Physiologically Attainable Concentrations of Lycopene Induce Mitochondrial Apoptosis in LNCaP Human Prostate Cancer Cells

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Prostate cancer is the second leading cause of cancer deaths among men in the United States. Studies show that people with diets rich in tomato-based foods have reduced risks of cancer, *viz.*, prostate cancer. This is attributed, in part, to lycopene, the most abundant carotenoid in tomatoes. Thus, we studied the effect of lycopene at physiologically attainable concentrations on apoptosis, cellular proliferation, and necrosis in LNCaP human prostate cancer cells. Cells at 37°C and >80% confluency were treated with media alone (0.32% tetrahydrofuran vehicle) or with increasing concentrations (0.3–3.0 μ*M*) of lycopene overnight. After washing monolayers, analyses by high-performance liquid chromatography (HPLC) showed that cellular accumulation of lycopene was 5.5 ± 0.8, 14.0 ± 3.2, and 36.7 ± 12.3 pmole/10^6 cells for 0.3, 1.0, and 3.0 μ*M*, respectively, and not detected in control cells. Lycopene did not alter cellular proliferation because bromodeoxyuridine (BrdU) incorporation and cell numbers were identical among groups. However, results of a 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay showed that mitochondrial function decreased 61%–83% with increasing concentrations of lycopene ($P < 0.001$). Cytotoxicity and necrosis did not contribute to this effect because lactate dehydrogenase (LDH) release (1.5%–1.8%) and trypan blue exclusion (89%–93%) were similar. Subsequently, we demonstrated that increasing concentrations of lycopene significantly ($P < 0.05$) reduced mitochondrial transmembrane potential, induced the release of mitochondrial cytochrome c, and increased annexin V binding, confirming induction of apoptosis. Thus, lycopene at physiologically relevant concentrations did not affect cellular proliferation or promote necrosis but clearly altered mitochondrial function and induced apoptosis in LNCaP human prostate cancer cells. Exp Biol Med 230:171–179, 2005

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Prostate cancer is the second leading cause of cancer death in men in the United States (1). Epidemiological studies demonstrate a clear inverse association between diets high in carotenoid-rich fruits and vegetables and reduced incidence of prostate cancer (2). Specifically, those who consume tomatoes and tomato-based foods have a significantly lower risk of prostate cancer (3, 4). This protective effect is attributed, in part, to the presence of lycopene, a potent antioxidant and the predominant carotenoid in tomatoes (5).

The accumulation of lycopene in the human prostate suggests that dietary sources of lycopene are protective against prostate cancer and particularly the more lethal forms of the disease (4). In one prospective human study, low levels of plasma lycopene, but not other carotenoids, were associated with an increase in prostate cancer (6). In a large prospective study, the effect of consumption of 46 fruits and vegetables on prostate cancer incidence was evaluated. Only four foods were significantly inversely associated with intake and three, including tomato sauce, tomatoes, and pizza, but not strawberries, increased plasma levels of lycopene and significantly lowered by 40% the risk of prostate cancer even when the contribution of fruit and vegetable consumption was controlled (7). Numerous other studies have suggested a protective role for dietary lycopene against prostate cancer (1, 8). A review of epidemiologic evidence showed that, of 72 studies, 57 demonstrated an inverse relationship and 35 of these demonstrated statistical significance (9). Although accumulating evidence is compelling that lycopene protects against prostate cancer, conclusive evidence that lycopene is the active agent is lacking. Indeed, in other epidemiological studies, data have been inconsistent with no reductions in prostate cancer, suggesting that lycopene is not protective (10, 11). As a result, lycopene and its potential mechanism(s) of action

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have received intense recent investigation to assess lycopene as a potential chemopreventive agent against prostate cancer.

Lycopene has been demonstrated to protect against carcinogenesis via modulation of numerous mechanisms associated with the cancer process (12–14). Studies show significant lycopene-associated reductions in cellular proliferation (15) and growth (16) of several cancer cell lines, including prostate cancer cell lines, as well as melanoma, lung, mammary, colon, and leukemia cells (15) . Other suggested protective roles have been induction of cell-cycle arrest (17), enhanced gap-junction communication (18), inhibition of malignant transformation (19), induction of differentiation (20), and induction of apoptosis (14, 21). Collectively, data suggest that lycopene may beneficially affect numerous, diverse cellular functions associated with carcinogenesis, with few suggesting negative effects (22).

While many studies support a protective role for lycopene in prostate cancer, the specific mechanism is unclear. Many studies have focused on alterations of cellular proliferation because neoplasia is generally defined as unregulated growth and clonal expansion of abnormal cells. However, fewer studies have examined the effects of lycopene on programmed cell death, or apoptosis. Studies that have focused on cell-number imbalances typically have used concentrations that are substantially higher (up to 50 μM) than attainable in normal human plasma, introducing the question of physiological relevance (16). Normal levels of lycopene in human plasma are 0.2–0.3 μM but may increase markedly above this level depending on foods consumed and the absorptive capacity of an individual (23). Thus, studies designed around a reasonably attainable concentration range of lycopene would likely reveal physiologically relevant mechanisms of action, if present.

