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EFFECTS OF RETINOIC ACID ON PROSTAGLANDIN BIOSYNTHESIS IN GUINEA-PIG SKIN

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Topical application of retinoic acid on guinea-pig skin resulted within 70 hours in erythema with a concomitant elevation of endogenous prostaglandin E_2 (PGE_2) in the treated areas of the skin. Prolonged daily treatment resulted in the development of severe scaly dermatoses and a corresponding decrease in the level of PGE_2 in the skin. Examination of retinoic acid effects on the in vitro biosynthesis of PGE_2 from arachidonic acid by extracts from guinea-pig skin and sheep vesicular gland demonstrated that retinoic acid inhibits prostaglandin synthesis in a concentration and time-dependent manner. These results indicate that retinoic acid may exert a regulatory role on prostaglandin biosynthesis in the skin.

Vitamin A (all-trans retinol) has long been recognized as an essential nutrient for man and vertebrates, and it performs specific visual functions in lower forms of life. In the dietary absence of this compound animals suffer from a variety of symptoms, including impaired vision, retarded growth,. increased susceptibility to infections, blindness, and ultimately death [1]. On the other hand, vitamin A-acid (all-trans retinoic acid) when administered to vitamin A-deficient animals stimulated their growth, although blindness still resulted in the experimental animals. The possibility that retinoic acid may be the form of active vitamin A in many nonvisual functions has, therefore, interested investigators for several years [2].

It has also been recognized that an adequate dietary level of retinol is essential in the maintenance of epithelial integrity although its precise biochemical function is unknown [3]. Retinoic acid, which is the most active form of vitamin A following topical application to skin, produces a characteristic epithelial hyperplasia. A persistent erythema often develops concomitantly.

Since excess retinol has been shown to produce diverse pathologic changes, particularly in the alteration of cellular membranes and release of lysosomal enzymes [4–7], we reasoned that the erythema associated with topical application of retinoic acid might be due to elevation of endogenous level of E prostaglandins in the skin. As a first step in our effort to elucidate the mechanism of action of retinoic acid in the skin, we have assayed the E prostaglandins in the skin of retinoic acid-treated guinea pigs and tested the effects of retinoic acid on prostaglandin E_2 biosynthesis from arachidonic acid by extracts obtained from guinea-pig skin and acetone powder prepared from vesicular gland of sheep.

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MATERIALS AND METHODS

PGE_2 was a gift from Dr. John Pike of Upjohn Co., Kalamazoo, Michigan. Acetone powder of a microsomal fraction prepared from sheep vesicular gland was a gift from Dr. Wallach of Upjohn Co. ω-nor PGE_2 and ω-homo PGE_1 were gifts from Professor Van Dorp of the Unilever Research Laboratory, Netherlands. Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) was purchased from Aldrich Chemical Company. BSA (N,O-bis-trimethylsilylacetamide) was from Pierce Chemical Co. Retinoic acid (0.05%), from Eastman-Kodak, was dissolved in propylene glycol:ethanol:ethylene glycol-monomethyl ether, 1:2:2. Reagents were of analytical grade and solvents were redistilled before use.

Topical treatment of guinea pigs with vehicle and retinoic acid. The guinea pigs used in these experiments were fed ad lib with Purina chow. The animals were divided into three groups: Group A was untreated, Group B was treated topically with the vehicle (propylene glycol:ethanol:ethylene monomethyl ether, 1:2:2) alone, and Group C was treated with retinoic acid (0.05%) dissolved in the vehicle. The hair on the dorsum of the animal was removed by clipping or depilating with sodium thioglycolate. The retinoic acid or vehicle was applied twice daily to the shaved skin for 2 weeks. The animals were kept in individual cages, inspected and weighed daily.

Isolation and purification of prostaglandin E_2 from skin. Skin biopsies (15–25 mg) were removed rapidly from the shaved area of the guinea pigs' skin on the 3rd, 6th, and 9th day, respectively. The samples were weighed and dropped immediately into preweighed vials containing a mixture of chloroform–methanol, 2:1 [8] to stop enzymatic release of precursor fatty acid and synthesis of prostaglandin in the skin. The skin was homogenized in chloroform–methanol using a motor-driven glass homogenizer at 4°C. Tissue debris was removed by filtration on sintered glass funnels and the filtrate evaporated to dryness in a rotary evaporator. The residue, containing total lipids, was dissolved in a small volume of chloroform–methanol 1:1 and subjected to thin-layer chromatography on silica gel according to Smith and Lands [9].

