Influence of Phospholipids on β-Carotene Absorption and Conversion into Vitamin A in Rats

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Summary This study determines the effect of lysophosphatidylcholine (lysoPC) and phosphatidylcholine (PC) in mixed micelles on β-carotene and retinyl palmitate levels in rats in order to delineate the role of micellar phospholipids in the intestinal uptake of β-carotene and its conversion into vitamin A. The rats were fed a single dose of β-carotene solubilized in lysoPC (LPC group), PC (PC group) or no phospholipids (NoPL, control group) in micellar form. The level of β-carotene and retinyl palmitate in plasma and β-carotene in liver was analyzed by HPLC after 1, 2, 3, 6 and 9 h of feeding. The postprandial levels of β-carotene in plasma (599.9 pmol/mL, Area Under Curve (AUC)) and in liver (1,161.3 pmol/g) were significantly (p<0.05) higher in the LPC group compared with its level in plasma (207.2 pmol/mL) and in liver (616.5 pmol/g) of the PC group and in plasma (119.1 pmol/mL) and in liver (626.2 pmol/g) of the NoPL group. No difference was seen between the PC and NoPL groups. The results demonstrate that β-carotene absorption and its accumulation in plasma and liver were unaffected by PC compared with NoPL, while lysoPC not only enhanced its accumulation but also increased cleavage of intestinally absorbed β-carotene into vitamin A as the AUC of plasma BC was higher and the AUC of retinyl palmitate in plasma of the lysoPC group was significantly higher than those of the other two groups. The results suggest that the luminal hydrolysis of PC to lysoPC is necessary for intestinal uptake of β-carotene solubilized in mixed micelles.

Key Words phospholipids, mixed micelles, β-carotene, retinyl palmitate, bioavailability

Malnutrition contributes to about 40–50% of infant mortality in India (1). Apart from protein malnutrition, micronutrient malnutrition poses a serious concern to the health of the vulnerable groups of population. The three important micronutrient deficiencies of public health concern in India are: 1) vitamin A deficiency leading to blindness in children, 2) iron deficiency anemia contributing to maternal mortality and 3) iodine deficiency disorders causing neurological impairments. Although long-term measures to control these deficiencies have made some progress, vitamin A deficiency is still prevalent among the rural population. This may be due to poor bioavailability of pro-vitamin A carotenoids from food. In developing and underdeveloped countries, where vitamin A deficiency is more prevalent, most dietary vitamin A is obtained from locally available green leafy vegetables/vegetables in the form of pro-vitamin A carotenoids (2, 3). Carotenoids are fat-soluble pigments and the importance of dietary fat in modulating the absorption, bioconversion into vitamin A and the bioavailability of provitamin A carotenoids from food or supplements has been well documented in humans (4, 5). Despite the interest in the beneficial role of dietary pro-vitamin A carotenoids in human health, little is known about the influence of dietary phospholipids on the intestinal absorption of carotenoids and their conversion into vitamin A in vivo. The mechanism of fat stimulated carotene absorption may involve enhanced incorporation of carotenoids into mixed micelles (6) and enhanced activity of β-carotene 15,15'-dioxygenase (7).

The absorption of dietary carotenoids from food involve several steps: the breakdown of the food matrix to release the carotenoids, dispersion in the lipid emulsion particles, solubilization in mixed micelles, movement across the unstirred water layer adjacent to the microvilli, uptake by the cells of intestinal mucosa and incorporation into chylomicron (8, 9). Thus the carotenoids must be solubilized in mixed micelles before cellular uptake. The mixed micelles are composed of bile acids, cholesterol and phosphatidylcholine, which are components of the bile fluid. Dietary fat induces the secretion of bile and its hydrolysis products such as monoacylglycerides and fatty acids are also present in the mixed micelles. Roels et al. (10) demonstrated the importance of dietary fat in the utilization of vegetable carotenoids in young boys, who showed clinical signs of vitamin A deficiency. Dimitrov et al. (5) studied the effect of dietary fat on the bioavailability of β-carotene

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supplements in healthy men and women. Thus the events up to the solubilization in the mixed micelles before intestinal absorption are dependent mostly on the physicochemical properties of dietary fat and carotenoids. However, studies with animals have lead to conflicting results regarding the effect of different types of fat on β-carotene absorption and its metabolism (11, 12).

