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13-HYDROXYOCTADECADIENOIC ACID REVERSES EPIDERMAL HYPERPROLIFERATION VIA SELECTIVE INHIBITION OF PROTEIN KINASE C-β ACTIVITY

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13-Hydroxyoctadecadienoic acid (13-HODE) is a major lipoxygenase metabolite of linoleic acid in epidermis. Employing a docosahexaenoic acid (22:6n-3) induced model of hyperproliferative guinea pig epidermis, we demonstrated reversal of hyperproliferation by topical 13-HODE. To delineate a possible mechanism for 13-HODE effect, we demonstrated that topical 13-HODE was incorporated into 13-HODE-containing diacylglycerol (13HODE-DAG). This novel substituted-DAG which was markedly depleted in the hyperproliferative skin paralled the increased activities of PKC-α and β. Replenishment of the hyperproliferative epidermis with topical 13-HODE resulted in the accumulation of tissue 13HODE-DAG and the selective suppression of PKC-β activity. These data taken together suggest that the generation of putative 13-HODE-DAG and the selective suppression of PKC-β isozyme activity may play a role in modulating epidermal hyperproliferation.

Protein kinase C (PKC) is a calcium/diacylglycerol/phospholipid-dependent kinase which now is recognized to play an important role in the regulation of cellular proliferation and differentiation (1, 2, 3, 4). Molecular cloning and biochemical analysis have demonstrated the diversity of PKC isozymes in distinct tissues, with multiple isotypes found in a single tissue (5). The PKC isotypes: PKC-α and PKC-β are identified as the main PKC isozymes in normal epidermis (6).

An analysis of lipoxygenase products in the model of docosahexaenoic acid (22:6n-3, DHA) induced hyperproliferative epidermis revealed a selective and markedly depressed level of 13-hydroxyoctadecadienoic acid (13-HODE), a 15-lipoxygenase metabolite of linoleic acid (18:2n-6, LA) (7). Topical application of 13-HODE on the DHA-induced hyperproliferative guinea pig (GP) epidermis caused a reversal to a normal state. The reversal paralleled the in vivo replenishment of tissue with 13-HODE (7). These initial findings suggested that 13-HODE, may be involved in the

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regulation of epidermal hyperproliferation. Since a number of reports indicate that lipoxygenase products of arachidonic acid (HETEs) and linoleic acid (13-HODE) are incorporated into tissue and cellular phospholipids (8,9,10) and can be released by receptor-mediated stimulation of the cells as putative substituted diacylglycerol (DAG) (9, 10), we tested the hypothesis that the mechanism of 13-HODE reversal of epidermal hyperproliferation maybe, at least in part, due to the generation of 13-HODE-substituted DAG in epidermis which in turn, may function to modulate epidermal hyperproliferation via signal transduction/protein kinase C processes.

MATERIALS AND METHODS

Animal Treatment: Male Hartley guinea pigs (400-450 g) were purchased from Simonsen Laboratory (Gilroy, CA). The hair from the dorsal skin was removed and the dorsal skin demarcated into two regions along the spine as described previously (7). The right side serving as vehicle control was treated with ethanol/propylene glycol [70:30], containing 1.0% vitamin E (as antioxidant), while the left side serving as the experimental was treated with vehicle, containing DHA (0.5%) and 1% vitamin E. The treatments were topically applied twice daily (5 ml per side) covering an area of approximately 4 x 12 cm^2 for 5 days as described previously (7). Evidence of epidermal hyperproliferation appeared approximately after five days.

Topical Application of 13-HODE on 22:6n-3 Induced Hyperproliferative Skin: The DHA-treated hyperproliferative left side was treated with vehicle containing 0.1% of 13-HODE. The right side was treated with vehicle and served as non-13HODE treated control. The applications were twice daily (5 ml per side) over an area of approximately 4 x 12 cm^2 for additional four days.

Histological Evaluation and Epidermal DNA Synthesis: Biopsies (4 mm^2) from the whole skin were collected, stained with hematoxylin and eosin (H & E), and evaluated for epidermal thickening (acanthosis) under light microscopy as described previously (7). Mitotic activities in each treated group was ascertained by uptake of [^{3}H]thymidine into tissue as described previously (7).

Quantitative Analysis of Endogenous 13-HODEContaining Diacylglycerol (13HODE-DAG): The total lipids were extracted from the homogenized epidermis and then subjected to separation by TLC in the solvent system of diethyl ether: hexane: acetic acid (30:70:3.5, v/v/v). The plate was visualized in iodine tank and the band with chromatographic mobility as standard diacylglycerol (DAG) was eluted. For qualitative identification of putative 13HODE-containing DAG, a portion of the eluted total DAG fraction was subjected to hydrolysis with bacterial lipase (C. viscosum) as described previously (19). The hydrolytic release of 13-HODE from 13HODE-DAG was identified after separation by TLC in the solvent system of: petroleum ether:diethylether:methanol:acetic acid (80:20:2.5:1, v/v/v/v) and finally confirmed by characteristic separation on HPLC as described previously (11,19).

