Evidence for a Lecithin-Retinol Acyltransferase Activity in the Rat Small Intestine*

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(Received for publication, November 19, 1987)

The intestinal absorption of vitamin A (reviewed in Ref. 1) is a complex process involving several enzymatic and transport steps. The final step requires esterification of retinol (vitamin A alcohol) with long chain fatty acyl moieties and incorporation of the resulting retinyl esters into the lipid core of chylomicrons. The chylomicrons exit the enterocyte to the lymphatic system and then the retinyl esters are subsequently delivered to the liver to be stored in the liver as chylomicron remnants. In vivo studies have demonstrated that the intestinal esterification of retinol produces predominantly retinyl palmitate and retinyl stearate, a ratio of approximately 2:1 (2). This retinyl ester composition is essentially independent of the lipid composition of the diet (2).

Several groups have examined intestinal activities that esterify retinol in vitro. Mahadevan et al. (3) described a CoA-independent activity that was stimulated by exogenous fatty acids in vitro. Helgerud et al. (4, 5) characterized an acyl-CoA-dependent activity present in microsome preparations obtained from rat (4) or human (5) intestinal mucosa. This activity has been named acyl-CoA-retinol:acyltransferase. However, those in vitro activities did not display the appropriate fatty acyl specificity expected from the in vivo observations.

Recently, the small intestine was found to contain a binding protein for retinol, cellular retinol binding protein, type II (CRBP (II)) (6). CRBP (II) is located in the mature enterocytes of the adult small intestine (7). All-trans-retinol was shown to be an endogenous ligand of the protein (6). When the ability of intestinal microsome preparations to esterify retinol complexed to CRBP (II) was examined, an acyl-CoA-independent activity was observed (8). This in vitro activity utilized an endogenous acyl donor associated with the microsomes to produce retinyl esters with a composition similar to that of exogenous fatty acids did not affect the reaction.

Huang and Goodman (2) had previously noted a similarity between the acyl composition of lymph retinyl esters and the fatty acids at the C-1 position of lymph phosphatidylcholine. They suggested that phosphatidylcholine might serve as a source of acyl groups for retinol esterification. We report here that the acyl-CoA-independent enzyme activity in intestinal microsome preparations will catalyze the transfer of acyl groups from exogenous phosphatidylcholine to retinol-CRBP (II) to produce retinyl esters. The acyl group transferred to retinol came from the C-1 position of phosphatidylcholine.

**EXPERIMENTAL PROCEDURES**

*Materials—Dodecanoic anhydride, tetradecanoic anhydride, decanoic anhydride, all-trans-retinol, all-trans-retinaldehyde, Triton WR-1339, NEM, pCMB, PMSF, and all diacyl- and lysophospholipids were obtained from Sigma. High purity methanol, chloroform, and acetone were from Burdick and Jackson Laboratories Inc. Aluminum oxide (activated, neutral, Brockmann I), 4-pyrrolidinopyridine, and dimethyl sulfoxide were from Aldrich. Thin layer chromatography was performed on Silica Gel IB2 flexible sheets (20 x 20 cm) from J. T. Baker Chemical Co. [3H]Retinol was prepared by reduction of all-trans-retinaldehyde with NaBH₄ (9) and was purified by HPLC (10). CRBP (II) was isolated from 1-day-old rat pups (6) or from the small intestine of adult rats. [3H]Retinol-CRBP (II) was prepared as described previously (8).

*Synthesis of Phosphatidylcholines of Positionally Defined Fatty Acyl Composition—1-Lauroyl-2-myristoylphosphatidylcholine, 1-myristoyl-2-lauroylphosphatidylcholine, 1-myristoyl-2-decanoylphosphatidylcholine, and 1-palmitoyl-2-decanoylphosphatidylcholine were synthesized as described previously (8).

*The abbreviations used are: CRBP (II), cellular retinol binding protein, type II; PC, phosphatidylcholine; DHPC, dihetepeano-1,2,3-trisphosphatidylcholine; DLPC, dilauroylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; HPLC, high performance liquid chromatography; NEM, N-ethylmaleimide; pCMB, p-chloromercuribenoic acid; PMSF, phenylmethylsulfonyl fluoride.

