

Mitochondrial β -Carotene 9',10' Oxygenase Modulates Prostate Cancer Growth via NF- κ B Inhibition: A Lycopene-Independent Function

Xiaoming Gong¹, Raju Marisiddaiah², Susan Zaripheh³, Doris Wiener⁴, and Lewis P. Rubin^{1,5}

Abstract

Despite numerous inquiries into protective roles of lycopene in prostate cancer prevention or therapy, little is known about mechanisms by which lycopene or its metabolites inhibit prostate cancer. The enzyme β -carotene 9',10'-oxygenase (BCO2), which catalyzes asymmetric cleavage of several carotenoids, is the principal regulator of lycopene metabolism, but the range of BCO2 biological functions is incompletely understood. This study investigated expression and functional roles of BCO2 in human prostate cancer. Expression of the *bco2* gene is dramatically decreased in prostate cancer tissue and in a range of prostate cancer cell lines as compared with nonneoplastic prostate tissue and normal prostatic epithelial cells, respectively. Inhibition of DNA methyltransferase activity restored *bco2* expression in prostate cancer cell lines tested. Treatment with lycopene or its metabolite, apo-10-lycopenal, also increased *bco2* expression and reduced cell proliferation in androgen-sensitive cell lines, but lycopene neither

altered *bco2* expression nor cell growth in androgen-resistant cells. Notably, restoring *bco2* expression in prostate cancer cells inhibited cell proliferation and colony formation, irrespective of lycopene exposure. Exogenous expression of either wild-type BCO2 or a mutant (enzymatically inactive) BCO2 in prostate cancer cells reduced NF- κ B activity and decreased NF- κ B nuclear translocation and DNA binding. Together, these results indicate epigenetic loss of BCO2 expression is associated with prostate cancer progression. Moreover, these findings describe previously unanticipated functions of BCO2 that are independent of its enzymatic role in lycopene metabolism.

Implications: This study identifies BCO2 as a tumor suppressor in prostate cancer. BCO2-mediated inhibition of NF- κ B signaling implies BCO2 status is important in prostate cancer progression. *Mol Cancer Res*; 14(10); 966–75. ©2016 AACR.

Introduction

Prostate cancer is the most common noncutaneous cancer among men and second leading cause of cancer-related death among men in the United States (1). When compared with progress made in other major cancers, therapeutic options in prostate cancer have remained more limited. Nevertheless, a high occurrence rate, slow tumor growth, and long latency to clinically significant disease suggest prostate cancer may be especially amenable to environmental (including nutritional) preventive and therapeutic interventions. In developed countries, epidemiologic studies and clinical trials have shown an inverse relationship exists between dietary lycopene intake and prostate cancer

risk (2–4). Lycopene, a non-pro-vitamin A carotenoid found in tomatoes, tomato-based products, and other foods, is the predominant carotenoid accumulated in prostate tissue (5). Men who consume tomatoes and tomato-based products have a significantly lower risk of prostate cancer (6). These putative effects have been attributed, in part, to lycopene's potent antioxidant properties (7), but the relevance of this mechanism has been challenged (8).

Several experimental studies suggest lycopene may suppress prostate cancer *in vivo* and *in vitro* (9, 10). As few randomized clinical trials (RCT) have been specifically directed to assess lycopene's effects in prostate cancer, and some study designs may have introduced bias, generally there has been insufficient evidence to draw firm conclusions with respect to dietary lycopene in prostate cancer prevention or treatment (10). Nevertheless, careful examination of study designs and data interpretation actually may suggest lycopene or related factors do inhibit prostate cancer growth (11). Whether lycopene itself or its metabolites are responsible for the reported beneficial effects on prostate cancer requires further investigation.

Carotenoid cleavage enzymes are crucial in many animal and plant physiologic processes (12). Oxidative cleavage of carotenoids is catalyzed by a large family of non-heme iron (II) containing oxygenases that yield a structurally diverse class of retinoids (vitamin A-related compounds) and apo-carotenoids. In mammals, the major central cleavage pathway, catalyzed by β -carotene 15,15'-oxygenase (BCO1), produces retinal, retinol, and retinoic acid (RA) from pro-vitamin A carotenoids,

¹Department of Pediatrics, Texas Tech University Health Sciences Center, Paul L. Foster School of Medicine, El Paso, Texas. ²Children's Research Institute, St. Petersburg, Florida. ³Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio. ⁴Moffitt Cancer Center, Tampa, Florida. ⁵Department of Biomedical Sciences, Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, Texas.

Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

Corresponding Author: Lewis P. Rubin, Department of Pediatrics, Texas Tech University Health Sciences Center, 4800 Alberta Avenue, El Paso, Texas 79905. Phone: 915-215-5708; Fax: 915-545-6975; E-mail: lewis.rubin@ttuhsc.edu

doi: 10.1158/1541-7786.MCR-16-0075

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including α -carotene, β -carotene, and β -cryptoxanthin (13, 14). The eccentric carotenoid cleavage pathway is catalyzed by β -carotene 9',10'-oxygenase (BCO2; ref. 15). BCO2 can cleave the 9',10' double bonds of several carotenoids to form apocarotenoids (e.g., apo-10'-lycopenoids) both *in vitro* and *in vivo* (16–18). Lycopene is a preferred physiologic substrate for this enzyme (19).

Expression of the *bco1* and *bco2* genes overlaps in the human gastrointestinal tract, but *bco2*, additionally, is expressed in several tissues neither known to be sensitive to vitamin A deficiency nor to express *bco1* (20, 21). The discovery that BCO1-null mice cannot metabolize β -carotene to vitamin A despite intact *bco2* expression (22) implicated BCO2 in biological processes other than vitamin A synthesis. Studies in BCO2-null mice show this enzyme protects cells against carotenoid-induced oxidative stress and verify that BCO2 metabolizes lycopene and xanthophyll carotenoids, such as lutein (18, 19).

BCO2 is expressed in normal prostate epithelium (20, 21). The observation that lycopene levels are significantly higher in cancerous than in normal or benign prostate (23) prompted our speculation that BCO2-mediated lycopene metabolism may be impaired in prostate cancer cells. BCO2 expression and its role in regulating lycopene action in prostate cancer have not previously been investigated.

In this study, we determined (i) BCO2 status in human prostate cancer and (ii) molecular mechanisms of BCO2 actions in prostate cancer. We found *bco2* gene expression is suppressed during prostate carcinogenesis. In addition, the BCO2 substrate lycopene regulates enzyme expression in prostate cancer cell lines. Restoration of BCO2 expression suppresses prostate cancer cell growth and tumor formation via downregulation of NF- κ B signaling.

Materials and Methods

Prostate tissue samples, cell lines and cell culture

Human prostate tissue samples were obtained from The Human Tissue Resource Network (http://www.pathology.med.ohio-state.edu/ext/SubDivision_page.aspx?ID=Tissuebank). The human normal prostate epithelial cell line (PrEC) was obtained from Lonza and cultured as recommended. Human prostate cancer cell lines (LNCaP, C4-2, DU145, and PC3) were obtained from the ATCC and were cultured in phenol red-free RPMI1640 with penicillin (100 U/mL), streptomycin (100 μ g/mL), and 10% FBS (HyClone) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. All above-mentioned reagents, with the exception of FBS, were purchased from Invitrogen.

Reagents and constructs

pNF- κ B-luc (p-NF- κ B *cis* reporter plasmid) was purchased from Stratagene. Expression vectors for pCMV- β -gal have been described previously (24). The pCMV-BCO1 and pCMV-BCO2 were gifts from the late Dr. A. Stefan Andersson (University of Houston, Houston, TX). Lycopene and apo-10'-lycopenal were gifts from Dr. Hansgeorg Ernst (BASF). 5-Aza-2'-deoxycytidine (5-aza-2dC) and DMSO were from Sigma-Aldrich. TNF α was purchased from BD Biosciences.

Antibodies

The synthetic peptide Ac-RLPVFKRYMGNTLPQKKAV-[C-Amide], corresponding to the N-terminal 19 amino acid residues 27-44

in human BCO2, was coupled to keyhole limpet hemocyanin and used for immunization of rabbits. Polyclonal antibodies to BCO2 were characterized by an ELISA using the synthetic peptide as an antigen as described previously and by Western blot analysis (25). Polyclonal and monoclonal antibodies to NF- κ B p65, I κ B α , and Histone H3 were obtained from Cell Signaling Technology. mAb to β -actin was from Sigma.

