. Subsequent studies of recombinant BCMO from different species revealed the more detailed biochemical properties of the enzyme.

Species and tissue distribution

BCMO activity has been measured in a wide variety of mammalian species including humans, rat, guinea pig, rabbit, hamster, chicken, sheep, goat, bovine and pig (Lakshman and Okoh, 1993; Goodman and Huang, 1965; Singh and Cama,1974; van Vliet et al., 1996a; Bachmann et al, 2002; Yang and Tume, 1993; Nagao et al., 1996). In these mammalian species, small intestinal mucosa has the highest BCMO activity, and other organs and tissues, i.e., liver, lung, kidney and brain, have comparably low enzyme activities (Wyss, 2004). In accordance with the BCMO enzyme activities, BCMO mRNA expression was subsequently determined in different human tissues. Higher levels of BCMO mRNA expression were present in the entire intestinal tract, liver and kidney, but lower levels BCMO mRNA were found in the prostate, testis, ovary, and skeletal muscle (Lindqvist and Andersson, 2002). BCMO mRNA was also reported to be expressed in monkey retina and human retinal pigment epithelium (RPE) (Bhatti et al., 2003). The data suggested that human RPE has higher levels of BCMO mRNA than other organs and tissues such as liver, kidney, intestine, or testes (Yan et al., 2001). However, high activities of BCMO in the small intestine could well explain the phenomenon in this tissue of the conversion of the majority of provitamin A to vitamin A, which is then transported to peripheral tissues via the circulation. The peripheral BCMO might play an alternative role in the conversion of the circulating provitamin A carotenoids to local vitamin A to meet special needs or during deficient status (Lindqvist and Andersson, 2002).

Optimal reaction conditions and kinetic parameters

As a cytosolic soluble protein, the BCMO enzyme preparation is usually prepared by centrifugation at 9,000 g for 0.5 h. Supernatant (S-9) was shown to be the cell fraction with

the highest BCMO cleavage activity (van Vliet T et al., 1996a). The optimal pH for BCMO activity is slightly alkaline, although the pH optimum for BCMO may vary across mammalian species. For example, the guinea pig BCMO enzyme has a pH optimum at 8.5, the rabbit enzyme has a slightly lower pH optimum at 7.8 (Singh and Cama, 1974), while the hog BCMO has a narrow pH optimum range (7.8-8.2) (Fidge et al., 1969). Human BCMO has the highest enzymatic activity at pH 8.0 (Lindqvist and Andersson, 2002). Bile salt or appropriate detergent is required for BCMO activity and the addition of appropriate combinations of detergent and lipid (i.e. Tween-40, sodium glycocholate and sphingomyelin) was shown to increase the activity of enzyme cleavage (Fidge et al., 1969). The Michaelis-Menten constant (Km) of BCMO was reported to be 1-10 μM depending on its tissue source. Some sulfhydryl reducing agents including cysteine and glutathione could protect or increase BCMO enzyme activity (Lindqvist and Andersson, 2002). Protein structure studies indicated that BCMO is a non-heme iron-containing oxygenase with conserved histidine and aspartate/glutamate residues across species. The conserved oxygenase structure might be the essential motif for binding iron (Lindqvist and Andersson, 2002; Poliakov et al., 2005). This concept could well explain why iron acts as a cofactor for BCMO activity (Singh and Cama, 1974).

Substrate specificity

BCMO is responsible for catalyzing the cleavage of β-carotene at the central double bond into 2 molecules of retinal. Substrate specificity studies suggest BCMO only attacks the 15, 15' double bond when it is not blocked by other structures such as aromatic rings (Woggon, 2002). Also, at least one unsubstituted β-ionone ring is imperative for cleaving the 15, 15'-double bond. Therefore, β-carotene acts as a substrate with high efficiency, since it has two unsubstituted β-ionone rings and no geometrical constraints at its central core. In addition to β-carotene, other carotenoids such as α-carotene, β-cryptoxanthin, β-apocarotenals, and β-apocarotenols which possess one β-ionone ring, can serve as a substrate of BCMO but with lower cleavage efficiency (Lindqvist and Andersson, 2002).

In addition, other studies on substrate specificity demonstrated that β-carotene acts as the optimal substrate for BCMO because any modification of the isoprenoid chain, i.e. shortening and cis-isomerization, may lead to a significant decrease in retinal formation.

Moreover, the structure containing two β-ionone rings may be beneficial for a higher efficiency of BCMO catalyzation. The first β-ionone ring binds to a hydrophobic area in the enzyme active site, which facilitates the second β-ionone ring to undergo chemical modifications and subsequently to induce conformational changes involved in the enzyme cleavage reaction. This phenomenon can explain why other provitamin A substrates with one β-ionone ring showed 16-46% of β-carotene activities (Grolier et al., 1997).

BCMO activity in human cell line

The activity of BCMO was first assayed in the human intestine *in vivo* using radioactive tracers (Goodman et al., 1966b), and then *in vitro* studies confirmed this enzyme activity in the intestinal mucosa of human neonates (Lakshman and Okoh, 1993). Due to a more specific tissue distribution of BCMO in humans and limited availability of such human tissues, BCMO studies using human tissues were rather limited until human cell lines containing BCMO activity were developed.

Although some cell lines, such as a human adenocarcinoma colon cell line (Caco-2) (Quick and Ong, 1990) and two human lung fibroblastic cell lines (WI38 and HLF) (Scita et al., 1992), are able to produce retinoids when incubated with β-carotene, no BCMO activity was detected in these cell lines. A breakthrough in 1998 enabled BCMO studies in human cell lines. During et al. (1998a) found significant BCMO activity in two subclones of Caco-2 cells (PF11 and TC7) using a sensitive HPLC method. Compared with PF11, TC7 has higher BCMO activities (14.7 pmol/h.mg vs 2.5 pmol/h.mg) and therefore is more suitable for research on BCMO activity. TC7 cells could be used as a convenient cell model for studying BCMO activity and β-carotene metabolism *in vitro*.

The human RPE cell line D407 was reported to possess BCMO activity (Chichili et al., 2005). BCMO mRNA, protein expression, and enzyme activity were detected in this cell line, and the effects of β-carotene and its metabolites on BCMO in this cell line have been investigated. These studies using the human RPE cell line D407 suggested that it is possible to study β-carotene and its metabolism in peripheral tissues.

Molecular characterization and identification of BCMO

Since the first cloned BCMO from *Drosophila melanogaster* was reported by von Lintig and his colleagues (2000), identification, molecular cloning, and functional

characterization of BCMO were widely described across different species including chicken (Wyss et al., 2000), mouse (Paik et al., 2001; Redmond et al., 2001), human (Yan et al., 2001), rat (Bachmann et al., 2002), and zebrafish (Lampert et al., 2003).

The encoded protein size varies to some extent in different species. For example, human (GenBank™ accession NP_059125) BCMO cDNA encodes a hydrophilic protein of 547 amino acids, whereas BCMO cDNAs of *Drosophila melanogaster,* chicken, mouse, rat and zebrafish (GenBank™ accession CAB93141, Q9I993, NP_067461, NP_446100, NP_571873, respectively) contain 620, 526, 566, 566 and 516 amino acids, respectively. Compared with the human BCMO amino acid sequence, the mouse counterpart showed the highest homology with 85% sequence identity, followed by rat with 84%, chicken with 67%, and zebrafish with 56%. The *Drosophila melanogaster* BCMO showed the largest discrepancy with the human enzyme with only 22% amino acid sequence identity (Lindqvist and Andersson, 2002).

After intensely exploring the encoding frame of BCMO, interest has shifted to its promoters and response elements in the promoter area which may be related to its transcriptional regulation. Three elements have been reported so far in different animals. In the mouse BCMO promoter, a direct repeat RA responsive element (RARE) with a space of two nucleotides was identified by Bachmann et al. (2002). Soon after that, the peroxisome proliferator response element (PPRE) was found in the mouse BCMO promoter, and can be specifically bound by the peroxisome proliferator activated receptor gamma (PPAR γ) (Boulanger, 2003). This PPRE was also found in the human BCMO promoter (Gong et al., 2006), and works synergistically with another element, myocyte enhancer factor (MEF2) binding element, to regulate BCMO expression. Mutation of either element could decrease basal promoter activity, and mutation of both results in the abrogation of BCMO expression in intestinal cells. The absence of MEF2 binding element in mouse promoter and the cooperation of MEF2 and PPAR in human BCMO expression may explain the different conversion efficiencies of β-carotene in intestinal mucosa across species. In rodents, almost all of absorbed β-carotene is converted into retinal in the small intestine, while in human small intestine, about 60-70% of β-carotene undergoes cleavage and the rest remains intact to be delivered to liver, adipose, and other tissues (Wyss, 2004).

Regulation of BCMO

Although the role of BCMO in conversion of β-carotene to retinal has been known for a long time, the molecular bases of regulation of the enzymatic activity are rather uncertain. The few existing reports about BCMO regulation mainly focused on two aspects. One is that the enzyme is regulated by some compounds which share a similar structure or function with β-carotene, such as other provitamin A and non provitamin A carotenoids, phytochemicals, and antioxidants. Another addresses some nutritional factors related to the absorption and/or metabolism of β-carotene, such as performed vitamin A, β-carotene and its metabolites, and dietary fatty acids. The underlying mechanisms of enzymatic regulation were also touched upon in these studies.

Regulation of enzyme activity

Dietary carotenoids were first found to have inhibitory effects on BCMO activity. Lycopene, lutein and astaxanthin competitively inhibit BCMO activity *in vitro* (Ershov et al., 1993; 1994). Canthaxanthin (mixed-type) and zeaxanthin (noncompetitive) could inhibit BCMO activity by 71% and 40%, respectively *in vitro* and their inhibitory effects were further confirmed in an *in vivo* study (Grolier et al., 1997). Competitive inhibition indicates that canthaxanthin, although not serving as a substrate, could compete with β-carotene and bind to the enzyme active site, whereas noncompetitive inhibition by zeaxanthin may be accounted for by the nonspecific interaction of fat-soluble carotenoids and a hydrophobic site other than the substrate binding site of enzyme. In addition, α-carotene and β-cryptoxanthin exhibited inhibitory effects *in vitro* (van Vliet T et al., 1996a). In general, most studies supported the ability of carotenoids to inhibit BCMO activity although there were some discrepancies. For example, the inhibitory effects of lycopene and lutein varied in different studies (van Vliet T et al.,1996a; Grolier et al., 1997).

Both antioxidants and dietary flavonoids were observed to inhibit BCMO activity *in vitro* (Nagao et al., 2000). In this study, pig intestinal homogenate was used as a BCMO enzyme source, and a variety of antioxidant and dietary flavonoids was applied to evaluate their inhibitory capacities. The results suggested that 2,6-Di-tert-butyl-4-methylphenol (BHT), a synthetic antioxidant, was a strong inhibitor of BCMO (mixed-type inhibition). BHT resulted in the largest reduction in retinal formation even at a very low concentration

(10^{-6} M), while other antioxidants, such as butylated hydroxyanisole, nor-dihydroguaiaretic acid, n-propyl gallate, and curcumin, showed moderate inhibitory effects on the BCMO cleavage activity. Flavonoids including luteolin, quercetin, rhamnetin,and phloretin also showed significant inhibition of the enzyme activity with a noncompetitive mechanism. Study of the structure-activity relationship indicated that the phenolic hydroxyl group and the carbon skeleton of BHT and catechol structure of ring B of flavonol are essential for their inhibitory potency. The inhibitory effects of BHT (2 μM) and rhamnetin (5 μM) on BCMO was also confirmed in a Caco-2 cell line model.