Details on the in vivo absorption of carotenoids after solubilization in mixed micelles have not been fully revealed. The early findings in perfused rat intestine and in hBRIE 380 rat intestinal cells support a simple diffusion mechanism for the cellular uptake of carotenoids (12, 13). This was also supported by the linear relationship found in our recent observation on the cellular uptake of micellar carotenoids and its hydrophobicity (14, 15). Thus, the size and lipid composition of the mixed micelles would influence the movement of carotenoids from micelles to the intestinal cells by diffusion (16). Recent studies have indicated that phospholipids in mixed micelles and phospholipase A₂ profoundly affect the cellular uptake of cholesterol and α-tocopherol (17, 18). We also have found that phosphatidylcholine (PC) suppresses the intestinal uptake of β-carotene and lutein solubilized in the mixed micelles by the human Caco-2 cells and mice, whereas lysophosphatidylcholine (lysoPC) significantly enhances their uptake (6, 15). Thus, phospholipids derived from bile and foods would affect the cellular uptake of carotenoids solubilized in mixed micelles formed in the intestinal tract. The present study was conducted to evaluate the influence of PC and lysoPC solubilized in mixed micelles on the concentration of β-carotene in plasma and liver and its conversion into vitamin A in rats. Another objective of the present report and the work in progress is to create awareness among the rural population towards the importance of consuming dark green leafy vegetables and phospholipids together for better bioavailability of carotenoids from the food matrix.

**MATERIALS AND METHODS**

**Materials.** All-trans-β-carotene (type IV, 95%), All-trans-retinol (95%), retinyl palmitate, monooleoyl glycerol, DL-α-tocopherol, sodium taurocholate, egg-yolk PC (99%) and lysoPC (99%), oleic acid (>99%) and α-apo-8′-carotenal were purchased from Sigma Chemical Co. (St. Louis, MO, USA). HPLC grade acetonitrile, hexane, methanol, ethyl acetate and dichloromethane were purchased from Sisco Research Laboratories (Mumbai, India). Other chemicals and solvents were of reagent grade purchased locally.

**Purification of reference standard chemicals.** All-trans-β-carotene was purified by a neutral alumina column (HiMedia Laboratories Ltd., Mumbai, India) using hexane and methanol as the mobile phase. Purified all-trans-β-carotene (98.8%) was dissolved in hexane/dichloromethane (2/1 v/v) and stored at −70°C until used. Retinyl palmitate (>99%) was purified by HPLC using TSK gel ODS 80TS column, 6.4 × 250 mm (Tosoh, Tokyo, Japan) with ethyl acetate/methanol (30/70 v/v) as the mobile phase. The purity of β-carotene and retinyl palmitate was checked by HPLC based on the peak area of each component absorbed at a specific wavelength.

**Animals.** Necessary clearance was obtained from the Institutional Animal Ethical Committee for conducting the animal experiments. Male albino rats of CFT strain, 28 d old and weighing 35 ± 2 g bred in the animal housing facility of the Institute were housed at room temperature (28±2°C) with a 12 h light/dark cycle. The animals received a commercial diet (Amrut Feeds, Sangli, India) and had free access to tap water. After 7 d of feeding, rats were deprived of food for 12–13 h before carotenoid administration.

**Preparation of micelles and feeding.** Mixed micelles in phosphate buffered saline containing monooleoyl glycerol (2.5 mM), oleic acid (7.5 mM), sodium taurocholate (12 mM), and β-carotene (200 μM) with PC (3 mM), lysoPC (3 mM) or no phospholipid was prepared as per the procedure given by Baskaran et al. (15). The components were dissolved in hexane or methanol. The solvent was evaporated to dryness with vigorous mixing using a vortex mixer to obtain an optically clear solution. The micelle composition chosen was based on the composition of the clear layer obtained by ultra centrifugation of the duodenal content of healthy adult human subjects given a triglyceride-rich meal (19). It would hypothetically produce a mixture of mixed micelles and small unvesicular vesicles (20). The vesicles can regroup spontaneously into the mixed micelles as the ratio of lipid to cholic acid decreases during the absorption process. Thus, the optically clear solutions obtained by the procedure described above were used as the mixed micelles in the present study. The concentration of β-carotene in the micelles was checked by HPLC before feeding to rats.

