β-Carotene Stability and Uptake by Prostate Cancer Cells Are Dependent on Delivery Vehicle

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Abstract: Cell culture systems provide an opportunity to evaluate the effects of carotenoids on molecular and cellular processes involved in proliferation and differentiation of prostate cancer cells. The stability and cellular uptake of β-carotene (BC) by prostate cancer cells were investigated in vitro by use of various delivery methods and three human prostate adenocarcinoma cell lines: PC-3, DU 145, and LNCaP. Recovery of BC from the media (prepared from water-dispersible BC beadlets) significantly (p < 0.05) decreased after 12 hours in culture and continued to significantly decrease (p < 0.05) after 24, 48, 72, and 96 hours, an observation primarily attributed to BC degradation rather than isomerization, metabolism, or cellular uptake. The uptake of BC by prostate cancer cells was compared when delivered by tetrahydrofuran, BC-enriched bovine serum, water-dispersible BC beadlets, and artificial liposomes. Recovery of BC after three days in culture from enriched bovine serum medium was significantly (p < 0.05) greater than recovery from medium prepared by beadlets, tetrahydrofuran, or artificial liposomes. We conclude that BC is relatively unstable in vitro and that degradation products may contribute to biological responses. Furthermore, our studies indicate that enriched bovine serum provides a stable and physiological approach for carotenoid treatment of cells in culture.

Introduction

Cell culture systems have been utilized to evaluate the effects of carotenoids on molecular and cellular processes involved in proliferation and differentiation of cancer cells (1,2). However, the delivery of carotenoids to cells in culture presents unique challenges. Investigators must address the hydrophobicity and sensitivity of carotenoids to degradation by light and heat that are typically encountered in normal cell culture manipulations and incubations. It is possible that the physical and chemical breakdown products of carotenoids could induce biological effects that are strictly in vitro phenomena. Degradation also reduces the desired concentrations of the carotenoid under examination and, unless quantitated by high-performance liquid chromatography (HPLC), prevents reliable assessment of dose-dependent biological responses. Therefore, the objective of our experiments was to determine how delivery techniques can be optimized to enhance stability of β-carotene (BC) in vitro and to facilitate cellular uptake of BC. For these studies, we utilized PC-3, DU 145, and LNCaP human prostate cancer cell lines, since prostate cancer risk may be related to specific dietary carotenoids (3–5).

Common in vitro methods employed to deliver BC include water-miscible solvents such as tetrahydrofuran (THF) (6,7), water-dispersible BC beadlets (8), and artificial liposomes (9–11). The incorporation of lipophilic compounds such as carotenoids into aqueous media by artificial vehicles may not produce biologically relevant subcellular distribution and molecular effects. We hypothesize that the in vitro delivery of BC to cells in culture by lipoproteins may be a more biologically relevant approach. Carotenoids are transported in the circulation and distributed to tissues in vivo via lipoprotein particles (12). With BC used as a model carotenoid, the following studies evaluate traditional delivery approaches and a novel delivery method, BC-enriched bovine serum, for the delivery of carotenoids to prostate cancer cells in vitro.

Materials and Methods

Cell Culture Methods

PC-3, DU 145, and LNCaP human prostate adenocarcinoma cells were obtained from the American Type Culture Collection (Rockville, MD), and culture supplies were purchased from Sigma Chemical (St. Louis, MO). All studies were conducted in early-passage (<10) cells in our laboratory. Cell lines were maintained at 37°C with 5% CO2 in Dulbecco's modified Eagle's medium (DMEM) supple-

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mented with 10% fetal calf serum (catalog no. 2442, containing <1.0 nmol BC/l), 60,000 U of penicillin, 60 mg of streptomycin (catalog no. P0781), and 2.4 mmol of L-glutamine (catalog no. G7513) per 500 ml of DMEM. BC was delivered to cells by one of four methods described in detail below: THF, water-dispersible BC beadlets (beadlets), liposomes, or BC-enriched bovine serum. Media and cell pellets were stored in polypropylene tubes at -20°C overnight before extraction and HPLC analysis. Cell culture experiments were carried out under subdued lighting to minimize carotenoid breakdown by light.