In this study, we selected the commonly used human prostate cancer cell line LNCaP as an *in vitro* model and investigated the effect of lycopene at physiologically relevant concentrations on biochemical processes important in cancer. Specifically, we used 0.3, 1.0, and 3.0 μM lycopene, representing plasma lycopene levels of the average American or individuals that might consume lycopene-rich fruits and vegetables or routinely supplement with lycopene. We analyzed cell-number balance, including assays of cellular proliferation and apoptosis, to determine potential beneficial effects of lycopene on these processes.

Materials and Methods

Chemicals. Tetrahydrofuran (THF; UV-grade), methanol, acetonitrile, ammonium acetate, triethylamine, 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), butylated hydroxytoluene (BHT), and lycopene were obtained from Sigma Chemical Company (St. Louis, MO). 3,3′-Dihexyloxacarbocyanine (DiOC6(3)) and 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide (JC-1) were obtained from Molecular Probes (Eugene, OR). Plasticware (plates and flasks) was purchased from Corning, Inc. (Corning, NY). All other chemicals and cell culture reagents were obtained from Sigma Chemical Company.

Cell Culture. LNCaP human prostate cells were purchased from American Type Culture Collection (Rockville, MD) and cultured in flasks at 37°C in a humidified 5% CO_2 atmosphere. Cells were grown in RPMI 1640 medium with 2 mM L-glutamine containing 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/l glucose, and 1.5 g/l sodium bicarbonate. RPMI 1640 media were further supplemented with 10% volume per volume (v/v) heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% amphotericin B. Stock flasks were grown to 70% confluency and subcultured routinely. Medium renewal was 2–3 times weekly.

For experiments, cells were grown in T25 and T75 flasks or multiwell plates. At 70% confluency, medium was removed and cells were treated with increasing concentrations of lycopene (0, 0.3, 1, and 3 μM) in RPMI 1640 medium using a THF vehicle. After incubating for 20 hrs, medium was removed, and cells were washed twice with HBSS and harvested. For experiments determining DNA banding, cells were also incubated in appropriate treatments for 72 hrs.

Carotenoid Solutions. Lycopene was dissolved in THF (2 mM) and stored at −70°C in an opaque glass container under an inert nitrogen blanket. Immediately before experiments, aliquots of stock solutions were added to cell culture media with vigorous stirring to attain the indicated concentrations of lycopene. The THF concentration was 0.32% (v/v) for all final working solutions. All procedures were conducted under subdued lighting to minimize degradation of lycopene.

Cellular Accumulation. After overnight incubation with medium alone or medium containing lycopene, medium was removed and monolayers (three T25 flasks per treatment) were washed twice with Hanks Balanced Salt Solution (HBSS; pH 7.4). Cells were collected into 2 ml harvest solution (0.1 M phosphate buffer, pH 7.4, with 10% (v/v) ethanol containing 0.45 mM BHT). Samples from the three replicates were pooled, the internal standard β-apo-8-carotenal added, and lycopene was extracted by adding 2 ml absolute ethanol, vortexing, and adding an equal volume of hexane. After vortexing 10 secs, the upper organic layer was transferred to a new tube and the extraction was repeated twice more. Accumulation was determined by high-performance liquid chromatography (HPLC) as described by Martin *et al.* (24) using a Waters 2690 Separations Module (Milford, MA) and Millennium^{32} Chromatography software (Milford, MA). The mobile phase consisted of 70% acetonitrile (with 1.3 mM triethylamine), 20% dichloromethane, and 10% methanol (with 0.13 mM ammonium acetate). A C18 column was used with an isocratic mobile-phase flow rate of 1.7 ml/min and lycopene detected at 450 nm using a Waters 996 Photodiode Array

Detector (Milford, MA). Values were normalized to the internal standard and calculated from an external standard curve using purified lycopene.

Cellular Proliferation. Cellular proliferation was quantitated by both a bromodeoxyuridine (BrdU) incorporation assay and hemacytometry (cell counts). For the BrdU assay, stock BrdU diluted 1:2000 in RPMI complete media was added to control and treated cells at the time of lycopene addition and incubated overnight. After washing monolayers and fixing cells, an enzyme-linked immunosorbent assay (ELISA) was performed as described by the manufacturer (Trevigen, Gaithersburg, MD) to measure DNA incorporation of BrdU. After addition of stop solution, samples were analyzed at 450 nm on a SpectroFluor Plus multiwell plate reader (Tecan, Research Triangle Park, NC).

To corroborate BrdU results, cell counts were determined by hemacytometry. Briefly, after overnight incubation as described above, monolayers were washed, trypsinized, neutralized with complete media, centrifuged, and resuspended in media. An aliquot (50 µl) of cell suspension was diluted 1:2 with trypan blue (50 µl) and cells were counted by microscopy. Dead cells, but not live cells, stained intensely with trypan blue and indicated cytotoxicity. Viability was determined using the formula: % viability = (no. live cells)/(no. live cells + no. dead cells) × 100.