**Animal feeding.** The rats were randomly divided into 16 groups. Groups 1 to 5 were fed with β-carotene solubilized in the mixed micelles containing no phospholipid (NoPL group), groups 6 to 10 were fed with micelles containing PC (PC group) and groups 11 to 15 were fed with micelles containing lysoPC (LPC group). Group 16 was not fed with mixed micelles (zero-time, control). The mixed micelles (0.2 mL/rat) were administered to each rat by direct intubation to the stomach. The volume size of intubation had no adverse effects on the rats. Rats in the zero time control (n=6) and in each treatment group (n=6/time point) at 1, 2, 3, 6 and 9 h after gavage were anesthetized with diethyl ether and sacrificed. Blood was collected directly from the heart into heparinised test tubes. Plasma was immediately separated from the blood by centrifugation at 1,000 × g for 15 min at 4°C. The livers were excised and washed with ice-cold saline. The plasma and liver were immediately stored at −70°C until analyzed. The amount of β-carotene fed was calculated to be 0.671 mg/kg body weight and was comparable to the amounts reported in studies in which β-carotene was supplemented to human subjects (21). In a preliminary study in rats,
one-tenth of the carotenoid level used in the present study was fed, but the level of β-carotene in plasma was undetectable (data not shown).

**Extraction of β-carotene from plasma and liver.** β-Carotene, retinol and retinyl palmitate were extracted from the plasma according to the procedures described by Baskaran et al. (15). Briefly, 0.6 mL of plasma was made up to 0.8 mL with ice-cold deionized water and 3 mL of dichloromethane/methanol (1/2 v/v) containing 0.2 μM α-tocopherol was added, mixed for 1 min using a vortex mixer, followed by addition of 1.5 mL of hexane, mixed well and centrifuged. The resulting upper layer of hexane and dichloromethane was withdrawn into a clean test tube. The extraction of the lower phase was repeated twice using 1 mL of dichloromethane and 1.5 mL of hexane. The pooled extract was evaporated to dryness under a stream of nitrogen and re-dissolved in 0.1 mL dichloromethane/methanol (2/1 v/v) for the HPLC analysis of β-carotene, retinol and retinyl palmitate.

Liver samples were homogenized with 9 parts of ice-cold isotonic saline using a Potter-Elvehjem homogenizer. Homogenate (0.8 mL) was extracted for β-carotene and retinol by the same procedure described above for plasma. Homogenization and extraction were done in dim yellow light at 4°C to minimize isomerization and oxidation by light.

**HPLC analysis.** The HPLC system consisted of an LC-10AD pump (Shimadzu, Kyoto, Japan), an SPD-10A UV-Visible (UV-VIS) absorbance detector (Shimadzu) and a personal computer with EZ Chrome Chromatography Data System software (Scientific Software, Pleasanton, CA, USA). β-Carotene, retinol and retinyl palmitate were separated on TSK gel ODS-80Ts (Tosoh), 4.6×250 mm, attached to a precolumn (2×20 mm) of Pelliguard LC-18 (Supelco, Bellefonte, PA, USA). Ethyl acetate/methanol (30/70 v/v) containing 0.1% ammonium acetate was used as a mobile phase for the analysis of β-carotene, retinol and retinyl palmitate. An isocratic analysis was performed at a flow rate of 1 mL/min. β-Carotene was monitored at 450 nm and retinol and retinyl palmitate were monitored at 325 nm. They were quantified from their peak areas in relation to the respective reference standard. The peak identity of these components was further confirmed by their characteristic UV-Vis spectra, recorded with a model 1100 HPLC system equipped with a photodiode array detector (Hewlett Packard, Palo Alto, CA, USA).

**Statistical analysis.** To quantify the postprandial β-carotene level in plasma and liver over 9 h, the area under the curve (AUC) was calculated by trapezoidal approximations. Data were tested for the homogeneity of variances by the Bartlett test. When homogeneous variances were confirmed, the data were tested by ANOVA and significant differences in means among groups by Tukey’s test. The values underwent log transformation before the tests if necessary. Differences were considered significant at a level of p<0.05.

