Vol. 156, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1988 Pages 157-163

A LECITHIN:RETINOL ACYLTRANSFERASE ACTIVITY IN HUMAN AND RAT LIVER

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Received August 29, 1988

Summary: This report demonstrates that exogenous phosphatidylcholine will serve as an acyl donor for the esterification of retinol complexed to cellular retinol-binding protein (CRBP) by human and rat liver microsomal preparations. The retinyl ester synthases utilized phosphatidylcholine but had little or no ability to transfer acyl groups from lysophosphatidylcholine, phosphatidylethanolamine, or phosphatidic acid to retinol-CRBP. The human and rat activities also demonstrated positional selectivity as only the fatty acyl group at the sn-1 position of phosphatidylcholine was transferred. This *in vitro* activity may have considerable physiological importance since the fatty acyl composition at the sn-1 position of phosphatidylcholine is remarkably similar to the hepatic retinyl esters observed *in vivo*.

Vitamin A is stored primarily in the liver, where it is present as long-chain fatty acid esters of retinol. A CoA-dependent activity in microsomal preparations obtained from human (1) and rat liver (2) has been suggested to carry out hepatic retinol esterification *in vivo*. This activity, designated acyl-CoA:retinol acyltransferase, was characterized *in vitro* with unbound or free retinol as the substrate. However, both human and rat liver contain a protein that binds retinol with high affinity and specificity, termed cellular retinol-binding protein, or CRBP (reviewed in Ref. 3). When retinol bound to CRBP was examined as a substrate for esterification by rat liver microsomal preparations, it was not available for the reaction catalyzed by acyl-CoA: retinol acyltransferase (4). Instead, the retinol-CRBP substrate was esterified by a different enzyme that used an endogenous acyl donor associated with the microsomal preparation to synthesize mainly retinyl palmitate, the predominant hepatic retinyl ester observed *in vivo* (5). Similar CoA-independent retinyl ester synthases are present in the bovine retina (6-8) and rat small intestine (9,10). Here, we report that the CoA-independent activity

Abbreviations used: CRBP, cellular retinol-binding protein; HPLC, high performance liquid chromatography; NEM, N-ethylmaleimide; PMSF, phenylmethylsulfonyl fluoride.

0006-291X/88 $1.50

described in rat liver (4) will catalyze acyl transfer from the C-1 position of exogenous phosphatidylcholine to retinol-CRBP to produce retinyl esters. Human liver preparations also catalyzed a similar reaction. These observations suggest that this lecithin:retinol acyltransferase activity may be of considerable importance in the metabolism and storage of vitamin A in the liver of human and rat.

EXPERIMENTAL PROCEDURES

Materials- all-trans-Retinol, all-trans-retinaldehyde, N-ethylmaleimide, phenylmethylsulfonyl fluoride, fatty acyl anhydrides, and all phospholipids were from Sigma. [^{3}H]-Retinol was prepared by reducing all-trans-retinaldehyde with sodium boro-[^{3}H]-hydride (11). 1-Myristoyl-2-lauroylphosphatidylcholine and 1-lauroyl-2-myristoylphosphatidylcholine were prepared and purified as described previously (10). CRBP was purified from rat liver (12). [^{3}H]-Retinol-CRBP was prepared by adding [^{3}H]-retinol to apo-CRBP (9).

Microsomal preparations- Frozen portions from two human livers (weighing approximately 1 and 4 gm) were provided by Dr. F. Peter Guengrich, Department of Biochemistry, Vanderbilt University, Nashville, TN. The material had been obtained at autopsy from two males, ages 22 and 36 years. Collection of the samples was approved by the Vanderbilt University Committee for the Protection of Human Subjects. Microsomal preparations were obtained from human and rat liver in 0.2 M KH_2PO_4, pH 7.2 by methods described previously (4). Protein concentrations were determined by the Pierce BCA protein assay with bovine serum albumin as the standard.