Mitochondrial Activity. As a measure of cellular proliferation and cytotoxicity, cells were analyzed using an MTT assay as previously described (24). After overnight incubation and subsequent washing, control and lycopene-treated cells were washed and incubated with medium containing 12.1 m*M* MTT without FBS or phenol red. After 1 hr, plates were centrifuged at 1500 *g* for 10 mins at 25°C to pellet precipitated MTT crystals. After carefully aspirating media, 1 ml dimethyl sulfoxide (DMSO) was added to each well to solubilize MTT crystals. The optical density of reduced MTT was measured at 560 nm by spectroscopy.

Necrosis. To determine necrosis, cells and media were tested for lactate dehydrogenase (LDH) activity to determine percent release by the cells into the media as described previously (24). Enzymatic activity of LDH in the medium and in cells was determined kinetically by measuring the oxidation of NADH at 340 nm for 10 mins. The percent release of LDH was determined using the formula: % release = (LDH activity in media)/(LDH activity in media + LDH activity in cells) × 100.

Measurement of Mitochondrial Transmembrane Potential. Changes in mitochondrial transmembrane potential were determined by the retention and fluorescence of DiOC6(3) and JC-1. Briefly, LNCaP cells grown in T25 flasks were treated overnight with vehicle alone or containing increasing concentrations of lycopene. DiOC6(3) in DMSO was added to media (final concentration 40 n*M*) for the last 30 mins of incubation at 37°C. Monolayers were washed with HBSS and trypsinized for approximately 5 mins before inactivation with complete media. Cells were collected, centrifuged (800 *g* for 10 mins) and the supernatant was discarded. Cells were resuspended in PBS and washed twice more with HBSS before resuspension in 500 µl phosphate-buffered saline (PBS) and storage on ice until analysis by flow cytometry. Cells undergoing apoptosis demonstrated decreased mitochondrial membrane potential, revealed by dulled fluorescence, compared to cells with normal mitochondria. For analysis with JC-1, monolayers were washed twice with HBSS and trypsinized for approximately 5 mins before inactivation with complete media. Cells were collected, centrifuged (1000 *g* for 6 mins) and the supernatant was discarded. After washing once with complete media, cells were resuspended in RPMI complete media containing JC-1 (final concentration 10 µ*M*) and incubated at 37°C for 10 mins. To remove unassociated JC-1, cells were centrifuged again, washed once with complete media, resuspended in 0.5 ml complete media, and stored on ice until analysis by flow cytometry.

Cytochrome *c* Release. As an indicator of apoptosis, the release of mitochondrial cytochrome *c* was analyzed as described previously (25). Briefly, monolayers grown in T75 flasks were washed once with ice-cold PBS (pH 7.5) followed by addition of lysis buffer (2 ml/flask) containing 250 m*M* sucrose, 1 m*M* EDTA, 0.1 m*M* phenylmethylsulfonylfluoride, 1 m*M* dithiothreitol, 1 m*M* benzamidine, 0.05% (w/v) digitonin, and 1 µg/ml protease inhibitor cocktail in 25 m*M* Tris-HCl (pH 6.8). Monolayers were scraped into lysis buffer and transferred to separate ice-cold tissue grinders and disrupted by 30 strokes. After transfer back to tubes, cell homogenates were centrifuged (700 *g* for 10 mins at 4°C) to pellet cellular debris. The supernatant was transferred to a second tube and centrifuged 30 mins at 10,000 *g* to pellet mitochondria. The cytoplasm was transferred to a third tube and the pellets resuspended in lysis buffer (0.8 ml). The mitochondrial fractions were lysed by 10 passages through a 21-gauge syringe. Protein levels were determined using a BCA protein kit according to the instructions of the manufacturer (Bio-Rad, Hercules, CA). To each lane of a 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel, 50 µg protein was added and cytochrome *c* was detected by Western blotting.

Western Blotting. Proteins from SDS-PAGE gels were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot SD Semi-Dry Transfer Cell (Hercules, CA). Membranes were blocked with 5% Blotto in TBST for 3 hrs and incubated with monoclonal mouse anti-cytochrome-*c* primary antibody (Zymed, San Francisco, CA) overnight. Membranes were washed in Tris-buffered saline Tween-20 (TBST) and incubated for 1 hr with HRP-conjugated goat antimouse secondary antibody (Zymed). After washing in TBST, protein bands were detected by autoradiography using Pierce SuperSignal West Pico Enhanced Chemiluminescent (ECL) Substrate (Rockford, IL).