Extraction of lycopene

Lycopene was extracted as described previously, with minor modifications (26). After incubation of PrEC and prostate cancer cells with known amounts of lycopene for the specified times, the cell culture plates were placed on ice, medium removed, and monolayers were washed once with 0.5 mL of 10 mmol/L sodium taurocholate in PBS to remove surface-bound carotenoids, followed by two additional PBS washes. The washed cells were harvested by brief trypsinization, and cell pellets were homogenized in 0.5 mL ice-cold PBS and transferred to glass tubes. An aliquot (0.1 mL from 0.2 mmol/L stock) of butylated hydroxytoluene was added to the homogenate. Lycopene extraction was performed by vigorous mixing with 1.5 mL dichloromethane/methanol (1:2, v/v) followed by 2 mL hexane. Following centrifugation, the resulting upper layer was collected; the lower layer was similarly extracted two more times and the hexane-dichloromethane layers were pooled. The combined extract was dried under a SpeedVac concentrator (model: Savant AS160), redissolved in 0.1 mL dichloromethane/methanol (1:4, v/v), and subjected to high-performance liquid chromatography (HPLC) analysis (described below). We also analyzed the concentrations of lycopene in the medium before and after incubation. Sample handling, homogenization, and extraction were carried out in low temperature and dim yellow light to minimize lycopene isomerization.

HPLC analysis of lycopene

Lycopene was analyzed and quantified as described previously, with minor modifications. A Shimadzu HPLC system (model: UFLC) equipped with PDA detector, SPD-M20A monitoring from 210 to 670 nm, comprising a gradient pump system, LC-20AT, and a personal computer equipped with LC Solution software (Shimadzu), was used for the detection and quantification of lycopene. Lycopene was separated on a C30 carotenoid column (5 μ m, 4.6 \times 150 mm, YMC; Waters), attached to a guard cartridge (5 μ m, 4.0 \times 20 mm, YMC; Waters).

Cell proliferation assays

For cell proliferation studies, cells were counted and seeded onto 6-well plates at a density of 8×10^4 cells per well for 12 hours to allow adhesion. The medium was then removed and replaced with fresh containing vehicle (DMSO) or 1 μ mol/L lycopene or apo-10'-lycopenal. At 24-hour intervals for 4 days, duplicate wells were trypsinized and cells counted by cytometry. In other experiments, cells were seeded in 96-well cell culture plates and transfected for 24 hours on the second day with pcDNA3 (control vector), pCMV-BCO1, or pCMV-BCO2, followed by treatment with lycopene (1 μ mol/L) or vehicle. At treatment end, cell growth was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Color absorbance was measured at 575 nm using a Spectra Microplate Reader (Molecular Devices). In separate

experiments, DMSO at the indicated concentration had no independent effects on cell proliferation.

Site-directed mutagenesis

A panel of *bco2*-mutant expression vectors was created using either pCMV-BCO2 (mammalian system) or pRSET-A-BCO2 constructs (*E. coli* system) as indicated. Histidine residues in the iron coordination (enzymatic) site of human BCO2 were targeted. His²²⁶, His²⁸⁶, His³⁵⁷, Glu⁴⁶⁵, and His⁵⁷⁴ singly and in combination were mutated to alanine using QuikChange multiple site-directed mutagenesis (Stratagene). The validity of all point mutations and integrity of the open reading frames were verified by DNA sequencing.

Transfection and luciferase assays

Various prostate cancer cell lines were seeded onto 6-well tissue culture plates at a density of 5×10^5 cells per well. Transfections were performed using Lipofectamine LTX reagent (Invitrogen) following the manufacturer's protocol using 0.5 μ g of luciferase reporter gene plus 0.1 μ g of internal control β -galactosidase (β -gal) expression plasmid, pCMV- β -gal. For cotransfection assays, the total amount of DNA for each transfection was kept constant using control vector (pcDNA3). At various time points, cell lysates were analyzed for luciferase and β -gal activities, luciferase activity being adjusted to activity of control β -gal. All transfections were performed in triplicate in at least three independent experiments.

Soft agar assay for colony formation

Prostate cancer cells (8×10^5 cells/well in 6-well formats) were transfected with 2.5 μ g of empty vector (pcDNA3), BCO2, or a mutated BCO2 (BCO2-mt) expression vector using Lipofectamine LTX reagent (Invitrogen) as described above. After 24-hour transfections, cells were reseeded at a density of 5.0×10^3 cells per agar-treated petri dish and grown for 12 days. Colonies were stained with 0.1% crystal violet (Sigma) for 1 hour and counted to establish the colony formation index.

Reverse transcription and real-time PCR

Total RNA was extracted using TRIzol (Invitrogen), and residual genomic DNA was removed by DNase I (Invitrogen) treatment. RNA was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) according to the manufacturer's protocol. Real-time PCR was performed using TaqMan gene expression human primer/probe sets for BCO2 (Hs00230564_n1) and 18S rRNA (Hs99999901_s1; Applied Biosystems). Relative gene expression was calculated using the $\Delta\Delta C_t$ method. The resultant mRNA was normalized to a calibrator, which, in each case, was the basal sample. Final results are expressed as *n*-fold difference in gene expression relative to 18S rRNA and calibrator as follows: $n\text{-fold} = 2^{-(\Delta C_t \text{ sample} - \Delta C_t \text{ calibrator})}$, where ΔC_t values of the sample and calibrator are determined by subtracting the average C_t value of the transcript under investigation from the average C_t value of 18S rRNA expression for each sample.

Subcellular fractionation and NF- κ B binding assay

Cytoplasmic and nuclear cellular extracts were prepared with NE Nuclear and Cytoplasmic Extraction Reagents (Sigma) as described previously (24) or NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, cat#78833). Nuclear

protein content was used to quantify DNA-binding activity of NF- κ B family members using an ELISA-based Trans NF- κ B family kit (Active Motif) according to the manufacturer's instructions.

Western blot analysis

Protein was isolated from tissues and cells in ice-cold M-PER Mammalian Protein Extraction Reagent (Pierce) that contained 1 mmol/L dithiothreitol, 1 mmol/L phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Roche Diagnostics GmbH) and subjected to centrifugation at $15,000 \times g$ for 10 minutes. Protein concentrations were determined by the BCA protein assay (Pierce). Protein lysates (40 μ g) from each treatment or transfection condition were subjected to 10% SDS-PAGE. After electrophoresis, the separated proteins were transferred to Immun-blot PVDF membranes (Bio-Rad) by semi-dry blotting and probed with the appropriate antibody, followed by incubation with the appropriate anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences). Immunoreactive proteins were detected using ECL Western blotting reagents (Amersham Biosciences). Alternatively, a 30 to 40 μ g aliquot of protein from each treatment was subjected to NuPAGE 4% to 12% Bis-Tris Gel (Life Technologies). After electrophoresis, the separated proteins were transferred to Immun-blot PVDF membrane (Bio-Rad) by semi-dry blotting and probed with the appropriate antibody, followed by incubation with Odyssey secondary antibodies according to the manufacturers' instructions (goat anti-rabbit IRDye 680 or 800 and goat anti-mouse IRDye 680 or 800, depending on required combinations). Blots were imaged using an Odyssey Infrared Imaging System (LI-COR Biosciences).

Statistical analysis

Data are expressed as mean \pm SD from at least three experiments. Two-tailed Student *t* tests were used to evaluate differences between groups; $P < 0.05$ was considered statistically significant.