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synthesized by acylation of 1-lauroyl-, 1-myristoyl-, and 1-palmitoyl-2-lysophosphatidyicholine with the corresponding fatty acid anhydride (11). Briefly, 25 mg of the lysophosphatidylcholine was dissolved in 300 μl of dry chloroform. Following the addition of 110 mg of anhydride and 8.5 mg of 4-pyrollidinopyridine in 600 μl of chloroform, the vial was sealed under N₂ and incubated for 2 h at 35 °C. Diacyl-phosphatidylcholine was purified following acid extraction of the organic phase by preparative thin layer chromatography developed in a solvent system of chloroform:methanol:28% ammonium hydroxide (65:35:5). Phosphatidylcholine was eluted from the scraped silica with chloroform:methanol:1(2), the eluant taken to dryness by rotary evaporation, and the residue recrystallized once from acetonechloroform (95:5). The dried crystals were sealed under N₂ and stored desiccated at -20°C.

Isolation of Intestinal Microsomes—Adult, male Sasco rats (approximately 300 g) were anesthetized with sodium pentobarbital. Cardiac perfusion was performed with 120–180 ml of 10 mM KH₂PO₄, 0.16 M NaCl, pH 7.4, until the mesentery vessels were clear. Microsomes were isolated from the scraped mucosa of the perfused intestines as described previously (8). Dithiothreitol was omitted from the final preparation.

Inhibitor Studies—To examine the effect of chemical modifiers on the esterification of retinol-CRBP(I1) with endogenous acyl donor, microsomes (approximately 12 μg of protein) were incubated with increasing concentrations of NEM, pCMB, or PMSF for 10 min at 37°C. The esterification reaction was then initiated by the addition of 0.3 nmol of [³H]retinol-CRBP(I1) to a final volume of 0.1 ml. The reaction was stopped after 10 min at 37°C by the addition of 4 vol of ice-cold ethanol and extracted into hexane (8). The hexane extract was analyzed for [³H]retinyl esters by batch analysis on columns of aluminum oxide (4).

The stability of retinol complexed to CRBP(I1) in the presence of NEM, pCMB, and PMSF was examined by monitoring the fluorescence excitation spectrum of the complex. Greater than 90% of the retinol fluorescence remained after a 30-min incubation with each of these reagents. Thus, decreased esterification of retinol-CRBP(I1) in the presence of inhibitors was not due to dissociation of the complex and/or destruction of the retinol.

Preparation of Lipid Substrates and Treatment of Microsomes—Microsomes were treated in the following manner to examine retinyl ester formation from exogenous phospholipid substrates. Generally, 500 nmol of phospholipid dissolved in chloroform was evaporated under N₂ and then lyophilized for 30 min to remove residual solvent. The residue was suspended in 1.0 ml of 0.2 M KH₂PO₄, pH 7.2, containing 9.5 mg of Triton WR-1339. This detergent-lipid suspension was dispersed by sonication with a Branson Sonifier fitted with a microtip for 60 × 1-s pulses at a power output of 2. Microsomes (approximately 6 mg/ml) were diluted 1:5 with this detergent-lipid preparation and incubated at room temperature for 15 min.

Esterification Assay—The esterification reaction was initiated by the addition of 50 μl (60 μg of microsomal protein) of the microsome preparation containing exogenous lipid and detergent to 1.5 nmol of retinol-CRBP(I1) to a final volume of 0.5 ml. The final concentration of lipid and detergent was 40 μM and 0.08% (w/v), respectively. The reaction was transferred to a 37°C shaking water bath. After 15 min, the reaction was stopped with ethanol, extracted into hexane, and the hexane extract analyzed by reverse-phase HPLC (8). All procedures were performed under dim yellow lights.