Annexin V Binding and Propidium Iodide Influx. In apoptotic cells, phosphatidylserine (PS) residues

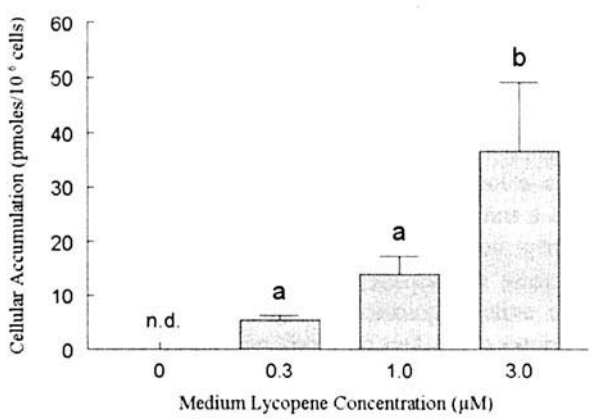

Figure 1. Cellular accumulation of lycopene in LNCaP human prostate cancer cells incubated overnight with increasing concentrations of lycopene in tetrahydrofuran (THF; 0.32% volume per volume) vehicle. Accumulation was determined by high performance liquid chromatography. Values were normalized to the internal standard β-apo-8-carotenal and calculated from an external standard curve using purified lycopene. Values are means ± SD from four experiments, each performed in triplicate. (n.d. = not detected). Bars with different letters are significantly different ($P < 0.05$).

relocate from the inner leaflet to the outer leaflet of the cell membrane, where annexin V conjugated to fluorescein isothiocyanate (FITC) can bind. Coupled with PI, disruption or increased permeability of the plasma membrane can also be determined. LNCaP cells were cultured as described above, harvested by centrifugation (1000 *g* for 6 mins), and transferred to polystyrene tubes. Cells were washed with RPMI complete media, resuspended in buffer containing propidium iodide (PI) (5 μg/ml) and annexin V-FITC, and incubated at 25°C for 15 mins as described by the manufacturer (Trevigen, Gaithersburg, MD). After incubation, buffer was added and unfixed cells were analyzed within 1 hr by flow cytometry.

Apoptosis by Agarose Gel Electrophoresis. Cells cultured in T25 flasks were washed twice with HBSS (2 ml/wash), trypsinized for ~5 mins, inactivated with complete media, resuspended, and transferred to sterile tubes. Cell suspensions were centrifuged, supernatant discarded, and pellets stored at –20°C until DNA extraction. DNA was extracted from control and lycopene-treated cells using a Qiagen DNeasy Tissue Kit (Valencia, CA) and quantified by UV spectroscopy at 260 nm. DNA (10, 30, or 50 μg) was loaded into wells for agarose gel electrophoresis (1.2% weight per volume [w/v]) and run for 2 hrs at 70 V using tris-borate-EDTA (TBE) running buffer. DNA banding was visualized by incorporation of ethidium bromide and the results were photodocumented with a PhotoDOC-IT Imaging station (UVP Model M-20; Upland, CA).

Statistics. Data were analyzed using one-way ANOVA and Fisher protected least significant difference (PLSD) *post hoc* analysis for multiple comparisons using StatView Software (Cary, NC). $P < 0.05$ was considered statistically significant.

Results

Cellular Accumulation of Lycopene. Lycopene was accumulated in a dose-dependent manner in LNCaP cells following overnight addition of increasing concentrations of lycopene (Fig. 1). Accumulated lycopene was highest following addition of 3 μ*M* lycopene ($P < 0.05$), reaching a level of 36.7 ± 12.3 pmole/10^6 cells. Supplemental lycopene at 0.3 and 1 μ*M* increased cellular levels to 5.5 ± 1.6 and 14.0 ± 6.5 pmole/10^6 cells, respectively. Lycopene was not detected in LNCaP cells cultured in medium alone

Cellular Proliferation. Cellular proliferation was determined indirectly as a measure of mitogenesis via BrdU incorporation. Cellular proliferation was expressed as a percentage of the response of control cultures. There were no significant differences in cellular proliferation when cells were treated with any concentration of lycopene (Table 1). To corroborate the BrdU results, we determined cellular proliferation by hemacytometry. We noted ~1 × 10^7 cells per T75 flask, with no differences in cell number between control cultures and those treated with increasing concentrations of lycopene (Table 1).

Necrosis. We determined LDH release as an indicator of necrosis because cytoplasmic LDH is released when the plasma membrane is disrupted. In control cultures, LDH released to the medium was 1.76% ± 0.12% of total LDH and treatment with increasing concentrations of lycopene did not significantly increase LDH release (Table 2). In contrast, incubation with 1 μ*M* lycopene significantly

Table 1. Effect of Lycopene on Mitogenesis and Cellular Proliferation[a]

Medium lycopene concentration (μ*M*)	BrdU[b] incorporation (% of control)[c]	Cell number per flask (x 10^7)[d]
0	100.0 ± 3.6	0.98 ± 0.08
0.3	100.0 ± 5.7	1.09 ± 0.17
1.0	100.0 ± 4.9	1.07 ± 0.03
3.0	100.0 ± 3.2	1.01 ± 0.05

[a] Values are means ± SD.
[b] BrdU, bromodeoxyuridine.
[c] $n = 36$.
[d] $n = 9$.

Table 2. Effect of Lycopene on Necrosis and Viability in LNCaP Cells[a]

Medium lycopene concentration (μ*M*)	LDH[b] release (% of total)	Viability[c] (%)
0	1.76 ± 0.12	89.4 ± 2.8
0.3	1.77 ± 0.03	89.7 ± 0.5
1.0	1.54 ± 0.07[d]	93.1 ± 0.6
3.0	1.66 ± 0.12	90.2 ± 2.1

[a] Values are means ± SD, $n = 9$.
[b] LDH, lactate dehydrogenase.
[c] Trypan blue exclusion.
[d] $P < 0.05$; control *vs.* 1.0 μ*M*.

