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Lycopene and apo-10'-lycopenal do not alter DNA methylation of GSTP1 in LNCaP cells

Ann G. Liu and John W. Erdman Jr.*

Division of Nutritional Sciences and Department of Food Science and Human Nutrition, 905 S. Goodwin Avenue, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

Abstract

DNA hypermethylation and silencing of tumor-suppressor genes are commonly seen in human cancers, and likely contribute to the process of carcinogenesis. A growing body of evidence suggests that dietary compounds may alter cancer risk through epigenetic modifications. Glutathione S-transferase P1 (GSTP1) is hypermethylated in >90% of prostate cancer cases making it one of the most common genome alterations in prostate cancer. LNCaP cells were treated either with lycopene or apo-10'-lycopenal for 7 days, and mRNA expression and DNA methylation of GSTP1 were evaluated. Neither compound significantly altered expression nor methylation of GSTP1 while treatment with 5-azacytidine decreased methylation by 78%. These findings demonstrate that lycopene and apo-10'-lycopenal are not effective demethylating agents of GSTP1 in the human LNCaP cell line.

Keywords

Lycopene; lycopene metabolites; prostate cancer; carotenoids; epigenetics

INTRODUCTION

Prostate cancer is the most commonly diagnosed male cancer and second leading cause of male cancer deaths in United States with approximately 186,000 new cases diagnosed each year [1]. Environmental exposures can alter gene expression through epigenetic modifications and these modifications may in turn alter disease susceptibility [2]. The two major types of epigenetic changes are modifications to DNA, in the form of DNA methylation, and modifications to chromatin packaging, specifically histone modification.

DNA methylation is critical for regulation of gene transcription and structure of the cell nucleus. Aberrant DNA methylation patterns are commonly seen in human cancers which often consist of global hypomethylation of DNA accompanied by hypermethylation of specific tumor-suppressor genes [3]. Currently, the methylation status of glutathione S-transferase P1 (GSTP1) is being investigated to determine if it can serve as an early and specific biomarker of prostate cancer [4, 5].

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*CORRESPONDING AUTHOR: Tel: 217-333-2527, Fax: 217-333-9368, jwerdman@illinois.edu .

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Gluathione S-transferases (GSTs) are a large family of detoxification enzymes which catalyze conjugation reactions with reduced glutathione. They play an important role in protecting DNA from reactive electrophilic compounds and oxidants. GSTP1 is hypermethylated in >90% of prostate cancer cases and 70% of prostatic intraepithelial neoplasia lesions making it one of the earliest and most commonly found genomic alterations in prostate cancer [5]. Mice that are null for GSTP1 develop skin tumors at significantly increased rates compared to wild type mice when exposed to the carcinogen 7,12 dimethylbenz[a]anthracene [6]. GSTP1 is completely silenced in LNCaP human prostate cancer cells due to hypermethylation [7]. When these cells are exposed to the carcinogenic heterocyclic amine 2-Amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), high levels of PhIP-DNA adducts are formed, but when the cells are modified to express GSTP1 they are resistant to the formation of PhIP-DNA adducts [8]. These data demonstrate that GSTP1 may play a key role in the prevention of cancer formation.