Results

BCO2 expression is decreased in human prostate cancer

BCO2 is expressed in human normal prostate (21). To determine whether BCO2 is expressed in prostate cancer tissue, we first compared BCO2 protein lysates isolated from normal prostate tissues, benign prostatic hyperplasia (BPH), and prostate cancer tissues by Western blot analysis using an antibody specific for human anti-BCO2 antibody (Fig. 1A). BCO2 was detected in normal prostate ($n = 10$) and BPH tissues ($n = 5$) examined (full data not shown). In contrast, expression was detected weakly or not at all in most prostate cancer tissue specimens ($n = 15$; full data not shown). To determine whether BCO2 expression is associated with prostate cancer transitions from early stage, androgen-sensitive to more aggressive, androgen-resistant cell phenotypes, we examined several human prostate cancer cell lines: androgen-sensitive LNCaP, C4-2 (a subline of LNCaP hypersensitive to low androgen levels), and androgen-resistant DU145 and PC3 cells. All prostate cancer cell lines expressed BCO2 protein (Fig. 1B), higher levels being detected in LNCaP and C4-2. BCO2 mRNA expression was assayed using qRT-PCR in normal PrEC and representative prostate cancer

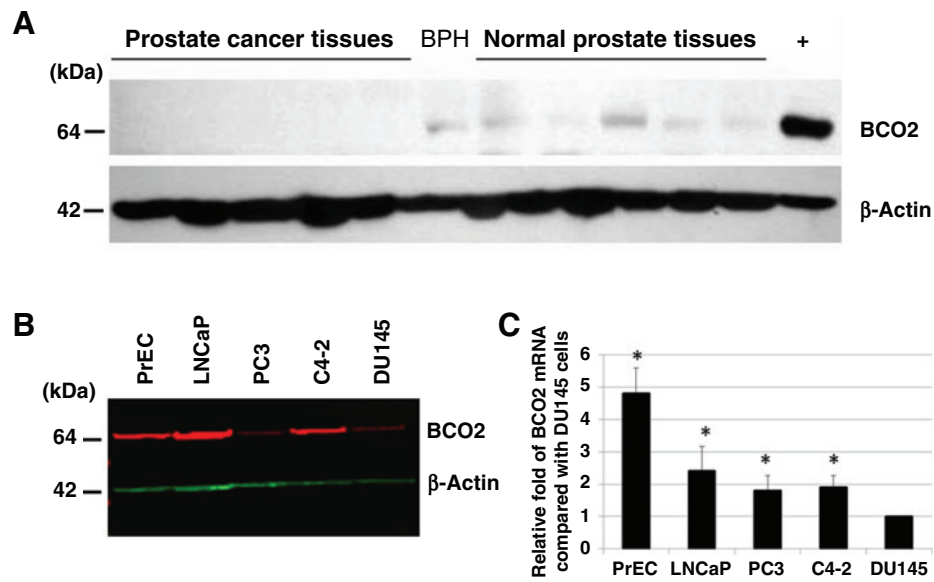


Figure 1.

BCO2 expression is downregulated in human prostate cancer tissues and cell lines. **A**, BCO2 protein expression in normal human prostate, BPH, and prostate cancer tissues. The positive control lane (+) contains protein extracted from HEK-293 cells engineered to overexpress the *bco2* gene. Western blot analysis was conducted using anti-rabbit antibody against BCO2 protein N-terminal peptide. A representative tissue blot is shown. **B**, BCO2 expression in normal PrEC and prostate cancer cell lines LNCaP, PC3, C4-2, and DU145. Cell lysates were isolated and 30 μ g of protein was subjected to NuPAGE. Equal loading of protein was confirmed by reprobing with a β -actin monoclonal antibody. **C**, real-time RT-PCR of *bco2* gene expression in normal PrEC and the prostate cancer cell lines LNCaP, PC3, C4-2 and DU145. Total RNA was isolated from indicated cell lines, and real-time RT-PCR was performed as described in "Materials and Methods." Relative fold *bco2* expression is presented as $2^{-\Delta\Delta C_t}$ relative to the $\Delta\Delta C_t$ of DU145 cells. Data, mean \pm SD ($n = 3$). *, $P < 0.05$.

cells. As shown in Fig. 1C, BCO2 mRNA was detected in these cell lines, most robustly in normal epithelium and least in DU145 cells.

Methyltransferase inhibition induces *bco2* expression in prostate cancer cells

Loss of gene expression during cancer progression may occur by mutation, loss of heterozygosity, or epigenetic silencing. Important mechanisms of epigenetic transcriptional downregulation are hypermethylation and histone deacetylation at a gene promoter that results in tightly packed chromatin and decreased DNA access to transcription factor binding. The finding that the relative abundance of BCO2 protein and mRNA in different prostate cancer cells is roughly correlated suggested BCO2 may be transcriptionally regulated. To assess the potential role of epigenetic mechanisms, prostate epithelial cells and prostate cancer cell lines were treated in the presence or absence of the methyltransferase inhibitor 5-aza-2dC. In the PrEC, treatment with 5-aza-2dC did not significantly alter an already robust *bco2* expression (Fig. 2, left lane). In contrast, treatment with 5-aza-2dC in prostate cancer cell lines (LNCaP, PC3, C4-2, and DU145) resulted in a robust (>5-fold for LNCaP, PC3, C4-2) induction of BCO2 mRNA levels (Fig. 2, middle and right lanes).

Lycopene is differentially accumulated in prostatic epithelial cells and induces *bco2* expression in androgen-sensitive prostate cancer cells

Before interrogating potential actions of lycopene, we examined uptake efficiency of lycopene in the different prostate

cell lines. HPLC analysis of cellular extracts and medium from lycopene-treated PrEC, LNCaP, C4-2, DU145, and PC3 cells showed there was differential lycopene accumulation among PrEC, androgen-sensitive, and androgen-resistant prostate cancer cells (27). Cellular lycopene uptake was time dependent, with a maximum accumulation by 24 hours in cell lines tested (data not shown). Concentration-dependent cellular

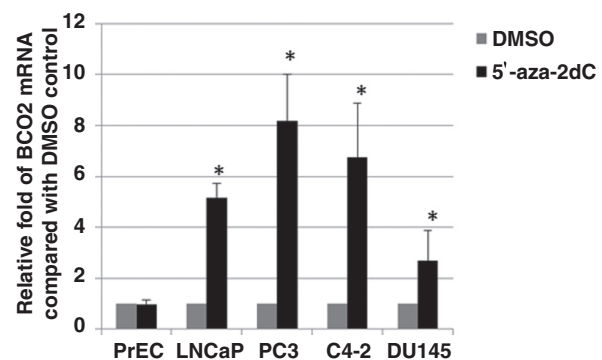


Figure 2.

Demethylation induces *bco2* expression in prostate cancer cells. Normal prostatic epithelial (PrEC) and prostate cancer (LNCaP, PC3, C4-2, DU145) cell lines were treated with 10 μ mol/L of the methyltransferase inhibitor 5-aza-2dC or vehicle (DMSO) for 96 hours. Total RNA was isolated and real-time RT-PCR was performed as described in "Materials and Methods." Relative fold *bco2* expression is presented as $2^{-\Delta\Delta C_t}$ relative to controls. Data, mean \pm SD ($n = 3$). *, $P < 0.05$ compared with controls (DMSO).

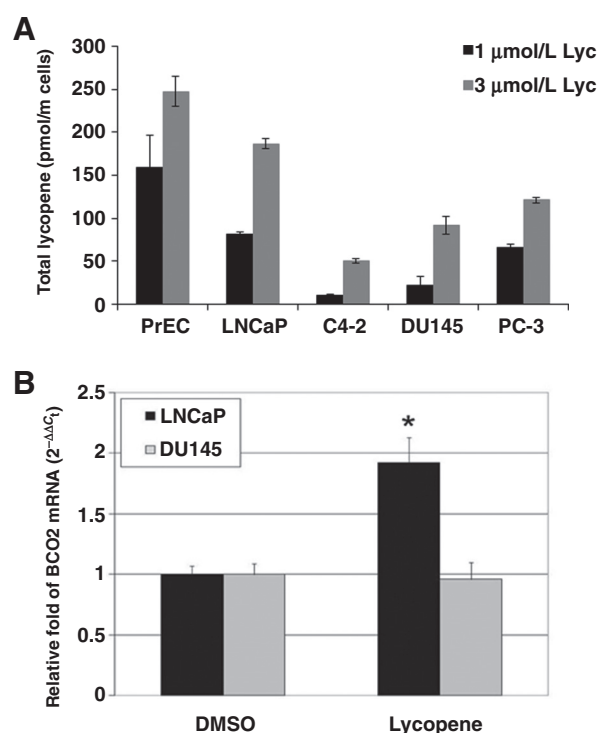


Figure 3. Lycopene is differentially accumulated in prostatic epithelial cells and upregulates *bco2* gene expression in specific prostate cancer cells. **A**, normal PrEC and prostate cancer cell lines were treated either with 1 or 3 μmol/L lycopene for 24 hours. Cellular lycopene was extracted and analyzed by HPLC in individual cell lines as described in "Materials and Methods." Lycopene concentrations in PrEC and prostate cancer cell lines are presented as pmol per million (m) cells. **B**, prostate cancer cell lines (LNCaP and DU145) were treated with 1 μmol/L lycopene or vehicle (DMSO) for 24 hours. Total RNA was isolated and real-time RT-PCR was performed as described in "Materials and Methods." Relative fold expression is presented as $2^{-\Delta\Delta C_t}$ compared with DMSO control. Data, mean \pm SD ($n = 3$). *, $P < 0.05$, lycopene concentration versus control.

