Absorption and subcellular localization of lycopene in human prostate cancer cells

Ang Liu, Natasa Pajkovic, Yan Pang, Dongwei Zhu, Barbara Calamini, Andrew L. Mesecar, and Richard B. van Breemen
Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, Chicago, Illinois

Abstract
Lycopene, the red pigment of the tomato, is under investigation for the chemoprevention of prostate cancer. Because dietary lycopene has been reported to concentrate in the human prostate, its uptake and subcellular localization were investigated in the controlled environment of cell culture using the human prostate cancer cell lines LNCaP, PC-3, and DU145. After 24 hours of incubation with 1.48 μmol/L lycopene, LNCaP cells accumulated 126.6 pmol lycopene/million cells, which was 2.5 times higher than PC-3 cells and 4.5 times higher than DU145 cells. Among these cell lines, only LNCaP cells express prostate-specific antigen and fully functional androgen receptor. Levels of prostate-specific antigen secreted into the incubation medium by LNCaP cells were reduced 55% as a result of lycopene treatment at 1.48 μmol/L. The binding of lycopene to the ligand-binding domain of the human androgen receptor was carried out, but lycopene was not found to be a ligand for this receptor. Next, subcellular fractionation of LNCaP cells exposed to lycopene was carried out using centrifugation and followed by liquid chromatography-tandem mass spectrometry quantitative analysis to determine the specific cellular locations of lycopene. The majority of lycopene (55%) was localized to the nuclear membranes, followed by 26% in nuclear matrix, and then 19% in microsomes. No lycopene was detected in the cytosol. These data suggest that the rapid uptake of lycopene by LNCaP cells might be facilitated by a receptor or binding protein and that lycopene is stored selectively in the nucleus of LNCaP cells. [Mol Cancer Ther 2006; 5(11):2879–85]

Introduction
Prostate cancer is the most frequently diagnosed male cancer and the second leading cause of cancer-related mortality in men in the Western world. In 2005, there were 232,090 new cases of prostate cancer diagnosed in the United States and 30,350 cancer deaths (1). Because prostate cancer tumors usually grow slowly, more men die “with prostate cancer” than “of prostate cancer” (2). The molecular mechanisms responsible for the initiation and progression of prostate cancer have not been fully elucidated, and the only established risk factors for this disease include age, ethnic group, and family history (3). In addition, the importance of diet in prostate carcinogenesis has been shown by epidemiologic studies of Asian immigrants to Hawaii or California (4). Among the dietary compounds implicated in the chemoprevention of prostate cancer, lycopene, the red pigment of tomatoes and tomato-derived products, is one of the most promising (5–7).

Lycopene (3,3-carotene) is an acyclic hydrocarbon carotenoid that contains 11 conjugated and 2 nonconjugated double bonds arranged linearly in the all-trans configuration (see structure in Fig. 1). Due to its hydrophobicity and sensitivity to degradation by light and heat, tetrahydrofuran, dimethylsulfoxide, and special vehicles, such as liposomes and water-dispersible beadlets, have been used to solubilize, stabilize, and deliver lycopene to cells growing in culture (8, 9). Lycopene is one of the most abundant carotenoids found in the diet, blood, and tissues, and in the United States, lycopene reaches plasma concentrations between 0.01 and 1.8 μmol/L with median plasma concentrations of 0.59 μmol/L (10–12). In addition to liver, the primary storage site, the highest lycopene levels have been detected in the adrenals, testes, and prostate (10). However, mechanisms for the selective accumulation of lycopene in organs such as the prostate are unknown.

In vitro (8, 9, 13, 14) and in vivo studies using animal models (15) or controlled clinical trials (16, 17) are consistent with cancer chemopreventive effects of lycopene. For example, a rat study using the MatLyLu Dunning prostate cancer model indicated that dietary lycopene prevented tumor growth and induced tumor necrosis (15). In a review of epidemiologic evidence relating cancer incidence to intake of tomatoes and tomato-derived products, 57 of 72 studies showed an inverse relationship between tomato intake or blood lycopene level and the risk of specific cancers, including prostate cancer (18–20). Although the evidence for the prevention of prostate cancer by lycopene is accumulating, exactly how lycopene exerts its protective effect is still unclear (15, 21–23).