**Extraction and HPLC Analysis of Cells and Media**

The cells were transferred from the polypropylene centrifuge tubes in which they were frozen to glass tubes by rinsing three times with 0.5 ml of distilled water. Aliquots for protein assays were removed and analyzed by the bicinchoninic acid method (kit TPRO-562, Sigma Chemical). The centrifuge tubes were further rinsed twice into the glass tubes with 1 ml of 100% ethanol [containing 1 g/l butylated hydroxytoluene (BHT)]. Saturated KOH (200 µl) was added, and the mixture was vortexed and saponified for 20 minutes at 60°C. On cooling, 200 µl of distilled water and an internal standard, echinenone (Hoffmann-La Roche, Nutley, NJ), were added. After addition of equal volumes of hexane, the samples were vortexed thoroughly and allowed to separate on ice. The hexane layer, which contained the carotenoids and retinoids, was removed, and the hexane addition and extraction were repeated. The samples were evaporated with a model AS 160 Speedvac (Savant, Farmingdale, NY) and stored at -20°C under argon gas before analysis, which was completed within 48 hours of the extraction. Reconstitution for reverse-phase HPLC analysis was in methylene chloride. Typically, 100 µl of media were extracted by addition of 100 µl of ethanol-BHT solution and echinenone. Without saponification, the extraction procedure proceeded with the addition of equal volumes of hexane, as stated above.

A Vydec 201TP54 C18 reverse-phase column (The Separations Group, Hesperia, CA) was used for carotenoid analysis (2 ml/min) with mobile phase of 88% methanol-9% acetonitrile-2% water with the addition of 1% 2,2,4-trimethyl pentane as a solvent modifier. Detection at 450 nm and integration utilized a model 170 UV-Vis detector (Bio-Rad, Richmond, CA) and a model CR601 Chromatopac integrator (Shimadzu, Kyoto, Japan). All HPLC solvents were obtained from Fisher Scientific (Pittsburgh, PA). Standard curves were prepared using crystalline BC (Fluka, St. Louis, MO) and internal standard (echinenone) plotting a ratio of BC area to echinenone area vs. nanograms of BC injected into the machine. This laboratory participates quarterly in the National Institute for Standards in Technology micronutrient analysis proficiency testing program. The coefficient of variation for BC analysis is <10%.

**THF**

Delivery of carotenoids by THF to cells in culture was previously described by Bertram and co-workers (6). Crystalline BC (Fluka; 97% purity) was solubilized in 10 ml of THF containing 0.01% BHT. Serial dilutions of the initial THF-BC solution were made to achieve lower concentrations. The THF-BC solutions were used at 100 µl/20 ml of prostate medium for a final solvent concentration of 0.5%. Control medium was prepared with 0.5% THF + BHT. Fresh HPLC-grade THF was necessary, since non-HPLC-grade THF was found to be more toxic to cells in culture. BC in THF will adhere to plastic surfaces; therefore, medium was prepared in glass containers. Actual concentrations for the vehicle comparison study determined by HPLC ranged from 0.66 to 0.83 µmol BC/l.

**Beadlets**

Water-dispersible BC beadlets and echinenone were provided as gifts from Hoffmann-La Roche. BC beadlets are of proprietary composition containing 10% (wt/wt) BC and 1.0% (wt/wt) α-tocopherol. A known quantity of BC beadlets containing 10% BC was dissolved in 10 ml of DMEM and placed in a bath sonicator for five minutes. Serial dilutions using DMEM were made for less-concentrated BC media. The BC beadlet solutions were added at 100 µl/20 ml of medium (8). Actual concentrations determined by HPLC for the vehicle comparison study ranged from 1.09 to 1.66 µmol BC/l.

**Liposomes**

This method for liposome preparation was adapted from the procedures of Verdon (9) and Grolier (10) and their co-workers. Chemicals were purchased from Sigma Chemical. Solutions of BC, cholesterol linoleate (50 mg/ml), and dicetyl phosphate (1 mg/ml) were prepared in methylene chloride; chloroform and ethanol were utilized for phosphatidylcholine (100 mg/ml) and α-tocopherol (1 mg/ml), respectively. Appropriate amounts of each solution were placed in a glass test tube (13 x 100 mm) to achieve 0.078 µmol of BC, 0.399 µmol of α-tocopherol, 0.185 nmol of cholesterol linoleate, 0.927 µmol of dicetyl phosphate, and 6.49 µmol of phosphatidylcholine. Contents were evaporated under a stream of argon gas, resolubilized with 2 ml of diethyl ether, and reevaporated; 2.5 ml of medium were added and the solution was allowed to equilibrate at room temperature for two hours. This suspension was further diluted to 50 ml with prostate medium to obtain 1.6 µmol BC/l of liposome medium. Actual concentrations determined by HPLC ranged from 0.8 to 1.2 µmol BC/l.