RESULTS AND DISCUSSION

Effect of Exogenous Phosphatidylcholines on the Esterification of Retinol-CRBP(I1)—The acyl donor involved in the intestinal esterification of retinol-CRBP(I1) has been suggested to be phosphatidylcholine (8). Initial experiments examined the in vitro esterification of retinol-CRBP(I1) with exogenous diarauroyl-, dimyristoyl-, and diheptadecanoylphosphatidylcholines as potential acyl donors. These phosphatidylcholines were chosen because their acyl moieties are different from the retinyl esters produced with endogenous acyl donor. Thus, any formation of retinyl esters from the exogenous phosphatidylcholine would be clearly distinct from the ester products of the endogenous donor. Intestinal microsome preparations were preincubated with detergent and with and without DLPC, DMPC, or DHPC as described under “Experimental Procedures.” That mixture was then combined with retinol-CRBP(I1) to initiate the reaction. The reaction mixtures were extracted and analyzed for retinyl esters by HPLC. Transfer of acyl moieties from DLPC, DMPC, and DHPC to retinol-CRBP(I1) to form retinyl laurate, retinyl myristate, and retinyl heptadecanoate, respectively, could be observed (Fig. 1). Also evident in each chromatogram of Fig. 1 are the retinyl esters synthesized from the endogenous acyl donor associated with the microsome preparation. These esters are retinyl linoleate, retinyl palmitate/oleate, and retinyl stearate. The use of heat-treated microsomes or omission of retinol-CRBP(I1) gave no retinyl esters. All peaks eluting prior to the indicated retinyl esters were independent of the presence of retinol-CRBP(I1).

Exogenous phosphatidylcholines of increasing acyl chain length yielded decreased incorporation of the acyl group into retinyl esters. This may indicate that the retinyl ester synthesis prefers shorter-chain phosphatidylcholines. However, experiments described in a later section suggest that the chain-length specificity observed here, lauroyl > myristoyl >...
heptadecanoyl, may be due more to solubility of the added phosphatidylcholine rather than an innate preference by the enzyme.

Ability of Various Lipids to Serve as Acyl Donor in Retinol-CRBP(II) Esterification—The previous experiment established that exogenous phosphatidylcholine could serve as acyl donor in retinol-CRBP(II) esterification. The selectivity of the ester synthase for various other lipids was then investigated. Intestinal microsome preparations were preincubated with detergent with and without DMPC, dimyristoylphosphatidylethanolamine, dimyristoylphosphatidic acid, myristoyl-CoA, or myristic acid and the esterifying reaction initiated with addition of retinol-CRBP(II). The retinyl ester products of these reactions were analyzed by HPLC (Table I). Reaction mixtures containing DMPC produced similar amounts of retinyl myristate from retinol-CRBP(II) as observed in the previous experiment. Again the production of retinyl linoleate, retinyl palmitate/oleate, and retinyl stearate from the endogenous donor was observed. Approximately 12% of the total retinyl ester synthesized was retinyl myristate. Microsomes in the presence of detergent and dimyristoylphosphatidylethanolamine, dimyristoylphosphatidic acid, or myristic acid produced little if any retinyl myristate. The pattern of retinyl esters produced from these microsomes was essentially identical to the pattern observed for microsomes incubated with detergent alone. When myristoyl-CoA was examined as a potential donor some formation of retinyl myristate was observed. The amount of retinyl myristate produced from myristoyl-CoA was 75% lower than when DMPC was examined under similar conditions. As will be shown later, utilization of acyl-CoA for esterification was due to a different enzyme activity.