accumulation of lycopene was greater in PrEC than in different prostate cancer cells (Fig. 3A), which suggests lycopene accumulation in prostate cancer may not be due to enhanced lycopene uptake. As lycopene can induce *bco2* expression in other biological systems, including ferret lung tissue (17), we examined whether lycopene affects *bco2* expression in prostate cancer cells and whether lycopene differently regulates its enzyme using an androgen-sensitive (LNCaP) and an androgen-resistant (DU145) cell line. LNCaP and DU145 cells were treated with 1 μmol/L of lycopene or vehicle (DMSO) for 24 hours. BCO2 mRNA was assayed by qRT-PCR. As shown in Fig. 3B, lycopene treatment significantly increased *bco2* expression in LNCaP cells, but lycopene had no effect on *bco2* expression in DU145 cells.

Lycopene and lycopene metabolites suppress cell growth in androgen-sensitive LNCaP cells

Lycopene has been shown to inhibit prostate cancer cell growth (9, 10). However, whether lycopene itself or its metabolites are responsible for this activity in different stages of prostate cancer has remained unclear. Consequently, we exam-

ined whether cell growth differs in response to lycopene or to a known biologically active lycopene metabolite (apo-10-lycopenal) in androgen-sensitive and androgen-resistant cells. As shown in Fig. 4A, lycopene and apo-10-lycopenal significantly reduced cell growth in LNCaP cells, but neither affected cell growth in DU145 (Fig. 4B), C4-2 (Supplementary Fig. S1), or PC3 cells (Supplementary Fig. S2).

Exogenous expression of BCO2 inhibits prostate cancer cell proliferation and colony formation

Prostate carcinogenesis involves a shift of balance from cell differentiation to proliferation (28). Prompted by the above-mentioned findings that (i) *bco2* expression appears to be suppressed during prostate carcinogenesis, and (ii) lycopene slows prostate cancer cell proliferation in lines where *bco2* expression

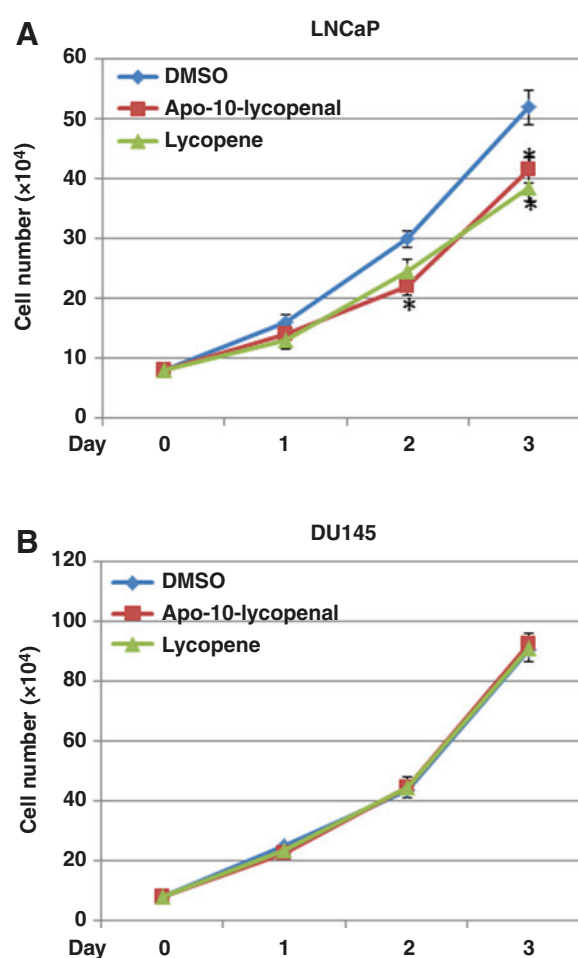


Figure 4. Lycopene and a lycopene metabolite inhibit cell growth in specific prostate cancer cells. In these representative experiments, LNCaP cells (8×10^4) were seeded onto 6-well plates, then treated with 1 μmol/L lycopene or apo-10-lycopenal, or vehicle (DMSO) for up to 3 days. Every 24 hours, duplicate samples were trypsinized and counted. Trypan blue exclusion showed no differences in cell death among different conditions (data not shown). Data, mean \pm SD ($n = 3$). *, $P < 0.05$, lycopene concentration versus control, or apo-10-lycopenal versus control. **A**, LNCaP cells. **B**, DU145 cells.

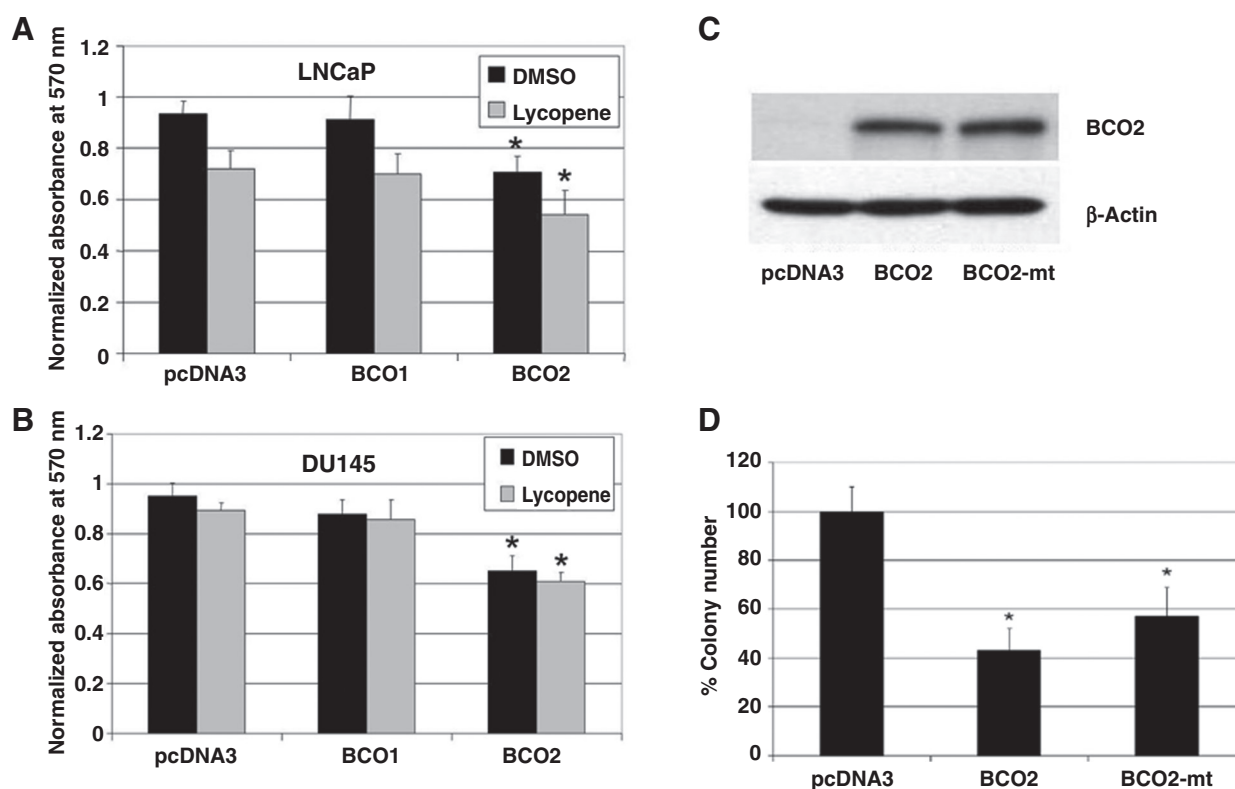


Figure 5.