Regulation of transcriptional level

Dietary β-carotene, which dissolves in lipid droplets and then is incorporated into mixed micelles containing bile salts and hydrolates of dietary triglycerides, enters the mucosa of the small intestine via a scavenger receptor class B type I(SR-BI) transporter facilitated diffusion. In the small intestine, retinal, formed by conversion of β-carotene via BCMO cleavage activity, is reduced to retinol and subsequently to retinyl ester. Retinyl ester is then incorporated into chylomicrons and delivered to tissues through the lymphatic circulation (During and Harrison, 2007). Cellular retinol-binding protein type II (CRBP II), as a protein with highly restricted tissue distribution (only identified in the small intestine in humans), could combine with retinal to facilitate the subsequent enzymatic reaction, leading to formation of retinyl ester (Ong, 1993).

Based on the absorption and metabolism of β-carotene, a few related nutritional factors were evaluated for their effects on BCMO regulation and possible mechanisms were proposed. Conversion of β-carotene was considered to be tightly regulated to maintain vitamin A homeostasis since high-dose β-carotene supplements in human did not to result in any signs of hypervitaminosis A or teratogenicity (Mathews-Roth, 1993). Therefore, most of studies regarding BCMO regulation focused their interest on the effect of β-carotene as well as its metabolites such as retinol, retinyl ester and retinoic acid.

In rats, intestinal BCMO activity was increased by vitamin A deficiency (Villard et al.,1986; van Vliet T et al., 1996b .), but decreased by β-carotene supplements (van Vliet et al., 1996b), and retinoic acid (Bachmann et al., 2002; Takitani et al., 2006). The underlying mechanisms may be explained by the recent finding of a direct repeat of RARE in the mouse

BCMO promoter. RA and 9-*cis*-RA were suggested to down-regulate the intestinal BCMO gene expression via their interaction with retinoid acid receptors (RAR) and/or retinoid X receptors (RXR) (Bachmann et al., 2002; Takitani et al., 2006). Exogenous β-carotene was found to temporarily up-regulate, followed by down-regulation of, BCMO mRNA expression in a human retinal pigment epithelial (RPE) cell line, while addition of RA down-regulated BCMO mRNA expression in the same cell line (Chichili et al., 2005). This finding indicated not only the presence of the pathway for retinal formation in peripheral tissues, but also regulation of BCMO in RPE by β-carotene and retinoic acid. In addition, the finding that RAR-α and RXR-α mRNA expression was markedly induced by retinoic acid *in vitro* further confirmed the previous hypothesis about the transcriptional regulation of BCMO by retinoic acid via interaction with RAR and/or RXR (Bachmann et al., 2002).

It was reported that dietary polyunsaturated fatty acids could enhance both BCMO activity and CRBP (II) level in rat intestine (During et al., 1998b), suggesting that the same mechanism may be involved in regulating both proteins. The presence of PPRE in the mouse BCMO promoter seems to provide the molecular basis for this phenomenon (Boulanger et al., 2003). PPARγ, a member of the steroid hormone receptor super family, can be activated by polyunsaturated fatty acids. It was shown that PPARγ specifically bind to PPRE in TC7 and PF11 cells. The binding of PPARγ to PPRE possibly needs the heterodimeric complex with RXRα. Treatment of mice with PPARα/γ agonist could increase the BCMO protein expression in liver. PPRE was also identified in the CRBP II gene, which could be up-regulated by fatty acids possibly via PPAR or by retinoic acid via RXR (Mangelsdorf et al., 1991; Takase et al., 2000; Suruga et al., 1999). The identification of PPRE in human BCMO further indicated that PPAR was an important transcriptional factor responsible for transcriptional regulation of the BCMO gene across different species (Gong et al., 2006).

Some hormones were also suggested to affect BCMO gene expression. Glucocorticoid enhanced the duodenal BCMO mRNA during the perinatal period in chick (Yamaguchi et al., 2007), while triiodothyronine (T3) elevated BCMO mRNA level in 7 day post-confluent Caco-2 BBe cells in both dose- and time- dependent manners. BCMO activity was also enhanced by T3 treatment in Caco-2 BBe cells incubated with medium containing fetal bovine serum (Yamaguchi and Suruga, 2008).

Other possible mechanisms

In addition to the above-mentioned regulatory mechanisms of BCMO, BCMO activity was found to be inhibited by protein deficiency, possibly due to limited availability of raw materials of protein synthesis (Gronowska-Senger and Wolf, 1970). Moreover, BCMO activity could be enhanced by fructose feeding, copper depletion, and high iron status. The underlying mechanism of effects on BCMO under these conditions was suggested to be related to the oxidative stress promoted by these three independent factors and the induced high iron concentration in the small intestine (During et al., 2000). Further studies are required to characterize those factors that influence BCMO activities and their regulation mechanisms.

Iron effect on BCMO regulation

Iron is required for the function of many proteins. These proteins are involved in multiple cellular physiological activities including DNA synthesis, oxygen transport and electron transport (Drakesmith and Prentice, 2008). As a crucial trace element for human health, the metabolism of iron has been well elucidated.

Iron uptake and metabolism

Under normal conditions, iron homeostasis is maintained via feedback regulation on intestinal iron absorption by the body's iron needs. Low iron leads to iron deficiency, but excess iron results in iron toxicity. Dietary iron absorption mainly occurs in the duodenum. The ferric iron is reduced to the ferrous state by a duodenal ferric reductase (Dcytb) on the apical surface of enterocytes before absorption. Ferrous iron is then transported into cells by divalent metal transporter 1 (DMT 1). Heme, another source of iron, is internalized into enterocytes via a heme carrier protein-1 (HCP1). Iron absorbed by enterocytes is either stored in ferritin or transported out of the basolateral membrane by a transporter called ferroportin -1 (FPN1). Extracellular iron is then bound by a type of serum iron-transport protein named transferrin (Tf) and released into the circulation. Circulating Tf-binding iron is recognized by Tf receptor 1 (TfR 1) and taken up by certain types of cells through receptor –mediated endocytosis for cellular use or storage. Iron is used for a wide variety of

metabolic activities in the main forms of heme and iron-sulfur (Fe-S) cluster (Fleming and Bacon, 2005; Dunn et al., 2007).

Iron effect on BCMO activity

The history of study of the relevance of iron to BCMO could date back to the mid 1960s. Two effective metal chelators, α,α'-bipyridyl and o-phenanthroline, were revealed *in vitro* to remarkably inhibit BCMO activity in homogenates of rat intestine, rat liver and hog intestinal mucosa (Olson and Hayaishi, 1965; Fidge et al., 1969). The inhibitory effects of these two chelators on BCMO were confirmed in guinea pig and rabbit models. *In vitro*, addition of ferrous iron stimulated BCMO in a concentration-dependent manner. The addition of ferric iron (Fe^{3+}) showed no effect on BCMO activity (Singh and Cama ,1974). Metal chelating agents, i.e. α,α'-bipyridyl and o-phenanthroline, were shown to have similar inhibitory effects on recombinant BCMO activity. These results suggested that iron is a metal ligand of BCMO (Lindqvist and Andersson, 2002). Although this evidence suggested that ferrous iron is an essential cofactor for BCMO protein, there was no further investigation of the molecular basis of iron binding to BCMO until the identification of the essential structure for iron-binding in recombinant mouse BCMO. Four conserved histidines and Glu405 residues were indicated to be necessary for iron coordination. Any one mutation in these five residues would cause complete loss of activity (Poliakov et al., 2005).

Although there are many *in vitro* experiments, few *in vivo* studies have been conducted in rats regarding the iron effect on intestinal BCMO activity. However, the iron concentration in the rat small intestine was found to be positively associated with intestinal BCMO activity in two *in vivo* studies (During et al., 1999 & 2000).

Certain progress has been made in researching the effect of iron on BCMO both *in vitro* and *in vivo*; most of these studies have been limited to animals or animal-derived cell lines. To our knowledge, the sole study regarding iron effect on BCMO using human *in vitro* models (human intestinal tissue preparation and TC7 cells) was carried out by During's group (2001). Desferrioxamine (DFO), a strong iron chelator which has been used to treat iron overload since the 1960s, was added to human intestinal enzyme preparations and the medium for TC7 cells at different concentrations. Inhibitory effects on BCMO activity were observed in both *in vitro* human models. The decreasing rate of the enzyme activity was

positively correlated with the concentration of DFO. Approximately 50% inhibition of the enzyme activity in TC7 cell model occurred when 0.5 mM of DFO was present, and the inhibitory effect reached a plateau (70%) as DFO concentration was raised to higher than 1 mM. A 50% inhibition in intestinal enzyme preparations from 3 different human subjects was found in the presence of 0.5-2 mM of DFO. Another observation was that iron addition in the medium could increase BCMO activity and reverse the inhibitory effect of DFO in the TC7 cell model. However, the increased enzyme activity and reversible DFO effect were not seen when iron was added in the reaction mixture containing the human intestinal preparation. Additionally, the enhancement of BCMO enzyme activity in TC7 cells by iron addition started at 12 h after incubation with medium containing iron (During et al., 2001).

The observation in the TC7 model is in agreement with the previous animal studies. These data suggested that human BCMO is also iron dependent. However, there are no consistent results with regard to the effects of iron on human intestinal preparation enzyme activity, and it is unclear as to what extent those iron effects of *in vitro* and animal studies can readily translate into human BCMO. Both the inconsistent observations on intestinal enzyme preparation and the delayed effect of iron on enzyme activity in TC7 cells prompts us to hypothesize the involvement of other mechanisms in regulation of human BCMO by iron. Considering the different metabolic efficiencies of β-carotene conversion to vitamin A in humans and animals (usually rats), i.e. partial conversion versus almost complete conversion, it is likely that β-carotene conversion in human intestine is more strictly regulated. In addition, the sensitivity of BCMO activity to iron may be variable across species.

The successful application of the TC7 cell culture model in During's study (2001) provided a useful human *in vitro* model for studying the regulation of BCMO. Measured Kms of the enzyme preparation from TC7 cells and the intestinal preparation from human subjects are in close agreement. The only difference between these two sources of enzyme is the Vmax. These similar biochemical properties suggest that these two enzyme preparations have similar affinity for the substrate (β-carotene), but differ only in the amount of the enzyme. In addition, TC7 cell is a subclone from Caco-2 cells which is a good cell culture model for studying iron absorption and transport (Garcia et al., 1996).

Based on these accumulated data, our hypothesis is that iron regulates the enzyme activity of BCMO by affecting its gene expression. This hypothesis will position us to investigate the iron effect on human BCMO at mRNA and enzyme activity levels using TC7 cells as a human *in vitro* model.

To our knowledge, this is the first study of the iron effect on human BCMO gene expression beyond the known role of iron as a cofactor. Previous studies of BCMO gene regulation mostly concentrated on β-carotene and its metabolites, as well as other dietary components such as fatty acids. No interaction of a mineral with expression of this enzyme has been reported to date.