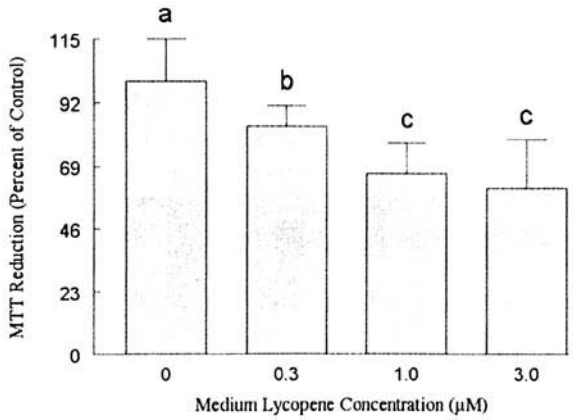

Figure 2. Mitochondrial function in LNCaP human prostate cancer cells incubated overnight with medium alone or with increasing concentrations of lycopene and analyzed for the ability of mitochondria to reduce 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT). Bars are means ± SD for three experiments, each with 12 replicates, representing MTT reduction as a percent of control. Significantly lower optical density in treatment groups indicates impaired mitochondrial function. Bars with different letters are significantly different ($P < 0.05$).

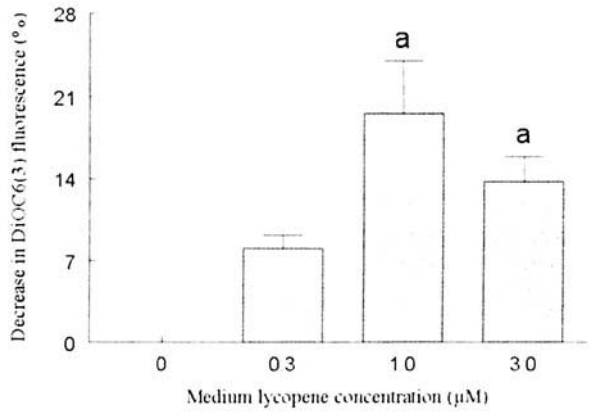

Figure 3. Mitochondrial transmembrane potential in LNCaP human prostate cancer cells incubated overnight with medium alone or with increasing concentrations of lycopene as assessed by the fluorescence of the cationic dye DiOC6(3) (40 n*M*). Data represent loss of fluorescence indicative of apoptosis induction. Values are the means ± SD of three experiments, each performed in triplicate. Bars with different letters are significantly different ($P < 0.05$).

reduced LDH release compared to control cells, suggesting enhanced protection. LDH release in vehicle (THF)-treated cells was not different from cells incubated with medium alone.

Cytotoxicity. Because cells may die without complete disruption of the plasma membrane, we corroborated LDH results by determining cytotoxicity, or loss of viability, using the trypan blue exclusion test. Viability in control cultures was 89.4% ± 2.8% and was not significantly different in cultures treated with increasing concentrations of lycopene (Table 2).

Mitochondrial Function. The MTT assay has been used interchangeably in other studies as an indicator of both cellular proliferation and cytotoxicity. However, the assay ultimately depends on a functioning mitochondrion. The chemical reduction of the MTT dye decreased significantly by 12%, 32%, and 40% (Fig. 2) when cells were incubated with 0.3, 1.0, and 3.0 μ*M* lycopene, respectively. Overall, lycopene induced a dose-dependent impairment of mitochondrial function even at the lowest concentration of lycopene (0.3 μ*M*).

Transmembrane Potential of the Mitochondria. To determine the cause for reduced mitochondrial activity in the presence of unchanging cell numbers, we analyzed additional markers of mitochondrial function often associated with apoptosis. We incubated cells with increasing concentrations of lycopene overnight, then added the cationic, mitochondria-specific dye DiOC6(3) to determine the impact of lycopene on mitochondrial transmembrane potential. DiOC6(3) oxidation was significantly decreased by 20% and 13% after preincubation with 1 μ*M* and 3 μ*M* lycopene, respectively (Fig. 3). For simplicity, data are expressed as loss of fluorescence, with increasing values indicating increased dysfunction of the mitochondrion. The lowest concentration of lycopene did not significantly decrease the mitochondrial transmembrane potential. To corroborate these results, we analyzed cells using the mitochondrial membrane potential sensing dye JC-1. JC-1 is also a cationic dye that indicates mitochondrial polarization but is more specific for mitochondrial *vs.* plasma membrane potential than is DiOC6(3). When compared to control cells, we noted a significant 4.5-fold increase in apoptotic cells incubated with the highest concentration of lycopene, but no significant changes at lower concentrations (Fig. 4).