The present cell culture study used the well-characterized human prostate cancer cell lines LNCaP, PC-3, and DU145, and was part of our ongoing investigation of the mechanisms by which lycopene might contribute to the prevention of prostate cancer.
of prostate cancer. LNCaP cells are an androgen-sensitive prostate cancer cell line with high expression of the androgen receptor (AR) and androgen-regulated genes, such as prostate-specific antigen. In contrast, PC-3 and DU145 cells, with low and no AR expression, respectively, are androgen insensitive. The cellular uptakes of lycopene by LNCaP, PC-3, and DU145 cells were compared using water-dispersible beadlets to solubilize the hydrophobic lycopene in cell culture medium. Because these studies indicated that LNCaP cells accumulated the most lycopene, the uptake kinetics and subcellular distribution of lycopene within LNCaP cells were investigated as well as the effect of lycopene on the secretion of prostate-specific antigen into the cell culture medium. To support these studies, we have developed quantitative assays for lycopene in biological matrices using high-performance liquid chromatography (HPLC) combined with mass spectrometry (MS), including liquid chromatography-MS (LC-MS) and LC-MS-MS (24, 25). Compared with HPLC with UV-visible absorbance detection, mass spectrometry–based assays are more sensitive and selective.

Materials and Methods

Chemicals
Lycopene, butylhydroxytoluene (2,6-di-tert-butyl-4-methylphenol), Tris-HCl, dithiothreitol (DTT), EDTA, ethanol, testosterone, and protease inhibitor cocktail were purchased from Sigma-Aldrich (St. Louis, MO). Water-dispersible lycopene beadlets (10% lycopene) and placebo beadlets were a gift from Hoffman-La Roche (Nutley, NJ). Echinone was purchased from CaroteNature (Lupsingen, Switzerland). Methanol, acetonitrile, methyl-tert-butyl ether, hexane, dichloromethane, and chloroform were HPLC grade and were purchased from Fisher Scientific (Fairlawn, NJ).

Cell Culture
The human prostate cancer cell lines LNCaP, PC-3, and DU145 were obtained from the American Type Culture Collection (Rockville, MD). Cell culture supplements and supplies were purchased from Invitrogen (Carlsbad, CA). According to the instructions from the supplier, LNCaP, PC-3, and DU145 cell lines were maintained in RPMI 1640 (with 2 mmol/L l-glutamine adjusted to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate), Ham’s F12K medium (with 2 mmol/L l-glutamine and adjusted to contain 1.5 g/L sodium bicarbonate), and Eagle’s MEM (with 2 mmol/L l-glutamine and Earle’s balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, and 1.0 mmol/L sodium pyruvate), respectively, supplemented with 10% fetal bovine serum and 1% antibiotics (10,000 IU penicillin and 10,000 μg/mL streptomycin) at 37°C with 5% CO2. These cell lines were passed once weekly.

Cellular Uptake of Lycopene
LNCaP, PC-3, and DU145 cells were each seeded at a density of 1 × 10^5/mL in 75-cm^2 flasks with three replicates for each incubation time point. After 48 hours of preincubation, the medium was replaced with fresh medium containing lycopene beadlets. The time course of lycopene uptake by each cell line was investigated by incubation with medium containing 1.48 μmol/L lycopene for up to 48 hours. Lycopene stability was also examined by dissolving water-dispersible lycopene beadlets in cell culture medium and incubating with or without cells under the same culture conditions. All experiments were carried out under dim light, and all samples were stored in amber vials to minimize the degradation and isomerization of lycopene by light exposure. The data were evaluated by two-way ANOVA in combination with post testing to identify the significant differences between groups and the effects of incubation times (GraphPad Prism, San Diego, CA). P < 0.05 was considered statistically significant.

Figure 1. The stability of 1.48 μmol/L lycopene at 37°C solubilized as water-dispersible beadlets in RPMI 1640 with (■) or without (□) LNCaP human prostate cancer cells for up to 72 h. The concentration of lycopene at each time point was measured using LC-MS. Points, mean of the percentage of remaining lycopene (n = 3); bars, SD. In the absence of cells, >80% of the lycopene remained in the cell culture medium after 72 h of incubation.

