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Rapid Report

Incorporation of carotenoids in aqueous systems: uptake by cultured rat hepatocytes

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The in vitro investigations about carotenoid functions and metabolism are hindered by their hydrophobicity. In order to mimic as close as possible the physiological events, we prepared by rapid and easy methods sterile liposomes and emulsions containing carotenoids which are absorbed by cultured rat hepatocytes as a function of time and temperature. The lipid composition of the vesicles was shown to influence the carotenoid encapsulation.

Experimental studies on carotenoid functions in biological systems are limited by the absence of an adequate animal model allowing accurate interpretation of in vivo investigations on absorption, metabolism and tissue distribution. The high hydrophobicity of carotenoids makes them insoluble in aqueous systems and therefore poorly available for cell cultures [1–3].

In most in vitro studies, carotenoids were provided as water dispersible beadlets, detergent solutions or diluted in various solvents such as alcohols, dimethyl sulfoxide, tetrahydrofuran and hexane [4–7]. These methods allowed the evaluation of the potential effects of the pigments but non-specific uptake and problems of miscibility, cristallization and toxicity could mislead the physiological significance of the observed phenomenon. Liposomes and emulsions have recently been used as nontoxic carriers to improve the delivery of drugs and macromolecules in cultured cells as well as in specific tissues in the body [8,9]. Moreover the use of these structures allowed to mime physiological events such as receptor–ligand interactions, endocytosis... [10]. The aim of this study was to develop stable and sterile lipid carriers that could trap large quantities of carotenoids and direct them to cultured rat hepatocytes.

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Abbreviations: Car, β-carotene; Chol, cholesterol; DCP, dicetyl phosphate; PC, phosphatidylcholine; PS, phosphatidylserine; SA, stearylamine; Zea, zeaxanthin.

Materials and methods. Zeaxanthin and phosphatidylcholine were gifts from Hoffmann La Roche, Basel, Switzerland and Lucas Meyer, Hamburg, Germany, respectively. Other reagents were obtained from Sigma and Boehringer Manheim.

Small oligolamellar liposomes were prepared as described elsewhere by H. Fessi [11]. The phospholipids, cholesterol, dicetyl phosphate and carotenoids (total lipids 200 mg) were dissolved in a mixture of dimethylformamide and ethanol. This solution was then gently added to a 10 mM phosphate buffer containing 150 mM NaCl, pH 7.4 (PBS) and the mixture was energically dispersed by ultra-Turrax for 20 min at room temperature. The solvents were removed slowly in a rotatory evaporator under vacuum. Large vesicles and non encapsulated β-carotene were removed by centrifugation.

Emulsions were prepared with 10% (w/v) soybean oil containing carotenoids (1.2 mg/ml), 1.2% (w/v) soya lecithin and 2.5% (w/v) glycerol. The lipids were mixed and heated to 60°C and double-distilled water (60°C) was added in a slow stream to the lipid phase underagitation. The agitation was achieved using an ultra-Turrax homogenizer at 15000 rpm during 30 min.

All prepared solutions were subsequently extruded through hydrophylic membranes with a final pore size of 0.2 μm and then assayed for carotenoids [12] and phospholipids contents (using a phospholipids B-test, Walko).

Mean vesicle diameters were determined by laser spectroscopy (submicron particle sizer N4 Coulter).

TABLE I

Effects of the lipid composition of the initial mixture on the carotenoid concentration and the particle size in the liposome solution

Liposomes are extruded on 1.2, 0.45 and 0.2 μm filters. Mean ± S.D., $n = 3$.

Lipid composition	Molar ratio	Particle size before filtration (nm)	Carotenoid concentration in the filtered solution (μg/ml)
Soya PC/Chol/ DCP/Car	10:0:0:0.2	231 ± 36	51 ± 9 (38) [a]
	8:2:0:0.2	238 ± 21	64 ± 6 (48)
	7:2:1:0.1	193 ± 22	33 ± 6 (49)
	7:2:1:0.2	184 ± 17	85 ± 10 (64)
	6.4:2:1:0.6	273 ± 29	70 ± 8 (17)
	6:2:1.1	337 ± 32	54 ± 9 (8)
	7:2:2:0.2	283 ± 19	62 ± 5 (46)
Egg PC/Chol/ DCP/Car	7:2:1:0.2	171 ± 11	81 ± 11 (61)
Soya PC/Chol/ PS/Car	7:2:1:0.2	214 ± 20	81 ± 15 (61)
Soya PC/Chol/ SA/Car	7:2:1:0.2	281 ± 13	66 ± 6 (52)
Soya PC/Chol/ DCP/Zea	7:2:1:0.2	147 ± 15	108 ± 16 (80)

[a] Final encapsulation yield.