Preparation of phospholipid substrate solutions- Briefly, 400 nmol of phospholipid in chloroform were evaporated to dryness under N_2. The residue was lyophilized for 30-45 min to remove residual solvent and then resuspended in 1 ml of 0.2 M KH_2PO_4, pH 7.2 by sonication with a Branson sonifier fitted with a microtip for 60 x 1 s pulses at a power setting of 2.

Esterification assays- Retinyl ester production from human and rat liver preparations was examined by methods described previously (10). Briefly, 60-120 μg of microsomal protein were incubated for 5 min at room temperature with or without 20-40 nmol of the sonicated phospholipid preparations. The reactions were then initiated with the addition of retinol-CRBP (generally 1.5 nmol) to a final volume of 0.5 ml. Following a 15 min incubation at 37°C, the reactions were quenched with 4 volumes of ice-cold ethanol, extracted into hexane, and the hexane extract analyzed for retinyl esters by reverse-phase HPLC (9). Control incubations used microsomal preparations that were heat-inactivated for 5 min at 100°C. All HPLC results were corrected for the levels of endogenous retinyl esters present in the microsomal preparations as determined in incubations that omitted retinol-CRBP. In some experiments, retinyl ester production was determined with [^{3}H]-retinol-CRBP and the [^{3}H]-retinyl esters were analyzed by batch elution on columns of alumina (9).

Positional selectivity of the acyltransferase activity in human microsomal preparations- The positional selectivity of the acyltransferase activity in human microsomal preparations was examined in 0.22 ml reactions that contained 3.0 μM [^{3}H]-retinol-CRBP and 53 μg of microsomal protein in the presence and absence of 40 nmol of 1-myristoyl-2-lauroylphosphatidylcholine or 1-lauroyl-2-myristoylphosphatidylcholine. Following a 15 min incubation at 37°C, the reactions were stopped with 4 volumes of ice-cold ethanol and then extracted from 44 % ethanol into 3.2 ml of hexane (9). [^{3}H]-Retinyl esters were separated from [^{3}H]-retinol by applying 5 x 0.5 ml aliquots of the hexane extract to 5 x 1.2 gm columns of alumina. The [^{3}H]-retinyl esters were eluted from each column with 5 ml of hexane containing 2% ether (13). The ester fractions were combined, taken to dryness under N_2, redissolved in methanol,

and analyzed by reverse phase HPLC (9). Fractions were collected at 20 s intervals (0.66 ml) and the radioactivity in each fraction was determined by scintillation counting.

Inhibitor Studies- N-Ethylmaleimide and phenylmethylsulfonyl fluoride were examined as chemical inhibitors of the human retinyl ester synthase by methods described previously (10).

RESULTS AND DISCUSSION

Esterification of retinol-CRBP by human liver microsomal preparations- Microsomal preparations obtained from rat liver use an endogenous acyl donor and retinol-CRBP to catalyze the production of retinyl esters (4). Here, we examined whether human liver preparations would esterify retinol-CRBP in a similar fashion.

Microsomal preparations from two separate human livers were observed to catalyze [^{3}H]-retinyl ester production from [^{3}H]-retinol-CRBP. No other factors were required for retinyl ester production. The synthesis of retinyl esters by both human preparations was linear up to at least 120 μg of microsomal protein per ml. Heat-treated microsomes were inactive. Figure 1 illustrates the saturable production of [^{3}H]-retinyl esters with increasing amounts of [^{3}H]-retinol-CRBP from one of these preparations. These data were transformed linearly to calculate the kinetic constants of the microsomal activity (Fig. 1, inset). The average Km and Vmax values for the two preparations were 0.5 μM and 16 pmol per min per mg microsomal protein, respectively. The corresponding kinetic constants reported for the rat liver preparations were 1 μM and 50 pmol per min per mg microsomal protein, respectively (4).

