Comparison of tetrahydrofuran, fetal calf serum, and Tween 40 for the delivery of astaxanthin and canthaxanthin to HepG2 cells

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Abstract The present investigation aimed to compare fetal calf serum (FCS) and Tween 40 with the commonly employed tetrahydrofuran (THF) with respect to cytotoxicity, stability of the solubilized carotenoids, and uptake and accumulation of the xanthophylls astaxanthin (AX) and canthaxanthin (CX) in cultured human liver cells (HepG2). Incubation of HepG2 cells for 24 h with THF ($\geq 1.25\%$) or FCS ($\geq 11.25\%$) with or without AX ($\geq 25 \mu\text{mol/L}$) or CX ($\geq 25 \mu\text{mol/L}$) did not affect cell viability. Tween 40 (0.25–1.25% in medium) reduced cell viability by 75–99%. The stabilities of AX and CX in

cell-free RPMI 1640 medium for ≤ 24 h were higher when delivered with THF instead of FCS. The dose- and time-dependent accumulations of AX and CX (1–10 $\mu\text{mol/L}$) in HepG2 cells were higher when carotenoids were delivered with FCS compared to THF. In conclusion, FCS and THF, but not Tween 40, were suitable solvent systems for the delivery of AX and CX to HepG2 cells. In our experiments FCS was superior with regard to the uptake and accumulation of both carotenoids.

Keywords Carotenoids · Astaxanthin · Canthaxanthin · Delivery vehicle · HepG2 cell culture · Fetal calf serum · Tween 40 · Tetrahydrofuran

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Introduction

Carotenoids are a group of more than 600 natural pigments that are synthesized *de novo* in higher plants, mosses, algae, bacteria and fungi. Based on their chemical structures carotenoids are classified into two major groups: carotenes, which are oxygen-free hydrocarbons, and xanthophylls, which are oxygenated derivatives containing hydroxyl- or oxygen-groups (Higuera-Ciupara et al. 2006). As pigments, carotenoids determine not only the colour of plant leaves, fruits and flowers, but also of certain birds, insects, fish and crustaceae (Goodwin 1980). Humans and animals are not capable of carotenoid biosynthesis, but ingest them as part of their diets. Of

the approximately 60 carotenoids present in the human diet, only about 20 have been detected in human blood and tissues (Yonekura and Nagao 2007).

There is increasing interest in studying carotenoid function in cellular systems. However, the delivery of carotenoids into cells in culture is impeded by the fact that they are, due to their sensitivity toward light and heat, instable as well as poorly soluble in aqueous solutions. Tetrahydrofuran (THF), a commonly used solvent for carotenoid delivery in cell culture, oxidizes readily in culture media, which may lead to instability of test compounds and result in cytotoxicity (Xu et al. 1999; Williams et al. 2000; Shahrzad et al. 2002; Hurst et al. 2004; O'Sullivan et al. 2007). Therefore, attempts have been made to improve carotenoid delivery by using vehicles other than THF, including dimethyl sulfoxide, cyclodextrin, lipoproteins, micelles and beadlets (Martin et al. 1996; Stivala et al. 1996; Garrett et al. 1999; Pfitzner et al. 2000; Lancrajan et al. 2001; Vertzoni et al. 2006). However, the employed vehicles are in part cytotoxic, poorly soluble, crystallize in the culture medium or need to be frequently prepared freshly (Stivala et al. 1996; Xu et al. 1999; Lancrajan et al. 2001; Da Violante et al. 2002; Shahrzad et al. 2002; O'Sullivan et al. 2004; Palozza et al. 2008).

The aim of the present investigation was to compare the two recently described carotenoid delivery systems fetal calf serum (FCS) (Lin et al. 2007) and Tween 40 (O'Sullivan et al. 2004; During and Harrison 2007) with the commonly used THF with respect to cytotoxicity, stability of the dissolved carotenoids, and uptake and accumulation of two of the major xanthophylls, namely astaxanthin (AX) and canthaxanthin (CX), in cultured human liver cells (HepG2).

Materials and methods

Chemicals

Human hepatoblastoma cells (HepG2) were obtained from the Institute of Applied Cell Culture (IAZ, Munich, Germany). Cell culture medium, supplements and cell culture reagents were all from PAA (Coelbe, Germany). Cell culture flasks and plates were obtained from Sarstedt (Nuembrecht,

Germany). Astaxanthin (AX, purity 98.7%) and canthaxanthin (CX, purity 96.1%) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Tetrahydrofuran (THF), acetonitril, *n*-hexane and chloroform (all of HPLC grade quality) were obtained from VWR International (Darmstadt, Germany). Tween 40 (polyoxyethylene sorbitan monopalmitate) was purchased from Sigma (Deisenhofen, Germany). Butylhydroxytoluol (BHT), ascorbic acid (AA) and neutral red were from Carl Roth (Karlsruhe, Germany).