The thin-layer plates were developed to 15 cm in benzene:dioxane:acetic acid:formic acid (82:14:1:1). The plates were air dried for 15 min and then further developed to 7.5 cm in acetone-methylene chloride (60:40). Under these conditions, reference E prostaglandins are effectively separated from F prostaglandins and other lipids and can be readily visualized in a tank of iodine vapor. Authentic samples of PGE_2 and $PGF_{2\alpha}$ were carried through the entire procedure.

Estimation of E prostaglandins by gas chromatography with electron capture detection. The E prostaglandins eluted from the thin-layer plates were evaporated to dryness under a stream of nitrogen, and subjected to analysis by gas liquid chromatography with an electron capture detector system according to Jouvenaz et al [10]. For identification, reference PGE_2, internal standards, ω-nor-PGE_2 (19:4, n-5), ω-homo-PGE_1 (21:4, n-7), and skin extracts were converted initially into PGB_2 by treating each sample with 3 ml of 0.5 N NaOH in 50% aqueous ethanol for 30 min. Quantification was by triangulation of gas chromatographic peaks. The results have been expressed as nanograms (ng) PGE_2 per gm wet weight of skin.

Preparation and incubation of microsomal pellet of guinea-pig skin for prostaglandin synthesis. Guinea pigs were killed by decapitation, and the skin free of underlying fat was minced and homogenized in 4 vol of ice-cold potassium phosphate buffer (pH 8.0) in an ice bath with a Polytron homogenizer as described previously [11]. Unbroken cellular debris was removed by filtration of the homogenate. The nuclear pellet was obtained by centrifugation of the homogenate at $800 \times g$ for 12 min at 4°C. The supernatant obtained after the removal of the pellet was subjected to differential centrifugation to obtain the microsomal pellet as described previously [12]. The pellet obtained was resuspended and homogenized in buffer (pH 8.0) and used for incubations.

[1-^{14}C] Arachidonic acid (2.8 μCi, 0.05 μmole) dissolved in acetone was incubated at 37°C for 15 min in a medium containing 5 ml of microsomal pellet (31.2 mg of protein) prepared from guinea-pig skin, glutathione (0.65 mM), hydroquinone (0.5 mM), and EDTA (30 mM). Fractionation and identification of radioactive PGE_2 by thin-layer chromatography were as reported previously [12].

Oxygen absorption measurement. Oxygen absorption measurements were done on a Yellow Springs Instrument Company Model 53 as reported previously [13]. The oxidation of arachidonic acid (20:4, n-6) by the phenol-activated vesicular gland oxygenase [9] was determined by measuring oxygen absorption at a constant temperature of 30° ± 0.5°C. In most experiments, the total volume of the reaction mixture in the sample chamber was 3.0 ml. Additions of enzyme preparation and various concentrations of retinoic acid were made through the side of the electrode holder.

RESULTS

Effects of Topical Application of Retinoic Acid on Skin of Guinea Pigs

The initial gross change in the skin of the guinea pigs after daily application with retinoic acid (0.05%) was erythema which appeared approximately 72 hr after treatment. Continued treatment resulted in a severe scaly dermatosis after 9 days. Histologic examination of the areas treated with retinoic acid showed characteristic acanthosis and parakeratosis.

Effects of Topical Application of Retinoic Acid on Prostaglandin Levels in Skin of Guinea Pigs

In preliminary experiments to develop the assay system by gas-liquid chromatography, two internal standards, ω-nor-PGE_2 (19:4, n-5) and ω-homo-E_1 (21:4, n-7), were carried through the entire procedure as described under *Materials and Methods* along with authentic PGE_2. A typical chromatogram of such a resolution is shown in Figure 1*A*. A typical chromatogram of an analysis of an extract from guinea-pig skin is shown to equal those of added ω-homo-PGB_1 and ω-nor-PGB_2, respectively, thus enabling a reliable estimation of recoveries after any series of assays. The linearity of the detector response for reference PGB_2-ME-TMS over 0–25 ng is shown in Figure 2.