Subcellular Distribution of Lycopene
After 48-hour incubation with medium containing 1.48 μmol/L lycopene as water-dispersible beadlets, LNCaP cells were washed twice with PBS and scraped from the flasks. Cell suspensions were collected by centrifugation for 10 minutes at 800 × g and 4°C. Subcellular fractionation was carried out as described previously (26, 27) with some modifications. Briefly, pellets were lysed by repeated freezing and thawing in hypotonic buffer [20 mmol/L Tris-HCl (pH 7.5), 5 mmol/L MgCl2, 5 mmol/L CaCl2, 1 mmol/L DTT, and 1 mmol/L EDTA] supplemented with...
protease inhibitor cocktail for 45 minutes. Cells were centrifuged for 25 minutes at 1,800 \times g, 4{\degree}C, to separate the nuclear pellet from the supernatant containing microsomes and cytosol. The supernatant was removed and then ultracentrifuged for 3 hours at 100,000 \times g, 4{\degree}C, to pellet the microsomal fraction. The cytosolic and the microsomal fractions were assayed immediately for lycopene content using LC-MS-MS as described below. The crude nuclear pellet from the low-speed centrifugation was resuspended in ice-cold low salt buffer containing 20 mmol/L Tris (pH 7.5), 5 mmol/L MgCl₂, 20 mmol/L KCl, 1 mmol/L DTT, and 1 mmol/L EDTA with protease inhibitor cocktail. Then, high-salt buffer consisting of 20 mmol/L Tris (pH 7.5), 5 mmol/L MgCl₂, 1.2 mol/L KCl, 1 mmol/L DTT, 1 mmol/L EDTA, and protease inhibitor cocktail was added dropwise to the suspension on ice and stirred for 30 minutes. The suspension was centrifuged at 25,000 \times g for 30 minutes at 4{\degree}C to separate nuclear membranes from the nuclear matrix. The nuclear membranes and nuclear matrix were assayed immediately for lycopene content using LC-MS-MS.

**Assay of Lycopene Binding to the AR**

The pGEX-KG plasmid encoding the cDNA for the ligand-binding domain of human cortisone/cortisol–responsive AR (ARc), which is a L701H and T877A double mutant binding domain of human cortisone/cortisol–responsive MS-MS.

**Lycopene Extraction and Quantitative Analysis**

Cell pellets and subcellular fractions were resuspended in 500 μL PBS (pH 7.4). All samples were mixed with the internal standard, echinone (20 μL, 10 μmol/L), and deproteinized with 500 μL ethanol. The mixtures were extracted thrice with 2 mL hexane containing 100 mg/L butyldihydroxytoluene. The hexane extracts were collected after centrifugation for 5 minutes at 800 × g, 4{\degree}C, combined, and evaporated to dryness under vacuum. The residues were reconstituted in 100 μL acetonitrile/methyl-tert-butyl ether (1:1, v/v). The samples were frozen at −20{\degree}C overnight or stored in the autosampler at 4{\degree}C for <4 hours while awaiting injection onto the HPLC.

Lycopene stock solution was prepared by dissolving ~1 mg all-trans lycopene in 10 mL chloroform and stored at −80{\degree}C. Concentration of the stock solution was measured using a Shimadzu (Columbia, MD) UV-2401PC UV-visible recording spectrophotometer and determined based on the absorbance at 502 nm using a molar absorptivity of 1.72 \times 10^5 L mol⁻¹ cm⁻¹ in hexane/CH₂Cl₂ (98:2; v/v). Fresh calibration solutions (0.25–25 μmol/L) in acetonitrile/methyl-tert-butyl ether (1:1, v/v) were prepared each day from the stock solution and calibration curves were constructed by linear regression analysis of the area ratios of lycopene/internal standard versus the amount of lycopene injected on-column.

LC-MS-MS or LC-MS was carried out using either a Thermo Electron (San Jose, CA) Surveyor HPLC system interfaced to a TSQ Quantum triple quadrupole mass spectrometer or an Agilent (Palo Alto, CA) 1100 pump system with a G1946A single quadrupole mass spectrometer. An aliquot of 10 μL was injected for each analysis. Lycopene eluted as a single HPLC peak (the sum of cis- and all-trans lycopene) from a Phenomenex (Torrance, CA) Luna C₁₈ HPLC column (5 μm, 2.0 × 150 mm) using isocratic acetonitrile/methyl-tert-butyl ether (85:15; v/v) at a flow rate of 0.4 mL/min. Negative ion atmospheric pressure chemical ionization mass spectrometry was used for detection. During LC-MS-MS, the atmosphere pressure chemical ionization capillary temperature was set to 300{\degree}C, the vaporizer temperature was 350{\degree}C, the discharge current was 20 μA, and the sheath gas (nitrogen) pressure was 40 psi. Argon at 1.5 T was used as the collision gas for collision-induced dissociation at 26 eV, and the transitions of m/z 536 to 467 and m/z 550 to 458 were monitored for lycopene and echinone (internal standard), respectively, at a dwell time of 2 seconds/scan. During LC-MS, selected ion monitoring of m/z 536 and 550 was used for lycopene and echinone, respectively, with a dwell time of 500 ms/scan during LC-MS, a nebulizer pressure of 40 psi, a vaporizer temperature of 350{\degree}C, a nitrogen drying gas temperature of 300{\degree}C, a drying gas flow rate of 7 L/min, a capillary voltage of 2,000 V, a fragmentor voltage of 100 V, and an atmospheric pressure chemical ionization corona current of 15 μA.
Results
Stability of Lycopene
To measure the stability of lycopene during the incubations, water-dispersible beadlets containing 10% lycopene were dissolved at different concentrations (0.17, 1.48, and 22.3 μmol/L) in cell culture medium and incubated with or without LNCaP cells for up 72 hours. More than 76% of lycopene remained in the cell culture medium after 72 hours (see Fig. 1 for incubations with 1.48 μmol/L lycopene). Similar results were obtained for the incubations with 0.17 and 22.3 μmol/L lycopene (data not shown). After 12 hours, the concentration of lycopene in the medium of incubations containing LNCaP cells was lower than that of the corresponding incubation without cells (Fig. 1). This difference was probably due to cellular uptake of lycopene. Overall, these studies showed that lycopene solubilized in beadlet form was stable during the cellular uptake studies.