Cell culture conditions and delivery of carotenoids

HepG2 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 µg/mL streptomycin under standard conditions (37 °C, 5% CO₂) in a humidified incubator. Culture medium was changed every second day and cells were subcultured every 4 days with a split ratio of 1:3–5. Cells were used from passage 13 to 30. For experiments, HepG2 cells were seeded at an initial density of 50,000 cells per cm² in 24- or 6-well plates for cytotoxicity and uptake experiments, respectively. At ≥90% confluence, HepG2 cells were incubated with either AX or CX, which were delivered to the medium by three different vehicles. Stock solutions of AX and CX were prepared by dissolving the carotenoids (2 mmol/L) in THF (containing 160 mg/mL BHT and 352 mg/mL ascorbic acid). Concentrations of the stocks were verified spectrophotometrically (Beckman DU 800, Beckman Coulter, Krefeld, Germany), after dilution in *n*-hexane, using the published molar extinction coefficients. Aliquots of the carotenoid stock solutions were stored at –80 °C and used within 8 weeks of preparation.

To prepare the THF vehicle, an appropriate aliquot of the AX or CX THF stock was diluted in cell culture medium to final concentrations of 10 or 25 µmol/L. The maximum THF concentration in the medium was 1.25%. For the FCS version, which was prepared according to Lin et al. (2007) carotenoid stock aliquots were added to FCS in a tenfold higher concentration than desired, mixed and added to FCS free medium. As a third delivery vehicle, Tween 40 was used as described by O'Sullivan et al. (2004, 2007). Briefly, aliquots of the THF stock solution

were concentrated at room temperature in a Jouan RC 10-10 centrifugal evaporator (Saint Herblain, France) and re-dissolved in identical volumes of Tween 40 (200 mL Tween 40 diluted in 1 L acetone). These solutions were dried again and reconstituted in RPMI medium and vortex-mixed.

Determination of cell viability

The cytotoxicities of AX, CX and the different delivery vehicles THF, FCS and Tween 40 were evaluated using the neutral red assay (Borenfreund and Puerner 1985). Briefly, HepG2 cells were incubated with AX- or CX-supplemented media (using all three delivery vehicles separately) at concentrations ranging from 1 to 25 $\mu\text{mol/L}$. To assess vehicle effects on the cell viability of liver HepG2 cells, THF, FCS and Tween 40 were added to the cell culture medium separately up to 1.25% (by volume). After 24 h, the culture medium was replaced by medium containing neutral red (50 $\mu\text{g/mL}$) and cells were incubated for 3 h. Neutral red medium was aspirated and cells washed with phosphate buffered saline (PBS). The incorporated neutral red dye was extracted using a bleaching solution (50% ethanol, 1% acetic acid, 49% H_2O) and the absorbance read at 540 nm using a plate reader (Labsystems, Helsinki, Finland). Cell viability was expressed as percent viability compared to untreated control cells. Cytotoxicity experiments were performed in triplicate in two independent passages.

Carotenoid uptake into HepG2 cells

To study the uptake of AX and CX into liver cells, HepG2 were incubated with 1, 5 and 10 $\mu\text{mol/L}$ AX or CX, delivered by THF or FCS, for 6, 12 and 24 h. Since the Tween 40 version appeared to be highly cytotoxic (see “Results”), this delivery version was omitted for subsequent uptake studies. At the designated time points, cells were washed twice with pre-warmed PBS, detached by scraping, centrifuged and the resulting cell pellets stored at -80°C . Uptake experiments were performed in duplicate in three subsequent cell passages.

Carotenoid stability in culture medium

To evaluate whether AX and CX are stable in cell culture medium and whether stability might be

affected by the delivery vehicle, AX and CX were added to RPMI medium at concentrations of 1, 5, 10, and 25 $\mu\text{mol/L}$ and incubated under cell culture incubation conditions (37°C , 5% CO_2) in three independent experiments. After 0, 6, or 24 h, aliquots were removed and stored at -80°C until quantification of carotenoids by HPLC.

