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EFFECT OF RETINOIC ACID ON THE ACYLATION OF PHOSPHOLIPIDS IN GRANULOCYTES IN VITRO

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SUMMARY: The influence of retinoic acid on the incorporation of [1-^{14}C]palmitic acid and [1-^{14}C]arachidonic acid into phospholipids was examined in guinea pig peritoneal granulocytes. All-trans-retinoic acid inhibited the incorporation of both fatty acids into phsophatidic acid and phosphatidylinositol. However, it stimulated the incorporation of both fatty acids into phosphatidylcholine but not other phospholipids. All-trans-retinoic acid was more effective than 13-cis-retinoic acid. The influence of all-trans-retinoic acid on the acylation of phospholipids was concentration-dependent with significant effect occurring at 2.1 μM. The loss of labeled fatty acids from prelabeled phospholipids and the transport of labeled fatty acids into granulocytes were not responsive to the presence of retinoic acid in the incubation media. These results suggest that retinoic acid may affect the activities of acyltransferases involved in the synthesis of phosphatidic acid, phosphatidylinositol and phosphatidylcholine.

All-trans-retinoic acid is a physiological metabolite of retinol and its esters. It has been found to inhibit tumor-promoter, phorbol-12-myristate-13-acetate-stimulated induction of orithine decarboxylase in mouse epidermal cells (1,2), collagenase production in rat kidney fibroblasts (3), and DNA synthesis in lymphocyte cultures (4,5). The mechanism by which retinoic acid reverses the effects of tumor-promoter has not been defined.

The initial action of phorbol myristate acetate has been suggested to be on the cell membrane (4). Studies in mouse skin (6) and in bovine lymphocytes (7) demonstrated that phorbol myristate acetate activated the metabolism of phosphatidylcholine. In granulocytes this tumor-promoter modified the incorporation of fatty acids into phospholipids (8). It is of interest to investigate whether retinoic acid also affects phospholipid metabolism in granulocytes. In the present study, the effect of retinoic acid on the metabolism of the fatty acyl groups of phospholipids in granulocytes was examined.

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MATERIALS AND METHODS

[1-^{14}C]Palmitic acid (specific activity, 51.0 Ci/mol) and [1-^{14}C]arachidonic acid (specific activity, 55.8 Ci/mol) were purchased from New England Nuclear Corp. Individual phospholipid standards were products of Supelco. Fatty acid-free bovine serum albumin was obtained from Miles Laboratories, Inc. Albumin-bound fatty acids were prepared as described previously (9). All-trans-retinoic acid was from Sigma. 13-cis-Retinoic acid was a gift of Hoffmann-La Roche Inc. Retinoic acid was dissolved as a stock solution in dimethylsulfoxide at a concentration of 10 mg per ml and stored at -20°C until use. Prior to use aliquots of the stock solutions were diluted with 0.9% NaCl containing 4 mg bovine serum albumin to 100 µg retinoic acid per ml.

Preparation of granulocytes. Granulocytes from guine pig peritoneal exudates were prepared as described previously (9). Cells were suspended in Krebs-Ringer phosphate buffer containing 5 mM glucose to give a concentration of 25 • 10^6 cells per ml and were kept at 37°C. Cell counts were made in a hemocytometer, and cell viability was measured by Trypan-blue exclusion. The cell preparations contained more than 95% polymorphonuclear leukocytes.

Incubations. All incubations were carried out at 37°C in glass-stoppered tubes under air with shaking. In a final volume of 2.0 ml, each tube contained 2.57 x 10^5 dpm (1 µM) of [1-^{14}C]palmitic acid or 2.08 x 10^5 dpm (1 µM) of [1-^{14}C]arachidonic acid in the presence of 0-10 µg (0-33.6 µM) of all-trans-retinoic acid. In tubes not containing retinoic acid, equivalent volume of dimethylsulfoxide diluted with 0.9% NaCl containing 4 mg of bovine serum albumin per ml was included. Incubations were initiated by the addition of 40 • 10^6 cells to each tube. The highest all-trans-retinoic acid concentration (33.6 µM) used contained 0.05% dimethylsulfoxide in the incubation medium. In control experiments, neither dimethylsufloxide nor all-trans-retinoic acid at the concentrations used affect cell viability as monitored by Trypan-blue exclusion and lactate dehydrogenase release into the incubation media (10).