Positional Specificity in the Transfer of Acyl Groups from Phosphatidylcholine to Retinol-CRBP(II)—To determine if the acyl transfer from phosphatidylcholine to retinol-CRBP(II) was positionally specific, phosphatidylcholines of positionally defined fatty acid composition were examined as substrates. 1-Myristoyl-2-lauroylphosphatidylcholine and 1-lauroyl-2-myristoylphosphatidylcholine were preincubated with microsomes in the presence of detergent. The microsomes were then assayed for retinol-CRBP(II) esterifying activity, and the retinyl esters were analyzed by HPLC (Fig. 2). Microsomes incubated with detergent alone catalyzed the formation of retinyl linoleate, retinyl palmitate/oleate, and retinyl stearate from the endogenous acyl donor (Fig. 2, upper panel). Inclusion of 1-myristoyl-2-lauroylphosphatidylcholine in the incubation resulted in the formation of significant levels of retinyl myristate as well as the expected amounts of retinyl ester from the endogenous donor (Fig. 2, middle panel). Only low levels of retinyl laurate (equivalent to 10% of the retinyl myristate produced) were observed. Conversely, when 1-lauroyl-2-myristoylphosphatidylcholine was provided as the acyl donor, the predominating ester formed from exogenous lipid was retinyl laurate (Fig. 2, lower panel). Only minor amounts of retinyl myristate were detected. Thus, a clear positional selectivity for the sn-1 moiety of phosphatidylcholine was demonstrated for the activity that esterified retinol-CRBP(II) with exogenous phosphatidylcholine. The positional purity of these synthetic phospholipids was not examined. Consequently, the small incorporation of the acyl group presumed to be at the 2-position may be due to some positional impurity.

Greater amounts of retinyl myristate were produced from 1-myristoyl-2-lauroylphosphatidylcholine than from DMPC (compare Fig. 1, middle panel and Fig. 2, middle panel). This observation suggested that the increased solubility of shorter-chain phosphatidylcholines may improve their utilization under these conditions. When DMPC, 1-myristoyl-2-lauroyl-

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**TABLE I**

Production of retinyl myristate from retinol-CRBP II using various exogenous lipids as acyl donor

<table>
<thead>
<tr>
<th>Exogenous lipid</th>
<th>RM</th>
<th>RL</th>
<th>RP/O</th>
<th>RS</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMPC</td>
<td>6.6 (±0.3)</td>
<td>2.7 (±0.1)</td>
<td>26.6 (±1.3)</td>
<td>18.2 (±0.1)</td>
</tr>
<tr>
<td>DMPE</td>
<td>&lt;0.7</td>
<td>3.2 (±0.3)</td>
<td>24.4 (±0.1)</td>
<td>19.0 (±0.8)</td>
</tr>
<tr>
<td>DMPA</td>
<td>ND</td>
<td>2.9 (±1.0)</td>
<td>25.8 (±0.6)</td>
<td>17.4 (±0.1)</td>
</tr>
<tr>
<td>M-CoA</td>
<td>1.5 (±0.7)</td>
<td>2.6 (±0.1)</td>
<td>25.0 (±0.1)</td>
<td>16.5 (±2.7)</td>
</tr>
<tr>
<td>M-Acid</td>
<td>ND</td>
<td>2.6 (±0.3)</td>
<td>24.9 (±1.0)</td>
<td>18.7 (±1.6)</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Positional specificity in the transfer of acyl groups from phosphatidylcholine to retinol-CRBP(II). 1-Myristoyl-2-lauroyl phosphatidylcholine (middle panel) and 1-lauroyl-2-myristoyl phosphatidylcholine (lower panel) were assayed for acyl transfer to retinol-CRBP(II) by methods described in the legend of Fig. 1. Retinol-CRBP(II) esterification in the presence of detergent without exogenous phosphatidylcholine is illustrated in the upper panel ("Endogenous Donor").
phosphatidylcholine, and 1-myristoyl-2-decanoylphosphatidylcholine were compared as acyl donors at a concentration of 40 \( \mu \text{M} \). Microsomes were observed to catalyze the production of 5.1 \( \pm 0.5 \), 14.1 \( \pm 1.3 \), and 24.4 \( \pm 0.4 \) pmol of retinyl myristate from each phosphatidylcholine, respectively. The amount of retinyl myristate produced with 1-myrstoyl-2-decanoylphosphatidylcholine represented 42\% of the total ester synthesized from exogenous and endogenous donors. Decreasing the length of the acyl chain at the C-2 position of phosphatidylcholine enhanced its solubility and clearly increased its effectiveness as an acyl donor. These results may explain why symmetrical long-chain phosphatidylcholines were not effective as exogenous substrates. No significant increase in the amount of retinyl palmitate or retinyl stearate was observed when dipalmitoyl-PC or distearoyl-PC were provided exogenously. However, when 1-palmitoyl-2-decanoylphosphatidylcholine was examined under identical conditions, considerable acyl transfer to retinol-CRBP(II) was observed. Retinyl palmitate production increased 7.9 pmol above the amount synthesized with the endogenous donor. However, these results do not rule out the alternative possibility that the ester synthase prefers shorter-chain acyl moieties at the C-2 position of phosphatidylcholine.