Carotenoid extraction and quantification by HPLC

Cell pellets were homogenized in 250 μL PBS containing 2 mmol/L EDTA. Two hundred μL cell suspension or cell culture medium was extracted twice with 1 mL hexane-ethanol-acetone (50:25:25, by volume) (Olives Barba et al. 2006; O’Sullivan et al. 2007). To prevent oxidation of carotenoids during extraction, the antioxidants BHT and ascorbic acid (AA; 160 mg/mL), respectively, were added to *n*-hexane and acetone, respectively. Samples were centrifuged at low speed and the supernatants transferred to a clean tube. The pooled extracts were dried at 40°C in a Jouan RC 10-10 centrifugal evaporator (Saint Herblain, France) and resuspended in 100 μL acetone/AA of which 20 μL were injected into a Jasco HPLC system (Gross-Umstadt, Germany) consisting of an autosampler (X-LC 3059-AS), a pump (PU-2085 Plus), a 3-line degasser (DG-2080-53), a ternary gradient unit (LG-2080-02), and a UV/Vis-detector (2070 Plus) operated at a wavelength of 470 nm. AX and CX were separated on a Vydac 218TP C18 column (250×4.6 mm; 5 μm) connected to a Vydac 218TP C18 guard column (7.5×4.6 mm; 5 μm ; both from Alltech Grom GmbH, Rottenburg-Hailfingen, Germany) with acetonitrile/methanol (92.5:7.5, v/v, containing 160 mg/L AA) as the mobile phase delivered at a flow rate of 1.5 mL/min. Peaks were recorded and integrated using the chromatography software ChromPass version 1.8.6.1 (Jasco). The concentrations of AX and CX were quantified against external standard curves with authentic carotenoids ranging from 0.1 to 10 $\mu\text{mol/L}$. The detector responses for both carotenoids were linear over the tested ranges (AX, 1–114 ng injected; CX, 1–113 ng injected) and standards were stable for at least 24 h at room temperature and 3 d at -80°C (<4% degradation). Results were adjusted for total cell protein, which was measured after appropriate dilution in PBS with

the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). Due to a shortage in acetonitrile, a modified mobile phase, which resulted in sharper peaks and a significantly shorter run time of <4.5 min (as opposed to ~11 min with the above described eluent containing acetonitrile as organic modifier; Fig. S1 provided online as supplemental data), was used for determination of carotenoid stability (see above). The mobile phase consisted of methanol and hexane (90:10, v/v) with 160 mg/L ascorbic acid and was delivered at a flow rate of 1.5 mL/min.

Statistical analysis

Results are expressed as mean with standard deviation. Statistical analyses were performed with the computer program SPSS 15.0 for Windows (SPSS GmbH Software, Munich, Germany). Group means were compared by student's *t* test or Mann–Whitney U-test as appropriate. Results were considered significant at $p < 0.05$.

Results

Cytotoxicity of astaxanthin and canthaxanthin solubilized in tetrahydrofuran, fetal calf serum, or Tween 40

The potential cytotoxicities of AX and CX and the three delivery vehicles THF, FCS, and Tween 40 were evaluated with the neutral red assay, a viability test which is based on the lysosomal accumulation of the neutral red dye by live cells. HepG2 liver cells were incubated for 24 h with increasing concentrations of up to 25 $\mu\text{mol/L}$ AX or CX delivered via each of the three vehicle systems. Neither THF alone, up to the maximum concentration of 1.25% in the cell culture medium, nor together with AX and CX (0–25 $\mu\text{mol/L}$) did affect the viability of HepG2 cells (Fig. 1). Similarly, neither FCS itself nor AX and CX dissolved in FCS affected the viability of HepG2 cells (Fig. 1c, d). In contrast, Tween 40 at concentrations of 0.50 and 1.25%, which correspond to the solvent concentrations required for delivery of 10 and 25 $\mu\text{mol/L}$ AX and CX, respectively, strongly reduced cell viability to 5.2 and 0.5%, respectively (Fig. 1e, f). Even a Tween 40 concentration of 0.25% (at 5 $\mu\text{mol/L}$ AX or CX) reduced cell viability to

22% (SD 15.4). Only at 1 $\mu\text{mol/L}$ AX or CX, corresponding to 0.05% Tween 40 in the medium, cell viability was not significantly affected (92 and 95%, respectively). Because of its strong cytotoxicity, Tween 40 was not used for the subsequent stability and uptake studies.

Stability of astaxanthin and canthaxanthin in cell-free systems

The stabilities of the carotenoids under culture conditions were studied by supplementing cell culture media with 1, 5, 10 or 25 $\mu\text{mol/L}$ AX or CX (final concentrations in culture media) dissolved in THF or FCS and incubation at 37 °C and 5% CO₂ for 0, 6 or 24 h. The stability of both carotenoids decreased with time and with decreasing concentrations in the culture media. Both carotenoids were less stable in FCS than in THF (Fig. 2). Maximum decay of AX was 62% in medium with 1 $\mu\text{mol/L}$ AX in FCS and 14% in medium with 10 $\mu\text{mol/L}$ AX in THF. Maximum loss of CX was 64 and 26% in medium containing 1 $\mu\text{mol/L}$ CX in FCS or THF, respectively (Fig. 2).