Lipid extraction and chromatographic resolution of phospholipids. Incubations were terminated with 10 ml CH_3OH containing 0.025 N hydrochloric acid, and total lipids wre extracted by the method of Bligh and Dyer (11). Phospholipids were resolved by two-dimensional thin-layer chromatography on silica gel H (Analtech) (12). The radioactivity and phosphorus content of each phospholipid, and cell-associated ^{14}C-labeled free fatty acid were measured as described previously.

Effect of all-trans-retinoic acid on the loss of labeled fatty acids from prelabeled phospholipids. Granulocytes (400 • 10^6 cells) in 20 ml of Krebs-Ringer phosphate buffer were incubated with 2.57 x 10^6 dpm of [1-^{14}C]palmitic acid or 2.07 x 10^6 dpm of [1-^{14}C]arachidonic acid at 37°C for 60 min. Then the excess label was removed by washing cells with buffer containing fatty acid-free bovine serum albumin (2 mg per ml). The labeled cells were resuspended in 20 ml Krebs-Ringer phosphate buffer. In a final volume of 2.0 ml, 40 • 10^6 labeled cells were incubated with 1 µM unlabeled palmitic acid or arachidonic acid in the presence or absence of 8.4 µM all-trans-retinoic acid. After cells were incubated at 37°C for 0-30 min, lipids were extracted and resolved, and the radioactivity and phosphorus content of each phospholipid were determined.

RESULTS

Fig. 1 illustrates that retinoic acid selectively inhibited [1-^{14}C]palmitic acid incorporation into phosphatidic acid and phosphatidylinositol, but it enhanced that into phosphatidylcholine. At 1 min incubation of cells with

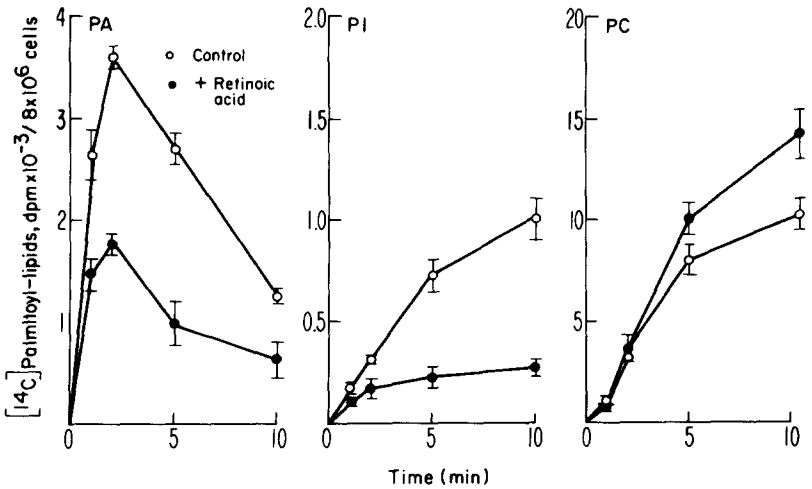

Fig. 1. Effect of all-trans-retinoic acid on [1-^{14}C]palmitic acid incorporation into phospholipids as a function of time. Granulocytes ($40 \cdot 10^6$) were incubated at the indicated period of time (1-10 min) with [1-^{14}C]palmitic acid $2.57 \cdot 10^5$ dpm, 1 μM) as albumin complex in the presence (●) or absence (○) of 8.4 μM all-trans-retinoic acid. Each point represents the mean ± S.E. from four experiments. PA, phosphatidic acid; PI, phosphatidylinositol; PC phosphatidylcholine.

8.4 μM retinoic acid, a 45% inhibiton of [1-^{14}C]palmitic acid incorporation into phosphatidic acid was observed, and there was no significant change in the degree of inhibition after longer periods of incubation. A 30% inhibition of [1-^{14}C]palmitic acid incorporation into phosphatidylinositol was shown at 1 min incubation of cells with retinoic acid; the inhibition reached 75% at 10 min incubation. An increased radioactivity of phosphatidylcholine was demonstrated only after 5 min incubation. Under identical experimental conditions, retinoic acid was without effect on the labeling of phosphatidylethanolamine, phosphatidylserine or sphingomyelin. In addition, the phosphorus content of individual phospholipids remained unchanged in the presence of retinoic acid (data not shown).