Rat hepatocytes were isolated according to Seglen [13] and plated at a density of $0.7 \cdot 10^6$ cells per cm^2 in Williams E medium containing 40.6 i.u./ml of penicillin, 40.6 g/ml of streptomycin, 4 mM of glutamine, 10^{-6} M of hydrocortisone, 10^{-6} M of insuline, 0.55 mg/ml of nicotinamide, and during the four first hours, 8.10% of foetal calf serum. After 24 h cells were incubated at 37°C with fresh media containing various concentrations of carotenoids, incorporated in liposomes or emulsions for 48 h. In order to remove non-cellular pigments, cells were intensively washed twice with cold PBS, scraped off and centrifuged. The cell pellet was again washed twice with PBS, sonicated and utilized for determination of carotenoids [12] and fluorimetric DNA measurements [14].

Results and discussion. In an initial experiment, we tested different preparations of liposomes. We found that the methods of Bangham (sonication of dried lipids in buffer) [15], the ether injection [16] and the reverse-phase evaporation [17] should be avoided due to the too low β-carotene concentration in the final solution. That was probably due to the formation of large unilamellar or multilamellar vesicles (mean diameter above 400 nm) which were higly retained on 0.2–0.45 μm filters. Too small unilamellar vesicles were also avoided due to bad initial encapsulation yield. As carotenoids were shown to be located in the hydrocarbon core of the lipid bilayer of liposomes [18], multilamellar vesicles with a medium diameter would be a good model for trapping β-carotene. The two-step method (solvent injection-evaporation) used here, allows to obtain rapidly homogeneous solution of small vesicles (180–280 nm) made of few lipid bilayers with a good β-carotene encapsulation yield (49–64%). After filtration, the mean diameters were ranged between 130 and 240 nm. Moreover, this method did not use sonication or chlorinated solvents that may damage the carotenoid pigments.

The lipid compositions of both liposomes and emulsions were shown to influence the β-carotene concentration in the filtered solutions (Tables I and II). No difference was observed in the particle size and the β-carotene content when soya and egg lecithins were tested. The addition of cholesterol up to 20% of total lipids induced a 29% increase in the pigment incorporation. Similar effects were shown when 10% dicetyl phosphate or phosphatidylserine were added, suggesting that the negative charges also favor the β-carotene interation with liposomes.

Final β-carotene concentration was shown to increase linearly as the pigment percentage increased from 0 to 2% in the initial lipid mixture. The addition of more β-carotene increased the liposome diameter up to 337 nm and did not further improve significantly its concentration in the filtered liposomal solution. This result could be related to the increased thickness of liposome lipid bilayer to carotenoid incorporation [18]. Zeaxanthin was better incorporated into liposomes (108 μg/ml) than β-carotene, suggesting a better interaction of the former with the phospholipid polar heads.

In emulsions, the main limiting parameter seemed to be the low solubility of carotenoids in oils (1 mg/ml). Lecithin was used as emulsifier. The β-carotene concentration in the filtered emulsions was shown to increase as a function of the relative percentage of

TABLE II

Effects of soya phosphatidylcholine on the final level of emulsion β-carotene

Phospholipids are mixed with 10 g of triglycerides and then dispersed in 100 ml of distilled water. The emulsion is filtered on 1.2, 0.45 and 0.2 μm membranes. Mean ± S.D., $n = 3$.

	% of PC in the initial lipid mixture (w/w)					
	10	15	20	23	26	30
β-Carotene in the filtered emulsion (μg/ml)	23.7 ± 1.8	26.1 ± 3.1	27.9 ± 3.7	36.7 ± 2.4	31.0 ± 4.1	19.8 ± 2.7

lecithin up to 23% to triglycerides (w/w). Hence the pigment solubilization was improved by 55% when the phospholipid fraction increased from 10 to 23% of total lipids. Higher levels of phospholipids produced a flocculation phenomenon which altered filtration and pigment concentration in the sterile solution. Thus, in excess, phospholipids could act as demulsifying agents. After filtration the particle sizes were ranged between 195 and 250 nm. It should be noticed that the poor oil solubility (< 0.5 mg/ml) of zeaxanthin prevented the production of submicron-emulsion of this pigment by our method.

Large multilamellar liposomes had already been used in order to direct carotenoids to cells with a high endocytosis potential as hamster pouch carcinoma [19] and human monocytes [20]. Our liposomes (PC/Chol/DCP/CAR, 7:2:1:0.2) and submicron-emulsions (10% triglyceride, 3% PC, 0.01% β-CAR) were tested on cultured rat hepatocytes and were not cytotoxic. This was attested as by cytosolic enzyme leakage or cell detachment over 48 h of experiment in the range of 1 to 7 μM of carotenoids, which was equivalent to 0.1 to 1 μmol of phospholipids per 10^6 cells The stability of the lipid carriers was not modified in culture media as judged by the evolution of pigment concentrations during the experiment.