Sensitivity of the human retinyl ester synthase to sulfhydryl and hydroxyl modifying reagents- The CoA-independent esterification of retinol-CRBP by rat liver preparations is sensitive to reagents that modify sulfhydryl or active serine residues (4). Here, the sulfhydryl reagent, NEM, and the anti-esterase, PMSF, were examined as potential inhibitors of the activity in human liver preparations. Retinyl ester production from retinol-CRBP was inhibited when human liver preparations were treated with increasing amounts of either NEM or PMSF (Fig. 2). The human retinyl ester synthase was extremely sensitive to NEM. Preincubating human preparations with 1 μM NEM resulted in essentially complete inhibition of esterifying activity (Fig. 2A). The esterification of retinol-CRBP was also decreased more than 90 % by 2 mM PMSF (Fig. 2B). In contrast, the acyl-CoA:retinol acyltransferase of various tissues is relatively insensitive to millimolar concentrations of PMSF (14,15). Thus, the inhibition observed with PMSF indicated that retinol-CRBP esterification with the endogenous donor was not the result of acyl-CoA:retinol acyltransferase using an endogenous pool of acyl-CoA.

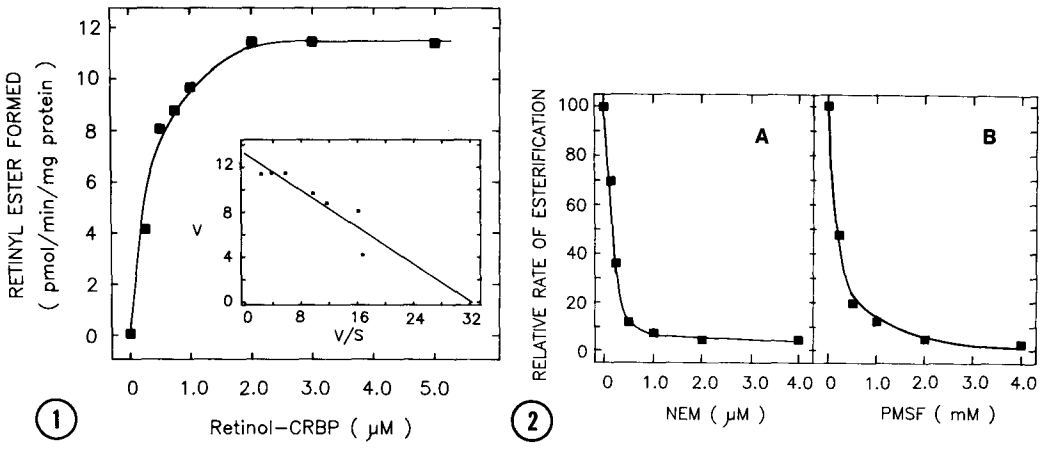

Fig. 1. Concentration dependence of retinol-CRBP esterification by human liver microsomal preparations. Increasing concentrations of [^{3}H]-retinol-CRBP were incubated with 14 μg of microsomal protein in 0.2 M KH_2PO_4, pH 7.2. The reaction volume was 0.12 ml. Following a 10 min incubation at 37°C, 0.1 ml of the reaction was removed into 0.4 ml of ice-cold ethanol and then extracted into hexane (9). The hexane extract was analyzed for [^{3}H]-retinyl esters on 1.2 gm alumina columns (13). Each point is an average of two determinations minus the background radiation obtained in reactions using heat-inactivated microsomal preparations. A linear transformation of this data is shown in the inset.

Fig. 2. Effect of N-ethylmaleimide and phenylmethylsulfonyl fluoride on the esterification of retinol-CRBP by human liver microsomal preparations. Increasing concentrations of either N-ethylmaleimide (panel A) or phenylmethylsulfonyl fluoride (panel B) were incubated in 0.2 M KH_2PO_4, pH 7.2 with 14.4 μg human liver microsomal protein for 10 min at 37°C. Esterification reactions were then initiated with the addition of 0.36 nmol of [^{3}H]-retinol-CRBP (3.5 Ci/mmol) to a final volume of 0.12 ml. Following a 10 min incubation at 37°C, the reactions were analyzed for [^{3}H]-retinyl esters as described in the legend to Figure 1.