The effect of retinoic acid on [1-^{14}C]arachidonic acid incorporation into phospholipids (Fig. 2) resembled that on [1-^{14}C]palmitic acid incorporation into phospholipids. The inhibition of 8.4 μM retinoic acid on [1-^{14}C]arachidonic acid incorporation into phosphatidic acid and phosphatidylinositol was observed at 1 min incubation. Phosphatidylcholine radioactivity was enhanced

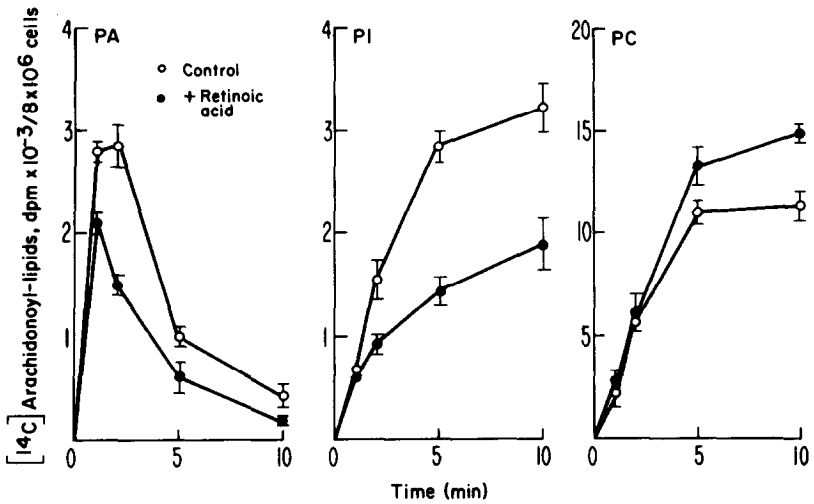

Fig. 2. Effect of all-trans-retinoic acid on [1-^{14}C]arachidonic acid incorporation into phospholipids as a function of time. Granulocytes (40 • 10^6) were incubated at the indicated period of time (1-10 min) with [1-^{14}]arachidonic acid 2.08 • 10^5 dpm. 1 μ M) as albumin complex in the presence (●) or absence (○) of 8.4 μM all-trans-retinoic acid. Each point represents the mean ± S.E. from four experiments. PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine.

by the presence of retinoic acid only after 5 min incubation. The radioactivity of other phospholipids was not affected by retinoic acid.

A rapid labeling of phosphatidic acid by both [1-^{14}C]palmitic acid (Fig. 1) and [1-^{14}C]arachidonic acid (Fig. 2) was demonstrated. The rapid decline in the radioactivity of phosphatidic acid appears to be consequences of an exhaustion of labeled fatty acids and a rapid metabolism of the labeled molecule, since it could be restored by a further addition of labels.

The influence of retinoic acid on labeled fatty acid incorporation into phospholipids was concentration-dependent with significant effect occurring at 2.1 μM. The degree of inhibition induced by retinoic acid on the labeling of phosphatidic acid by [1-^{14}C]palmitic acid and [1-^{14}C]arachidonic acid was similar. However, the degree of inhibition obtained by retinoic acid at all concentrations on the labeling of phosphatidylinositol by [1-^{14}C]palmitic acid was greater than that by [1-^{14}C]arachidonic acid. In a concentration-dependent manner, retinoic acid produced similar magnitude of stimulation on the incorporation of both fatty acids into phosphatidylcholine. Table I shows the

TABLE I

Effect of all-trans-retinoic acid concentration on [1-^{14}C] arachidonic acid incorporation into phospholipids*

Retinoic acid (μM)	PA	PI	PC
	(% of Control)		
2.1	91.5 ± 2.9	87.1 ± 5.0	112 ± 3.7
4.2	85.6 ± 3.5	76.0 ± 2.7	125 ± 4.6
8.4	72.6 ± 5.2	59.0 ± 5.1	145 ± 7.7
16.8	64.7 ± 1.7	55.2 ± 4.4	165 ± 5.4
33.6	49.0 ± 4.9	52.7 ± 3.5	178 ± 10.2

*Granulocytes ($40 \cdot 10^6$) were incubated for 10 min at 37°C with [1-^{14}C]arachidonic acid (2.08×10^5 dpm, 1 μM) and indicated concentrations of all-trans-retinoic acid. Data represent mean ± S.E. of four experiments. PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine.

effect of varying concentrations of retinoic acid on [1-^{14}C]arachidonic acid incorporation into phospholipids. The response of phospholipid metabolism to varying concentrations of retinoic acid was not due to cytotoxic effect of retinoic acid since exposure of granulocytes to 33.6 μM retinoic acid for 30 min at 37°C did not cause the release of lactate dehydrogenase into the incubation media (data not shown).