The presence of at least two retinol esterifying activities in intestinal microsome preparations has been demonstrated (8). Unbound retinol is available for esterification by the CoA-dependent activity, acyl-CoA-retinol:acyltransferase. However, retinol-CRBP(II) is essentially restricted to the acyl-CoA-independent retinyl esterase under study here. Acyl-CoA-retinol:acyltransferase is sensitive to reagents that modify sulphydryl residues (4,12-14) but is relatively insensitive to anti-esterases such as PMSF (13, 14). To attempt to clearly distinguish between the CoA-dependent and CoA-independent activities in intestinal microsomes, the effect of these protein modifying reagents on the CoA-independent esterification of retinol-CRBP(II) was examined. Fig. 3 illustrates the effect of the anti-esterase PMSF (panel A) and the sulphydryl reagents NEM (panel B) and pCMB (panel C) on the in vitro esterification of retinol-CRBP(II) by endogenous acyl donor. A concentration-dependent decrease in retinyl ester synthesis was observed using microsomes preincubated with each of the reagents. NEM and pCMB were the most potent compounds, resulting in complete inhibition of retinol-CRBP(II) esterification at 3 and 5 \( \mu \text{M} \), respectively. PMSF gave approximately 90\% inhibition at 1.0 mM. Torma and Vahlquist (14) observed only a 20\% reduction in the CoA-dependent acyl-CoA-retinol:acyltransferase activity at 1.0 mM PMSF. Higher concentrations were not tested. These observations suggested that PMSF might be used to distinguish acyl-CoA-retinol:acyltransferase activity from the acyl-CoA-independent activity described here.

Comparison of Phosphatidylcholine and Acyl-CoA as Substrates for the Esterification of Retinol-CRBP(II) and Unbound Retinol—The esterification of retinol-CRBP(II) and unbound retinol was examined in the presence and absence of PMSF (Fig. 4). DLPC and lauryl-CoA were used as exogenous acyl donors. Detergent was not included in this study since DLPC readily participated in the reaction in the absence of detergent.

When DLPC was provided as an exogenous acyl donor, lauryl groups were transferred to retinol-CRBP(II) and to unbound retinol to produce retinyl laurate (Fig. 4, A and B, upper tracings). The retinyl esters synthesized from the endogenous acyl donor were also apparent. The relative proportion of retinyl laurate produced from DLPC was similar using either retinol-CRBP(II) or unbound retinol as substrate. This indicated that the phosphatidylcholine-dependent activity would utilize both bound and free retinol as substrate. The synthesis of retinyl esters from both the endogenous acyl donor and exogenous DLPC decreased more than 85\% when the microsomes were preincubated with the anti-esterase PMSF (Fig. 4, A and B, lower tracings). This suggested the
activity that utilized exogenous phosphatidylcholine was the same as the activity utilizing endogenous donor.