Ability of various exogenous phospholipids to participate in retinol-CRBP esterification with human and rat liver preparations- The serum enzyme, lecithin:cholesterol acyltransferase, and the lecithin:retinol acyltransferase activity described in the rat small intestine are also sensitive to reagents that modify active serine and sulfhydryl residues (10,16-18). These activities catalyze the transfer of fatty acids from lecithin to cholesterol or retinol presumably via serine and sulhydryl residues in their active sites. Thus, we examined the ability of the human and rat liver microsomal preparations to catalyze a similar reaction.

In the absence of exogenous phospholipid, only those retinyl esters synthesized from the endogenous acyl donor were observed. These esters were mainly retinyl palmitate/oleate and retinyl stearate (Table 1). Minor amounts of retinyl linoleate and retinyl myristate were also observed (not tabulated). Including dilauroylphosphatidylcholine in the incubation resulted in the appearance of an additional retinyl ester peak in the chromatogram that co-eluted with standard retinyl laurate. Retinyl laurate equalled 30-40 % of the

TABLE I

Selectivity of Hepatic IRAT for Various Exogenous Phospholipids[a]

	Rat Liver Preparations[b]			Human Liver Preparations[b]		
	RL[c]	RP/O	RS	RL	RP/O	RS
NO ADDITION	0	65	27	0	85	15
DLPC	40	41	12	28	61	11
L-PC	4.2	67	20	0	80	20
DLPE	> 2	67	26	0	91	9
DLPA	2.3	69	23	0	88	12

a. Rat and human liver microsomal preparations (60 and 120 μg of microsomal protein, respectively) were incubated with and without 20 nmol of various phospholipid preparations and 1.5 nmol of retinol-CRBP in a volume of 0.5 ml. After 15 min at 37°C, the reactions were extracted into hexane and analyzed by HPLC.
b. All values are expressed as a percentage of the total retinyl ester produced.
c. RL, retinyl laurate; RP/O, retinyl palmitate/oleate; RS, retinyl stearate; DLPC, dilauroylphosphatidylcholine; L-PC, 1-lauroyl-2-lysophosphatidylcholine; DLPE, dilauroylphosphatidylethanolamine; DLPA, dilauroylphosphatidic acid.

total ester generated (Table 1). Boiled microsomal preparations gave no retinyl ester formation from either endogenous or exogenous acyl donors. Other phospholipids such as dilauroylphosphatidylethanolamine, dilauroylphosphatidic acid, or 1-lauroyl-2-lysophosphatidylcholine demonstrated little if any ability to participate in the production of retinyl laurate (Table 1). Thus, of the exogenous lipids examined, both the human and rat activities demonstrated a marked preference for phosphatidylcholine.

Positional selectivity of the acyltransferase- The predominant retinyl ester found in the liver is retinyl palmitate (5). Hepatic phosphatidylcholine contains palmitate almost exclusively at the C-1 position; the C-2 position contains mainly unsaturated fatty acids (19). If the physiological enzyme of the liver is a lecithin:retinol acyltransferase, then one would expect that the transferase is positionally selective for the C-1 fatty acyl moiety. In contrast, lecithin:cholesterol acyltransferase is positionally selective for the fatty acid at the C-2 position (16,20). To examine this possibility, phosphatidylcholines of positionally defined fatty acid composition were synthesized and examined as acyl donors for the esterification of retinol-CRBP.