Reexpression of BCO2 inhibits prostate cancer cell proliferation and colony formation. LNCaP (**A**) or DU145 (**B**) cells were transiently transfected with an empty vector (pcDNA3), or BCO1 or BCO2 expression constructs for 24 hours, then treated with 1 μ mol/L lycopene or DMSO control for 24 hours. Cell number was estimated by the MTT method as described in "Materials and Methods." Data, mean \pm SD ($n = 3$). *, $P < 0.05$, BCO2 expression versus vector control. **C**, Western blot analysis of BCO2 protein expression in pcDNA3, BCO2, or BCO2-mt transfected DU145 cells at 24 hours posttransfection (β -actin detection as loading control). **D**, colony formation assay for transfected cell lines described in **C**. Quantification of colonies per well from nine 3-cm dishes for transfected cell lines as above. Data, mean \pm SD ($n = 3$). *, $P < 0.05$.

remains somewhat active, we tested whether restoring BCO2 expression affects cell proliferation. Representative cell lines, LNCaP and DU145, were transfected with DNA constructs in which a CMV promoter drove expression of either carotenoid enzyme, BCO1 (pCMV-BCO1) or BCO2 (pCMV-BCO2). Transfections with empty vector (pcDNA3) served as vector controls. Transfection efficiencies of $>50\%$ were verified by cotransfections with a GFP expression vector (data not shown). After 24 hours, cells were treated for an additional 24 hours \pm lycopene (1 μ mol/L). As shown in Fig. 5A, lycopene treatment plus BCO2 (but not BCO1) overexpression significantly reduced LNCaP cell proliferation. In contrast, although lycopene itself had no significant effect on DU145 cell proliferation, exogenous BCO2 overexpression in these cells significantly inhibited cell growth, even in the absence of lycopene (Fig. 5B). These findings prompted an examination whether BCO2 might be antiproliferative in prostate cancer independent of its enzymatic activity.

In these experiments, we designed mutant BCO2 expression constructs in which the enzymatic site is disrupted. Sequence alignment of carotenoid oxygenase family members (BCO1, BCO2, and RPE65) reveals four conserved histidines and seven acidic amino acid residues (His²²⁶, His²⁸⁶, His³⁷⁵, His⁵⁷⁴, Asp¹⁰⁵, Glu¹⁹⁴, Glu³⁶³, Glu⁴⁶⁵, Glu⁵¹⁷, Glu⁵²⁹, and Asp⁵³⁰) in human BCO2 (Supplementary Fig. S3). As mutation of any one

of the four conserved histidines results in total loss of BCO1 activity (29), we created BCO2-mt constructs of the four histidine residues required for the enzymatic pocket by site-directed mutagenesis (Supplementary Fig. S4A and S4B). To further characterize the effects of these proteins on cell growth in prostate cancer, we compared growth of prostate cancer cells expressing either wild-type BCO2 or mutated BCO2 using a colony formation assay. DU145 cells were transfected either with empty vector (pcDNA3), BCO2, or BCO2-mt. After transient transfection, cellular production of BCO2 and BCO2-mt was confirmed by Western blot analysis (Fig. 5C). As shown in Fig. 5D, colony formation was significantly decreased in both BCO2 and BCO2-mt-transfected cells compared with the empty vector control. Similar results were obtained in C4-2 cells after transient transfection of above constructs (Supplementary Fig. S5A and S5B). These data indicate the multifunctional BCO2 protein inhibits prostate cancer cell growth by mechanisms that are structurally and functionally distinct from its enzymatic activity.

Restoration of BCO2 inhibits NF- κ B transcriptional activity in prostate cancer cells

NF- κ B is a key regulator of cell proliferation and survival, and constitutive activation of NF- κ B is an important survival

pathway in many cancer cell types. The NF- κ B transcription complex also is constitutively activated in several prostate cancer cell lines and prostate carcinoma xenograft models. To determine whether BCO2-antiproliferative effects in prostate cancer are associated with NF- κ B activity, we asked whether restoring BCO2 expression inhibits NF- κ B signaling. First, we determined whether BCO2 suppresses NF- κ B activity in DU145 cells, a cell line in which NF- κ B is constitutively activated. DU145 cells were transfected with an NF- κ B luciferase reporter construct plus one of the following: pcDNA3 empty vector, BCO2, or BCO2-mt. Cells were then treated \pm lycopene for an additional 24 hours. As shown in Fig. 6A, lycopene treatment alone had no effect on NF- κ B luciferase reporter activity. In contrast, NF- κ B luciferase activity was significantly reduced in cells that expressed either the wild-type or the mutated BCO2. As indicated above, lycopene exposure itself had no growth effects in this lycopene-resistant prostate cancer line.

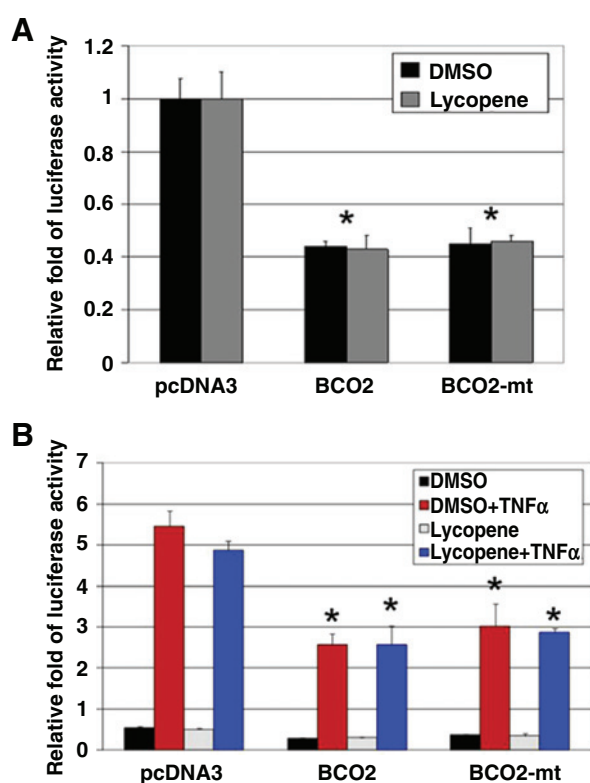


Figure 6. Exogenous BCO2 expression inhibits NF- κ B transcriptional activity in prostate cancer cells. **A**, DU145 cells were transiently transfected with a NF- κ B luciferase reporter, β -gal (transfection efficiency control), and the indicated expression constructs for 24 hours followed by treatment with either 1 μ mol/L lycopene or vehicle for 24 hours; cells were lysed and luciferase assays performed. Data represent mean \pm SD ($n = 3$) normalized to β -gal activity (*, $P < 0.05$, BCO2, or BCO2-mt expression vs. control). **B**, C4-2 cells were transiently transfected with the NF- κ B-luciferase reporter gene, β -gal, and the indicated constructs for 24 hours, and then treated with 1 μ mol/L lycopene or DMSO for 20 hours. After stimulation with TNF α (10 ng/mL) for 3 hours, cells were assayed for luciferase activity. Data represent mean \pm SD ($n = 3$) normalized to β -gal (*, $P < 0.05$, BCO2 or BCO2-mt expression vs. control).

Activation of NF- κ B is a central event in cellular responses to inflammatory signals; cytokines, such as TNF α , rapidly increase NF- κ B transcription. In subsequent experiments, C4-2 cells, in which NF- κ B is not constitutively activated but do respond stimulation with TNF α , were selected. We first exogenously expressed BCO2 or BCO2-mt in C4-2 cells and then stimulated the cells with TNF α . In cells transfected with the empty vector, TNF α caused the expected robust increase in NF- κ B transactivation (Fig. 6B), but in cells transfected with either BCO2 or BCO2-mt, TNF α -stimulated NF- κ B activity was significantly blunted. Collectively, these results suggest BCO2 suppresses basal and inducible NF- κ B activity, independent of its enzymatic activity.

