Intracellular β-Carotene Transport in Bovine Liver and Intestine Is Not Mediated by Cytosolic Proteins[1,2]

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ABSTRACT The mechanisms responsible for the movement of absorbed carotenoids between intracellular organelles and for the incorporation of carotenoids into serum lipoproteins are unknown. It was the objective of this study to use bovine liver and intestine as a model to study the possible cytosolic protein-mediated carotenoid transfer between liposomes and mitochondria in vitro. Liposomes containing β-[^{3}H]carotene were incubated with isolated bovine hepatic mitochondria (1 mg protein) and various quantities of liver cytosol or intestinal mucosal cell cytosol (0–6 mg protein) for up to 1 h at 37°C. The β-[^{3}H]carotene activity in liposomes was then measured over time to determine percent transfer of β-carotene to mitochondria. Over the time period studied, the rate of β-carotene transfer was unaffected by increasing levels of either hepatic or intestinal cytosolic protein. These results suggest that unlike many other lipid species, intracellular transport of β-carotene is not mediated by cytosolic transport proteins and must occur by other mechanisms such as vesicular transport or by membrane-bound proteins. J. Nutr. 126: 1470–1474, 1996.

INDEXING KEY WORDS:

• *β-carotene* • *transport* • *bovine* • *liver* • *intestine*

Many epidemiological studies indicated that a diet rich in carotenoid-containing fruits and vegetables is associated with a decreased risk of chronic disease conditions, including certain cancers (Ziegler 1989), cataracts (Knekt et al. 1992), and heart disease (Gaziano and Hennekens 1993). It has been difficult, however, to determine biological functions of carotenoids in mammalian systems because of the fact that the absorption, transport, intracellular distribution and metabolism of carotenoids is poorly understood (Erdman, Bierer and Gugger 1993).

The mechanisms responsible for the movement of absorbed carotenoids between intracellular organelles and for the incorporation of carotenoids into serum lipoproteins are unknown. Intermembrane transport of several lipid species, including phospholipids, cholesterol and α-tocopherol, has been shown to be mediated by cytosolic transport proteins (Bloj and Zilversmit 1977, Sato et al. 1991, Verdon and Blumberg 1988, Wirtz and Gadell 1990). The current study was designed to determine the possibility of cytosolic protein-mediated β-carotene transport between artificial and natural membranes by using established methods for other lipid species. Bovine tissues were chosen for this work because this species readily absorbs β-carotene and apparently metabolizes β-carotene in a way similar to humans (Poor et al. 1992 and 1993). Mitochondria were chosen as an acceptor membrane system because of their successful use in numerous lipid transport assays, including those for phospholipids and cholesterol (Bloj and Zilversmit 1977) and α-tocopherol (Sato et al. 1991, Verdon and Blumberg 1988). In addition, mitochondria are easy to separate from liposomes by centrifugation and have been reported to contain carotenoids when isolated from tissues of carotenoid-fed bovine animals (O'Fallon and Chew 1984, Patton and Kelly 1979). Liposomes containing β-[^{3}H]carotene were incubated with isolated bovine mitochondria (1 mg protein) and various quantities of liver cytosol or intestinal mucosal cell cytosol (0–5.72 mg protein) for up to 6 h at 37°C. The β-[^{3}H]carotene activity in liposomes was measured over time to determine percent transfer of β-carotene to mitochondria.

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MATERIALS AND METHODS

Animals. Liver sections (2 kg) and small intestine (upper third) were obtained from the University of Illinois abattoir from adult Angus cross steers immediately after killing. For one experiment, a steer was fed 200 mg β-carotene/d (10% water-dispersible beadlets, compliments of Hoffmann LaRoche, Nutley, NJ) along with normal rations for 7 d before slaughter. Liver sections were placed on ice, perfused with cold 0.154 mol NaCl/L and immediately processed for isolation of mitochondria and cytosol. Intestinal sections were washed with 0.154 mol NaCl/L to remove intestinal contents and immediately processed for cytosol isolation. Procedures with animals were approved by the University of Illinois Laboratory Animal Care Advisory Committee.