**BC-Enriched Steer Serum**

In addition to its usual diet, an Angus steer was fed 200 mg of BC from BC beadlets daily for one week before slaughter. Serum was collected, analyzed for BC by HPLC,
and added to DMEM at 12.5% with an additional 2.5% fetal calf serum. Final BC concentration of the steer serum was 16.9 μmol/l. Steer serum and fetal calf serum were filter sterilized using a 0.22-μm cellulose acetate filter before addition to DMEM. Final media concentrations ranged from 1.1 to 1.3 μmol BC/l.

Comparison of BC Uptake by DU 145, PC-3, and LNCaP Cell Lines

Three flasks (75 cm²) of each cell line, DU 145, PC-3, and LNCaP, were plated at 0.7 × 10⁶ cells/ml in 22.4 μmol BC/l of medium from beadlets. After three days of incubation, cells were rinsed with DMEM, trypsinized, counted by trypan blue exclusion, pelleted, and frozen at −20°C until extraction and HPLC analysis. Media samples were collected before and after incubation. Cells and media were analyzed for BC.

Uptake and Stability of BC

DU 145 cells were plated at approximately 1.2 × 10⁶ cells/ml in 75-cm² flasks and incubated for 48 hours before BC treatment. At Time 0, fresh medium containing 7.3–7.7 μmol BC/l as beadlets was added. Cells and media were harvested at 0, 6, 12, 24, 48, 72, and 96 hours. The medium was removed for HPLC analysis, and the cells were washed with DMEM to remove any remaining BC nonspecifically adhering to the cell surfaces. Three flasks were harvested at each time point. The cells were counted by trypan blue exclusion, pelleted, and frozen at −20°C for subsequent HPLC analysis of BC content.

BC Vehicle Comparison Study

Media were prepared at a target concentration of 1.0 μmol BC/l by THF, BC beadlets, artificial liposomes, and BC-enriched steer serum. Three 75-cm² flasks with PC-3 or DU 145 prostate tumor cells at 3.5 × 10⁶ cells/ml were employed for each delivery method. Cells were incubated for 72 hours, rinsed with DMEM, harvested by trypsinization, counted by trypan blue exclusion, pelleted, and frozen at −20°C for evaluation of BC by HPLC. Additionally, media samples were collected before and after incubation for BC quantitation.

Statistics

For cellular uptake and vehicle comparison assays, group means were compared using one-way analysis of variance. Pairwise testing among groups was conducted using the post hoc Fisher’s protected least squares difference test (Statview, Brain Power, Calabassas, CA).

Table 1. Comparison of BC Uptake in DU 145, PC-3, and LNCaP Human Prostate Cancer Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Avg 3-Day Cell Counts, 10⁶</th>
<th>BC Uptake, nmol/mg protein</th>
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</thead>
<tbody>
<tr>
<td>DU 145</td>
<td>24.5 ± 0.22</td>
<td>0.227 ± 0.027</td>
</tr>
<tr>
<td>PC-3</td>
<td>15.5 ± 0.34</td>
<td>0.341 ± 0.054</td>
</tr>
<tr>
<td>LNCaP</td>
<td>16.4 ± 0.37</td>
<td>1.370 ± 0.130</td>
</tr>
</tbody>
</table>

a: Media β-carotene (BC) was prepared by beadlet method. Medium was quantitated by high-performance liquid chromatography to contain 22.4 μmol BC/l.

b: Values in columns with different symbols (*, †) are significantly different (p < 0.05) by analysis of variance and Fisher’s protected least squares difference comparisons.

c: Cell counts are means; n = 3 flasks. Each cell line was plated at 0.7 × 10⁶ cells/ml, and cell counts were quantitated after three days.

d: Values are means ± SD; n = 3 flasks.