BCO2 reduces NF- κ B p65 subunit nuclear translocation and DNA-binding activity

A common mechanism in NF- κ B activation is enhanced degradation of the I κ B α protein, which mediates NF- κ B release from its complex and nuclear translocation. As a consequence, NF- κ B DNA binding and gene transcription increases. To further explore the role of BCO2 in regulating NF- κ B activity, we tested whether BCO2 affects I κ B α levels in C4-2 cells (which do not contain constitutively active NF- κ B). C4-2 cells were transiently transfected with pcDNA3, BCO2, or BCO2-mt for 24 hours and treated with TNF α for 3 hours. Neither wild-type nor mutated BCO2 expression significantly affected basal or TNF α -stimulated I κ B α levels (Fig. 7A).

Cytoplasmic events, including sequential phosphorylation, polyubiquitination, and degradation of I κ B proteins result in nuclear translocation of the NF- κ B transcription factor complex. We next examined whether BCO2-mediated NF- κ B inhibition is associated with inhibited nuclear translocation of the NF- κ B complex subunit, p65. Subcellular localization of p65 was assayed in prostate cancer cells transfected with pcDNA3, BCO2, or BCO2-mt. As shown in Fig. 7B, transient overexpression of BCO2 or BCO2-mt decreased p65 nuclear translocation.

DNA-binding activity of NF- κ B was also assayed in the presence or absence of BCO2 or BCO2-mt expression using an ELISA-based DNA-binding assay. Protein-DNA binding of the NF- κ B family subunits (p65, p50, p52, c-Rel, and RelB) was identified using specific antibodies. Nuclear extracts prepared from prostate cancer cells expressing exogenous BCO2 or BCO2-mt contained less p65 and p50 capable of binding DNA (Fig. 7C). DNA-binding activities of the NF- κ B complex subunits, p52, c-Rel, and RelB, were not altered. Figure 7C shows a representative experiment with DU145 cells. The suppression of p65 subunit translocation and DNA binding is consistent.

Discussion

These findings help explain why lycopene inhibits cancer cell growth in some but not all prostate tumors. We also provide the first evidence that BCO2 inhibits prostate cancer cell growth. BCO2 had antiproliferative effects in all prostate cancer cell lines tested, irrespective of whether cell proliferation was sensitive to lycopene or not. Three lines of evidence support the role of BCO2 in prostate tumor suppression. First, reduction or loss of BCO2 expression is associated with progression from normal prostatic epithelium to androgen-resistant, aggressive prostate cancer. Second, restoring expression of the *bco2* gene inhibits

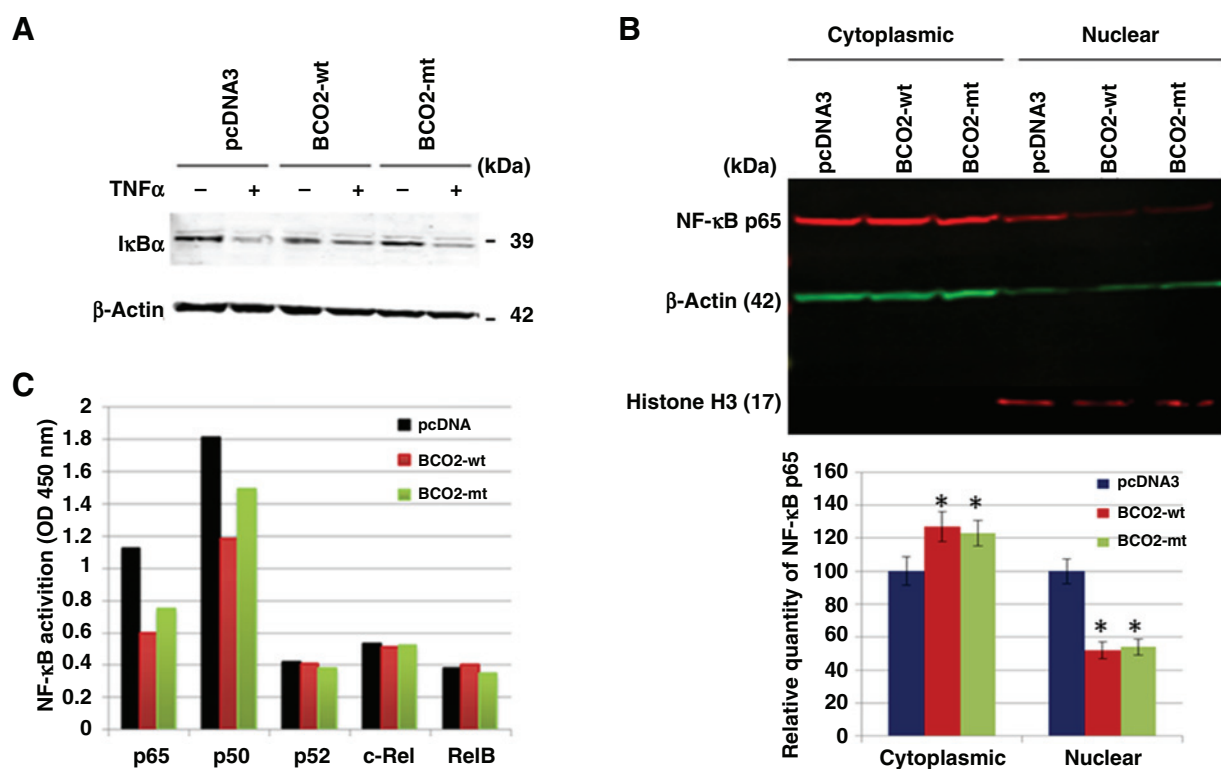


Figure 7. BCO2 expression does not affect I κ B α levels but reduces NF- κ B nuclear translocation and DNA binding. **A**, C4-2 cells were transiently transfected with the indicated constructs for 24 hours and treated with TNF α (10 ng/mL) for 3 hours. Total cellular protein lysates were isolated and I κ B α was detected by Western blot analysis using an I κ B α -specific antibody and β -actin detection as a loading control. **B**, DU145 cells were transiently transfected with the indicated constructs for 24 hours. The cellular extracts were separated into nuclear and cytoplasmic fractions and probed for NF- κ B p65; β -actin was used as a cytoplasmic marker and loading control; Histone H3 was used as nuclear marker (top). Bottom, quantification was performed using the results from three different transfections and immunoblots (*, $P < 0.05$, BCO2-wt or BCO2-mt expression vs. pcDNA3, respective subcellular fraction control). **C**, DU145 cells were transiently transfected with the indicated constructs for 24 hours and nuclear protein fractions were extracted. OD, optical density. The NF- κ B family protein DNA-binding assay is described in "Materials and Methods."

prostate cancer cell growth. Finally, BCO2 blocks NF- κ B activation, a critical pathway in prostate cancer cell growth. An unanticipated finding was BCO2 regulates cell proliferation apparently independently of this protein's enzymatic activity.

Unlike the related carotenoid cleavage enzyme BCO1, BCO2 is expressed in cardiac and skeletal muscle, prostate, endometrial connective tissue, and endocrine pancreas (21). In our study, prostate cancer tissues and cell lines demonstrated reduced or lost BCO2 expression compared with normal prostate. These findings suggest there may be an inverse association between BCO2 expression and prostatic carcinogenesis *in vivo* (Fig. 1A) and a similar association with aggressiveness (and possibly androgen resistance) *in vitro* (Fig. 1B).

One mechanism of loss of BCO2 expression in prostate cancer may occur by epigenetic silencing. Epigenetic silencing via promoter CpG island hypermethylation is a common way of inactivating tumor suppressors in cancer (30, 31), and hypermethylation of tumor suppressor genes in prostate cancer has been reported (32). Hypermethylation of relevant regulatory sites or transcription factors regulating *bco2* promoter may be involved in suppressing *bco2* gene expression in prostate cancer cells. The methylation status of the *bco2* promoter in prostate tissue specimens and in prostate cancer has not yet been interrogated.

In humans, although lycopene is not the most abundant dietary or circulating carotenoid, it is the chief carotenoid found in prostate tissue (5). The extent of lycopene accumulation in human prostate tissue has been controversial. A previous study indicated lycopene accumulation in human prostate cancer tissue was higher than that in normal prostate tissue (23). Recently, a human study suggested prostatic lycopene concentrations below 1 ng/mg prostate protein threshold are associated with prostate cancer at 6-month follow-up biopsy (33). The observation that lycopene tissue levels are significantly greater in malignant than in benign prostate tissue suggests either that lycopene uptake is increased or lycopene metabolism is diminished in prostate tumors. Close examination of the former possibility was beyond the scope of this study. However, our data on lycopene uptake in PrEC and prostate cancer cell cultures do not support this hypothesis. In contrast, the discovery that BCO2 expression is significantly reduced in prostate cancer may account for, at least in part, why lycopene accumulates in prostate tumors.