Zeaxanthin was selected because, in contrast to β-carotene, it could not be further metabolized into vitamin A. As shown by Fig. 1, cellular levels of carotenoids were low in the first hour and then progressed afterwards as a function of incubation time up

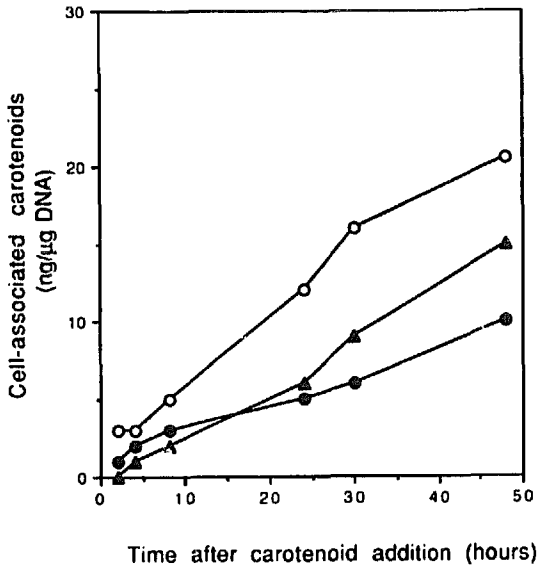

Fig. 1. Uptake of β-carotene from β-carotene liosomes (○) and emulsion (▲), and of zeaxanthin from zeaxanthin liposomes (●) by cultured rat hepatocytes at 37°C as function of exposure time. The carotenoid concentration was 7 μM and 0.9 to 1.2 μmol/10^6 cells of phospholipids were added. Data are the means of three determinations and the highest standard deviations are less than 15% of the value.

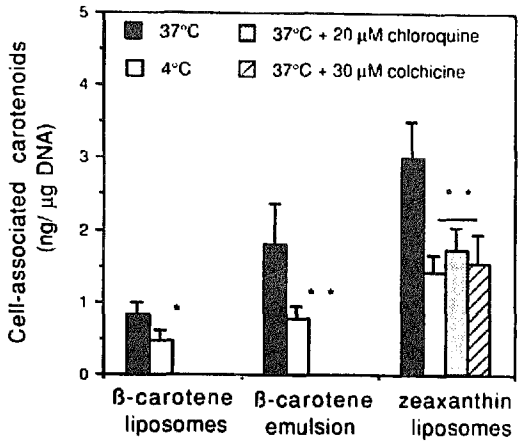

Fig. 2. Effects of temperature and metabolic inhibitors on carotenoids uptake by cultured rat hepatocytes after 8 h incubation. Mean ± S.D., $n = 4$. * $P < 0.05$, ** $P < 0.01$ as determined by the Student's t-test.

to 48 h. Interestingly, β-carotene absorption is higher when administred in emulsion at similar concentration as compared with liposomes.

After 48 h, the cell-associated β-carotene concentrations reached 15 ng/μg DNA. Rundhaug et al. [6] and Bertram et al. [7] reported a higher cellular accumulation of carotene (5–10-fold) when the pigment was administred at the same concentration in water dispersible beadlets or in solvent. However, their cell models were quite different and it could not be excluded that absorption was non-specific in these cases.

Incubation at 4°C makes it impossible for cells to undergo endocytosis, thus, to permit any distinction between the membrane binding and the active uptake by cells. Zeaxanthin and β-carotene uptake were decreased by 57% when hepatocytes were incubated 8 h at 4°C as compared with 37°C (Fig. 2). The same effect was observed when pigments were carried in either liposomes or emulsion. Moreover, the addition of metabolic inhibitors such as chloroquine 20 μM or colchicine 30 μM induced a 46% reduction in the liposomal zeaxanthin uptake at 37°C. All these results suggested that a fraction of cell-associated pigments was inside the cells and that the mechanism of absorption should be energy dependent. Our lipid vehicles contained no protein ligand which can interact with cellular receptors but it could be proposed that specific lipids at the carrier surface could mediate the binding and then the absorption of liposomes and emulsion. The data of Lee et al. [21] supported the hypothesis of a receptor-mediated endocytosis involving a putative membrane component that recognized and bound the negatively charged polar head of liposomal phospholipids.

In conclusion, we have prepared lipidic carriers for carotenoids with an easy and rapid method. These carriers can be sterilized by filtration and then used for in vitro investigations on cultured rat hepatocytes. Now,

this model is being improved by incorporation of apoproteins or galactoproteins in the lipid particles to enhance their specificity.

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