Results

Stability and Cellular Uptake of BC

To simultaneously determine whether PC-3, DU 145, or LNCaP cell lines exhibited different abilities to accumulate BC, 22.4 μmol BC/l of medium was prepared employing beadlets. After three days of incubation, LNCaP cells accumulated 1.370 nmol BC/10⁶ cells (p < 0.05 vs. PC-3 and DU 145) compared with PC-3 and DU 145 cells, which accumulated 0.341 and 0.227 nmol BC/10⁶ cells, respectively (Table 1).

The time course of BC disappearance from medium and cellular BC uptake was examined using DU 145 cells. Figure 1A illustrates the disappearance of all-trans BC recovered from the medium during the 96-hour incubation with the cells. Significant (p < 0.05) decreases in media BC were seen beginning at 12 hours and continuing through 96 hours. The all-trans BC content of DU 145 prostate tumor cells increased with time when incubated with 7.7 μmol BC/l of medium prepared from beadlets (Figure 1B). Significant (p < 0.05) increases in cellular BC compared with Time 0 were seen at 12–48 hours. No further increase in cellular BC was seen between 48 and 96 hours. Isomerization of all-trans BC was not significant during 96 hours and, therefore, cannot account for the disappearance (data not shown). Additionally, the decrease of BC cannot be accounted for by the content of BC recovered in the cells, since the amount accumulated in cells was <1% of the total media BC. We also investigated the hypothesis that the presence of cells contributed to the loss of BC by completing a similar quantitation of media BC over time but without cells in the flask. Similar loss of BC was observed, suggesting that physical and chemical factors inherent in the in vitro system account for BC loss (data not shown).

BC Vehicle Comparison Study

PC-3 cells were used to compare four different approaches for the delivery of BC to cultured cells. The stabili-
ity and efficiency of BC uptake for each method are shown in Table 2. Similar results were observed using DU 145 cells (data not shown). After three days of incubation, the recovery of BC from the medium was greater when delivered in the lipoprotein particles of enriched steer serum than when delivered by any of the three other methods. Liposomes were the least stable, and the inclusion of vitamin E in the liposome preparation did not prevent substantial BC loss. After incubation, 95% of the all-trans BC was preserved when delivered in bovine serum, whereas beadlet, THF, and liposome deliveries resulted in retention of only 59%, 37%, and 13%, respectively (p < 0.05 vs. bovine serum). Cellular BC concentrations (in pmol/10^6 cells) are also shown in Table 2. Efficiency of uptake [(nmol BC/10^6 cells) / (nmol BC/flask)] × 100 was higher for beadlet (8.3 × 10^-4) and enriched bovine serum (5.0 × 10^-4) delivery than for THF (1.8 × 10^-4) and liposome (1.7 × 10^-4) delivery. The uptake efficiency of the beadlet delivery method was significantly greater than that of the enriched bovine serum delivery and resulted in significantly more cellular BC. In contrast, THF and liposome delivery methods were less efficient and resulted in significantly less cellular BC than the beadlet or enriched serum approaches.

Discussion

To evaluate the potential cellular and molecular processes modulated by carotenoids, it is desirable to establish precisely defined in vitro cell culture systems. The present studies show that investigators must consider the method of BC delivery and the formation of degradation products in

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Table 2. Comparison of BC Stability and Cellular Uptake by PC-3 Human Prostate Cancer Cells by Different Delivery Methods

<table>
<thead>
<tr>
<th>Delivery Method</th>
<th>Media BC, μmol/l</th>
<th>%Media BC Remaining After Incubation</th>
<th>BC Uptake, pmol/10^6 cells</th>
<th>%Uptake Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time 0</td>
<td>3 days</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beadlets</td>
<td>1.09</td>
<td>0.64</td>
<td>59f</td>
<td>13.55*</td>
</tr>
<tr>
<td>Bovine serum</td>
<td>1.11</td>
<td>1.06</td>
<td>95*</td>
<td>8.36*</td>
</tr>
<tr>
<td>THF</td>
<td>0.83</td>
<td>0.30</td>
<td>37f</td>
<td>2.24f</td>
</tr>
<tr>
<td>Liposomes</td>
<td>0.80</td>
<td>0.10</td>
<td>13f</td>
<td>2.06f</td>
</tr>
</tbody>
</table>

a: Values in each column with different symbols (*, †, ‡, §) are significantly different (p < 0.05) by analysis of variance and Fisher’s protected least squares difference comparison.

b: Values are means; n = 3 flasks.

c: Uptake efficiency = [(nmol BC/10^6 cells) / (nmol BC/flask)] × 100.

d: Tetrahydrofuran.