Additionally, the co-existence of vitamin A and iron deficiency has long been a focus of attention. Vitamin A deficiency is frequently companied with indices of iron deficiency. Mejia et al. (1977) reported that low serum iron and low hemoglobin (Hb) were associated with low serum retinol in children, and a lower percentage of transferrin saturation was shown in children with low serum retinol. Other observations further showed that serum retinol was positively correlated with Hb, serum iron, and percentage of transferrin saturation (Mejia & Chew,1988; Bloem et al., 1990; van Stuijvenberg et al., 1997). The interaction between vitamin A and iron is complicated and reciprocal. Vitamin A deficiency was suggested to decrease the mobilization of iron from body stores since iron supplementation could not correct the low Hb that appeared in people with advanced vitamin A deficiency (van Stuijvenberg et al., 1997). Two recent studies conducted in rats found that iron deficiency may affect vitamin A distribution between plasma and liver, thus leading to low plasma retinol as well as elevated liver retinol storage (Rosales et al., 1999; Strube et al., 2002). Considering the complex interaction of iron and vitamin A and the limited preliminary studies in this field, further studies are needed to clarify the underlying mechanisms. We expect our study will provide necessary new information for better understanding of the iron effect on vitamin A metabolism.

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CHAPTER 2. IRON DEPENDENCY OF β-CAROTENE 15, 15'-MONOOXYGENASE IN CACO-2 TC7 CELLS

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Abstract

Iron is an essential cofactor of β-carotene 15, 15´-monooxygenase (BCMO) which catalyzes the formation of vitamin A from β-carotene. In addition to being a cofactor, iron may play a role in the regulation of BCMO. In the present study, we investigated the effects of iron on the gene expression and enzyme activity of BCMO in the cultured TC7 clone of Caco-2 human intestinal cells, a human *in vitro* model. Cells on day 15 post-confluency were treated with ferrous sulfate ($FeSO_4$) at 0, 0.01 or 0.5 mM for 2, 4, 6 or 12 h for analysis of mRNA expression, or 2, 3, 4, 6, 24, or 48 h for determination of enzymatic activity. The results showed that, compared with the consistent control, higher dose iron (0.5 mM) significantly increased BCMO mRNA expression in a time-dependent manner during 12 h of treatment with a peak at 2 h (2.3-fold increase). Lower dose iron (0.01 mM) showed an enhancing effect after 2 h and 4 h incubation with iron, but was weaker in stimulating BCMO mRNA expression than higher dose iron, suggesting a dose-dependent enhancing effect of iron. Despite the increased mRNA expression, BCMO enzyme activities were not increased by iron treatment at any of the observed time points (2-48 h). These data suggest that iron could up-regulate BCMO mRNA level but not its catalytic activity under our cell culture conditions.

Key words: β-carotene, β-carotene 15, 15'-monooxygenase, iron, gene expression, regulation.

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Introduction

Due to their inability to synthesize vitamin A *de novo*, humans have to meet the nutritional requirements for vitamin A through dietary sources either directly by consuming animal-derived retinyl esters or indirectly by cleaving plant-derived carotenoids (mainly β-carotene, α-carotene and β-cryptoxanthin) (During and Harrison, 2007). As the most abundant carotenoid in vegetables and fruits as well as the provitamin A carotenoid with the highest conversion efficiency, β-carotene, is believed to be responsible for a substantial portion of vitamin A formation in humans (During and Harrison, 2004). Moreover, β-carotene seems to be more important for people in the developing countries due to a reliance on plant-based foods. This is also the case for vegetarians, as the name implies, whose sole source of vitamin A is plants.

The conversion of β-carotene into vitamin A has been well documented. Once absorbed in the small intestine, β-carotene undergoes a cleavage process at the central double bond (15, 15' position) to generate two molecules of retinal in mucosa cells. Retinal will then be reduced to retinol or oxidized to retinoic acid (RA; During and Harrison, 2007). Vitamin A (retinol) and its derivatives (i.e. retinal and retinoic acid) are essential for mammals to maintain many normal physiological functions, such as vision, growth, cell differentiation, immunity and embryonic development (Napoli, 1996; Biesalski, 1997).

Although the phenomenon that β-carotene is converted into vitamin A *in vivo* was first reported in 1930 (Moore, 1930), the underlying pathways and mechanisms had not been described until the 1960s. Two independent research groups found, in the presence of oxygen molecules, a soluble protein from cell-free homogenates of rat intestine and liver was involved in the central cleavage (15, 15´ double bond) of β-carotene (Goodman and Huang, 1965; Olson and Hayaishi, 1965). Later, this soluble protein was termed β-carotene 15,15´-oxygenase (E.C. 1.13.11.21) and a dioxygenase mechanism was suggested for the enzyme (Goodman et al.,1966; Fidge et al., 1969). However, recent studies showed that a monooxygenase mechanism could better explain the enzyme-mediated oxidative cleavage (Leuenberger et al., 2001). The newly proposed mechanism suggested that the oxygen molecules in the two cleavage products (retinal) of β-carotene are derived from different sources - $^{17}O_2$ and H_2O^{18}. Therefore, the enzyme responsible for the central cleavage of β-

carotene was renamed β-carotene 15, 15'-monooxygenase (BCMO, E.C. 1.14.99.36) (Wirtz et al., 2001).

BCMO has a differential tissue distribution. Small intestinal mucosa contains the highest expression of BCMO activity. However, lower enzyme activities can be seen in many other tissues and organs such as liver, lung, kidney and brain (Wyss, 2004). The active BCMO in the small intestine can well explain that the conversion of provitamin A to vitamin A mainly occurs in this tissue from which vitamin A is transported to peripheral tissues. It should be noted that BCMO in peripheral tissues plays an essential but alternate role in converting the circulating provitamin A to local vitamin A to meet special needs or during deficiency states (Lindqvist and Andersson, 2002). Nevertheless, intestinal BCMO plays a predominant role in deciding the nutritional status of vitamin A in the body. Precisely because of this, the molecular regulation of intestinal BCMO activity has been the subject of extensive investigations and some underlying mechanisms have been elucidated.

In rats, intestinal BCMO activity was increased by vitamin A deficiency (Villard et al., 1986; van Vliet et al., 1996), but decreased by supplements of β-carotene (van Vliet et al., 1996) and retinoic acid (Bachmann et al., 2002; Takitani et al., 2006). The mechanistic basis for these observed phenomena may be a direct repeat of the retinoic acid response element (RARE) in the mouse BCMO promoter. RA produced from the metabolism of β-carotene or vitamin A and 9-*cis*-RA was suggested to down-regulate the intestinal BCMO gene expression via interaction with retinoid acid receptors (RAR) and/or retinoid X receptors (RXR) (Bachmann et al,. 2002; Takitani et al., 2006).

It was reported that dietary polyunsaturated fatty acid could enhance both BCMO activity and CRBP (II) levels in rat intestine (During et al., 1998a), suggesting that the same mechanism may be involved in the regulation of these two proteins. The finding of peroxisome proliferator response element (PPRE) in the mouse BCMO promoter seems to provide a clue for explaining the phenomena at the molecular level (Boulanger et al., 2003). Peroxisome proliferator activated receptor gamma (PPARγ), a member of steroid hormone receptor super family and activated by polyunsaturated fatty acids, has been shown to bind to PPRE in TC7 and PF11 cells. The binding of PPARγ to PPRE possibly needs a heterodimeric complex with RXRα. Treatment of mice with PPARα/γ agonist increased the

protein expression of BCMO in liver (Boulanger et al., 2003). Interestingly, PPRE was also identified in the CRBP II gene, which could be up-regulated by fatty acids possibly via PPAR or retinoic acid via RXR (Mangelsdorf et al., 1991; Suruga et al., 1999; Takase et al., 2000).

Due to the limited availability of human intestinal specimens, fewer studies of BCMO regulation were performed with human tissues. In addition, although minerals, i.e. iron, were found to have certain effects on the rat intestinal BCMO activity, for example, iron concentration in rat small intestine showed a positive association with intestinal BCMO activity in *in vivo* studies (During et al., 1999 & 2000), the mechanistic basis for the cofactor role of iron and the iron effect on human intestinal BCMO activity remains unclear. Iron has long been recognized as a cofactor of BCMO based on *in vitro* studies. Two effective metal chelators, α,α'-bipyridyl and o-phenanthroline, were revealed *in vitro* to remarkably inhibit BCMO activity in the homogenates of rat intestine, rat liver and hog intestinal mucosa (Olson and Hayaishi, 1965; Fidge et al., 1969). The inhibitory effects of these two chelators were further confirmed by observing intestinal BCMO from guinea pig and rabbit. In addition, the metal chelating agents, α,α'-bipyridyl and o-phenanthroline, were found to have similar inhibitory effects on recombinant BCMO activity (Lindqvist and Andersson, 2002). In contrast, addition of ferrous iron stimulated BCMO activity in a concentration-dependent manner *in vitro*. However, addition of ferric iron (Fe^{3+}) showed no enhancing effect on BCMO activity (Singh and Cama, 1974). These results indicated iron to be a metal ligand of BCMO. No further investigations were carried out to explore the molecular basis of the cofactor ferrous iron in regulation of BCMO protein until the finding of the essential structure for iron-binding in recombinant mouse BCMO. In the protein structure, four conserved histidines and a Glu405 are considered to be necessary for iron coordination. Any one mutation in these five residues will result in complete loss of the enzymatic activity (Poliakov et al., 2005).

A recent study (During et al., 2001) suggested that iron may have multiple roles in the BCMO metabolism. TC7 cells which derived from the Caco-2 cell line were used successfully as a human *in vitro* model since apparent substrate affinity in TC7 subclones of Caco-2 cells was found to be similar to that in human intestinal preparations. Additionally,

elevated or decreased BCMO activities were observed depending upon addition of iron or desferrioxamine (DFO), respectively, into the cell culture medium. Moreover, the inhibitory effect of DFO could be reversed by iron addition to the medium. Also, a linear increase in the BCMO activity was shown with the increasing incubation time up to 48 h in the presence of 0.5 mM of ferrous sulfate ($FeSO_4$), but no change in the enzyme activity was found during the first 12 h incubation with iron. Although these data provided more evidence for iron as a cofactor of BCMO, the delayed effect (the latency of 12 h) of iron on BCMO activity strongly suggested that there is (are) mechanism(s) involving the regulation of BCMO by iron.

Vitamin A and iron deficiency have long been recognized to be interrelated with each other. For example, serum retinol has been found to be positively correlated with hemoglobin (Hb), serum iron and percentage of transferrin saturation (Mejia and Chew, 1988; Bloem et al., 1990; van Stuijvenberg et al., 1997). Moreover, the interaction of vitamin A with iron is complex and reciprocal. Vitamin A deficiency was suggested to decrease the mobilization of iron from body stores since iron supplementation could not completely correct the low Hb appearing in people with vitamin A deficiency (van Stuijvenberg et al., 1997). Two recent animal studies in rats suggested that iron deficiency may affect vitamin A distribution between plasma and liver, leading to lower serum retinol levels but elevated liver retinol storage (Rosales et al., 1999; Strube et al., 2002). Considering the poorly defined mechanism of the interaction of iron with vitamin A, further studies are needed.