Recent data suggest that dietary compounds may prevent cancer through epigenetic modifications in the cell [9, 10]. Tomatoes are the second most commonly consumed vegetable in the American diet second only to potatoes [11]. In 2007, the average American consumed 19.2 pounds of fresh tomatoes and 68.8 pounds of processed tomato products [12]. Epidemiological studies have linked higher consumption of tomatoes and tomato products with decreased risk of prostate cancer [13]. In the Health Professionals Follow-Up Study, men who consumed >2 servings of tomato sauce per week had a 23% reduction in risk of prostate cancer vs men who consumed <1 serving per month (RR = 0.77; 95% CI = 0.66 to 0.90; $P_{\text{trend}} < .001$) [14]. The anti-carcinogenic effects of tomatoes are often associated with lycopene, the major carotenoid found in tomatoes. Lycopene has been shown to be a potent antioxidant and exert several other anti-carcinogenic effects *in vitro* including induction of cell cycle arrest [15]. However, the ability of lycopene to act through epigenetic alterations has thus far only been examined in one study where lycopene was shown to partially demethylate the GSTP1 promoter and restore GSTP1 expression in breast cancer cell lines [16]. Thus lycopene may also be able to prevent or slow prostate cancer growth through alterations in DNA methylation patterns. Here we investigate whether lycopene or one of its reported metabolites, apo-10'-lycopenal, can restore expression of GSTP1 in prostate cancer cell lines through demethylation of the GSTP1 promoter.

MATERIALS AND METHODS

Cell lines and Culture Conditions

The LNCaP cell line was originally isolated in 1977 by J.S. Horoszewicz, et al., from a needle aspiration biopsy of the lymph node of a 50-year-old Caucasian male with a confirmed diagnosis of metastatic prostate carcinoma [17]. The cells are androgen receptor positive and responsive to dihydrotestosterone. LNCaP cells were purchased from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 media (Sigma-Aldrich, St, Louis, MO) containing 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, and 5.6 mg/ml amphotericin B. Cells were maintained at 37°C with 5% CO₂ and atmospheric oxygen partial pressure.

Carotenoid Preparation

Doses of lycopene and apo-10'-lycopenal were prepared fresh daily. For lycopene, water-soluble lycopene beadlets (5.7% pure lycopene, DSM Nutritional Products, Basel, Switzerland) were dissolved in deionized water and added to 10 mL of media. Apo-10'-lycopenal (gift from Hansgeorg Ernst, BASF) is not available in beadlet form and was dissolved in dimethyl sulfoxide (DMSO) and added to 5 mL of media. The resulting stock

solutions were sonicated and filtered through a 0.22 μ m sterile filter. Solubilization was confirmed through quick observation under a light microscope. Stock lycopene or apo-10'-lycopenal concentrations were determined by extracting 100 μ L of media and measuring absorbance via a spectrophotometer (lycopene $\lambda_{\text{max}} = 472$ nm, $A_{1\text{ cm}}^{1\%} = 3450$ in hexane; apo-10'-lycopenal $\lambda_{\text{max}} = 460$ nm, $A_{1\text{ cm}}^{1\%} = 2684$ in hexane). All carotenoid extracts were kept on ice and under yellow lights throughout the extraction process.

Experimental Design

In the first experiment, cells were dosed daily with lycopene at 1, 2, or 4 μ M for seven days. Placebo beadlets were used as a vehicle control. In the second experiment, cells were dosed daily with apo-10'-lycopenal at 0.01, 0.1, or 1 μ M for seven days, and 0.1 % DMSO was used as a vehicle control. In both experiments, media were changed daily to minimize carotenoid degradation. Three independent replicates were performed for each assay, with $n=3$ per dose level.

5-azacytidine (Sigma-Aldrich, St. Louis, MO) is a known demethylating agent and was used as a positive control. Cells were dosed with 5 M 5-azacytidine every other day [18]. All experiments were carried out under dim light.

Carotenoid Analysis

Cells were rinsed with phosphate buffered saline and pelleted. For cell extraction, cells were resuspended in 100 μ L of water, and 500 μ L of acetone was added. Samples were sonicated for 10 min, centrifuged at 7000 $\times$ g for 5 min, and the supernatant was transferred to new tube. The acetone extract was dried down with argon and stored at -20°C for ≤ 24 h prior to HPLC-PDA analysis. For medium extraction, 1.0 mL ethanol containing 0.1% butylated hydroxytoluene was added to 1.0 mL medium and vortexed. Media samples were extracted 3 times with 2.0 mL hexane. Hexane extracts were dried in a Speedvac evaporator (model AS160; Savant, Farmingdale, NY), flushed with argon, and stored at -20°C for ≤ 24 h prior to HPLC-PDA analysis. All carotenoid extracts were kept on ice and under yellow lights throughout the extraction process.