13-cis-Retinoic acid was less effective than all-trans-retinoic acid in the inhibition of the acylation of phosphatidic acid and phosphatidylinositol, and in the stimulation of the acylation of phosphatidylcholine (Table II).

In cells prelabeled with either [1-^{14}C]palmitic acid or [1-^{14}C]arachidonic acid, retinoic acid did not induce measurable changes in the radioactivity or phosphorus content of individual phospholipids during 1-30 min chase with unlabeled fatty acids (data not shown). In addition, cell-associated free fatty acids were not affected by the presence of retinoic acid.

DISCUSSION

The present study presented the first evidence that retinoic acid has marked effect on the incorporation of both palmitic acid and arachidonic acid into granulocyte phospholipids *in vitro*. It appears that retinoic acid

TABLE II
Effect of 13-cis-retinoic acid on [1-^{14}C]palmitic acid incorporation into phospholipids*

Addition	PA	PI	PC
	(% of Control)		
13-cis-Retinoic acid	88.6 ± 6.0	50.7 ± 3.2	115 ± 5.5
All-trans-retinoic acid	63.5 ± 4.3	26.3 ± 1.6	130 ± 7.6

*Granulocytes ($40 \cdot 10^6$) were incubated for 10 min at 37°C with 2.57×10^5 dpm palmitic acid (1 μM) in the presence of 8.4 μM of the indicated retinoic acid. Data represent mean ± S.E. of three experiments. PA, phosphatidic acid; PI, phosphatidylinositol; PC, phosphatidylcholine.

affects the activities of specific acyltransferases involved in the synthesis of phosphatidic acid, phosphatidylinositol and phosphatidylcholine, because the labeling of phosphatidylethanolamine, phosphatidylserine or sphingomyelin was not affected. Cell-associated free fatty acids were not affected by the presence of retinoic acid, suggesting that the changes in fatty acid incorporation into phospholipids were not secondary to changes in the transport of fatty acids into cells. In addition, the loss of label from prelabeled phospholipids was not responsive to the presence of retinoic acid, indicating that the hydrolysis of phospholipids was not affected.

The inhibition of retinoic acid on the labeling of phosphatidylinositol by palmitic acid was consistently greater than that on the labeling of phosphatidic acid, suggesting that the decreased radioactivity of [1-^{14}C]palmitoyl-phosphatidylinositol was not totally inherited from [1-^{14}C]palmitoyl-phosphatidic acid. Retinoic acid may affect the acylation of lysophosphatidylinositol by palmitoyl-CoA.

Time course studies of the incorporation of [1-^{14}C]arachidonic acid into granulocyte phospholipids performed in the present study demonstrated that [1-^{14}C]arachidonic acid was rapidly incorporated into phosphatidic acid and the labeled phosphatidic acid was rapidly metabolized. However, it is not

certain whether part of the labeled arachidonoyl-phosphatidylinositol is derived from arachidonoyl-phosphatidic acid, since identification of labeled arachidonoyl-CDP-diacylglycerol was not performed in the present study. A rapid turnover of arachidonic acid in phosphatidic acid was also reported in horse platelets (13).

13-cis-Retinoic acid was less effective than all-trans-retinoic acid in affecting the acylation of phospholipids. It is not known whether 13-cis-retinoic acid has to be isomerized to all-trans-retinoic acid which is in turn metabolized to an active form in order to exert metabolic action. Recent studies indicated that 13-cis-retinoic acid was partially isomerized to all-trans-retinoic acid which was rapidly metabolized to highly polar compounds in tissues and blood of vitamin A-sufficient rats (14). More work is therefore needed to determine the mechanism by which retinoic acid regulates the acylation of phospholipids in granulocytes and to examine whether the effect of retinoic acid on the membrane metabolism contributes to part of its anti-tumor promoter action.

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