Comparison of Lycopene Uptake by LNCaP, PC-3, and DU 145 Cells
The human prostate cancer cell lines LNCaP, PC-3, and DU 145 were incubated up to 48 hours in medium containing 1.48 μmol/L lycopene, which is within the range of plasma concentrations of lycopene (0.01–1.8 μmol/L) normally found in the U.S. population (11). At each time point, cells were harvested and extracted, and the lycopene levels were measured. The time courses of lycopene uptake by LNCaP, PC-3, and DU 145 cells are shown in Fig. 2. The initial uptake of lycopene was rapid, followed by a slower but sustained uptake that reached a plateau after 24 hours. At all time points, the levels of cellular lycopene were significantly (P < 0.001) higher in the androgen-sensitive LNCaP cells than in the androgen-insensitive PC-3 and DU 145 cells. After 24 hours, LNCaP cells accumulated 126.6 pmol lycopene/million cells, which was 2.5 times higher than PC-3 cells (50.6 pmol/million cells), and 4.5 times higher than DU145 cells (28.0 pmol/million cells). These results indicated a preferential uptake of lycopene by LNCaP cells compared with PC-3 or DU145 cells.

Uptake Kinetics of Lycopene in LNCaP Cells and AR Binding
Because LNCaP cells showed the highest uptake of lycopene, a 6-hour incubation study was carried out to determine the rate of this process, and the results are shown in Fig. 3. The 6-hour time point was selected because it is before the plateau level and within the linear range of rectangular hyperbola indicated by the study shown in Fig. 2. Based on the data shown in Fig. 3, the kinetics of lycopene uptake by LNCaP cells was shown to be saturable and concentration dependent. In particular, the rate of lycopene uptake as a function of initial concentration followed the Michaelis-Menten model (Fig. 3). Analysis of these kinetics data indicated that the Vmax was 66.3 pmol/million cells/h, and that the apparent Km was 7.72 μmol/L. Finally, the Lineweaver-Burk plot derived from the double-reciprocal transformation of these data produced a straight line (r² = 0.96; see Fig. 3, inset).

BecauselycopeneuptakebyLNCaPhumanprostatecancercells. LNCaP cells were incubated for 6 h with lycopene at concentrations from 0.17 to 22.3 μmol/L. The rate of lycopene uptake was measured as (cellular lycopene) million cells⁻¹ h⁻¹. Data were evaluated using Michaelis-Menten kinetics and Lineweaver-Burk analysis (see inset). Points, mean (n = 3); bars, SD.

Subcellular Distribution of Lycopene in LNCaP Cells
Next, the intracellular localization of lycopene within LNCaP cells was investigated to provide information regarding the storage of lycopene within these cells. LNCaP cells were separated into four subcellular fractions...
by lysis in hypotonic buffer and then differential centrifugation, and then quantitative analysis of lycopene in these fractions was carried out using LC-MS-MS. As shown in Table 1, 55% of the cellular lycopene was stored in nuclear membranes, followed by 26% in the nuclear matrix, and then 19% in microsomes. No lycopene was detected in the cytosol.

Discussion

Cell pellets, subcellular fractions, and aliquots of cell culture medium were collected, extracted, and analyzed for total lycopene using LC-MS-MS or LC-MS. Because the levels of lycopene in subcellular fractions were low, the high sensitivity and low background noise of MS-MS detection was particularly helpful in carrying out these measurements. The use of LC-MS (a lower-cost instrument) was sufficient for all other measurements. It should be noted that although all-trans-lycopene was added to the cell culture medium, we and others have reported that lycopene isomerizes rapidly both in solution and in vivo to an equilibrium mixture of cis and trans isomers (25). Therefore, only total lycopene was measured and represented the sum of all isomers. The measurement of total lycopene also enhanced sensitivity, because all isomers eluted as a single peak, and facilitated more rapid HPLC separations.