Lycopene concentrations were analyzed by a reverse-phase HPLC-PDA system previously described [19]. Apo-10'-lycopenal concentrations were analyzed using a similar system. The system consisted of two Ranin Dynamax pumps (Model SD-200; Varian, Walnut Creek, CA), a C30 column (4.6 $\times$ 250 mm, 3 μ m, YMC, Wilmington, NC) with a guard column, an 18 $^{\circ}\text{C}$ column oven and a photodiode array detector (Model 2996, Waters, Milford, MA) and Millennium³² software (Waters). Mobile phase A (methanol: 15% ammonium acetate aqueous solution= 98:2, v/v) and mobile phase B (methanol: MTBE: 15% ammonium acetate aqueous solution= 8:90:2, v/v) were used, and the gradient profile was as follows: 0 min, 0%B; 30 min, 100%B; 40 min, 100%B; 45 min, 0%B; 50 min, 0%B. Samples were reconstituted in MTBE for injection, and the apo-10'-lycopenal was measured at 460 nm. All analyses were performed in duplicate, and the quantification of carotenoid isomers was carried out by comparing the UV spectra and retention times to analytical standards of lycopene or apo-10'-lycopenal (gifts from Hansgeorg Ernst, BASF, Ludwigshafen, Germany).

Real-time quantitative PCR

Total RNA was extracted from cells using Trizol (Invitrogen, Carlsbad, CA) per the manufacturer's instructions. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). mRNA expression of selected genes was measured via real-time PCR using SYBR Green Master Mix (Applied Biosystems, Foster City, CA). Reactions were monitored by an ABI Prism 7900HT. Primer

pairs were designed to measure glutathione-S-transferase π 1 (GSTP1) [8] and ribosomal protein L7a (RPL7A) [20]. A validation experiment was performed on each set of primers (Integrated DNA Technologies, Coralsville, IA) to confirm efficiency and product specificity. A serial dilution was used to create a standard curve for quantification. RPL7A was used as a housekeeping gene [20].

Methylation-specific PCR

Total genomic DNA was extracted from cells using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO). DNA was bisulfite treated using the EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA). MethPrimer (<http://www.urogene.com/methprimer>) was used to design primer pairs to measure methylated GSTP1 forward-5'-GGAAAGAGGGAAAGGTTTTTTC-3' and reverse-5'-GCCCTAAAATCCCCGAA-3' and unmethylated GSTP1 forward-5'-GGAAAGAGGGAAAGGTTTTTTT-3' and reverse-5'-AACACCCTAAAATCCCCAAA-3'. A serial dilution was used to create a standard curve for quantification. Reactions were monitored by an ABI Prism 7900HT.

Statistical Analysis

Dose-response effects were analyzed by linear regression, using the REG procedure of SAS Statistical Software (version 9.2; SAS Institute, Cary, NC), and values were considered different at $P < 0.05$. 5-azacytidine treated cells were compared among treatments by two-tailed analysis of variance, using the MIXED procedure. Values were considered different from controls at $P < 0.05$ using Tukey's procedure.

RESULTS AND DISCUSSION

Cellular uptake of carotenoids

Lycopene was provided at doses considered to be physiologically relevant (1 and 2 μ M) and super-physiological (4 μ M). Lycopene was taken up by cells in a dose-dependent manner as shown in Figure 1 ($P = 0.008$). The average lycopene content was 42, 61, and 78 pmol per million cells for 1 μ M, 2 μ M, and 4 μ M dose levels, respectively. These levels are similar to those observed in previous studies with lycopene dosing of LNCaP cells [21].