Subcellular fractionation. Liver (400 g) was cut into small pieces and homogenized with 1 L of SET buffer (0.25 mol/L sucrose, 1 mmol/L EDTA, 0.05 mol/L, Tris, pH 7.4) by using two 5-s bursts of a Waring blender. The homogenate was then filtered through four layers of SET-moistened cheesecloth and centrifuged at 1500 × *g* for 10 min. The pellets were resuspended in SET, recentrifuged and the supernatants were combined. The supernatant fraction was centrifuged at 15,000 × *g* for 20 min to sediment crude mitochondria. The pellets were washed with SET and supernatants combined. The supernatants were then centrifuged at 105,000 × *g* for 1 h to sediment crude microsomes. The fatty layer floating on top of the supernatant (cream) was separated from the remainder of the supernatant (cytosol).

Mitochondria were further purified on a sucrose step gradient (Dounce, Christensen and Bonner 1972) consisting of 1.0 mol sucrose/L layered over 1.45 mol/L sucrose (1 mmol/L EDTA, 50 mmol/L Tris, 0.1% bovine serum albumin, pH 7.4). Crude mitochondrial suspensions were layered on top of the gradients and centrifuged at 50,000 × *g* for 45 min in a SW 28 swinging bucket rotor (Beckman Instruments, Palo Alto, CA). The interface layer was removed, diluted with SET and pelleted at 9000 × *g* for 15 min.

For preparation of cytosol from small intestine, the upper third of the small intestine was sliced open longitudinally, washed with cold 0.154 mol NaCl/L and the inside surface scraped with a glass slide. Combined scrapings were mixed with 2.5 volumes of SET and homogenized with four 5-s bursts in a Waring blender. The procedure for isolation of intestinal cytosol was identical to that described previously for liver cytosol.

Protein concentrations of mitochondria and cytosol preparations were determined by using a bicinchorinic acid assay (Smith et al. 1985) with bovine serum albumin as a reference protein.

Liposome preparation. Liposomes consisting of egg-yolk phosphatidylcholine, dihexadecyl phosphate and BHT (molar ratio 1:0.1:0.01) with a trace (300 kBq) of β-[^{3}H]carotene (sp. act. 3.25 GBq/mg, compliments of Hoffmann LaRoche, Nutley, NJ) and glycerol tri[1-^{14}C]oleate (40 kBq) (sp. act. 2.22 MBq/mg, Amersham, Arlington Heights, IL) as a nonexchangeable marker were prepared as described previously (Verdon and Blumberg 1988). For one experiment, [1a,2a(n)-^{3}H]-cholesterol (300 kBq) (4.37 GBq/mg, Amersham) was used as the tritiated liposomal component. Briefly, lipids were dissolved in anhydrous ethyl ether (2 mL), and the ether was then evaporated under a stream of nitrogen. The SET buffer (2.5 mL) was added, and the lipids were allowed to swell for 2 h under nitrogen. The solution was then vortexed for 2 min and placed in a bath sonicator (Fisher Scientific, Pittsburgh, PA) for 35 min to obtain a clear solution. The preparation was then filtered through a 0.22-m cellulose acetate syringe filter and used immediately for transfer experiments. A sample of liposomes (10 mL) was counted for ^{3}H and ^{14}C in a liquid scintillation counter (Beckman LS9000) before incubation to determine a reference value for use in transfer calculations.