Cytochrome *c* Release. Cytochrome *c* release from mitochondria to the cytosol is an indicator of apoptosis. The results demonstrate, in control cells, an intense band for cytochrome *c* in the mitochondria with less intense banding

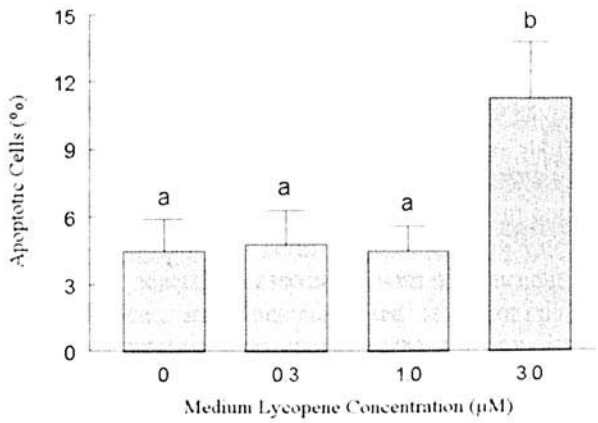

Figure 4. Mitochondrial membrane potential was determined as described above using the mitochondria-specific dye JC-1 (10 μ*M*) added for the last 15 mins of incubation. Data are means ± SD of four independent experiments. Bars with different letters are significantly different ($P < 0.05$).

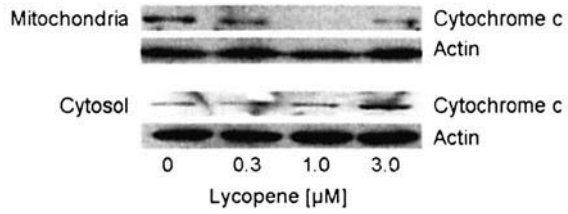

Figure 5. Cytochrome *c* release from mitochondria to cytosol in LNCaP cells incubated overnight with media alone or with 0.3, 1, or 3 μ*M* lycopene. Protein (50 μg/well) was loaded, electrophoresed, and detected as described previously. B-actin was included as a control for loading. Bands for cytochrome *c* and actin are shown. The top two and bottom two panels represent mitochondrial and cytosolic fractions, respectively, from a representative Western blot of three independent experiments.

in the cytoplasmic fraction (Fig. 5). Conversely, after overnight incubation with increasing concentrations of lycopene, there is a clear decrease in cytochrome *c* levels in the mitochondria of control cells and a marked increase of cytochrome c in the cytoplasm of treated cells, indicative of apoptosis.

Annexin V Binding and PI Influx. To corroborate apoptosis, we analyzed the transmigration of PS residues from the inner to outer plasma membrane leaflet characteristic of programmed cell death. Lycopene increased the percentage of annexin V-positive cells by 2.7-, 1.6-, and 3.3-fold in cells treated with 0.3, 1.0, and 3.0 μ*M* lycopene, respectively, compared to control cells. The influx of PI was not different among groups treated with <3.0 μ*M* lycopene, corroborating a lack of marked necrosis or increased plasma membrane permeability at lower concentrations. There was, however, a significant 2-fold increase in PI influx in cells treated with 3 μ*M* lycopene, suggesting increased membrane permeability (Fig. 6)

DNA Fragmentation by Agarose Gel Electrophoresis. We examined the effect of increasing concentrations of lycopene on induction of the DNA banding characteristic of late apoptosis. We noted no differences in DNA banding between control lanes and those treated with increasing concentrations of lycopene after 20 hrs. Different amounts (10, 30, and 50 μg) of DNA were loaded to maximize the sensitivity of the assay. When cells were incubated with lycopene for 72 hrs, we did note substantial apoptotic banding in all lycopene-treated groups, but not the control group (data not shown).

Discussion

Due to disparate results and differences of *in vitro* model systems of various investigators, the purported mechanisms of protection by lycopene have become more complicated rather than more lucid. The results of this study are novel in demonstrating alteration of mitochondrial function and induction of apoptosis by lycopene at physiologically relevant concentrations in human prostate cancer cells. Interestingly, we did not observe changes in cellular proliferation or necrosis as repeatedly shown by

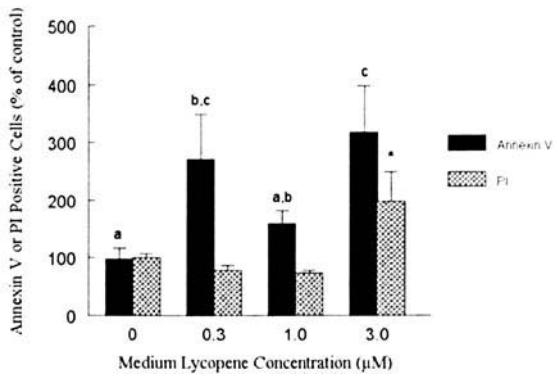

Figure 6. Annexin V binding and propidium iodide (PI) influx in LNCaP cells after incubation with media alone or with increasing concentrations of lycopene. Data represent means ± SEM of four independent experiments and indicate the percentage of cells staining positively for annexin V or PI. Bars with different letters are significantly different ($P < 0.05$). Asterisks indicate a significant difference ($P < 0.05$) between treated and control cells regarding PI staining.

others, but instead the clear induction of apoptosis supported a chemoprotective function for lycopene. The data suggest that lycopene can function as an anticancer agent through modulation of cellular signaling and function associated with critical cellular processes such as apoptosis (26).