Lycopene accumulated in human prostate cancer cells in the order LNCaP > PC-3 > DU 145 cells. Note that LNCaP cells are an androgen-dependent prostate cancer cell line with high expression of AR and androgen-regulated genes, whereas PC-3 cells show low AR expression, and DU 145 cells are androgen insensitive with no AR expression. Therefore, the relative levels of lycopene accumulation by these cell lines suggest that lycopene uptake might be related to androgen signaling. Furthermore, these results are consistent with in vivo studies that indicate that lycopene accumulates in hormonally regulated tissues, such as adrenals, testes, and prostate (2, 31, 32). However, because no binding of lycopene to the AR was detected (Fig. 4), binding of lycopene to the AR is not responsible for the uptake or storage of lycopene in LNCaP cells. Although the AR-binding studies reported here indicate that lycopene is not a ligand for AR, androgen signaling pathways might still be involved in lycopene uptake or storage.

Next, how the cellular uptake of lycopene changes with its initial concentration was investigated using LNCaP cells and lycopene beadlets at different concentrations up to 22.3 μmol/L, which is the maximum concentration obtained in the cell culture medium after filter sterilization. The Michaelis-Menten kinetics of cellular uptake was characterized by saturation at high lycopene concentration (see Fig. 3), which suggests that the process of lycopene uptake by LNCaP cells might be through facilitated diffusion. The K_m value for the uptake of lycopene is high, relative to the concentration used in this study and in human plasma, which indicates that the putative facilitating protein has a low affinity for lycopene. To the best of our knowledge, there have been no reports of lycopene uptake or storage in human cells.

Table 1. Subcellular distribution of lycopene in LNCaP human prostate cancer cells after incubation for 48 hours with 1.48 μmol/L lycopene and −5.0 × 10^6 cells

| Subcellular compartment | Lycopene (%) |
|-------------------------|--------------|
| Cytosol                 | 0 ± 0        |
| Microsomal fraction     | 34.0 ± 4.0   |
| Nuclear matrix          | 45.5 ± 2.2   |
| Nuclear membrane        | 95.9 ± 2.6   |

NOTE: LNCaP cells were fractionated into cytosol, microsomes, nuclear membranes, and nuclear matrix followed by quantitative analysis of lycopene using LC-MS-MS.

*Data represent means ± SD, n = 3.

†Lycopene was not detected in the cytosol (limit of quantitation = 22.8 fmol on-column).
no reports of lycopene receptors or binding proteins that facilitate its uptake by prostate cells, nor have there been other reports of the subcellular localization of lycopene in prostate cells. Passive diffusion is also a probable mechanism of cellular uptake that might be involved in the uptake of lycopene by all the cell lines tested, including LNCaP, PC-3, and DU145. Because lycopene accumulation does not seem to be a direct result of binding to the AR, perhaps some other receptor or protein in the hormonal signaling pathway is involved in this process.

Because lycopene uptake was significantly higher in LNCaP cells than in PC-3 or DU145 cells, the subcellular distribution of lycopene was investigated within LNCaP cells using cell fractionation followed by selective quantitative analysis of lycopene using LC-MS-MS. More than 80% of the cellular lycopene was localized to the nucleus with the majority of all lycopene in the cells being found in the nuclear membrane. This finding supports the hypothesis that prevention of DNA oxidation is a chemoprevention mechanism of lycopene (16, 33). Lycopene, due to its high number of conjugated double bonds, is one of the most efficient antioxidants, with the ability to quench singlet oxygen that is twice that of β-carotene and 10 times higher than that of α-tocopherol (34). As an antioxidant, dietary lycopene might prevent oxidative stress by trapping reactive oxygen species, increasing the overall antioxidant potential, and reducing oxidative damage to nuclear lipids and proteins as well as DNA (35).

Our clinical data from a 21-day dietary intervention study (16, 25) showed that lycopene supplementation in the diets of men with prostate cancer produced 3-fold and 2-fold enhancement of lycopene levels in prostate tissue and serum, respectively. These values are consistent with the high levels of cellular uptake of lycopene by prostate cells described here. In addition, lycopene administration to these men reduced DNA oxidation in prostate tissue, which is consistent with the subcellular localization of lycopene to the nucleus reported here. Although several mechanisms by which lycopene might prevent prostate cancer are under investigation (36, 37), our data are consistent with antioxidant activity in the nucleus as a mechanism of chemoprevention.

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