To quantitate total epidermal DAG, a second portion of the eluted DAG fraction from silica gel was phosphorylated by incubating with diacylglycerol-kinase and [γ-^{32}P]-ATP as described previously (12). The radioactivity which co-migrated with authentic standard of phosphatidic acid was ascertained by TLC using the solvent system, $CHCl_3$: Acetone: MeOH: HOAc: H_2O (50:20:15:10:5, v/v/v/v/v). An aliquot of the eluted fraction was counted in scintillation counter.

Subcellular Distribution of Epidermal Total Protein Kinase C: To determine subcellular distribution of tissue PKC, epidermis was homogenized and subjected to high speed (105,000 x g) centrifugation to obtain the high speed supernatant (cytosolic) and particulate (membrane) fractions as described previously (13). The resulting cytosolic fraction was applied onto DE-52 ion exchange

column. Partial purification of the cytosolic total PKC was achieved by gradient elution as described previously (13). The particulate fraction (containing the membrane-associated PKC) was solubilized with buffer containing 0.3% Triton X-100 as described previously (13). Solubilized membrane PKC was subjected to fractionation on DE-52 as described above for the cytosolic total PKC.

Determination of Epidermal PKC Isozymes: Epidermis was homogenized as described previously and the total PKC partially purified on DE-52 ion exchange chromatography. The pooled eluents were subjected to chromatography on hydroxylapatite column coupled to gradient elution as described previously (14). The column eluents containing PKC activity were pooled into separate fractions. A portion of each fraction (30 μg protein) was subjected to polyacrylamide gel electrophoresis (SDS-PAGE) (10%). Western Blot Assay was performed with specific monoclonal antibodies against specific PKC isozymes (α and β) as described previously (15). Another portion of each chromatographed fraction was also used to assay protein kinase C activity in incubation mixtures as previously described (13).

Statistics: Standard statistical methods were used to determine the mean values. For comparison between the observations, the two-tailed t-test (n=12) were used and are expressed as mean ± SEM. The probability (P) that statistical significance was reached was at 0.05 or below.

RESULTS

Histologic Evaluation and Epidermal DNA Synthesis of DHA-treated and 13-HODE-treated Epidermis: Histologically, the DHA-treated skin revealed marked epidermal hyperplasia characterized by acanthosis (thicking of the spinous layer) when compared with control ethanol/propylene glycol (vehicle) treated skin (Fig. 1 A and B). Topical application of 13HODE (0.1%) to DHA-treated skin reversed the hyperproliferative state (Fig. 1C).

Uptake of [^{3}H]thymidine ([^{3}H]Td) revealed that DHA-treated epidermis markedly incorporated [^{3}H]Td (233%) when compared to vehicle treated skin (Fig. 2). The DNA synthesis of DHA-treated

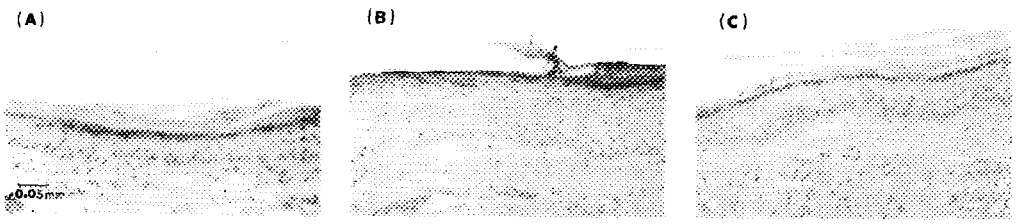

Figure 1. **Histologic evaluation for 22:6n-3 induced hyperproliferation and reversal by 13-HODE (magnification x 175).**
Skin in Fig. 1A was treated with vehicle (ethanol/propylene glycol [30:70] containing as antioxidant 1% vitamin E); Skin in Fig. 1B was treated with 0.5% of DHA in vehicle containing 1% vitamin E. Note acanthosis and hyperkeratiosis in Fig. 1B ; Data in Fig. 1C represents skin in Fig. 1B treated with 0.1% 13-HODE in vehicle. Note the reversal of acanthosis in Fig. 1C.