Lycopene supplementation induces *bco2* expression in lung tissues (17). This study shows lycopene also upregulates *bco2* gene expression in those prostate cancer cells that retain lycopene-inhibited cell growth. These observations are consistent with a

previous report (34) that lycopene induces quiescence and apoptosis in androgen-sensitive, but not in androgen-resistant prostate cancer cells.

Lycopene is among the most potent antioxidant carotenoids. In fact, the inverse association between prostate cancer risk and consumption of tomatoes and tomato paste generally has been attributed to this antioxidant property (35). For example, in short-term dietary interventions in prostate cancer patients (35), increased lycopene intake reduced markers of DNA-oxidative damage in leukocytes and prostate tissue. However, despite a common assumption that carotenoids, including lycopene, are biologically and clinically important nutritional antioxidants, rigorous *in vivo* and *in vitro* experimental support for this conclusion has been limited, and the physiologic significance of lycopene's antioxidant action as its major anticarcinogenic activity remains largely speculative (8). Other postulated mechanisms for lycopene action in biological systems include growth inhibition (36–39), induction of differentiation (40), induction of apoptosis (9, 39, 41), and increased gap junction intercellular communication via upregulation of the tumor suppressor connexin 43 (42).

In prostate, it has not been firmly established whether lycopene itself, its metabolites, or, as we now propose, its metabolic enzyme BCO2 is responsible for growth inhibition. The lycopene metabolite, apo-10'-lycopenoic acid, inhibits lung cancer cell growth *in vitro* and suppress murine lung tumorigenesis *in vivo* (43). More recently, supraphysiologic concentrations of apo-12'-lycopenal were shown to inhibit androgen-resistant prostate cancer cell growth (10, 19). As lycopene metabolites may be antitumorigenic, loss of lycopene metabolism in advanced prostate cancer may explain some of the inconsistent findings among lycopene trials. Indeed, recent clinical data have called into question whether lycopene supplementation has any well-defined clinical benefits in patients who have advanced hormone-refractory prostate cancer (44, 45).

Another novel finding is BCO2 inhibits prostate cancer cell growth independently of any lycopene interactions or lycopene effects. Along these lines, we observed that an enzymatically inactive BCO2 mutant can evoke the same antiproliferative effects as does the wild-type enzyme. Of note, we have detected naturally occurring isoforms of BCO2 (via alternative splicing) that have drastically reduced enzymatic activity (data not shown). A recent report on the differences in expression, localization, and activity between mouse and human BCO2 indicates inactivity of a human BCO2 expressed via alternative splicing and the presence of four amino acid residues, GKAA (46).

Finally, this report proposes the effects of BCO2 on prostate cancer cells are associated with cellular NF- κ B activity. The NF- κ B

family of transcription factors regulates expression of a wide range of genes that, in turn, regulate cell proliferation and cell survival (47). NF- κ B signaling is constitutively activated in androgen-resistant prostate cancer cell lines, and NF- κ B pathway activation can suffice to maintain androgen-resistant tumor growth via regulation of androgen receptor action (48). We demonstrate that BCO2, a predominantly mitochondrial (18) protein, reduces NF- κ B p65 subunit nuclear translocation and DNA binding. We speculate BCO2 may sequester NF- κ B at the mitochondrial surface via a protein–protein interaction. The NF- κ B p65 subunit, together with IKK subunits, also has been identified as a mitochondrial protein (11, 49, 50). The molecular interaction(s) by which downregulation of mitochondrial BCO2 contributes to enhanced NF- κ B activity has not yet been ascertained. Our current model involves BCO2 inhibition of NF- κ B activity by retaining p65 in mitochondria. The exact mechanisms are currently under investigation.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Gong, S. Zaripheh, L.P. Rubin
Development of methodology: X. Gong, R. Marisiddaiah, S. Zaripheh, D. Wiener, L.P. Rubin
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Zaripheh, D. Wiener, L.P. Rubin
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Gong, R. Marisiddaiah, L.P. Rubin
Writing, review, and/or revision of the manuscript: X. Gong, L.P. Rubin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Gong, D. Wiener
Study supervision: L.P. Rubin

Acknowledgments

We thank the late Dr. Stefan Andersson (University of Houston, Houston, TX) for providing the pCMV-BCO1 and pCMV-BCO2 expression vectors, Dr. Hansgeorg Ernst (BASF) for the gift of lycopene beadlets and purified apo-10'-lycopenal, and Lucy Li (Paul L. Foster School of Medicine, Texas Tech University Health Sciences Center, El Paso, TX) for help in preparation of cells and Western blotting.

Grant Support

This work was supported in part by grants from the NIH, HD42174 and RR18722, and the Muma Family Foundation.

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Received March 4, 2016; revised June 6, 2016; accepted June 27, 2016; published OnlineFirst July 12, 2016.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. *CA Cancer J Clin* 2016;66:7–30.
2. Giovannucci E, Rimm EB, Liu Y, Stampfer MJ, Willett WC. A prospective study of tomato products, lycopene, and prostate cancer risk. *J Natl Cancer Inst* 2002;94:391–8.
3. Dahan K, Fennal M, Kumar NB. Lycopene in the prevention of prostate cancer. *J Soc Integr Oncol* 2008;6:29–36.
4. Key TJ, Appleby PN, Travis RC, Albanes D, Alberg AJ, Barricarte A, et al. Carotenoids, retinol, tocopherols, and prostate cancer risk: pooled analysis of 15 studies. *Am J Clin Nutr* 2015;102:1142–57.
5. van Breemen RB, Xu X, Viana MA, Chen L, Stacewicz-Sapuntzakis M, Duncan C, et al. Liquid chromatography-mass spectrometry of *cis*- and *all-trans*-lycopene in human serum and prostate tissue after dietary supplementation with tomato sauce. *J Agric Food Chem* 2002;50:2214–9.
6. Giovannucci E. Tomato products, lycopene, and prostate cancer: a review of the epidemiological literature. *J Nutr* 2005;135:2030S–1S.
7. Clinton SK. Lycopene: chemistry, biology, and implications for human health and disease. *Nutr Rev* 1998;56:35–51.
8. Erdman JW Jr, Ford NA, Lindshield BL. Are the health attributes of lycopene related to its antioxidant function? *Arch Biochem Biophys* 2009;483:229–35.