Examination of lauroyl-CoA as an acyl donor for retinol-CRBP(II) esterification revealed little formation of retinyl laurate (Fig. 4C, upper tracing) compared to DLPC as acyl donor (Fig. 4A, upper tracing). Over 5-fold more retinyl laurate was produced from DLPC compared to that produced from lauroyl-CoA. Similar observations were made in the previous section which examined DMPC and myristoyl-CoA as acyl donors in retinol-CRBP(II) esterification (Table I). Lauroyl-CoA was a much better acyl donor for the esterification of unbound retinol (Fig. 4D, upper tracing). Approximately 5-fold more retinyl laurate was produced from unbound retinol than from retinol-CRBP(II). Preincubating the microsomes with PMSF resulted in greater than 95% inhibition of retinyl ester synthesis from the endogenous donor (Fig. 4, C and D, lower tracings) but had little effect on the synthesis of retinyl laurate from lauroyl-CoA (Fig. 4, C and D, lower tracings). Microsomes pretreated with PMSF retained up to 75% of the activity responsible for retinyl laurate synthesis from lauroyl-CoA using retinol-CRBP(II) or unbound retinol as substrates. Thus, the activity responsible for retinyl ester synthesis using acyl-CoA substrates, acyl-CoA-retinolacyltransferase, was clearly distinct from the synthase that used endogenous donor or exogenous phosphatidylcholine. Moreover, this indicates that the endogenous donor could not be acyl-CoA associated with the microsomes since PMSF had such a dramatic effect on the endogenous reaction. The selectivity of the acyl-CoA-independent retinyl ester synthase for phosphatidylcholine and the ability of PMSF to block incorporation from both exogenous phosphatidylcholine and endogenous acyl donor suggests that the endogenous microsomal acyl donor may well be phosphatidylcholine.

The presence of two esterifying activities raises the question of which is the physiologically important reaction. CRBP(II) is an abundant protein representing over 1% of the total soluble protein in extracts of small intestinal mucosa (6). All of the CRBP(II) is confined to the mature absorptive cells (7). Coupled with the high affinity of CRBP(II) for retinol (8, 15), this suggests that any retinol in the absorptive cell will be complexed to CRBP(II). Retinol bound to CRBP(II) is virtually restricted from the acyl-CoA-dependent esterification catalyzed by acyl-CoA-retinolacyltransferase (8) but would be readily available for esterification by the phosphatidylcholine-dependent ester synthase. This ester synthase selectively transfers the sn-1-acyl group of phosphatidylcholine to retinol-CRBP(II) to produce retinyl esters. The acyl composition of the retinyl esters produced in vivo closely matches the acyl composition at the C-1 position of lymph phosphatidylcholine (2). Consequently, we believe this phosphatidylcholine-dependent activity may be the retinyl ester synthase utilized in vitamin A absorption.

Similar acyl transferase activities may be involved in retinol esterification in other tissues. Futterman and Andrews (16, 17) described acyl-CoA-independent esterification of retinol with microsome preparations obtained from cat liver and bovine retina. Retinol in complex with cellular retinol-binding protein, CRBP, was esterified by an acyl-CoA-independent activity in retinal pigment epithelium preparations (18). Recently, we have described an acyl-CoA-independent activity in the rat liver that esterifies retinol complexed with CRBP (19). Preliminary experiments indicate that the activity in liver also will transfer acyl moieties from the C-1 position of exogenous phosphatidylcholine to retinol to produce retinyl esters.8

The mechanism of retinol esterification may proceed in a manner similar to lecithin-cholesterol acyltransferase. The mechanism of lecithin-cholesterol acyltransferase involves a phospholipase cleavage of the C-2 acyl moiety of phosphatidylcholine, transfer of the acyl group to a cysteine residue, and a subsequent transfer to cholesterol (20). The active site of lecithin-cholesterol acyltransferase contains a reactive serine hydroxyl and two reactive sulfhydryl residues (20-22). The retinol ester synthase under study here was also sensitive to anti-esterase and sulfhydryl reagents (20-22). The retinol ester synthase in other tissues. Futterman and Andrews (16, 21) described acyl-CoA-independent esterification of retinol using serine hydroxyl and two reactive sulfhydryl residues (20-22). The retinol ester synthase under study here was also sensitive to anti-esterase and sulfhydryl reagents (20-22). The retinol ester synthase under study here was also sensitive to anti-esterase and sulfhydryl reagents (20-22). The retinol ester synthase under study here was also sensitive to anti-esterase and sulfhydryl reagents (20-22).

REFERENCES

8 P. N. MacDonald and D. E. Ong, unpublished observations.