β-Carotene transfer assay. The possibility of cytosolic protein-mediated β-carotene transport from liposomes to mitochondrial membranes was investigated by incubating mitochondria (1 mg protein, 0.7 mmol phospholipid) with liposomes (10 mL, 0.028 mmol phospholipid), various quantities of intestinal or liver cytosolic protein (0–5.72 mg protein) and SET buffer to achieve a final volume of 1 mL. Cytosol was added last in all experiments. The preparations were allowed to incubate at 37°C with shaking for up to 6 h with samples removed at 0, 0.33, 0.5, 0.66, 1 and 6 h for determination of β-carotene transfer. Results are only shown for incubation periods of up to 1 hr. For transfer determination, samples were centrifuged at 15,000 × *g* for 10 min to pellet mitochondria, and the supernatant (0.9 mL) was removed and counted for ^{3}H and ^{14}C by using a liquid scintillation counter. Percentage transfer of β-carotene was calculated using the following formula:

$$1 - \frac{^3\text{H}/^{14}\text{C of liposomes after incubation}}{^3\text{H}/^{14}\text{C of liposomes before incubation}} \times 100$$

In a separate experiment, conditions were identical except that liposomes containing [1a,2a(n)-^{3}H]cholesterol instead of β-[^{3}H]carotene were incubated with mitochondria and various quantities of liver cytosol to monitor protein-mediated cholesterol transfer between liposomes and mitochondria.

Statistical analysis. One-factor ANOVA and Fisher's protected least significant difference test, $P < 0.001$, were used to determine differences between groups at different time points and at the same time points when more than two groups were compared. Unpaired *t*-tests were used to determine differences between two groups at the same time points (Ott 1988). Values in the text are means ± SD.

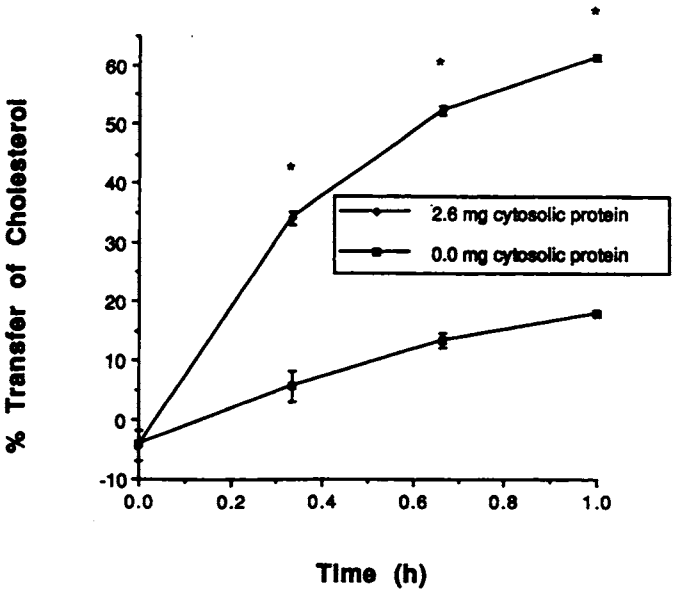

FIGURE 1 Percent transfer of [1a,2a(n)-^{3}H]cholesterol from liposomes to mitochondria from bovine liver over time in the absence or presence of liver cytosolic protein. Each point is the mean of three independent replications with error bars representing the SD. Nonprotein specific cholesterol transfer was mathematically corrected for with the use of a nonabsorbable glycerol tri[1-^{14}C]oleate. Differences between groups at each time point were analyzed by using unpaired *t*-tests, $^*P < 0.05$.