In preliminary experiments, skin biopsies were taken from 3 guinea pigs in groups A, B, and C,

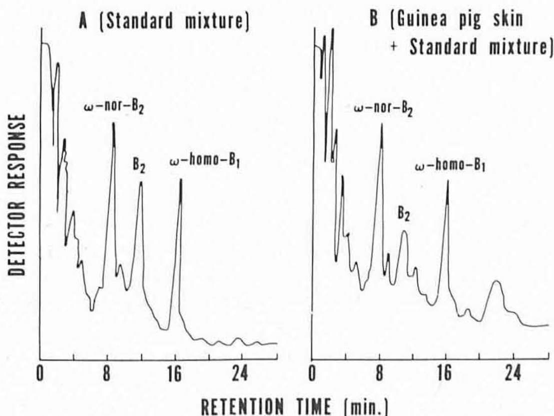

FIG. 1. Gas-liquid chromatography of prostaglandin derivatives. *A:* Standard mixture, 10 ng each. *B:* Analysis of guinea-pig skin. The ω-nor and ω-homo prostaglandins were added as internal standards. Details of the chromatographic analysis are described in the text.

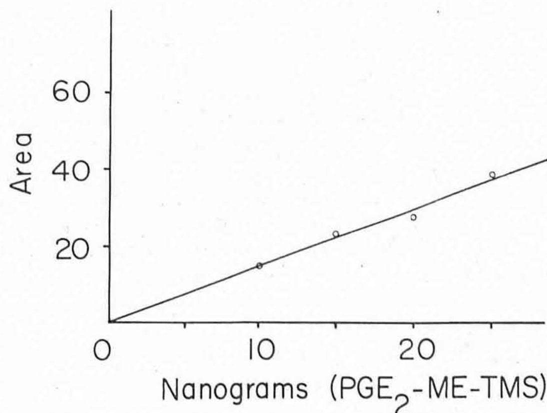

FIG. 2. Plot of area versus amount of PGB_2-ME-TMS injected into the gas chromatographic instrument equipped with ^{63}Ni-electron capture detector. Area was estimated by triangulation (Peak height $\times$ width at half-peak height). The data are averages of duplicate experiments.

respectively, and prostaglandin levels were determined according to the procedure described previously. After 3 days of daily topical treatment with retinoic acid, biopsies were again removed from the dorsa for prostaglandin determinations. Increases in E-prostaglandin levels were observed in group C guinea pigs that had been treated daily with 0.05% retinoic acid. The elevated level of E prostaglandins also paralleled the appearance of erythema on the skin of animals. Group B animals, treated with vehicle alone, showed no significant increase over the control group A and no marked erythema developed. Although our data of elevation of endogenous PGE_2 are associated with the appearance of erythema on the skin, the results do not exclude the possible contribution of some PGE_2 transported to the site of inflammatory reaction in the tissue. Studies are in progress to clarify this point.

The effect of topical indomethacin on retinoic acid-induced erythema was also investigated in our preliminary studies. Two guinea pigs were treated topically with 1% indomethacin dissolved in vehicle 30 min before the application of 0.05% retinoic acid. In another experiment, 2 animals were treated with a solution of 0.05% retinoic acid containing 1% indomethacin. Control animals were treated with 1% indomethacin in vehicle alone. Appearance of retinoic acid-induced erythema was suppressed in the first group of animals pretreated with indomethacin, whereas mild erythema developed in the second group. Development of scaling was enhanced in both groups of animals (5th day).

In view of the development of scaly lesions in the dorsa of the animals on prolonged treatment with retinoic acid, the release of prostaglandins at the different phases of the skin changes was carried out with another group of animals. Skin biopsies were taken before treatment, on the 3rd, 6th, and 9th day, respectively, after topical application of .05% retinoic acid. The skin specimens were subjected to prostaglandin analysis as described under *Materials and Methods*. The level of the E prostaglandins increased 5-fold after 3 days of daily treatment (Fig. 3). This increase coincides with the development of erythema in the skin of the guinea pigs. By the 6th day, the skin had become hyperplastic and scaly with a decrease in erythema and prostaglandin levels. By the 9th day, the lesions had become severe and analysis of the skin specimens revealed a decrease in the level of prostaglandin in the tissue below control levels. The significance of this decrease is not immediately clear.

Effects of Topical Application of Dodecyl Sodium Sulfate on Skin of Guinea Pigs

In order to determine whether the effects of retinoic acid on guinea-pig skin were specific or common to all irritants on the prostaglandin-synthesizing enzymes, varying concentrations of dodecyl sodium sulfate (0.05–5.0%) dissolved in the same vehicle as retinoic acid were applied to skin of 2 guinea pigs at each concentration studied.