**RESULTS**

**β-Carotene response in plasma**

The typical HPLC chromatogram obtained from the plasma of rats after the administration of β-carotene in mixed micelles is presented in Fig. 1A. β-Carotene was not detected in the plasma and liver of rats before the feeding (zero-time control), whereas it reached a maximum in plasma at 1 h in the LPC and at 2 h in the PC and NoPL groups fed mixed micelles containing β-carotene (Fig. 2A). The maximum level in the LPC, PC and NoPL groups were 161.58, 58.26 and 27.58 pmol/mL, respectively. A decline in plasma β-carotene was seen at 3 h after gavage in all the groups, which was significantly lower than the maximum level. No significant differences were found between the NoPL and PC groups by two-way ANOVA, whereas, the β-carotene level of the LPC group was significantly higher at all the time points than those of the NoPL and PC groups. The AUC of plasma β-carotene was calculated from the curve shown in Fig. 2A and the values are given in Table 1. The PC (374.6 pmol/mL/9 h) and NoPL (355.4 pmol/mL/9 h) groups had significantly lower AUC values than that of the LPC group (965.9 pmol/mL/9 h).

**Retinyl esters in plasma**

HPLC chromatogram obtained at 325 nm for retinyl esters in plasma of rats fed a single dose of β-carotene is presented in Fig. 1B. The response of retinyl palmitate to β-carotene administration is shown in Fig. 3. No significant difference was noticed between the NoPL and PC groups by two-way ANOVA, but significant differ-
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Fig. 2. β-Carotene level in plasma (A) and liver (B) of rats after the administration of β-carotene solubilized in mixed micelles. Micelles were composed of 2.5 mM monooleoyl glycerol, 7.5 mM oleic acid, 12 mM sodium taurocholate, and 200 μM β-carotene with 3 mM PC (△), 3 mM lysophosphatidylcholine (lysoPC) (○) or NoPL (○). Rats were fed a single dose of micelles and then sacrificed after various time intervals. β-Carotene in the plasma was analyzed by HPLC. Data represent the mean±SD (n=6). The values at each time point not sharing a common letter are significantly different (p<0.05) between groups as determined by one-way ANOVA and Tukey’s test after log transformation. No β-carotene was detected at 0h in plasma or liver of any group.

ence (p<0.05) was found between the LPC group and the other two groups. On the other hand, the plasma retinyl palmitate in the LPC group was significantly (p<0.05) increased from the baseline level (24 pmol/mL) to 215.2 pmol/mL at 2 h and then declined to 32.64 pmol/mL at 9 h after β-carotene administration. There was no significant difference in the levels of free retinol in plasma among the treated groups (data not shown).

Retinyl palmitate to β-carotene ratio in plasma

To get an indirect idea of the intestinal β-carotene cleavage activity, the ratio of retinyl palmitate to β-carotene in plasma was calculated. The ratio of the LPC group (1.2) was significantly (p<0.05) lower compared to the PC (1.61) and NoPL (3.96) groups. An efficient conversion of absorbed β-carotene to vitamin A expressed as a low ratio of retinyl palmitate to β-carotene was related to high AUC for β-carotene in plasma (Table 1).

β-Carotene in liver

β-Carotene level in the livers after the administration of micellar β-carotene is shown in Fig. 2B. The level of β-carotene in the liver of the PC group was higher at 1 h (171.46 pmol/g) and then declined to 34.97 pmol/g at 9 h after administration of β-carotene. On the other hand, β-carotene levels in the livers of the LPC and NoPL groups reached the maximum at 1 and 2 h after administration. The maximal levels in these groups were 282.39 and 125.3 pmol/g, respectively. The levels then decreased markedly at 9 h after administration (p<0.05). The AUC value of β-carotene in the LPC group was 85.5% higher than that of NoPL and 88.4% higher than the PC groups (Table 1). Thus, the effects of micelles on the accumulation of β-carotene in the liver were more or less similar to those observed in plasma.

DISCUSSION

This study was performed to evaluate the effects of phospholipids in mixed micelles on the intestinal uptake

Table 1. Area under the curve for β-carotene in plasma and liver and for retinyl palmitate in plasma of rats over 9 h after administration of β-carotene solubilized in mixed micelles.