Based on accumulated data, we hypothesized that iron regulates BCMO enzyme activity not only as a cofactor but also by modulating its gene expression. We investigated the effects of iron on mRNA expression and enzyme activity of human BCMO using TC7 cells as a human *in vitro* model. The results derived from this study will enhance understanding of the underlying mechanism of the complicated relationship between iron and vitamin A.

Materials and Methods

Chemicals

All-trans retinal (≥98%), all-trans retinyl acetate (synthetic, type I), and (±) α-tocopherol (≥96%, HPLC) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

All-trans β-carotene (≥ 97%, UV) was purchased from Fluka (Milwaukee, WI, USA). Acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, USA). Anhydrous ethyl alcohol was obtained from Dawn Scientific Inc. (Newark, NJ, USA). The sources of other chemicals and reagents are indicated elsewhere.

Cell culture

TC7 cells were a generous gift from Drs. T. Michael Redmond and Eugenia Poliakov (National Institutes of Health, Bethesda, MD). Cells (passage 65-75) were seeded at 3 $\times 10^3/cm^2$ into different tissue culture wares depending on the various experiments. Cells were plated into 6-well clusters for RNA isolation, in 75 cm^2 flasks for enzyme activity, and in 96-well culture plates for determination of cell viability. TC7 cells were first grown in advanced Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen Corporation, Carlsbad, CA, USA) containing 4,500 mg/L D-glucose and 110 mg/L sodium pyruvate at 37°C in a 5% CO_2 incubator for 5-7 days. The medium was also supplemented with 10% of fetal bovine serum (FBS), 1% of antibiotic-antimycotic, 10 mM of HEPES and 2 mM of L-glumatine. When cells reached 100% confluency, the medium was removed and replaced by serum-free medium supplemented with 1% insulin–transferrin-selenium-G supplement (Invitrogen) (Jumarie and Malo, 1991), 4 mg/L hydrocortisone (HC) (Sigma), 34 µg/L triiodothyronine (T3) (Sigma) and 20 µg/L epidermal growth factor (EGF) (Sigma) in advanced DMEM (Zhu et al., 2006), as well as with the same concentrations of antibiotic-antimycotic, HEPES and L-glumatine. The cells were allowed to undergo cell differentiation for 2 weeks. On day 15 post-confluency, cells were treated with different concentrations of iron (0, 0.01, and 0.5 mM of ferrous sulfate) for the indicated periods of time and then harvested for further experiments.

Cell Viability

Iron cytotoxicity in TC7 cells was measured using a Vybrant MTT cell proliferation assay kit (Invitrogen) according to the manufacturer's recommended protocol with minor modifications. After 15 days of incubation with serum-free medium post-confluency, medium was removed and 300 µl of serum-free medium containing ferrous iron (0, 0.01, and 0.5 mM) were added to individual wells in triplicate. At selected time points, the medium were removed, and the cells were washed once with phosphate buffered saline (PBS). Then

100 µl of fresh serum-free and phenol red-free DMEM medium and 10 µl of 12 mM MTT were added. Plates were allowed to continue incubation at 37°C and 5% CO_2 for 2 h. After removal of 85 µl medium, 50 µl of dimethyl sulfoxide were added to the remaining 25 µl of medium and agitated thoroughly with continued incubation for 10 min at room temperature. The extent of blue formazan formation from yellow MTT was determined photometrically with absorbance 562 nm using ELX 808 microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

RNA isolation and quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was isolated from the iron treated TC7 cells (0,0.01, and 0.5 mM) at 15 day post-confluency (two wells were pooled together) in affinity columns using the RNeasy Mini Kit (Qiagen, Valencia, CA). DNase I (Qiagen) digestion was included in order to remove DNA contamination. The RNA was reverse-transcribed and cDNA abundance was measured in a two-step quantitative real-time PCR reaction using the AffinityScript™ Multiple Temperature cDNA Synthesis Kit combined with the SYBR® Green Brilliant® II QPCR Master Mix (Stratagene, La Jolla, CA). The cDNA abundance was quantified with the Mx4000 multiplex quantitative PCR system (Stratagene). The following primer pairs were used (Chichili et al., 2005): BCMO (GenBank accession no. NM_017429) forward, 5'-CATCTTCCTTGAGCAGCCTTTC-3', reverse, 5'-GCAGCCGTCCTCTTCGTAG-3'; glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (NM_002046) forward, 5'-ATGACATCAAGGTGGTGA-3', reverse, 5'-CTGTAGCCAAATTCGTTGTCA-3'. BCMO and GAPDH products obtained from PCR are 217 and 196 bp, respectively. The amplification reaction was performed with the following thermal profile: 10 min at 95 °C for enzyme activation, followed by 40 cycles, which included 30 s at 95 °C for denaturation, 1 min at 56 °C for annealing, and 30 s at 72 °C for extension. After the 40 cycles were completed, an additional 5 min at 72 °C was included for further extension. The cDNA amplification reaction was performed in triplicate for both BCMO and GAPDH. The effect of iron treatment on BCMO gene expression, expressed as fold increase over 0 h untreated controls, was calculated after normalization to GAPDH. BCMO and GAPDH gene amplification efficiencies were determined using amplification of 0.01, 0.05, 0.1, 0.5, 1, 2, 6

dilution of the original copies of 0 h no iron control cDNA in triplicate. These amplification efficiencies (both of them are close to 1) were applied to calculate the BCMO gene fold increase according to the manufacturer's instructions which are based on the $2^{-\Delta\Delta Ct}$ Method (Livak ea al, 2001).

BCMO activity assay

BCMO enzyme preparation and activity assay were performed as previously described by During et al. (1998b) with minor modification.

TC 7 cells were treated with different levels of iron (0 or 0.5 mM) on day 15 post-confluency. At set time points, TC7 cells in flasks were washed 3 times with 15 ml of cold PBS, and then scraped in 10 ml PBS. Following centrifugation at 2000 rpm at 4 °C for 10 min, supernatants was discarded and cell pellets lysed with 650 µl of CelLytic M cell lysis reagent (Sigma) containing protease inhibitor (1 tablet/10 ml lysis buffer) (Roche Diagnostics GmbH, Mannheim, Germany) in ice-cold water for 15 min with gentle shaking. Cell lysate was centrifuged at 9,000 g at 4 °C for 30 min, and the supernatants were collected and pooled from 2 flasks of cells for the same iron treatment and incubation time point. Protein concentration was determined using a Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL, USA).

The enzyme preparations were immediately used for enzyme assay. The final reaction mixture (total volume 360 µl) contained 40 µM tricine-KOH buffer (pH 8.0), 0.2 µM dithiothreitol, 0.3 mg Tween 40, 1.6 µM sodium cholate, 20 nM α-tocopherol, 3 nM all-trans β-carotene, and the enzyme preparation (0.4 mg total protein). Forty µl of 75 µM β-carotene solution prepared according to During et al. (1998b) were added to initiate the reaction. The enzyme reaction was performed at 37 °C with gentle shaking for 1 h. The enzymatic reaction was terminated by adding 50 µl of 37% (w/w) formaldehyde and followed by incubation for 10 min (During et al., 1998b). Each enzyme assay was performed in triplicate.

Reaction products were extracted as previously described with slight modifications (Wei et al., 1998). Upon termination of the enzyme reaction, 15 ng of retinyl acetate was added as an internal standard. After 1.5 ml ethanol was added, and the mixture was vortexed 1 min; 4 ml hexane was used to extract twice and the supernatants were pooled. The

supernatants were dried under vacuum using a SPD speedvac (Thermo Electron Corporation, Waltham, MA, USA). The residue was reconstituted in 300 µl of acetonitrile, 200 µl of which was injected into a high performance liquid chromatography (HPLC) system equipped with Waters 717 plus autosampler, Waters 515 HPLC pump, Waters 2996 photodiode array detector and Nova-pak C18 reverse phase column 3.9 ×150 mm (Waters Corporation, Milford, MA, USA) under isocratic method using 90% acetonitrile and 10% water as mobile phase. The flow rate was 1 ml/min and the retention time for retinal was 20 min. Retinal was integrated at 380 nm, while retinyl acetate was monitored at 325 nm. Retinal was quantified based on the standard curve created with retinyl acetate as internal standard.

Data analysis

All the experiments were conducted at least three times and results were presented as mean ± standard error of the mean. Statistix software (version 8.0, Analytical Software, Tallahasee, FL, USA) was used for the statistical analysis. Differences between the control and iron treatment were tested by two-way analysis of variance (iron concentration × incubation time). A value $P < 0.05$ was considered significant.

Results

Iron effect on cell viability of TC7 cells

We analyzed the viabilities of TC7 cells upon exposure to different concentrations of ferrous iron (0.01 and 0.5 mM) for selected incubation times using the MTT assay. Our MTT assay showed that neither 0.01 mM nor 0.5 mM of ferrous iron addition in the medium significantly affected the viability of TC7 cells during the various incubation times (Table 1). Additionally, cell viability did not change significantly in the control over time. Compared with their corresponding control cells (no added iron), the low level of iron treatment (0.01 mM) did not change the cell viability except for a slight decrease at 24 h incubation (P = 0.49), which was not statistically significant. For the high concentration of iron (0.5 mM), the cell viability of TC7 cells showed slight nonsignficant declines at 2 h (P = 0.2), 24 h (P = 0.46) and 48 h (P = 0.54) but no significant difference from control cells.

Regulation of BCMO gene expression by iron in TC7 cells

The changes of BCMO mRNA expression induced by ferrous iron in 15-day post-confluent TC7 cells were investigated using real-time RT-PCR over time- and concentration-course (Fig. 1). Whereas a consistent BCMO mRNA level was shown in the control cells (no added iron), the BCMO mRNA levels were significantly increased upon exposure to 0.5 mM of $FeSO_4$ in a time-dependent manner. Addition of 0.01 mM of $FeSO_4$ resulted in up-regulation of BCMO expression only at 2 h and 4 h incubation. After 6 h and 12 h incubations with iron, elevated BCMO mRNA levels were only shown in the cells incubated with the higher level of added iron (0.5 mM $FeSO_4$). In summary, in the presence of 0.5 mM $FeSO_4$, up-regulation of BCMO1 mRNA levels occurred during the entire incubation period including 2 h, 4 h, 6 h, and 12 h. In the presence of 0.01 mM $FeSO_4$, up-regulation occurred only at 2 h and 4 h.

In the presence of 0.5 mM $FeSO_4$, BCMO mRNA was up-regulated to a maximal extent at 2 h (2.3-fold), and to lesser extents at 4 h (2.1-fold), 6 h (1.6-fold) and 12 h (1.8-fold). Compared with the induction of BCMO mRNA in the presence of 0.5 mM $FeSO_4$, the increase in BCMO mRNA in TC7 in the presence of 0.01 mM $FeSO_4$ was lower at all time points, including 2, 4, 6 and 12 h (1.8, 1.7, 0.8 and 0.9 –fold, respectively).