RESULTS

Cholesterol transfer experiments demonstrating protein-mediated cholesterol transfer from liposomes to biological membranes were described previously (Bloj and Zilversmit 1977). Cholesterol was initially used in place of β-carotene in the transfer assay as a positive control to ensure that the experimental conditions were appropriate to demonstrate transfer of another lipid species. Results of the cholesterol transfer assay are shown in **Figure 1.** With 2.6 mg of cytosolic protein, 65.9 ± 0.58% transfer of cholesterol from liposomes to mitochondria had occurred at 1 h vs. 0 h compared with 22.2 ± 0.18% transfer in the absence of cytosolic protein after adjustment of nonprotein specific cholesterol transfer using glycerol tri[1-^{14}C]oleate. Cholesterol transfer was significantly enhanced with the addition of 2.6 mg cytosolic protein compared with transfer without cytosolic protein at 0.33, 0.66 and 1 h ($P < 0.05$). For comparison, an identical transfer assay for β-carotene using the same cytosol and mitochondrial preparations as those used for the cholesterol assay showed that after 1 h, there was no significant transfer of β-carotene compared with baseline values from liposomes to mitochondria with the addition of 2.6 mg of cytosolic protein (**Fig. 2,** $P < 0.05$). In addition, there were no significant differences between addition of 0 and 2.6 mg cytosolic protein at any time point ($P < 0.05$).

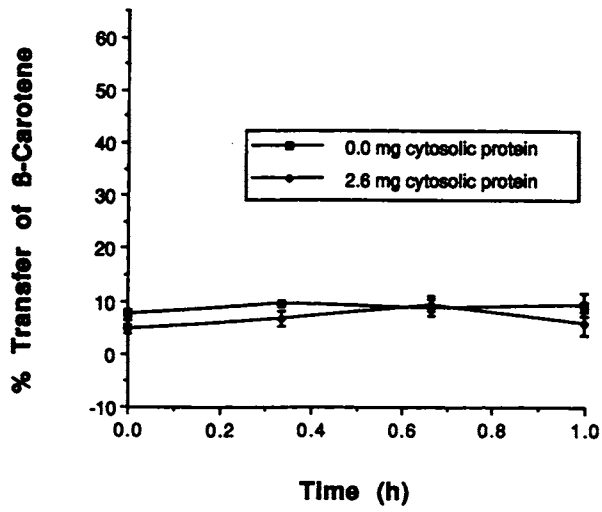

FIGURE 2 Percent transfer of β-[^{3}H]carotene from liposomes to mitochondria from bovine liver over time in the absence or presence of liver cytosol. Each point is the mean of three independent replications with error bars representing the SD. Nonprotein specific β-carotene transfer was mathematically corrected for with the use of a nonabsorbable glycerol tri[1-^{14}C]oleate. Percent β-[^{3}H]carotene transfer was not significantly different between groups at each time point when analyzed by using unpaired *t*-tests.

Results of a typical β-carotene transfer assay using various quantities of liver cytosolic protein are shown in **Figure 3.** Identical experiments were performed using mitochondria and liver cytosol from four additional steers, one of which was fed 200 mg β-carotene/d for

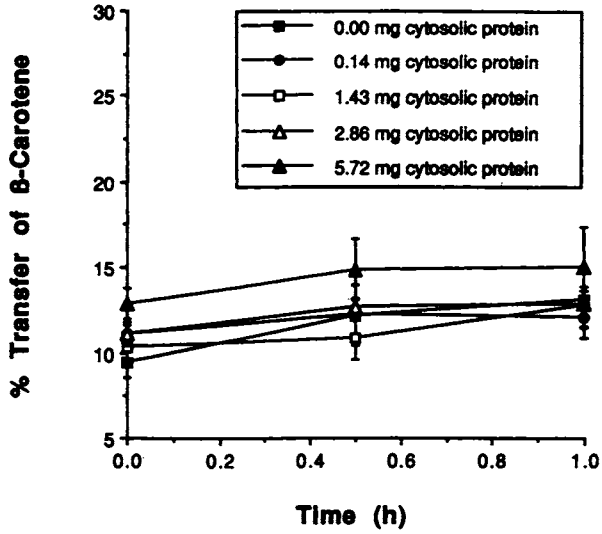

FIGURE 3 Percent transfer of β-[^{3}H]carotene from liposomes to mitochondria from bovine liver over time in the absence or presence of different quantities of liver cytosolic protein. Each point is the mean of three independent replications with error bars representing the SD. Nonprotein specific β-carotene transfer was mathematically corrected for with the use of a nonabsorbable glycerol tri[1-^{14}C]oleate. Percent β-[^{3}H]carotene transfer was not significantly different between groups at each time point when analyzed by using one-factor ANOVA.