<table>
<thead>
<tr>
<th>Group</th>
<th>β-Carotene (pmol/mL)</th>
<th>Retinyl palmitate (pmol/mL)</th>
<th>β-Carotene (pmol/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NoPL</td>
<td>119.1±15.8</td>
<td>472.6±23.5</td>
<td>626.2±32.5</td>
</tr>
<tr>
<td>PC</td>
<td>207.2±18.6</td>
<td>338.4±28.5</td>
<td>616.5±38.5</td>
</tr>
<tr>
<td>LPC</td>
<td>599.9±42.8</td>
<td>732.0±44.7</td>
<td>1,161.3±48.5</td>
</tr>
</tbody>
</table>

Data represent the mean±SD (n=6). Values not sharing a common superscript within a row are significantly different (p<0.05) between groups as determined by one-way ANOVA. NoPL, group fed mixed micelles containing no phospholipids; PC, group fed mixed micelles containing PC; LPC, group fed mixed micelles containing lyso-phosphatidylcholine.
of β-carotene and its metabolism in rats. We have followed the postprandial appearance of β-carotene and its cleavage product, retinal that may be reduced to retinol and later esterified with palmitic acid to form retinyl palmitate in plasma, as a measure of intestinal β-carotene absorption and cleavage after feeding β-carotene with phospholipids micelles to rats. Earlier studies on intestinal β-carotene absorption in animals and humans have relied on the changes in the β-carotene level in plasma as the final outcome (15, 21). However, van Vliet et al. (7) and Hu et al. (22) evaluated β-carotene in plasma chylomicron and plasma triacylglycerol-rich lipoprotein fractions as a vehicle for assessing intestinal absorption and cleavage of β-carotene. The present study indicates that mixed micelles containing lysoPC when intubated to rats profoundly influences the intestinal uptake of β-carotene and cleavage of newly absorbed β-carotene into retinal. The plasma level of β-carotene in the PC group was significantly lower than that of the LPC group. The influence of lysoPC on the intestinal uptake of β-carotene in rats was found to be four-fold higher than the results compared with mice (15) and this is in agreement with the observations of Shapiro et al., who studied the kinetics of β-carotene uptake and clearance in rat tissue (23). Although the exact mechanism by which micellar lysoPC influences the intestinal uptake and plasma level of β-carotene still remains unclear, evidence from in vitro studies by Sugawara et al. (14) and Noh and Koo (24) suggest that the inhibitory effects of PC or structurally similar sphingomyelin on β-carotene and α-tocopherol absorption may be mostly mediated within the intestinal cells. These phospholipids are hydrolyzed more slowly and incompletely in the intestinal lumen (24, 25). Thus, the slow and incomplete hydrolysis of PC in the upper segment of the intestine, where much of the lipid hydrolysis occurs, may allow for interaction between intact PC and other lipids in the intestinal environment, influencing the rate of hydrolysis, micellar solubilization and transfer of lipids from mixed micelles into the enterocyte. Evidence from an in vitro study (17) shows that the presence of intact PC in mixed micelles slows the transfer of more hydrophobic lipids such as cholesterol and α-tocopherol from the micellar matrix. Although no direct evidence is available for such interactions between PC and other lipids or lipophilic components in micelles, studies with lipid vesicles and membrane system indicate that PC interacts more tightly with fat-soluble components (26).

β-Carotene levels in the plasma and liver after oral administration were not significantly different between the NoPL and PC groups, but were significantly higher in the LPC group. The higher mean level of β-carotene in the liver and its AUC value for the LPC group compared with those in the other two groups indicated that lysoPC also enhanced accumulation of β-carotene in the liver. Similarly, plasma retinyl palmitate in the LPC group increased significantly to a level higher than the base line level at 2 h after β-carotene administration. The increase in retinyl palmitate might be due to the enhanced uptake of β-carotene and its metabolism in the intestinal cells (16). The enhancement in the level of retinyl palmitate in the plasma was estimated to be 30.5 pmol/mL. This value corresponds to ca. 15 pmol β-carotene, on the assumption that one molecule of β-carotene is converted to two molecules of retinal by central cleavage enzyme β-carotene 15,15'-dioxygenase in the intestinal cells (27). As the enhanced level of β-carotene measured in the plasma of LPC group was 36.4 pmol, a considerable amount of β-carotene might have been converted to retinyl palmitate. It is clear from the data on the retinyl palmitate that lysoPC enhanced the uptake of β-carotene, because significant differences in the retinyl palmitate levels in the plasma were found between the groups. In particular, the ratio of the retinyl palmitate to β-carotene may be a good indicator for intestinal conversion (7). These results suggest that LPC in mixed micelles may enhance the intestinal conversion of newly absorbed β-carotene, whereas PC had no effect on the conversion of β-carotene to vitamin A.