When lycopene is enzymatically cleaved by carotenoid monooxygenase II, it yields one molecule of apo-10'-lycopenal. Concentrations of apo-10'-lycopenal are much lower in human serum than lycopene. In human subjects consuming a high tomato juice diet for 8 weeks, the average concentration of apo-10'-lycopenal was 0.28 nM [22]. Even though the dose levels used in our study are relatively low compared to the concentrations sometimes used by others in cell culture experiments, our dose levels would still be considered super-physiological. The average apo-10'-lycopenal content of was 2.5 and 2.3 pmol per million cells for 1 μ M and 0.1 μ M dose levels, respectively, indicating very poor absorption by LNCaP cells. At the 0.01 μ M dose level, no apo-10'-lycopenal could be detected in the cells (data not shown). Thus the cellular concentration in LNCaP cells was considerably below what has been reported in human serum. There have been no reports of cellular levels of apo-10'-lycopenal, but our lab has previously identified apo-8'-lycopenal and apo-12'-lycopenal in the liver of rats following lycopene feeding [23].

GSTP1 expression and promoter methylation

Expression of GSTP1 and DNA methylation of the GSTP1 promoter were not significantly altered by treatment with either lycopene or apo-10'-lycopenal (Figures 2 and 3). Treatment with 5-azacytidine resulted in significant demethylation and increased expression of GSTP1 as expected ($P < 0.05$). These results indicate that lycopene is not an effective

demethylating agent in LNCaP cells. It is possible that lycopene could have epigenetic effects in different prostate cancer cell lines or on different genes. It is well known that the degree of hypermethylation of various genes varies by cell line [24]. King-Batoon et al. demonstrated that a single dose of 2 μ M lycopene partially demethylated the GSTP1 promoter in MDA-MB-468 cells but not in MCF-7 cells. The effect was also specific to the GSTP1 as RAR β 2, another gene hypermethylated in breast cancer, was not affected by lycopene treatment [16].

Using lycopene in cell culture can be difficult due to issues with stability and solubility. Multiple systems have been used to deliver carotenoids *in vitro*, and differences could potentially arise due to different delivery methods. Here we used water-soluble lycopene beadlets containing approximately 65% all-trans lycopene and 35% cis-isomers, and media was changed daily to minimize carotenoid degradation. However, King-Batoon et al. used tetrahydrofuran (THF) as a solvent and gave a single lycopene dose at the beginning of the 7-day study [16]. Lycopene solubilized in THF degrades rapidly in cell culture conditions with a half-life of less than 2 hr [21]. In contrast, when water-soluble lycopene beadlets are used as a delivery vehicle, approximately 80% of the original dose remains in the medium after 24 hours [25]. This variation with *in vitro* techniques can make interpretation of the literature somewhat confusing.

In summary, we have shown that lycopene and apo-10'-lycopenal are taken up by LNCaP cells but do not alter DNA methylation of GSTP1. Further research is needed to determine whether lycopene might exert epigenetic effects on other tumor suppressor genes in LNCaP cells or in other prostate cancer cell lines.

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Highlights

- The GSTP1 promoter is hypermethylated in over 90% of prostate cancer cases.
- The epigenetic effects of lycopene on prostate cancer have not been previously reported.
- Neither lycopene nor its putative metabolite, apo-10'-lycopenal, altered DNA methylation of GSTP1 in LNCaP cells.

Figure 1.
Lycopene concentration in LNCaP cells. Values are means $\pm$ SEM, n=3.

Figure 2. GSTP1 mRNA expression (A) and GSTP1 promoter methylation (B) of LNCaP cells treated with lycopene. Values are means of three independent experiments $\pm$ SEM, $n=3$. Asterisk indicates significant differences ($P < 0.05$).

Figure 3. GSTP1 mRNA expression (A) and GSTP1 promoter methylation (B) of LNCaP cells treated with apo-10'-lycopenal. Values are means of three independent experiments $\pm$ SEM, $n=3$. Asterisk indicates significant differences ($P < 0.05$)