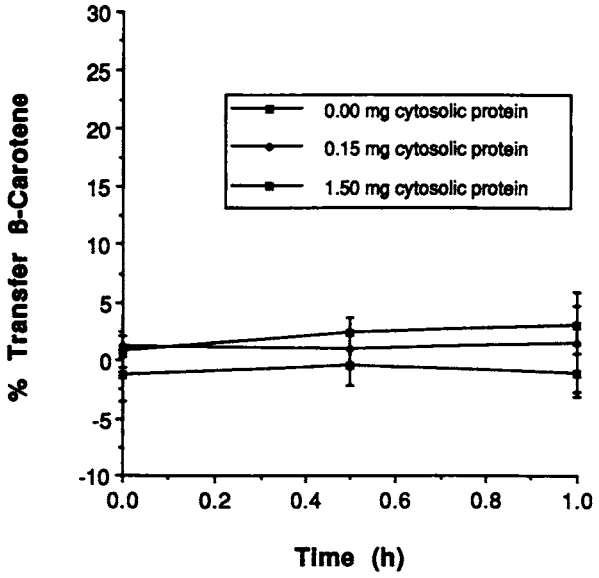

FIGURE 4 Percent transfer of β-[^{3}H]carotene from liposomes to mitochondria from bovine liver over time in the absence or presence of different quantities of intestinal cytosolic protein. Each point is the mean of three independent replications with error bars representing the SD. Nonprotein specific β-carotene transfer was mathematically corrected for with the use of a nonabsorbable glycerol tri[1-^{14}C]oleate. Percent β-[^{3}H]carotene transfer was not significantly different between groups at each time point when analyzed by using one-factor ANOVA.

1 wk, with essentially the same results. Upon addition of various quantities of liver cytosolic protein, the percent transfer of β-carotene from liposomes to mitochondria did not increase significantly compared with baseline values over the course of 1 h (or 6 h, data not shown), nor did values obtained with different levels of cytosolic protein differ significantly from one another at any time point.

For one study, cytosol was prepared from the upper third of the small intestine of one steer to investigate possible tissue differences in protein-mediated β-carotene transport. The results of this study (**Fig. 4**) similarly showed no significant increase in β-carotene transport from liposomes to mitochondria compared with baseline values over the course of 1 h because of the addition of cytosolic protein. Further, results obtained by using 0, 0.5 or 1.5 mg cytosolic protein did not differ significantly from one another at any time point.

DISCUSSION

Despite using liver cytosol and mitochondrial preparations from five different steers, there was no significant increase in β-carotene transfer from liposomes to mitochondria for up to 1 h after the addition of up to 5.72 mg of cytosolic protein. Transfer assays for other lipid species such as cholesterol (Fig. 1) (Bĭoj and Zilversmit 1977) and α-tocopherol (Murphy and Mavis 1981, Sato et al. 1991, Verdon and Blumberg 1988) using essentially identical conditions clearly demonstrate significant transfer over time periods less than 1 h with the addition of as little as 0.01–5 mg cytosolic protein.