Cellular uptake of astaxanthin and canthaxanthin from media with tetrahydrofuran or fetal calf serum as delivery vehicle or Tween 40 as delivery vehicle

HepG2 cells incubated with media containing 1, 5 or 10 $\mu\text{mol/L}$ AX or CX (dissolved in FCS or THF) for 6, 12 and 24 h, time- and dose-dependently accumulated AX and CX (Fig. 3). Intracellular concentrations of AX and CX were similar when FCS was employed as vehicle. Use of THF as delivery vehicle, however, resulted in significantly lower cellular accumulation of CX than AX (Fig. 3).

Discussion

Carotenoids are accumulated in the liver, from where they are secreted with lipoproteins and distributed throughout the organism (Schmitz et al. 1991). Due to the central role of the liver in carotenoid metabolism and trafficking, mechanistic cell culture studies with hepatocytes are warranted. However, because of the

Fig. 1 Viability of HepG2 cells incubated with 1–25 µmol/L astaxanthin (AX) or canthaxanthin (CX) dissolved in THF (**a**, **b**), FCS (**c**, **d**) or Tween 40 (**e**, **f**) in comparison to cells incubated with the respective solvents alone for 24 h. The solvent concentrations of 0.5 and 1.25% were chosen to represent their medium and maximum concentrations in the

culture medium upon incubation of the cells with the carotenoids. Viability was determined with the neutral red assay and calculated as percentage of cells grown in culture medium without addition of test substances or solvents. Data are mean $\pm$ SD of triplicate experiments performed in two different passages ($n = 6$)

poor solubility of carotenoids in the aqueous culture media and the lack of well-characterised solvent systems for the delivery of carotenoids to cultured liver cells, such studies are scarce. We therefore compared the three delivery systems THF, FCS, and Tween 40 for their suitability to deliver the carotenoids astaxanthin (AX) and canthaxanthin (CX) to HepG2 liver cells. The delivery systems, with and

without added carotenoids, were characterised for their impact on cell viability, stability of the carotenoids at culture conditions, and cellular accumulation of the carotenoids.

Plasma concentrations of carotenoids in general and AX and CX in particular are in the low micromolar range (e.g. β -carotene, ~ 0.5 µmol/L; AX and CX, 0.1–0.3 µmol/L) (Albanes et al. 1995;

Fig. 2 Stability (% of initial concentration) of astaxanthin (a) and canthaxanthin (b) dissolved in THF or FCS and re-suspended in cell culture medium (final concentrations of

1, 5, 10 and 25 $\mu\text{mol/L}$) and incubated at 37 °C for up to 24 h. Values represent the means of three independent experiments ($n = 3$) determined in duplicate by HPLC

Fig. 3 Time- and dose-dependent uptake of carotenoids in HepG2 cells incubated for 6, 12 or 24 h with increasing concentrations (1, 5 or 10 $\mu\text{mol/L}$) of AX (a) or CX (b). Carotenoids were extracted and analysed by HPLC as described in “Materials and methods”. Values reflect the cellular carotenoid content normalized to total cell protein and are given as mean $\pm$ SD of three independent experiments performed in duplicates ($n = 6$). * Significant difference between THF and FCS versions at indicated time/concentration ($p < 0.05$)

Schmidt et al. 1996; Ziouzenkova et al. 1996; Kompauer et al. 2006; Xiang et al. 2008). Ingestion of a single dose of 100 mg AX equivalents resulted in

maximum AX plasma concentrations (C_{max}) of 0.47 $\mu\text{mol/L}$ (Coral-Hinostroza et al. 2004). Mean AX plasma concentrations in the range of 0.27–0.52 $\mu\text{mol/L}$

were observed after daily consumption of 250 g of wild or aquacultured salmon for 4 weeks (Rufer et al. 2008). No high-dosage AX or CX supplementation trials with human subjects have been published to date. Ingestion of high daily doses (0, 15, 45, 180, or 300 mg) of β -carotene for 1 month, on the other hand, resulted in fasting β -carotene plasma concentrations ranging from ~ 0.5 to ~ 6.0 $\mu\text{mol/L}$ (Ringer et al. 1991). Hence, the carotenoid concentrations used in our cell culture experiments may not reflect normal dietary intake of AX and CX, but might well be achievable upon prolonged consumption of high doses of the carotenoids.