Effect of iron on BCMO activity in TC7 cells

BCMO activity changes induced by iron in TC7 cells under the same growing conditions were examined using a sensitive HPLC method. Considering the higher mRNA level changes with the 0.5 mM $FeSO_4$ treatment, we only compared the control and 0.5 mM $FeSO_4$ treatments. Because we anticipated a delay between up-regulation of BCMO mRNA expression and protein expression, we added more time points (2, 3, 4, 6, 24 and 48 h after incubation with iron) to capture the possible changes in enzyme activity. The TC7 cells grown in serum-free medium for 15 days after confluency possess BCMO activity and retinal formation is 27.6 pmol/h/mg protein (Fig. 2). Although we showed a significant up-regulation of BCMO mRNA levels across various incubation times, no significant difference in BCMO enzyme activity was found between control and iron treatments at any observed time point. The BCMO activity was consistent throughout the entire observed period; the

activity values in both control and iron treatment group were within the range of 26.2-29.3 pmol/h.mg protein.

Discussion

Iron is a recognized cofactor of BCMO, which catalyzes the formation of vitamin A from β-carotene. This role has been shown in an indirect way, for example, the chelators of ferrous iron in vitro inhibit BCMO cleavage activity in rat and hog intestinal preparation (Olson and Hayaishi, 1965; Fidge et al., 1969), and in a direct way, for example, ferrous iron but not ferric iron stimulates BCMO activity in guinea pig and rabbit intestinal preparation (Singh and Cama, 1974). Animal studies indicate that intestinal iron concentration is positively associated with intestinal BCMO activity in rats and high intestinal BCMO activity is attributable to the presence of the cofactor iron (During et al., 1999 & 2000). However, a finding in a human *in vitro* cell model indicated that iron addition exhibited a delayed effect (12 h latency) on BCMO activity (During et al., 2001). This inspired us to hypothesize that iron may regulate BCMO activity not only through the catalytic process as a cofactor of this enzyme but also via other undefined mechanisms.

In the present study, we examined the ferrous iron effect on BCMO mRNA expression in the TC7 cell line. We believe this is the first study to explore the regulation of BCMO by iron at the transcriptional level. We used two different concentrations of ferrous iron (0.01 and 0.5 mM $FeSO_4$) to treat the differentiated TC7 cells. A significant up-regulation of BCMO mRNA was found in cells exposed to 0.5 mM $FeSO_4$ at all observed time points (2, 4, 6, and 12 h) and in cells treated with 0.01 mM $FeSO_4$ for 2 h and 4 h. When compared with the 0.01 mM $FeSO_4$ treatment, 0.5 mM FeSO4 resulted in a greater increase in BCMO mRNA expression. Our results suggest that iron-induced BCMO mRNA expression mainly occurs soon (2-4 h) after iron addition and the inductive effect is dose-dependent. The MTT assay indicated that the iron-induced increase in mRNA expression is not due to cytotoxicity because iron had no effect on cell number. Although we cannot elucidate the underlying mechanisms based on the present data, it is possible that iron stimulates BCMO mRNA expression through certain upstream signal pathways. Previous studies suggested that peroxisome proliferator-activated receptor gamma (PPARγ) and RAR

and/or RXR are involved in the regulation of BCMO expression. Upon stimulation by fatty acids and retinoic acid, these transcriptional factors will regulate BCMO mRNA expression through interacting with the elements in BCMO promoter such as peroxisome proliferator response element (PPRE) (Mangelsdorf et al., 1991; Suruga et al., 1999; Takase et al., 2000) and retinoic acid response element (RARE) (Bachmann et al., 2002; Chichili et al., 2005; Takitani et al., 2006). However, no iron response element was reported in the BCMO promoter and up-regulation by iron could not be explained via direct activation of the enzyme promoter. Certain mediators, i.e. transcriptional factors, might be involved in the observed up-regulation.

In contrast to the up-regulation of BCMO mRNA, iron treatment showed no positive effects on the BCMO cleavage activity although we added a 3-h time point and extended the duration of iron treatment to 48 h. The protein expression and post-translational modification of BCMO may need some time and, thus, the presence of increased BCMO activity might be expected to lag behind its mRNA expression. However, we anticipated that increased enzymatic activity would begin at 3-5 h and then remain at higher levels for more than 24 h. In fact, earlier studies showed that the time course of elevated BCMO protein expression and enzyme activity are consistent with the time course of the increase in its mRNA expression. A study of the human retinal pigment epithelial (RPE) cell line D407 demonstrated that BCMO mRNA and protein expression exhibited the same kinetics of increase and then decline upon stimulation with exogenous β-carotene (Chichili et al., 2005). It was also found in a human intestinal Caco-2 BBe cell model that triiodothyronine (T3) induced a similar kinetic pattern for changes in both BCMO mRNA and enzyme activity. Although BCMO mRNA up-regulation did not result in an increase in the enzyme activity when Caco-2 BBe cells were incubated in serum-free medium and T3, both BCMO mRNA level and enzyme activity were indeed significantly increased in Caco-2 BBe cells incubated with 10% FBS-supplemented medium and T3 (Yamaguchi and Suruga, 2008). Therefore, it is possible that special experimental conditions are required for a concerted change in BCMO mRNA level, protein expression and enzyme activity. The above-mentioned finding that Caco-2 BBe cells grown in serum-free medium could not synthesize more BCMO protein despite increased mRNA levels is likely due to the lack of some crucial components present in FBS. In our

study, these ingredients necessary for cell protein synthesis might be completely depleted when TC7 cells were cultured in serum-free medium for 15 days after confluency. Therefore, enhanced BCMO mRNA expression did not result in a subsequent increase in BCMO cleavage activity.

Stable enzymatic activities following up-regulation of BCMO mRNA might be partially due to the posttranscriptional regulation of BCMO. Posttranscriptional regulation is a type of gene regulation mechanism which is mediated through a rapid turnover of mRNA in order to control the translation of certain proteins. These proteins usually have transient function during a certain period such as a specific phase of the cell cycle (Williams et al., 1993). Particular regions, i.e. AU-rich sequences in the 3'-UTR of eukaryotic mRNAs, and other specific regions are commonly found in the mRNAs of these proteins. These regions could bind to certain proteins and then trigger quick degradation of mRNAs (Shaw and Kamen, 1986; Jones et al., 1987; Shyu et al., 1989). However, there is no report with regard to these types of regions correlated with posttranscriptional regulation in BCMO mRNA. BCMO activity in humans seems more tightly regulated than its counterpart in rodents. In rodents, almost all of the absorbed β-carotene is converted into retinal in small intestine, while in humans, about 60-70% of β-carotene undergoes cleavage in small intestine and the rest remains intact to be delivered to liver and adipose tissue (Wyss, 2004). The absence of myocyte enhancer factor (MEF2) binding element in mouse promoter and the cooperation of MEF2 and PPAR in human BCMO gene expression were suggested to partially explain the different conversion efficiencies of β-carotene in intestinal mucosa across species at gene expression level (Gong et al., 2006). Since increased mRNA was shown in our study, some mechanisms after mRNA expression such as posttranscriptional regulation may affect the stability of mRNA and then act as further regulators of BCMO protein expression or activity.

However, this speculation is not consistent with earlier findings regarding the iron effect on BCMO activity in which BCMO activity was found to increase linearly with increasing incubation time (up to 48 h) in the presence of 0.5 mM of $FeSO_4$ after an initial 12-h latency period (During et al., 2001). Another mechanism may apply in this observation. Since no mRNA and protein levels were determined and BCMO protein synthesis is limited in Caco-2 cells grown in serum-free medium (Yamaguchi and Suruga, 2008), it is possible

that increased activity is not in conjunction with mRNA level change. Therefore, the enhanced BCMO activity may be attributable to the cofactor role of iron. In other words, the interaction of iron with BCMO may be responsible for the increased enzyme activity. In addition, the observed 12 h latency may be related to the cell culture condition. In During's (2001) paper, cells were cultured in medium without supplements of FBS and other chemicals. It was highly possible that after 15 days of culture, iron could be completely depleted and thus no trace of iron was accessible to the cells. Thus, when supplemented into the medium, iron will be first taken up to meet the basic needs of cellular growth and then used as a cofactor for BCMO. On the other hand, in our study, 1% ITS supplement was substituted for 10% FBS in serum-free medium to allow TC7 cell differentiation after confluency, and T3 and EGF were also added to benefit cellular differentiation. Transferrin contains a trace of iron and its introduction into our serum-free medium might result in iron deficiency instead of total iron depletion in TC7 cells. Transferrin-derived iron may be sufficient to maintain a certain concentration inside cells required for basic functions and to bind to BCMO. Thus, under our cell culture conditions, the iron supplement could not effectively stimulate BCMO enzyme activity, and instead, only up-regulated the mRNA level.

Moreover, the enzyme preparation procedure may affect BCMO activity and cause the 12 h latency period. In During's study (2001), ethylenediaminetetraacetic acid (EDTA) was introduced into the BCMO enzyme preparation extracted from TC7 cells because cell pellets were homogenized in washing buffer containing EDTA. EDTA is a metal chelator which is able to bind to various metals including lead, calcium, magnesium and ferric iron with high affinity (International Nutritional Anemia Consultative Group, 1993). This chelator may affect BCMO activity since it can bind to iron, which is an essential cofactor for BCMO. After 15 days of incubation in serum-free medium, intracellular BCMO is deficient in iron. Once supplemented with iron, BCMO recruits iron continuously with increasing incubation time. However, the iron binding in the BCMO structure in the enzyme preparation could be reversed by EDTA. Perhaps this effect was overcome when there was enough iron bound in BCMO after an extended period of incubation. After this time point, BCMO activity appeared to increase.

Taken together, we observed increased BCMO mRNA expression without corresponding changes in the enzyme activity upon iron exposure. Further studies are necessary to help understand the underlying mechanism. Cell culture conditions, enzyme preparation procedures and enzyme reaction conditions should be carefully considered to obtain positive results.

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Table 1. Cell viability of differentiated TC7 cells (15 day after confluency) upon iron exposure.

Incubation time (hour)	Iron concentration (mM)	
	0.01	0.5
2	102.79 ± 6.25	89.25 ± 5.19
4	102.62 ± 5.57	103.41 ± 4.18
6	106.16 ± 6.28	114.05 ± 12.94
12	113.35 ± 8.82	108.51 ± 6.01
24	94.20 ± 2.21	93.80 ± 5.53
48	100.40 ± 5.68	94.91 ± 1.78

Note: Values are percentage (mean ± standard error of the mean of three independent experiments) of corresponding control cells.