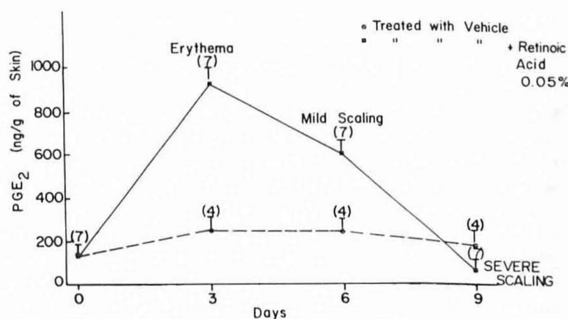

FIG. 3. Time course of prostaglandin E_2 from skin of guinea pigs after topical treatment with retinoic acid. Prostaglandin levels were estimated by the method of triangulation and the data are averages of experiments as indicated. *Vertical bars* indicate ranges. The number of animals from which skin specimens were taken for PGE_2 analysis at each point is indicated *in parentheses*.

No erythema or gross changes were observed in the skin of the 4 guinea pigs with low concentrations of 0.05% and 0.10% of the dodecyl sodium sulfate. However, at high concentrations 1% and 5%, respectively, the animals developed scaly dermatosis after 9 days but with minimal erythema.

Skin specimens were also removed on the 3rd, 6th, and 9th day, respectively, for prostaglandin assay. Results showed that topical dodecyl sodium sulfate at low concentrations had no effect on endogenous levels of PGE_2 in the guinea-pig skin. However, on the 9th day after daily topical treatment, the levels of PGE_2 in the skin specimens were decreased. Although the mechanism of this inhibitory effect is unclear, it is reasonable to suggest that effects at such high concentrations may imply nonspecific, detergent effects of this substance on the enzyme systems in the guinea-pig skin.

Effects of Retinoic Acid on the Biosynthesis of PGE_2 From Arachidonic Acid

In view of the decrease in prostaglandin levels with prolonged topical treatment of the guinea pigs with retinoic acid, experiments were carried out to test the effects of retinoic acid on the biosynthesis of PGE_2 in vitro by a microsomal fraction prepared from guinea-pig skin. The effects of retinoic acid on the incorporation of ^{14}C from [1-^{14}C] arachidonic acid into PGE_2 are shown in Table I. Low concentrations of retinoic acid did not inhibit the incorporation of ^{14}C from arachidonic acid into PGE_2. However, with increasing concentrations, retinoic acid inhibited the incorporation of ^{14}C from arachidonic acid into PGE_2. The results indicate a concentration-dependent inhibitory effect of retinoic acid on PGE_2 biosynthesis in guinea-pig skin.

Inhibition of Sheep Vesicular Gland Oxygenase by Retinoic Acid

In order to ascertain the nature of the inhibitory effect of retinoic acid on the biosynthesis of PGE_2

from arachidonic acid, experiments were carried out to test the effects of retinoic acid on the activity of the oxygenase prepared from acetone powder of sheep vesicular gland. Previous studies have shown that the enzyme from sheep vesicular gland and that from skin are responsive to similar agents [13]. This enzyme catalyzes the initial and rate-limiting step in the prostaglandin biosynthesis pathway from arachidonic acid [9]. The oxygenation of arachidonic acid by activated acetone powder of sheep vesicular gland was inhibited by retinoic acid by an instantaneous, concentration-dependent effect. Incubation of the enzyme with retinoic acid for 8 min prior to addition of substrate resulted in further loss of enzyme activity (Tab. II). The time-dependent destruction of the enzyme by retinoic acid is similar to that observed for eicosatetraynoic acid, an acetylenic analog of arachidonic acid [14] and inhibition by cis, trans conjugated unsaturated fatty acids [15].