The decreased reduction in the formazan MTT, in the presence of unchanging cell numbers, suggested altered cellular function and not reduced cell number, as often reported by others. It is noteworthy that, although the MTT assay has been used as an index for both cytotoxicity and cellular proliferation, the assay ultimately depends on mitochondrial function, a fact often not noted in the literature (27). Our initial results suggested cytotoxicity, but neither LDH release nor trypan blue exclusion were altered, ruling out overt necrosis. However, PI influx was significantly increased at the highest lycopene concentration, suggesting increased membrane permeability. This effect may be a function of size, as PI has the lowest molecular weight of the three molecules used for detection of permeability. This lack of necrosis concurs with others, who have shown that the viability of LNCaP cells was not significantly different from control cells when cultured for 24 hrs with up to 20 μ*M* lycopene, although significant reductions occurred in PC-3 and DU 145 prostate cells (15). Our observations agree with these findings because we observed no loss of viability. However, we did note changes in mitochondrial function characteristic of apoptosis.

As an early event in apoptosis, mitochondria undergo two key alterations in function. First, the outer mitochondrial membrane becomes permeable to proteins such as cytochrome *c* (28). Second, the inner mitochondrial transmembrane potential is disrupted (29). We corroborated apoptosis suggested by altered mitochondrial function (MTT assay) with immunoblotting, showing that mitochondrial cytochrome *c* release to the cytoplasm was markedly

increased after incubation with increasing concentrations of lycopene. This was associated with a concomitant decrease in mitochondrial cytochrome *c*. Moreover, the mitochondrial transmembrane potential was altered after lycopene preincubation, as demonstrated by decreased fluorescence of DiOC6(3), although JC-1 produced different results.

It is somewhat perplexing that the JC-1, DiOC6(3), MTT, and annexin V data do not align perfectly. We propose that there are several coexisting, yet interdependent, phenomena at work here, with binding specificities of the dyes (JC-1 and DiOC6[3]) contributing, in part, to the results. Although both JC-1 and DiOC6(3) associate with the mitochondrion, JC-1 associates more tightly and is less prone to nonspecific organelle binding. DiOC6(3) can associate with other organelles, including the plasma membrane. We propose that JC-1 indicates mitochondrial abnormalities at the highest concentrations of lycopene, although sensitivity and permeability of the cell to the JC-1 dye is a consideration because PI influx is significantly increased with 3 μ*M* lycopene. Plasma membrane integrity is not disrupted, as shown by LDH release and trypan blue exclusion. Interestingly, the greatest loss of DiOC6(3) fluorescence occurs with the least amount of external annexin V binding, suggesting a role for plasma membrane dysfunction. The capacity of extremely hydrophobic carotenoids, such as lycopene, to alter membrane fluidity and function has been documented. Last, the clear decrease in mitochondrial function as indicated by MTT data correlates well with cytochrome *c* release supporting increased apoptosis via the mitochondrial route. In sum, it appears that lycopene is inducing several coexisting phenomena consistent with induction of apoptosis.

In our experiments, we did not detect DNA banding after treatment with lycopene for 20 hrs. However, when incubation time was extended to 72 hrs, we did observe banding in all lycopene-treated groups. The nuclease-positive control induced banding at both time points. Because DNA banding is a late apoptotic event, it is likely that our results more readily displayed early apoptosis. Moreover, the relative lack of sensitivity of agarose gel electrophoresis may have precluded detection of DNA bands when, in fact, DNA fragmentation was occurring. Lack of DNA banding in LNCaP cells may be cell type-specific because many have shown greater susceptibility to DNA banding in other prostate cell lines, such as PC3 and DU145 cells (15, 30). In a recent report, lycopene at concentrations as low as 0.1 μ*M* significantly reduced cell viability and increased apoptosis as determined by annexin staining in LNCaP cells after 24 hrs, in partial agreement with our results (31). In this study, a confounding factor was delivery of lycopene in a beadlet formulation containing the antioxidants ascorbyl palmitate and α-tocopherol. Interestingly, antioxidant inclusion did not prevent lycopene degradation over 24 hrs and the beadlet vehicle control significantly increased apoptosis at the highest concentration.