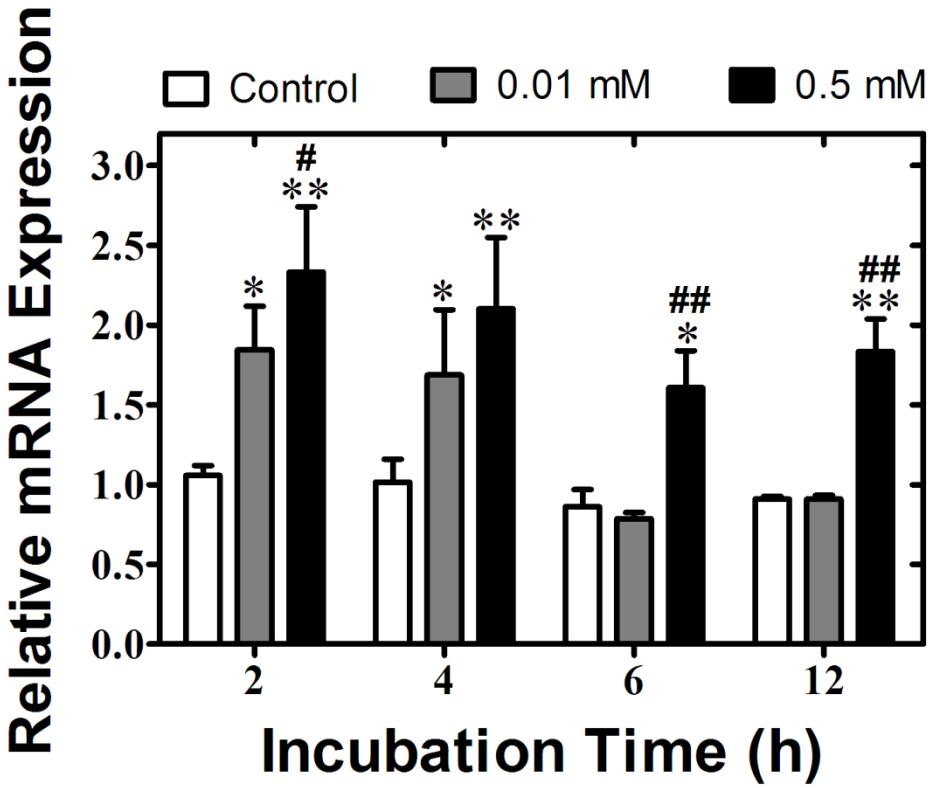


Fig. 1. Time-dependent transcriptional regulation of BCMO by two levels of ferrous sulfate (0.01 and 0.5 mM). On day 15 post-confluency, TC7 cells were incubated with serum-free medium containing 0.01 or 0.5 mM of $FeSO_4$ for 2, 4, 6, or 12 h. Total RNA was isolated, and the relative BCMO/GAPDH mRNA levels were quantified using real-time RT-PCR. Each mRNA measurement was determined in triplicate and three independent experiments were conducted. Values are mean ± standard error of the mean of relative BCMO/GAPDH mRNA expression (folds) collected from three independent experiments. * Indicates a significant difference of iron treatment (0.01 or 0.5 mM) group from the non-treated control group (*P<0.05; **P<0.001). # Indicates a significant difference between the two iron treatment groups ($^{\#}P$<0.05 and $^{\#\#}P$<0.001).

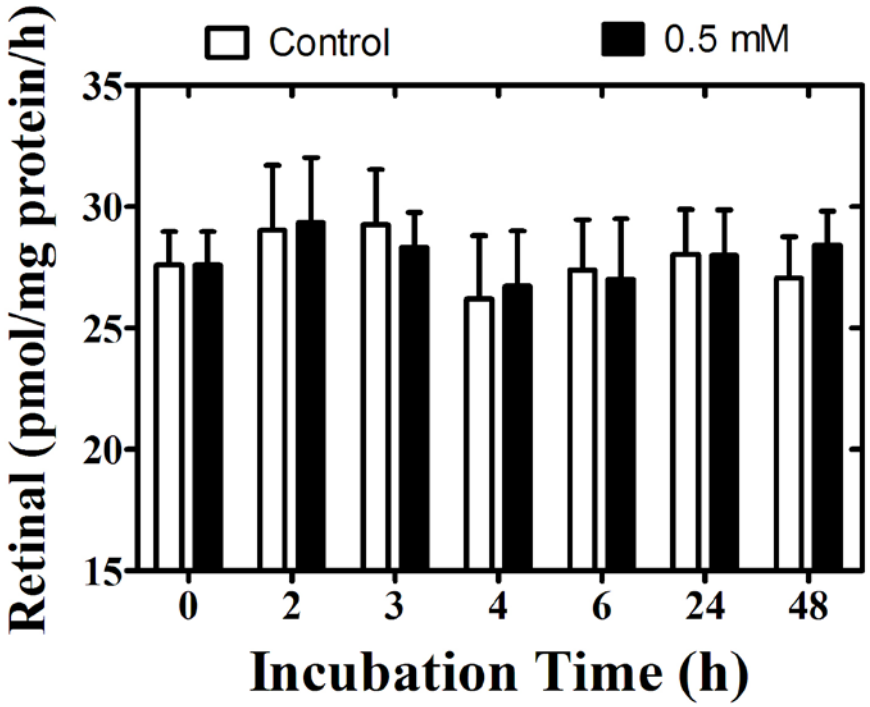


Fig. 2. Time-dependent regulation of BCMO activity by iron in TC7 cells. TC7 cells at 15 days post-confluency were treated with 0.5 mM of $FeSO_4$ for selected intervals from 0 to 48 h. Cytosolic protein (0.4 mg) extracted from TC7 cells was used to determine the BCMO activity and the enzyme activity is expressed as pmol of retinal formed per mg protein per hour. Values indicate mean ± standard error of the mean of three independent experiments performed in triplicate.

CHAPTER 3. GENERAL CONCLUSIONS

Ferrous iron was identified as an essential cofactor of BCMO which catalyzes the formation of vitamin A from β-carotene. In addition to serving as a cofactor, iron may play another role in the regulation of BCMO.

In the present study, we found ferrous iron could increase BCMO mRNA expression but not BCMO activity. The results showed that compared with the untreated control, the higher concentration of iron (0.5 mM $FeSO_4$) significantly increased BCMO mRNA expression in a time-dependent manner during 12 h of treatment with a peak at 2 h (2.3-fold increase). The lower concentration of iron (0.01 mM $FeSO_4$) also showed an enhancing effect after 2 h and 4 h incubation with iron, but was significantly weaker in stimulating BCMO mRNA expression than the higher concentration of iron, suggesting a dose-dependent enhancing effect of iron. Despite the increased mRNA expression, BCMO enzyme activities were not increased by iron treatment at any of the observed time points (2-48 h). These data suggest that iron could up-regulate BCMO mRNA expression level but not its catalytic activity under our cell culture conditions.

In conclusion, this study provided the first evidence of an iron effect on BCMO mRNA expression and enzyme activity. Iron at nontoxic levels up-regulated BCMO mRNA expression but had no effect on BCMO enzyme activity in the cultured TC7 subclone of Caco-2 human intestinal cells under our cell culture conditions. In addition, the higher concentration of iron (0.5 mM of $FeSO_4$) showed a stronger effect in stimulating BCMO mRNA expression than the lower concentration of iron (0.01 mM of $FeSO_4$). The TC7 cell model was shown to be an effective model to study BCMO cleavage activity and β-carotene metabolism *in vitro*. However, further experiments are necessary to explore the optimal cell culture conditions for study of the regulation of the BCMO gene and activity in this model.