DISCUSSION

The present studies have demonstrated that daily topical applications of retinoic acid on guinea-pig skin resulted in: (1) a marked increase in endogeneous E prostaglandin in retinoic acid-treated portions of the skin after 3 days; (2) a decrease in E-prostaglandin level below that of untreated skin specimens following prolonged daily treatment which was associated with the concomitant development of a scaly dermatosis. Although the mechanisms of action of retinoic acid in effecting these changes in the skin of guinea pigs are presently unknown, studies in other tissues of the effects of excess vitamin A on cellular membranes [6,16] suggest a possible role of this vitamin in the induction of tissue phospholipases which release fatty acid precursors from tissue phospholipids which could then be transformed into prostaglandins by tissue prostaglandin synthetase. For example, it has been suggested that during endocytosis, phospholipases are released from lysosomes of phagocytes [17]. These phospholipases in turn attack the cell membrane phospholipids which yield the arachidonic acid precursor which is converted into prostaglandin E_2 by the tissue prostaglandin-synthesizing enzymes. Furthermore, phospholipase A_2 contained in cobra venom has been shown to activate the synthesis of prostaglandins from tissues [18] by first releasing fatty acid precursors from tissue phospholipids.

The action of retinoic acid on skin phospholipase is unknown. The mechanism of action of retinoic acid in vivo on the synthesis of PGE_2 is also unclear. Nevertheless, at high concentrations, retinoic acid in vitro inhibits the transformation of arachidonic acid to PGE_2 by enzyme preparations from skin and sheep vesicular gland in a concentration and time-dependent manner (Tabs. I, II). These results suggest that retinoic acid at elevated levels in vivo may inhibit the transformation of endogenous arachidonic acid released from tissue phospholipids into PGE_2. This view will be consistent with the decrease in PGE_2 observed after 9 days of topical treatment with retinoic acid (Fig. 3). Since we have no data on tissue levels of retinoic acid, such an in vivo action of retinoic acid can remain only speculative at this time.

The in vitro studies on the biosynthesis of prostaglandins also showed that retinoic acid at low concentrations had no effect on the transformation of arachidonic acid to PGE_2 by enzyme preparations from skin and sheep vesicular gland (Tabs. I, II). It is interesting, however, to point out that no decrease in prostaglandin level was observed during the first 3 days of topical treatment of guinea-pig skin with retinoic acid. On the contrary, marked elevation of PGE_2 in the skin was observed on the 3rd day, suggesting the possible activation of a skin phospholipase A_2, followed by

TABLE I. *Effects of retinoic acid on biosynthesis on PGE_2 by microsomal fraction from guinea-pig skin*

[1-^{14}C]Arachidonic (2.8 μCi, 0.05 μmole) was incubated with 5 ml of microsomal fraction (31.2 mg of protein) obtained from skin homogenized in 0.1 M potassium phosphate buffer (pH 8.0) containing 30 mM EDTA. Incubation mixture contained 0.65 mM glutathione, 0.55 mM hydroquinone, and retinoic acid dissolved in 0.01 ml ethanol. Incubation was at 37°C for 30 min. Identification of radioactive products is described in the text. Results are expressed as percentages of added substrate recovered in PGB_2 fraction after correction for boiled enzyme control.

Retinoic acid (μM)	^{14}C in PGB_2 (%)		Inhibition (%)	
	(1)	(2)	(1)	(2)
None	8.6	9.0	—	—
40	8.8	9.4	—	—
80	8.0	8.8	—	—
160	5.2	6.0	39.6	33.4
200	4.4	5.4	48.9	40.0

TABLE II. *Effects of retinoic acid and arachidonic acid on sheep vesicular gland oxygenase*

Phenol-activated acetone powder preparation of sheep vesicular tissue was added to an assay mixture containing 0.67 mM phenol, 0.1 M Tris-HCl buffer, pH 8.5, and the indicated concentrations of retinoic acid. After the indicated times of incubation, arachidonic acid was added and the reaction rate (μMO_2/min = nmoles/ml per min) determined from continuous measurements of oxygen uptake with an oxygen electrode. The data are from the mean of triplicate experiments.

Retinoic acid (μM)	Preincubation time (rate (μM O_2/min) after 20:4 addition)	
	1 min	8 min
0	57	60
80	43	32
160	32	19
320	23	9

the release of arachidonic acid from tissue phospholipids for conversion into PGE_2. The hydrolysis of endogenous arachidonic acid from tissue phospholipids is the rate-limiting step in the endogenous biosynthesis of prostaglandins. Further studies to elucidate the mechanism of action of retinoic acid on skin phospholipases are in progress. Nevertheless, the association of the development of erythema and scaly dermatosis in the guinea-pig skin with the elevation and decrease of endogenous prostaglandins after daily topical treatment with retinoic acid suggests, at least in part, a selective mode of action of retinoic acid in this tissue.

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