A surprising observation in this study was the lack of an effect of lycopene on cellular proliferation and growth. Numerous studies have repeatedly demonstrated antiproliferative effects of lycopene and other carotenoids in prostatic cell lines as well as primary cultures of human prostate cells (14, 17, 32). Kim *et al.* (33) report that lycopene at 10–1000 μ*M* significantly reduced proliferation in LNCaP cells by 24%–43% after 48 hrs using a water-dispersible, microemulsion of lycopene, although the components of the preparation and vehicle were not identified. Lycopene dose dependently inhibited cellular proliferation by 20% at 1 μ*M* after 24 hrs, similar to the results reported in our study. Levy *et al.* (32) compared the antiproliferative properties of lycopene to other carotenoids and found that lycopene delivered in THF strongly inhibited cellular proliferation of endometrial, mammary, and lung cancer cells with a half-maximal inhibitory concentration of 1–2 μ*M* after 24 hrs. In another study, incubation of LNCaP cells with lycopene at 1 and 10 μ*M* for 24, 48, and 72 hrs showed no reductions in cell number at any time or dose, although the delivery vehicle was not identified (34). Pastori *et al.* (16) studied the growth of two different human prostate carcinoma cell lines (DU45 and PC3) and found that lycopene alone was not a potent inhibitor of prostate carcinoma cell proliferation, as determined by hemacytometry and ^{3}H-thymidine incorporation.

Accumulation of lycopene by LNCaP cells was dose dependent and readily detectable when cells were preincubated with physiologically attainable concentrations of lycopene. The cellular uptake reported here for LNCaP cells (5.5–36.7 pmole/10^{6} cells) is similar to the levels reported by others (17, 35, 36). While we detected total (*cis* and *trans*) lycopene in LNCaP cells, the stability of lycopene in cell culture medium is reported to be low, with a calculated half-life of 8 hrs under standard cell culture conditions (35). Others report that lycopene degrades in 2 hrs by 80% under standard cell culture conditions, although others have determined a half-life of 12–20 hrs. Moreover, it has been suggested that THF is unsuitable as a vehicle because of solvent toxicity, poor solubility, and instability of lycopene. We used THF as a cosolvent because it was effective in delivering lycopene and did not induce cytotoxicity, in agreement with others. (36). Our experiments were conducted at 20 hrs, thus it is likely that some degradation occurred although we quantitated presumably intact lycopene in cells by HPLC using a purified standard.

It is intriguing that oxidized metabolites of lycopene, but not lycopene itself, have been reported to occur *in vitro* and can, in fact, inhibit cell growth and stimulate apoptosis in HL-60 cells (37). The coadministration of vitamin E, a potent antioxidant, protected lycopene from degradation, supporting the occurrence of oxidative modification (16), although this is in contrast with the report of Hwang and Bowen (31). Interestingly, in one study, lycopene in LNCaP cells inhibited cell growth, but the oxidized mixtures displayed markedly more potent growth inhibition (37).

Many oxidized metabolites of lycopene exist in human plasma, including, but not limited to, 5,6-dihydroxy-5,6-dihydrolycopene, 2,6-cyclolycopene-1,5-diol, and lycopene epoxide, suggesting *in vivo* oxidations occur (37, 38). It has been demonstrated that lycopene oxidation stimulates gap-junction communication, an anticarcinogenesis mechanism, in rat liver epithelial cells (18). It is also noteworthy that when prostate cancer cells including PC-3, DU-145, and LNCaP cell lines were cultured with acyclo-retinoic acid, an *in vitro* lycopene oxidation product, the viability was reduced in all cells except LNCaP (30). Moreover, DNA fragmentation patterns were detected in PC-3 and DU-145 cell lines, but not in LNCaP cells, suggesting cell-type specificity. A key consideration is that PC-3 and DU-145 cells are androgen insensitive and LNCaP cells are hormone responsive (16). It has been shown that tissue lycopene concentrations and isomer patterns are affected by androgen status in rats, suggesting this may be an important factor (39).

It is possible that the observed effects are not lycopene-specific and other carotenoids would have produced the same effect. While this is arguably an important point, lycopene is preferentially accumulated in the human prostate and exhibits anticarcinogenic effects, suggesting a clear potentially chemoprotective role in prostate cancer by inducing neoplastic cell death before malignant progression (5). Indeed, in clinical trials, the biological and clinical effects of supplementation before prostatectomy with lycopene-rich oleoresin have been determined in patients with localized prostate cancer. In supplemented patients, tumors were smaller and the cancer involved less extraprostatic tissues and high-grade neoplasia occurred less frequently (40, 41). Moreover, supplementation with lycopene-rich tomato sauce in men with prostate cancer before prostatectomy showed decreased prostate-specific antigen (PSA) levels, substantially reduced DNA damage to leukocytes and neoplastic cells, as well as enhanced induction of apoptosis in neoplastic cells in resected tissues (21). Our data clearly support observations such as these at the mechanistic level because we demonstrate lycopene-induced apoptosis in cultured human prostate cells.

In summary, we have demonstrated that physiologically relevant concentrations of lycopene can in fact, in the presence of unchanging cellular proliferation or necrosis, induce apoptosis, or programmed cell death, in prostatic cancer cells. The potential for dietary constituents such as lycopene to initiate programmed cellular responses such as apoptosis suggests that a better understanding of this mechanism could potentially lead to the development of effective new therapies against cancer (42). This contention, along with accumulating data, continues to support the notion that increased consumption of fruits and vegetables, particularly those that are lycopene-rich, remains a logical and efficacious dietary practice. Further studies are, however, needed to elucidate and confirm other specific roles of lycopene in protecting prostate tissue against carcinogenic events.

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