APPENDIX A. RT-PCR RESULTS

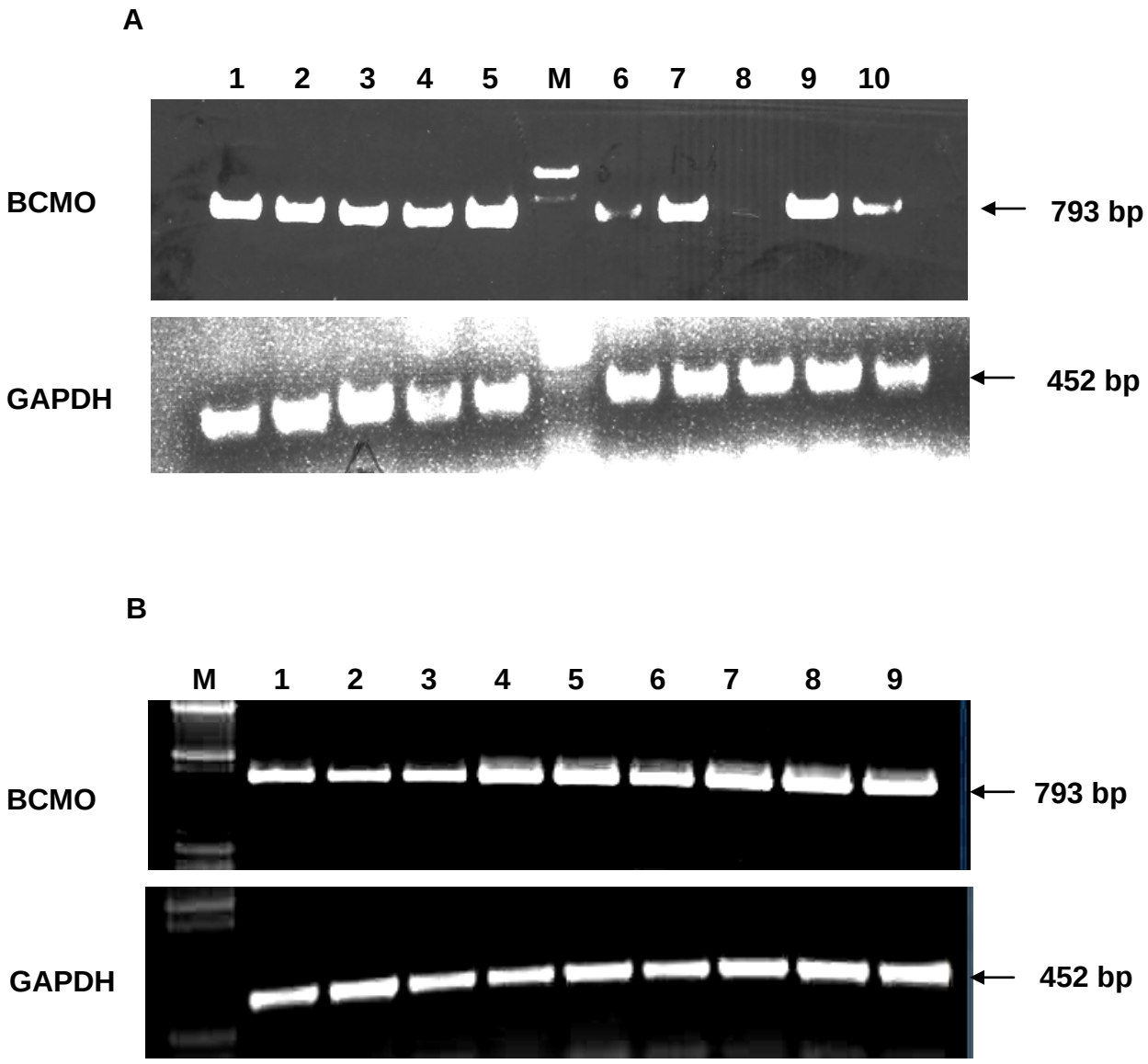


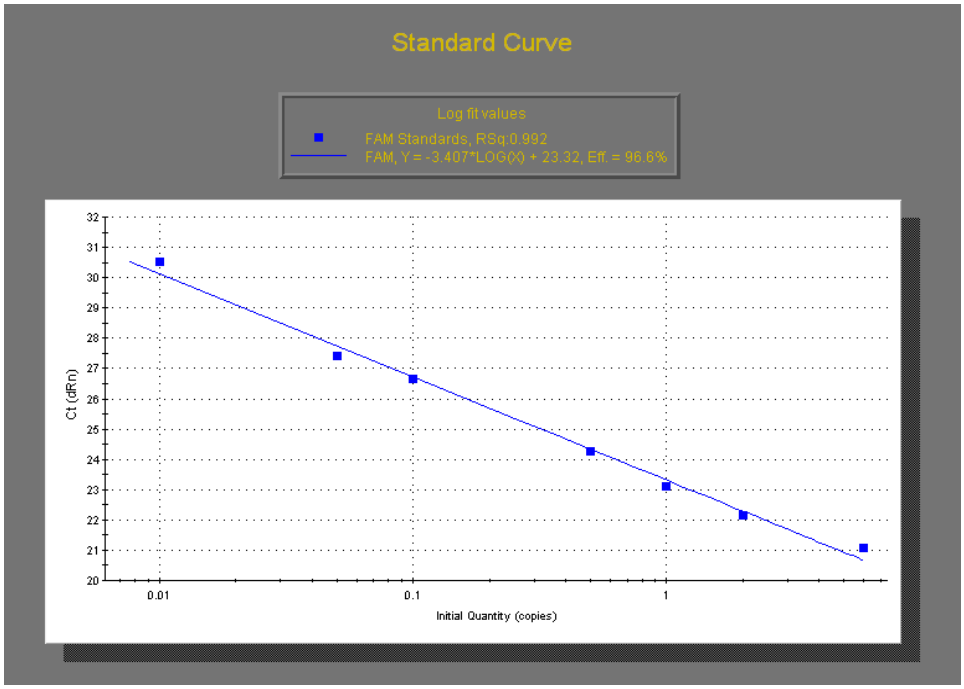
Fig. 1. β-Carotene 15,15'-monooxygenase (BCMO) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene expression in differentiated TC7 cells analyzed by RT-PCR. Total RNA was extracted from differentiated TC7 cells (15 days after confluency) that were incubated with $FeSO_4$ (0, 0.01 or 0.5 mM) for 1 h, 2 h, 4 h, 6 h, 12 h,

24 h, 36 h, and 48 h. Then 2 µg of total RNA was reverse transcribed into cDNA and PCR amplification was performed with either human BCMO gene-specific primers (forward 5'-GTCATCTTCC TTGAGCAGCCTTTC-3', reverse 5'-GCATATCGACATCAACAGAGGCAC-3') or GAPDH primers (forward 5'-ACCACAGTCCATGCCATCAC-3' and reverse 5'-TCCACCACCCTGTTGCTGTA-3') (Bhatti et al, 2003). The sizes of the PCR products obtained from BCMO and GAPDH were 793 bp and 452 bp, respectively. The PCR products were separated by electrophoresis on a 0.8% agarose gel, and the DNA bands were stained with ethidium bromide. A 1 kb plus DNA ladder was used to identify the size of the PCR products. **(A)** Lane M: 1 kb plus DNA ladder; lanes 1-5: TC7 cells incubated with 0 mM $FeSO_4$ for 6 h, 12 h, 24 h, 36 h, and 48 h; lanes 6-10: TC7 cells incubated with 0.5 mM $FeSO_4$ for 6 h, 12 h, 24 h, 36 h, and 48 h. (B) Lane M: 1 kb plus DNA ladder; lanes 1-3: TC7 cells incubated with 0 mM $FeSO_4$ for 1 h, 2 h, and 4 h; lanes 4-6: TC7 cells incubated with 0.01 mM $FeSO_4$ for 1 h, 2 h, and 4 h; lanes 7-9: TC7 cells incubated with 0.5 mM $FeSO_4$ for 1 h, 2 h, and 4 h.

We investigated BCMO mRNA gene expression changes at late time points to correspond with time points used to study BCMO enzyme activity in the literature (During et al 2001). However, the results shown in **Fig**. **1A** indicate that BCMO mRNA expression in the cells incubated with 0.5 mM $FeSO_4$ was unstable at the observed time points from 6 h to 48 h, whereas GAPDH mRNA expression was consistent over time. Therefore we changed our strategy to investigate effects of $FeSO_4$ on BCMO gene expression at earlier time points including 1 h, 2 h, and 4 h. **Fig. 1B** shows more intense bands from cells incubated with 0.01 or 0.5 mM of $FeSO_4$ for 1 h, 2 h, and 4 h when compared with the corresponding bands from control cells. These results suggest iron may induce expression of the BCMO gene within 4 h after iron addition. Based upon these results, we decided to quantify BCMO mRNA expression at earlier time points using real-time RT-PCR.

APPENDIX B. REAL-TIME RT-PCR

A



B

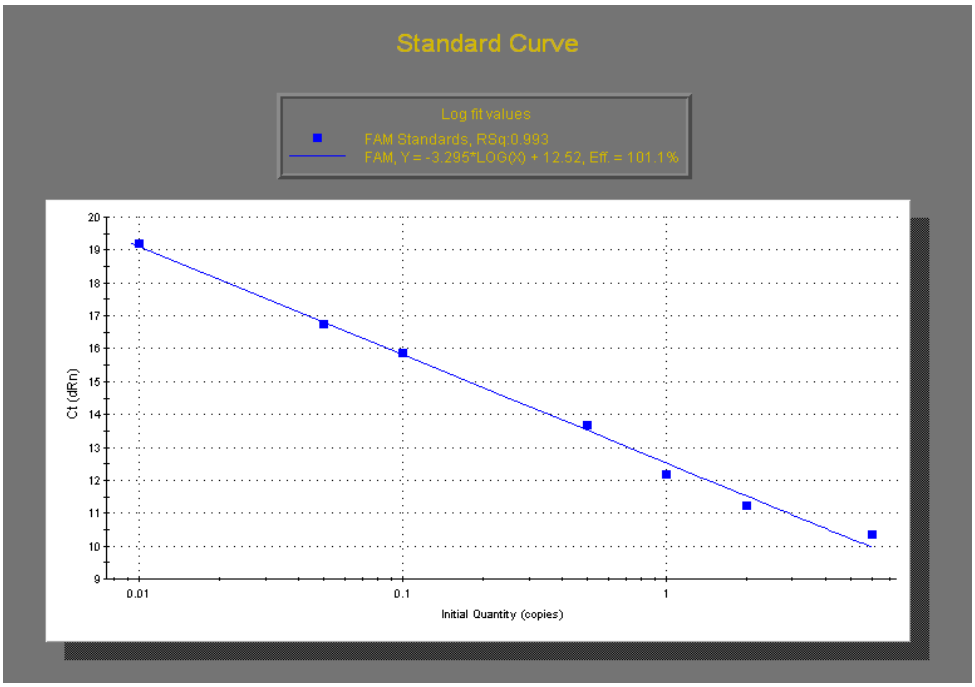
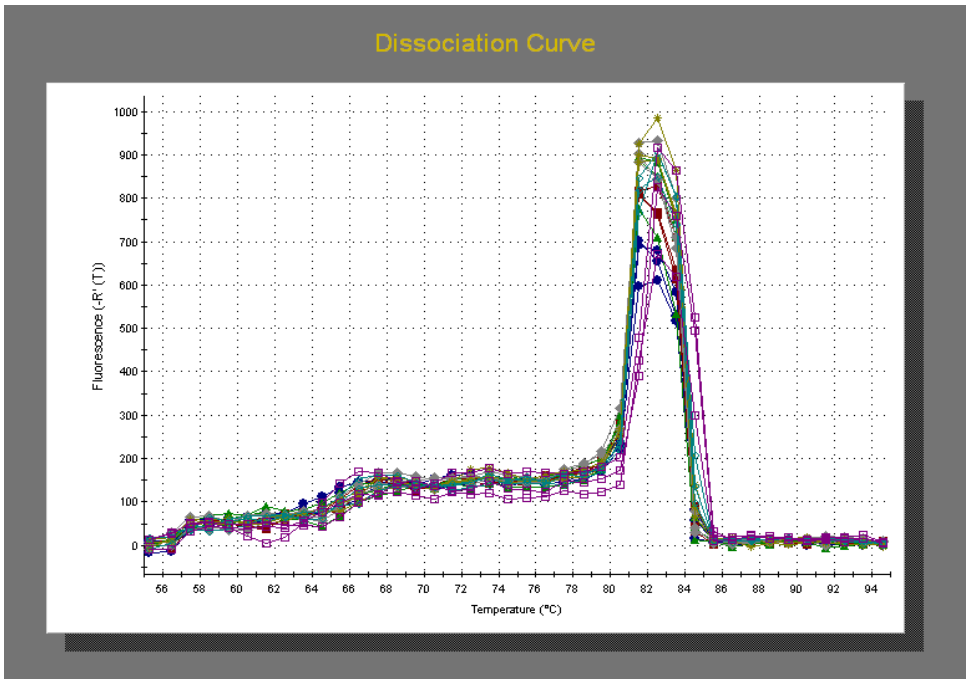


Fig. 2. BCMO and GAPDH amplification standard curves. The amplification standard curves were created by diluting the cDNAs for BCMO and GAPDH from the 0 h time point 0 mM $FeSO_4$ control cells. The corresponding threshold cycle (Ct) values were plotted and the amplification efficiencies were generated by the software. Data were collected with v. 4.2 Stratagene Mx4000™ software and analyzed with MxPro software. **(A)** BCMO amplification standard curve; **(B)** GAPDH amplification standard curve. BCMO amplification efficiency was 96.6%; GAPDH amplification efficiency was 101.1%. Both amplification efficiencies approached 100%, indicating similar high amplification efficiencies for these two genes under our experimental conditions.