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the assessment of in vitro investigations. The possibility that BC degradation products may contribute to reported biological effects should be considered. Furthermore, our previous work indicates that the potential cleavage of BC to retinol by prostate cells in vitro may also influence biological outcomes (13).

Uptake and Stability of BC

The mechanism of BC uptake into cultured cells is poorly understood and has been hypothesized to occur by receptor-mediated endocytosis (10) or by passive diffusion (14). Uptake into human lung fibroblasts plateaued after eight hours in 2 μmol BC/1 of medium prepared by THF, with cellular BC levels reaching 100 pmol BC/10⁶ cells (14). Conversely, a plateau was not observed in BALB/c 3T3 cells until three days in 3 μmol/l medium prepared with BC beadlets (8). Cellular BC concentration achieved at three days in this study was 0.57 pmol BC/10⁶ cells. In the present study, cells were incubated in 7.7 μmol BC/1 of medium prepared with BC beadlets, and the cellular uptake plateaued at 48 hours, achieving cellular BC levels of 0.030 nmol BC/10⁶ cells (Figure 1B).

Information regarding the stability of BC in cell culture systems is sparse. One study monitored BC degradation from a micellar solution of aqueous medium at 37°C and 5% CO₂. After 48 hours, pigment loss as measured by HPLC was reported as 10–15% (15). However, in a similar study, ¹⁴C-labeled BC was incorporated in medium by use of ethanol or hexane, incubated for 30 minutes at 37°C and 5% CO₂, and analyzed for ¹⁴C-labeled BC by scintillation counting (16). After incubation, only 50% of the labeled BC was recovered from the medium in the low- (67%) and high-density-lipoprotein (25%) fractions of the fetal bovine serum. Thus, after 30 minutes of incubation, 50% of the BC could not be accounted for. The authors did not report on the fate of the lost radiolabel. In addition, higher BC recoveries were observed with ethanol than with hexane as the delivery method or when increased levels of fetal bovine serum were used (16). Obara and co-workers (17) also reported substantial loss (62%) of BC as measured by HPLC after 24 hours of incubation with the THF. On the basis of chromatographic data collected at 340 nm, they concluded that the disappearance of BC in cell culture systems was the result of oxidative decomposition reactions.

The substantial degradation of carotenoids in cell culture medium during incubation is a concern. In our studies, the disappearance of carotenoids cannot be explained by isomerization or cellular uptake. Significant isomerization of all-trans BC was not detected in our studies, and the cells absorb <1% of the media BC. We observed that carotenoid degradation increased with incubation time and varied with the delivery method utilized. Overall, we conclude that carotenoids are relatively unstable in culture medium at 37°C. Recently, Bertram (18) suggested that degradation products of carotenoids caused by oxidative reactions or cellular metabolism may be more biologically active than the parent carotenoid. We propose that these unidentified degradation products of carotenoids produced during in vitro incubations may influence or directly mediate the biological effects investigators attribute to the parent carotenoid.

BC Vehicle Comparison

The enriched bovine serum delivery method was found to be the most stable vehicle and resulted in efficient BC uptake. The BC incorporated in vivo into lipoprotein particles in steer serum appears to be relatively protected from degradation under cell culture conditions. One hypothesis is that α-tocopherol is preferentially oxidized within lipoproteins, resulting in a preservation of carotenoids (19). Preparation of carotenoid-enriched bovine serum is not practical for most investigators, and beadlets offer a reasonable compromise in terms of stability and uptake efficiency. Of the delivery methods compared, carotenoid-enriched bovine serum and BC beadlets provided the greatest accumulation of BC by prostate cells and minimized degradation.

Conclusions

Our studies suggest that a high percentage of media BC degrades over the course of a three-day in vitro incubation with use of established cell culture techniques. Furthermore, lipoprotein particle and beadlet methods of BC delivery are reasonable approaches at this time. When using in vitro systems to identify cellular and molecular processes modulated by carotenoids, investigators should consider carotenoid degradation, metabolism, and cellular uptake in the interpretation of their data.

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