Figure 3A,D show the retinyl esters generated by the enzyme with acyl moieties derived from the endogenous acyl donor in the absence of exogenous phosphatidylcholine. The retinyl palmitate/oleate peak was the predominant ester peak observed with both human and rat preparations. The radioactivity elution profile of the reaction products from the human preparations also

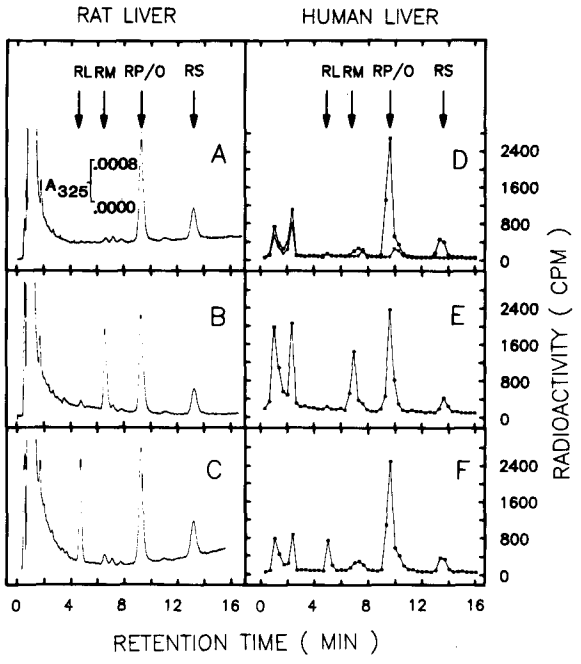

Fig. 3. Positional selectivity of the rat and human retinyl ester synthase for the C-1 moiety of phosphatidylcholine. Rat liver microsomal preparations (60 μg of microsomal protein) were incubated in the absence (panel A) and presence of 40 nmol of 1-myristoyl-2-lauroylphosphatidylcholine (panel B) or 1-lauroyl-2-myristoylphosphatidylcholine (panel C) in 0.2 M KH_2PO_4, pH 7.2 at room temperature for 5 min. Esterification reactions were initiated with the addition of 1.5 nmol of retinol-CRBP to a final volume of 0.5 ml. Following a 15 min incubation at 37°C, the reactions were analyzed for retinyl esters by reverse phase HPLC. Human liver microsomal preparations (40 μg of microsomal protein) were incubated in the absence (panel D) or presence of 18 nmol of 1-myristoyl-2-lauroylphosphatidylcholine (panel E) or 1-lauroyl-2-myristoylphosphatidylcholine (panel F) at room temperature for 5 min. Esterification reactions were initiated with the addition of 0.66 nmol of [^{3}H]-retinol-CRBP to a final volume of 0.22 ml. The reaction time and temperature was 15 min and 37°C, respectively. The [^{3}H]-retinyl esters were isolated on alumina columns and then analyzed by reverse phase HPLC as described in Experimental Procedures.

contained two peaks that eluted in the first three minutes. These peaks were present in the boiled control preparations as well (Fig. 3D, lower tracing). This radioactivity may represent some degradation of the retinol during processing of the extracts.

When human and rat liver preparations were incubated with retinol-CRBP and 1-myristoyl-2-lauroylphosphatidylcholine, the appearance of a new ester peak that coeluted with authentic retinyl myristate was observed (Fig. 3B,E). Only minor amounts of retinyl laurate were detected. Conversely, reactions containing 1-lauroyl-2-myristoylphosphatidylcholine produced retinyl laurate (Fig 2C,F). Thus, both the human and rat retinyl ester synthases displayed a clear positional selectivity for the C-1 acyl moiety of phosphatidylcholine.

Consequently, microsomal preparations from both human and rat liver catalyzed acyl group transfer from the C-1 position of exogenous phosphatidylcholine to retinol-CRBP to produce retinyl esters. The possible physiological relevance of such an activity is indicated by the observations that the predominant fatty acid at the C-1 position of phosphatidylcholine is palmitate and the predominant retinyl ester observed *in vivo* is retinyl palmitate. In support of this reaction as a general mechanism of retinol esterification is the recent demonstration of a similar, but not identical lecithin:retinol acyltransferase activity in the rat small intestine (10). The results here suggest that hepatic lecithin:retinol acyltransferase may play an important role in the storage of vitamin A in the liver of all species.

ACKNOWLEDGMENTS: We wish to thank Angela Gubitosi and Lee Bullock for excellent technical assistance as well as Dr. Frank Chytil and Dr. Tim Quick for a critical reading of the manuscript.

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