A



B

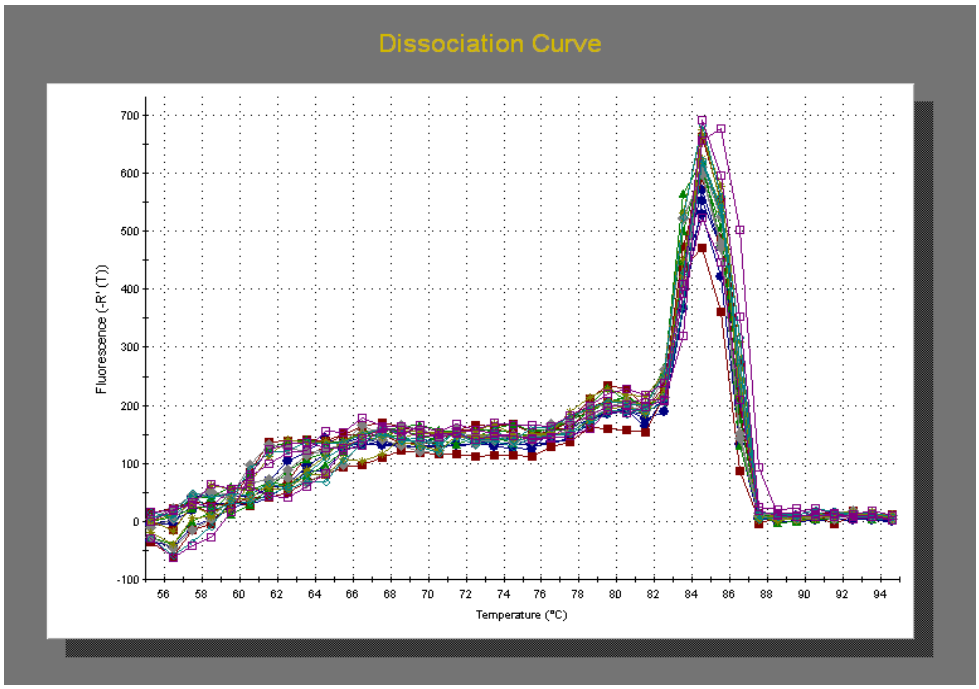
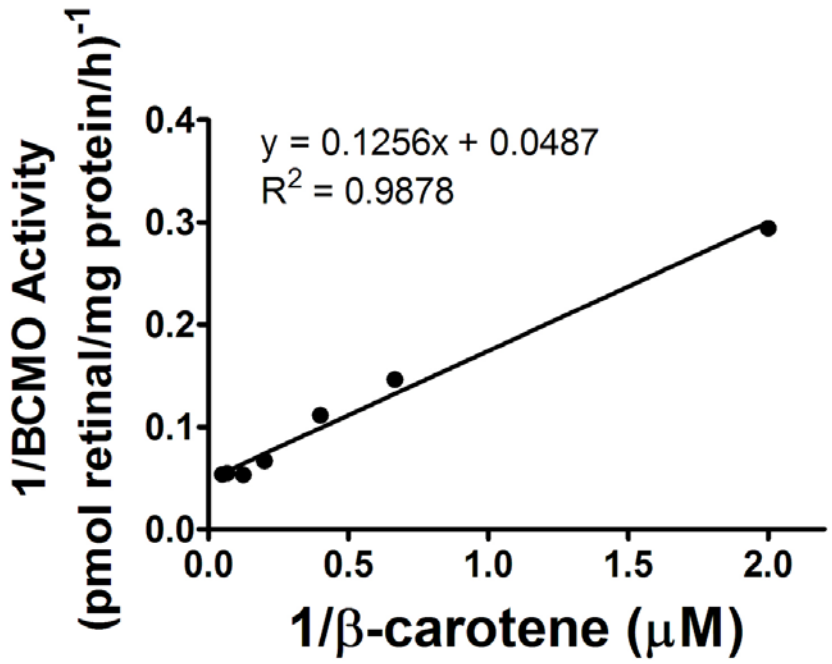


Fig. 3. BCMO and GAPDH dissociation curves. Dissociation curves were generated by the software after each PCR cycle was completed. **(A)** BCMO dissociation

curve; **(B)** GAPDH dissociation curve. Both curves show only a single dissociation peak. These results indicated there was no DNA contamination in our template.

APPENDIX C. BCMO KINETIC PARAMETERS

A



B

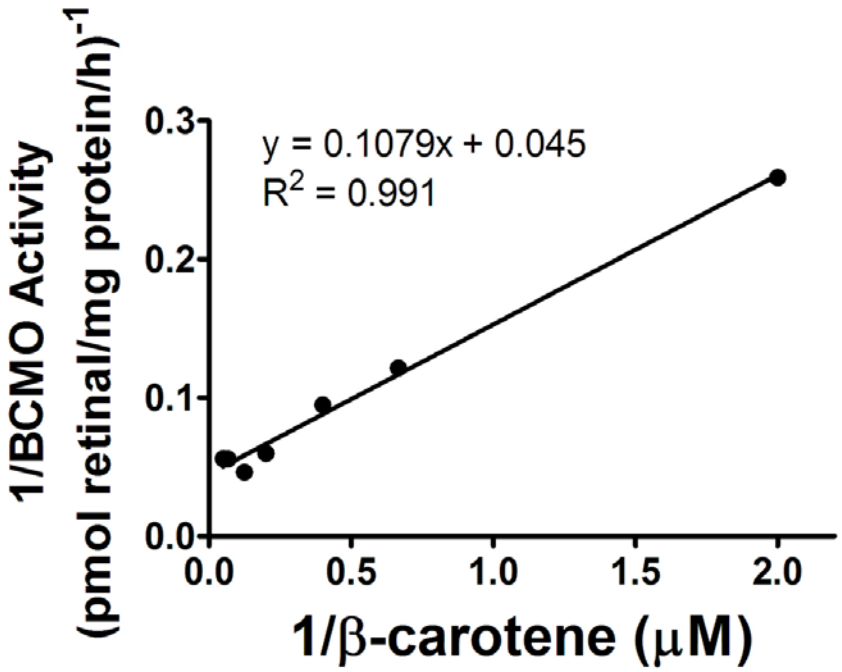


Fig. 4. BCMO activity in 15 day post-confluent TC7 cells incubated with increasing substrate (β-carotene) concentrations represented by Lineweaver–Burk plot (1/reaction velocity *versus* 1/substrate concentration) (Lineweaver and Burk, 1934). The cytosolic fraction was isolated from differentiated TC7 cells (15 days post-confluency) and the enzyme activity assay was performed based on the method described in Chapter 2. The β-carotene concentrations used in the assay were 0.5 μM, 1.5 μM, 2.5 μM, 5 μM, 8 μM, 15 μM, and 20 μM. BCMO activity plateaued when the β-carotene concentration was 8 μM. **(A)** BCMO activity in differentiated TC7 cells incubated for 3 h with 0 mM $FeSO_4$. **(B)** BCMO activity in differentiated TC7 cells incubated for 3 h with 0.5 mM $FeSO_4$. For the cells incubated with 0 mM $FeSO_4$, the estimated enzyme K_m was 2.6 μM of β-carotene and the V_{max} was 20.8 pmol retinal/mg protein/h. For cells incubated with 0.5 mM $FeSO_4$, the estimated enzyme K_m was 2.4 μM β-carotene and the V_{max} was 22.2 pmol retinal/mg protein/h. The BCMO V_{max} values in both 0 mM $FeSO_4$ control and 0.5 mM $FeSO_4$-treated cells were consistent with V_{max} values reported in the TC7 cells from literature (23.8 pmol retinal/mg protein/h) (During et al, 2001). Our K_m values (2.6 or 2.4 μM) were slightly higher than those reported in the literature (1.57 μM) (During et al, 2001). However, our V_{max} and K_m values were based on a single determination. To obtain more definitive values, the experiment would need to be replicated.

Based upon this experiment, we concluded that our substrate concentration was not limiting for BCMO enzyme activity in either the 0 mM or 0.5 mM $FeSO_4$-treated TC7 cells.

APPENDIX D. THE EFFECT OF CELL CULTURE MEDIA CONTAINING CHELATED FETAL BOVINE SERUM (FBS) ON BCMO ACTIVITY

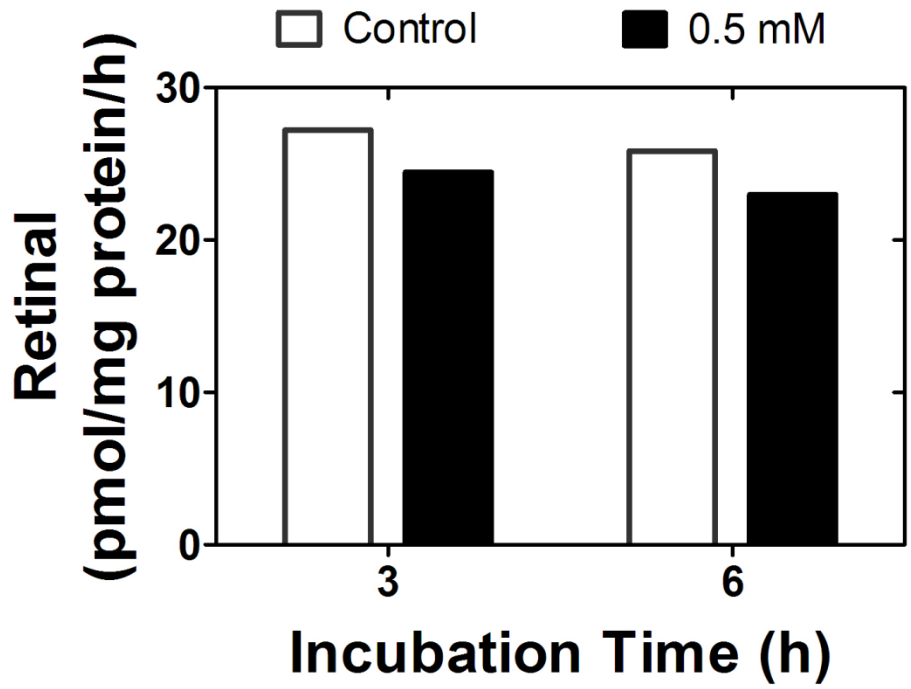


Fig. 5. BCMO activity in differentiated TC7 cells incubated initially with Chelex 100-treated FBS and then in serum free media containing 0 mM or 0.5 mM $FeSO_4$ for 3 h and 6 h. FBS was treated with 10% Chelex 100 at 4 ºC overnight and then filtered. Differentiated TC7 cells were incubated with cell culture medium containing 10% of the Chelex 100-treated FBS for 3 h at 37 ºC. The medium containing Chelex 100-treated FBS was removed. TC7 cells were then incubated in serum free medium containing 0 mM or 0.5 mM $FeSO_4$ for 3 h or 6 h. The enzyme activity assay was performed on the cytosolic fraction of the cell lysates as described in Chapter 2. There was no apparent difference in BCMO activity between 0 mM and 0.5 mM FeSO4 treatments with incubation for either 3 h or 6 h.

The objective of this experiment was to expose the cells to nutrient- and growth factor-rich but iron-poor cell culture medium in an effort to optimize expression and activity of the BCMO enzyme. As shown in Fig. 5, pretreatment with the chelated FBS did not result in greater enzyme activity in the iron-treated cells. Perhaps the incubation time with the chelated FBS was too brief or perhaps other minerals needed for BCMO activity were also chelated by the Chelex 100. Future experiments are needed to determine whether iron chelation of FBS is a viable approach to provide a nutrient-rich but iron-poor culture medium to the cells. The objective would be to determine whether factors missing from the serum free culture media could account for the lack of the expected increase in expression and activity of the BCMO enzyme in the $FeSO_4$-treated cells. (During et al. showed increased BCMO enzyme activity in TC7 cells incubated with 0.5 mM $FeSO_4$. However, this is the only published report that iron addition enhances BCMO activity in these cells.)

APPENDIX E. CYTOXICITY DATA

Table 1: Microplate reader absorbance data[1] for the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay in differentiated TC7 cells incubated in media containing different concentrations of $FeSO_4$.

Incubation time (hour)	$FeSO_4$ concentration (mM)		
	0	0.01	0.5
2	0.68±0.09	0.71±0.14	0.60±0.04
4	0.74±0.01	0.76±0.04	0.76±0.04
6	0.62±0.06	0.65±0.05	0.69±0.05
12	0.66±0.09	0.76±0.15	0.70±0.07
24	0.73±0.02	0.69±0.02	0.69±0.04
48	0.80±0.03	0.81±0.08	0.76±0.01

[1]Values are absorbance readings (mean ± SE of three independent experiments) taken from the microplate reader.

Differences between the 0 mM, 0.01 mM, and 0.5 mM $FeSO_4$ treatments were tested by two-way (iron concentration × incubation time) ANOVA. No significant differences were found.

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Lineweaver, H and Burk, D (1934). The determination of enzyme dissociation constants. J. Am. Chem. Soc. 56:658–666.

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