Under these experimental conditions, the results suggest that unlike most types of lipids, β-carotene transport between intracellular biomembranes does not occur via a cytosolic transport protein. In these experiments, nonprotein-mediated β-carotene transfer between liposomes and mitochondria, possibly occurring by collisional mechanisms or adhesion of liposomes to mitochondria, was corrected for mathematically by using glycerol tri[1-^{14}C]oleate as a nonprotein transferable lipid. As would be expected because of the greater hydrophobicity of β-carotene, spontaneous transfer (uncorrected for nonspecific trioleate transfer; data not shown) in the absence of cytosolic protein is slower for β-carotene (19%) than for cholesterol (36%) after 1 h under the same conditions. Regardless of this hydrophobicity difference, the addition of a cytosolic protein, which is specific for β-carotene transport between biological membranes, should have resulted in an increase in transfer between liposomes and mitochondria over the 1-h incubation (and, in fact, after the 6-h incubation in data not shown) if such a mechanism is an important means by which β-carotene is transported within mammalian cell systems. Therefore, although we are not suggesting that protein transport mechanisms do not exist for mediating β-carotene movement within cells, we do contend that cytosolic protein-mediated transport is not a major mode of transport under the above experimental conditions.

However, simple nonspecific collisional or adhesion type transfer would not be sufficient to account for the substantial intracellular movement of β-carotene into lipoproteins directly after a carotenoid-rich meal, for instance. It has been shown that after an oral dose of β-carotene, the β-carotene content of newly synthesized chylomicrons begins to rise 2–3 h postprandially and peaks between 3–6 h in both humans (Johnson and Russel 1992, Traber et al. 1994) and preruminant calves (Bierer, Merchen and Erdman 1995). For both chylomicrons and VLDL, triglycerides and other lipids present in the lipoproteins are generally assumed to be synthesized in the endoplasmic reticulum (ER), where they combine at the junction of rough and smooth ER with apolipoprotein B (Alexander, Hamilton and Havel 1976, Vance and Vance 1990). The newly formed lipoprotein particles are transferred to the Golgi apparatus by means of vesicles and finally to the plasma membrane, where the lipoproteins are released into the extracellular space. It is unclear how or where carotenoids enter into the lipoprotein assembly process before extracellular secretion.

Recent evidence from preruminant calves (Bierer, T.L., personal communication) suggests that after a carotenoid-containing meal, the serum β-carotene peak

in chylomicrons coincides with the cholesterol peak but is delayed for up to 1 h after the chylomicron triglyceride peak. Because newly synthesized, rather than preexisting triglyceride, has been shown to be preferred for lipoprotein secretion (Ide and Ontko 1981) and carotenoids as well as triglyceride precursors should arrive simultaneously at the enterocyte, this delay in the carotenoid peak may suggest a different processing mechanism for carotenoids with respect to their assembly into chylomicrons. In addition, it was recently shown that β-carotene may behave similarly to g- or SRR-α-tocopherol rather than RRR-α-tocopherol with respect to resecretion into VLDL (Traber et al. 1994). The RRR-α-tocopherol, which appears to associate with a tocopherol-binding protein, is preferentially incorporated into VLDL, whereas g- and SRR-α-tocopherol isomers, which do not apparently associate with transport proteins, are not efficiently incorporated into VLDL. This supports our findings that intracellular β-carotene transport does not appear to be mediated by cytosolic transport proteins.

If cytosolic carrier proteins do not appear to account for β-carotene movement in our study, one possible mechanism for intracellular transport of carotenoids is by small carrier vesicles, which have also been described for the rapid intracellular transport of cholesterol (Liscum and Dahl 1992). It seems plausible that similar carrier vesicles could be responsible for the movement of carotenoids between intracellular membrane compartments. The Niemann-Pick type C fibroblast cell line has also been described, which contains a transport defect specific for LDL-derived cholesterol (Liscum, Ruggiero and Faust 1989). This cell line has played an important role in the elucidation of cholesterol transport mechanisms and could potentially have a similar impact in the field of carotenoid transport.

The results of these experiments also do not exclude the possible involvement of membrane-bound proteins in the transfer of β-carotene between subcellular compartments. Membrane fusion occurs as a natural cellular process, and the involvement of fusion proteins in this process was described (Burger and Verkleij 1990). If such a process is important in carotenoid transport, the use of liposomes would not detect this phenomenon unless the appropriate fusion proteins could be incorporated.

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