It is not certain whether the mixed micelles fed directly to the stomach by gavage in the present study reached the intestine or were reconstituted as micelles in the intestinal tract after digestion in the stomach. However, the results of the present study were basically consistent with in vitro results of Sugawara et al. (6), who reported the solubilization of carotenoids in mixed micelles when directly incubated with cultured human intestinal Caco-2 cells. The decline in the β-carotene level in the plasma and an increase in the liver would suggest its transport to the liver or to other tissues and a decline in the liver after reaching maximum would suggest its transport to other tissues. Since liver has the second highest activity of β-carotene dioxygenase among the tissue (28), conversion of β-carotene to retinal may be involved in the decline of β-carotene levels in the liver.

The results of the present investigation and the reported in vitro and in vivo studies using human intestinal Caco-2 cells and mice models suggest that the hydrolysis of phospholipids in the intestinal tract by PLA₂ is vital for the efficient uptake of β-carotene by the intestinal cells, although PC plays a significant role in the solubilization of carotenoids in lipid emulsions (29). The mechanism underlying these effects is not clearly understood. Physical properties like low viscosity and larger size of the PC micelles compared to that of lysoPC and NoPL micelles (data not shown) may also be one of the reasons for the slower uptake of carotenoids by the intestinal cells. Rodgers and O’Connor (30) and Rampone and Long (31) reported that micellar PC suppressed the intestinal uptake of oleic acid and cholesterol in vitro. They have suggested that the effect was probably due to the amphipathic properties of PC causing it to incorporate and increase the size of the mixed lipid-bile acid micelles, thus reducing the free diffusion of the micelles through the unstirred water layer. This hypothesis explains in part how micelle formation affects the efficiency of absorption of lipophilic components since by being transported in the form of polar
aggregates the micelles overcome some of the disadvantages associated with the low aqueous solubility of β-carotene. The lysoPC micelles, therefore, may help to establish better contact or integration between β-carotene and the cell membrane by bridging the uninterred water layer. PC with two long chain acyl moieties is more hydrophobic than lysoPC, with one acyl moiety and a free hydroxyl group. Therefore, PC has a greater affinity for hydrophobic carotenoid molecules than does lysoPC (32). The uptake of PC by intestinal cells is itself known to be much lower than that of lysoPC (17, 33). Thus, PC can strongly retain the hydrophobic carotenoids like β-carotene in the mixed micelles so that their uptake by the intestinal cells is suppressed. The cells of the jejunum can take up lysoPC across the unstirred water layer covering the epithelial surface, an important barrier to lipid uptake (34), whereas bile acids are taken up in the ileum. Further, lysoPC might strongly associate with β-carotene and facilitate its diffusion across the water layer from mixed micelles to the brush border membrane of intestinal cells. Further, the lysoPC taken by the intestinal cells is quickly converted to PC and triglycerides, which then stimulate the synthesis of triglycerides and the secretion of chylomicrons (35, 36).

In conclusion, the present and earlier in vitro (6) and in vivo (15) studies with human intestinal Caco-2 cells and mice clearly demonstrate that PC in mixed micelles suppress the intestinal uptake and accumulation of β-carotene in plasma and liver, while lysoPC enhances their levels. Thus, the present results suggest that the hydrolysis of PC to lysoPC is an important step for the intestinal uptake of β-carotene solubilized in mixed micelles and that dietary lysoPC modifies the bioavailability of β-carotene. The mechanism of these effects of phospholipids in mixed micelles on the intestinal uptake of β-carotene and, in particular, its relationship to intestinal lipid metabolism deserves further study.

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