9. Hwang ES, Bowen PE. Cell cycle arrest and induction of apoptosis by lycopene in LNCaP human prostate cancer cells. *J Med Food* 2004;7:284–9.
10. Ford NA, Elsen AC, Zuniga K, Lindshield BL, Erdman JW Jr. Lycopene and apo-12'-lycopenal reduce cell proliferation and alter cell cycle progression in human prostate cancer cells. *Nutr Cancer* 2011;63:256–63.
11. Bottero V, Rossi F, Samson M, Mari M, Hofman P, Peyron JF. Ikappa b-alpha, the NF-kappa B inhibitory subunit, interacts with ANTI, the mitochondrial ATP/ADP translocator. *J Biol Chem* 2001;276:21317–24.
12. Moise AR, von Lintig J, Palczewski K. Related enzymes solve evolutionarily recurrent problems in the metabolism of carotenoids. *Trends Plant Sci* 2005;10:178–86.
13. von Lintig J, Vogt K. Filling the gap in vitamin A research. Molecular identification of an enzyme cleaving beta-carotene to retinal. *J Biol Chem* 2000;275:11915–20.
14. Wyss A, Wirtz G, Woggon W, Brugger R, Wyss M, Friedlein A, et al. Cloning and expression of beta,beta-carotene 15,15'-dioxygenase. *Biochem Biophys Res Commun* 2000;271:334–6.
15. Kiefer C, Hessel S, Lampert JM, Vogt K, Lederer MO, Breithaupt DE, et al. Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A. *J Biol Chem* 2001;276:14110–6.
16. Gajic M, Zaripheh S, Sun F, Erdman JW Jr. Apo-8'-lycopenal and apo-12'-lycopenal are metabolic products of lycopene in rat liver. *J Nutr* 2006;136:1552–7.
17. Hu KQ, Liu C, Ernst H, Krinsky NI, Russell RM, Wang XD. The biochemical characterization of ferret carotene-9',10'-monooxygenase catalyzing cleavage of carotenoids *in vitro* and *in vivo*. *J Biol Chem* 2006;281:19327–38.
18. Amengual J, Lobo GP, Golczak M, Li HN, Klimova T, Hoppel CL, et al. A mitochondrial enzyme degrades carotenoids and protects against oxidative stress. *FASEB J* 2011;25:948–59.
19. Ford NA, Clinton SK, von Lintig J, Wyss A, Erdman JW Jr. Loss of carotene-9',10'-monooxygenase expression increases serum and tissue lycopene concentrations in lycopene-fed mice. *J Nutr* 2010;140:2134–8.
20. Lindqvist A, Andersson S. Cell type-specific expression of beta-carotene 15,15'-mono-oxygenase in human tissues. *J Histochem Cytochem* 2004;52:491–9.
21. Lindqvist A, He YG, Andersson S. Cell type-specific expression of beta-carotene 9',10'-monooxygenase in human tissues. *J Histochem Cytochem* 2005;53:1403–12.
22. Hessel S, Eichinger A, Isken A, Amengual J, Hunzelmann S, Hoeller U, et al. CMO1 deficiency abolishes vitamin A production from beta-carotene and alters lipid metabolism in mice. *J Biol Chem* 2007;282:33553–61.
23. Clinton SK, Emehiser C, Schwartz SJ, Bostwick DG, Williams AW, Moore BJ, et al. cis-trans lycopene isomers, carotenoids, and retinol in the human prostate. *Cancer Epidemiol Biomarkers Prev* 1996;5:823–33.
24. Gong X, Tsai SW, Yan B, Rubin LP. Cooperation between MEF2 and PPARgamma in human intestinal beta,beta-carotene 15,15'-monooxygenase gene expression. *BMC Mol Biol* 2006;7:7.
25. Gong X, Marisiddaiah R, Rubin LP. beta-carotene regulates expression of beta-carotene 15,15'-monooxygenase in human alveolar epithelial cells. *Arch Biochem Biophys* 2013;539:230–8.
26. Sugawara T, Kushihiro M, Zhang H, Nara E, Ono H, Nagao A. Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human intestinal cells. *J Nutr* 2001;131:2921–7.
27. Liu A, Pajkovic N, Pang Y, Zhu D, Calamini B, Mesecar AL, et al. Absorption and subcellular localization of lycopene in human prostate cancer cells. *Mol Cancer Ther* 2006;5:2879–85.
28. Bonkhoff H, Remberger K. Differentiation pathways and histogenetic aspects of normal and abnormal prostatic growth: a stem cell model. *Prostate* 1996;28:98–106.
29. Poliakov E, Gentleman S, Cunningham FX Jr, Miller-Ihli NJ, Redmond TM. Key role of conserved histidines in recombinant mouse beta-carotene 15,15'-monooxygenase-1 activity. *J Biol Chem* 2005;280:29217–23.
30. Herman JC, Baylin SB. Gene silencing in cancer in association with promoter hypermethylation. *N Engl J Med* 2003;349:2042–54.
31. Egger G, Liang G, Aparicio A, Jones PA. Epigenetics in human disease and prospects for epigenetic therapy. *Nature* 2004;429:457–63.
32. Hoque MO. DNA methylation changes in prostate cancer: current developments and future clinical implementation. *Expert Rev Mol Diagn* 2009;9:243–57.
33. Mariani S, Lionetto L, Cavallari M, Tubaro A, Rasio D, De Nunzio C, et al. Low prostate concentration of lycopene is associated with development of prostate cancer in patients with high-grade prostatic intraepithelial neoplasia. *Int J Mol Sci* 2014;15:1433–40.
34. Ivanov NI, Cowell SP, Brown P, Rennie PS, Guns ES, Cox ME. Lycopene differentially induces quiescence and apoptosis in androgen-responsive and -independent prostate cancer cell lines. *Clin Nutr* 2007;26:252–63.
35. Chen L, Stacewicz-Sapuntzakis M, Duncan C, Sharifi R, Ghosh L, van Breemen R, et al. Oxidative DNA damage in prostate cancer patients consuming tomato sauce-based entrees as a whole-food intervention. *J Natl Cancer Inst* 2001;93:1872–9.
36. Kotake-Nara E, Kushihiro M, Zhang H, Sugawara T, Miyashita K, Nagao A. Carotenoids affect proliferation of human prostate cancer cells. *J Nutr* 2001;131:3303–6.
37. Heber D, Lu QY. Overview of mechanisms of action of lycopene. *Exp Biol Med* 2002;227:920–3.
38. Obermuller-Jevic UC, Olano-Martin E, Corbacho AM, Eischer JP, van der Vliet A, Valacchi G, et al. Lycopene inhibits the growth of normal human prostate epithelial cells *in vitro*. *J Nutr* 2003;133:3356–60.
39. Hantz HL, Young LF, Martin KR. Physiologically attainable concentrations of lycopene induce mitochondrial apoptosis in LNCaP human prostate cancer cells. *Exp Biol Med* 2005;230:171–9.
40. Karas M, Amir H, Fishman D, Danilenko M, Segal S, Nahum A, et al. Lycopene interferes with cell cycle progression and insulin-like growth factor I signaling in mammary cancer cells. *Nutr Cancer* 2000;36:101–11.
41. Bowen P, Chen L, Stacewicz-Sapuntzakis M, Duncan C, Sharifi R, Ghosh L, et al. Tomato sauce supplementation and prostate cancer: lycopene accumulation and modulation of biomarkers of carcinogenesis. *Exp Biol Med* 2002;227:886–93.
42. Aust O, Ale-Agha N, Zhang L, Wollersen H, Sies H, Stahl W. Lycopene oxidation product enhances gap junctional communication. *Food Chem Toxicol* 2003;41:1399–407.
43. Lian F, Smith DE, Ernst H, Russell RM, Wang XD. Apo-10'-lycopenoic acid inhibits lung cancer cell growth *in vitro*, and suppresses lung tumorigenesis in the A/J mouse model *in vivo*. *Carcinogenesis* 2007;28:1567–74.
44. Jatoi A, Burch P, Hillman D, Vanyo JM, Dakhil S, Nikkevich D, et al. A tomato-based, lycopene-containing intervention for androgen-independent prostate cancer: results of a phase II study from the north central cancer treatment group. *Urology* 2007;69:289–94.
45. Schwenke C, Ubrig B, Thurmann P, Eggersmann C, Roth S. Lycopene for advanced hormone refractory prostate cancer: a prospective, open phase II pilot study. *J Urol* 2009;181:1098–103.
46. Li B, Vachali PP, Gorusupudi A, Shen Z, Sharifzadeh H, Besch BM, et al. Inactivity of human beta,beta-carotene-9',10'-dioxygenase (BCO2) underlies retinal accumulation of the human macular carotenoid pigment. *Proc Natl Acad Sci U S A* 2014;111:10173–8.
47. Hayden MS, Ghosh S. Signaling to NF-kappaB. *Genes Dev* 2004;18:2195–224.
48. Jin RJ, Lho Y, Connelly L, Wang Y, Yu X, Saint Jean L, et al. The nuclear factor-kappaB pathway controls the progression of prostate cancer to androgen-independent growth. *Cancer Res* 2008;68:6762–9.
49. Cogswell PC, Kashatus DF, Keifer JA, Guttridge DC, Reuther JY, Bristow C, et al. NF-kappa B and I kappa B alpha are found in the mitochondria. Evidence for regulation of mitochondrial gene expression by NF-kappa B. *J Biol Chem* 2003;278:2963–8.
50. Guseva NV, Taghiyev AF, Sturm MT, Rokhlin OW, Cohen MB. Tumor necrosis factor-related apoptosis-inducing ligand-mediated activation of mitochondria-associated nuclear factor-kappaB in prostatic carcinoma cell lines. *Mol Cancer Res* 2004;2:574–84.