In our experiments, incubation of HepG2 cells with RPMI 1640 medium containing up to 1.25% THF or 11.25% FCS, and 25 $\mu\text{mol/L}$ AX or CX did not alter cell viability, which is in agreement with our own experiences with this cell line as well as previously published literature using different cell lines (Bertram et al. 1991; Stivala et al. 1996; Pfitzner et al. 2000; O'Sullivan et al. 2004). Unexpectedly, Tween 40, at concentrations ranging from 0.25 to 1.25% in the cell culture medium was cytotoxic and reduced the viability of HepG2 cells by 75–99% (Fig. 3). O'Sullivan et al. (2004), on the other hand, in agreement with During and Harrison (2007), did not report cytotoxic effects of either Tween 40 or Tween 80 to Caco-2 cells. This discrepancy may be due to the use of a different cell line (derived from a colon carcinoma instead of a liver carcinoma) and the fact that lower maximum concentrations of Tween in the cell culture medium (0.1%) were employed (O'Sullivan et al. 2004; During and Harrison 2007). Similarly, the viability of freshly prepared primary rat hepatocytes was not impaired by incubation for 1, 4 or 24 h with $\leq 0.03\%$ Tween 80, but significantly reduced when 0.3% Tween 80 were used (Bravo Gonzalez et al. 2004). Interestingly, the extent of the cytotoxic effect of Tween 40 was slightly reduced upon addition of AX or CX (Fig. 3). A similar phenomenon was observed by Palozza et al. (2008), who studied the suitability of Tween 60:cholesterol niosomes as a delivery vehicle for β -carotene to immortalized RAT-1 fibroblasts (Palozza et al. 2008). At concentrations of 2.5 and 5.0 $\mu\text{mol/L}$, β -carotene inhibited the cytotoxic effects of the niosomes (Palozza et al. 2008). Due to the apparent sensitivity of HepG2 cells to Tween 40, this vehicle was not employed in the further experiments.

At all studied concentrations (1–25 $\mu\text{mol/L}$), AX and CX were more stable in RPMI 1640 medium when first dissolved in THF rather than in FCS (Fig. 2). Lycopene, on the other hand, was reported to be more stable in culture media when delivered by FCS than by THF (Lin et al. 2007). In that study, however, a different cell culture medium (DMEM) and a different carotenoid were employed and may partly explain the observed differences in stability (Lin et al. 2007). Furthermore, lycopene was previously reported to be more stable in cell culture medium than CX (Bertram et al. 1991). While we did not observe significant differences in the relative stabilities of AX and CX depending on their concentrations in the culture media (Fig. 2), CX and lycopene, among other carotenoids, were more stable in basal Eagle's medium at concentrations of 1 $\mu\text{mol/L}$ than 10 $\mu\text{mol/L}$ in a previous study (Bertram et al. 1991).

At the employed cell culture conditions in our experiments, the dose- and time-dependent cellular accumulation of both AX and CX was much higher when the carotenoids were delivered with FCS rather than THF (Fig. 3), despite the carotenoids' lower stability in FCS compared to THF (Fig. 2). This is in agreement with findings from experiments employing different carotenoids and a different cell line. Lin et al. (2007) reported a better uptake of lycopene into DU145 prostate cancer cells from FCS- compared to THF-supplemented media. Lipoproteins appear to play an important role in the cellular uptake of carotenoids (During and Harrison 2007; O'Sullivan et al. 2007). The involvement of the scavenger receptor class B type I (SR-BI) in carotenoid uptake has been demonstrated (van Bennekum et al. 2005; During and Harrison 2007). SR-BI recognizes and binds lipoproteins, both in HepG2 cells (Rhainds et al. 1999) and in vivo (Van Eck et al. 2008), and mediates the internalization of their lipid contents (Rhainds and Brissette 2004). AX and CX may have been incorporated into lipoproteins present in the FCS-supplemented media, thus alleviating their active transport across the cell membrane.

In summary, our experiments suggest that, because of its cytotoxicity, Tween 40 is not suited as a delivery vehicle for test compounds to HepG2 cells. While both THF and FCS were without toxicity to HepG2 cells, carotenoids were less stable in FCS than in THF in a cell free system under culture conditions.

Nevertheless, accumulation of astaxanthin and canthaxanthin was more pronounced when FCS was used as vehicle. In conclusion, while both FCS and THF appear suitable as solvent systems for the delivery of carotenoids to HepG2 cells, FCS is superior with regard to the uptake and accumulation of the compounds, while THF is superior with regard to carotenoid stability. Researchers need to consider these trade-offs when choosing the appropriate delivery vehicle for carotenoids for their planned cell culture studies.

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