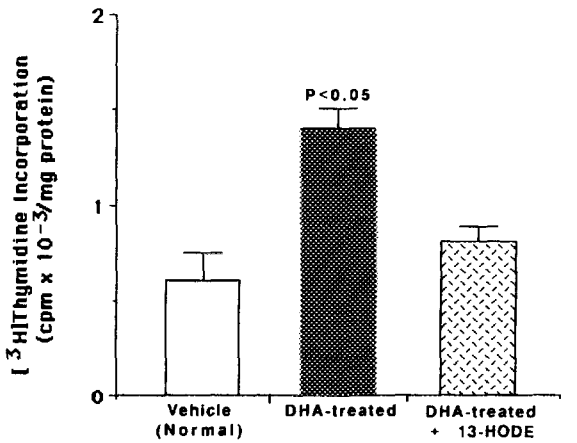

Figure 2. **[^{3}H] Thymidine incorporation into epidermal DNA in vehicle-treated (normal), DHA-treated (hyperproliferative) and DHA-treated plus 13-HODE (reversal) guinea pig epidermis.**

To measure relative rates of DNA synthesis epidermal discs from the vehicle-treated, DHA-treated and DHA-treated plus 13-HODE epidermis were incubated with [^{3}H] thymidine as described under Methods. DNA was analyzed by fluorescent reaction with ethidium bromide as described previously (7). Values represent Means $\pm$ SEM (n=12) from three separate experiments.

epidermis paralleled its hyperproliferative activity. Topical treatment with 13-HODE (0.1%) resulted in suppression of the tissue DNA synthesis as well as promote the clinical clearing of the lesion.

Quantitative Analysis of 13-HODE containing Diacylglycerol: To delineate a possible mode of action for 13-HODE in the reversal of DHA-induced epidermal hyperplasia, we investigated whether 13-HODE was incorporated into epidermal DAG (13HODE-DAG) since a recent report from this laboratory indicated that 13-HODE is incorporated into phosphatidyl 4,5-bisphosphate (19). Initially, total endogenous DAG and putative 13HODE-DAG in the epidermis were determined using the DAG-kinase assay. The data in Table 1 revealed that control vehicle-treated epidermis contained approximately, 480 pmol of total DAG per gram wet weight of epidermis when compared to 5.42 pmol of 13HODE-DAG. In contrast, total DAG in DHA-treated (hyperproliferative) epidermis was markedly elevated (970 pmol/g wet epidermis) while 13HODE-DAG was depressed markedly (0.97 pmol/g wet epidermis). Treatment with 13-HODE suppressed total DAG level to control 410 pmol/gram weight of epidermis while the level of 13HODE-DAG in the tissues was elevated to 24.6 pmol/gram wt. of epidermis.

TABLE 1. Altered Endogenous Levels of Total Diacylglycerol (DAG)/13HODE-Diacyglycerol (13HODE-DAG) in DHA-treated (hyperproliferative) and DHA-treated plus 13-HODE (reversal) Guinea Pig Epidermis

	Total DAG	13HODE-DAG	Ratio
	(pmol/g wet weight of epidermis)		total DAG:13HODE-DAG
Vehicle (normal)	480 ± 75.1	5.42 ± 0.3	88.56
DHA-treated	970 ± 36.5	0.97 ± 0.01	1000.0
DHA-treated + 13-HODE	410 ± 43.2	24.6 ± 0.83	16.67

The total lipids from each treated epidermis was extracted and fractionated for diacylglycerol (DAG) by TLC as described in Methods. To confirm that 13-HODE is incorporated into DAG, a portion of the eluted total DAG fraction was subjected to hydrolysis with bacterial lipase (C. viscosum). The release of 13-HODE as the hydrolytic product from 13HODE-DAG was confirmed first by TLC and secondly by HPLC as described in methods. The data represent the Mean ± SEM (n=12) samples from three separate experiments.

Total PKC activity of vehicle-treated, DHA-treated and 13-HODE treated Skin: To discern whether or not total tissue PKC was altered during the various skin treatments, total epidermal PKC activity from each treated group was determined. Our data revealed marked elevation of total PKC activity in the epidermal homogenate from DHA-treated epidermis when compared to vehicle-treated epidermis. Treatment with 13-HODE suppressed the elevated total PKC activity (data not shown). However, to localize the cellular compartments of the PKC activity, subcellular fractionation of the homogenate into cytosolic and particulate fractions revealed no significant alteration of cytosolic total PKC activities in the vehicle-treated, DHA-treated and the 13HODE-treated epidermis. In contrast the 105,000 g particulate (membrane) fraction revealed marked elevated total PKC activity (350% over control) in DHA-treated hyperproliferative epidermis when compared to vehicle treated epidermis (Fig. 3A). Topical treatment with 13-HODE markedly suppressed elevated membrane-PKC activity.

Immunological Identification of the PKC Activity: To determine the expression of total PKC in the epidermis after various treatments, an aliquot containing 30 μg protein from each of the epidermal homogenate of the treated animals was subjected to SDS-PAGE/Western-blot analysis. The data in Fig. 3B revealed a strong expression of an 80 KD membrane-associated (M) total PKC

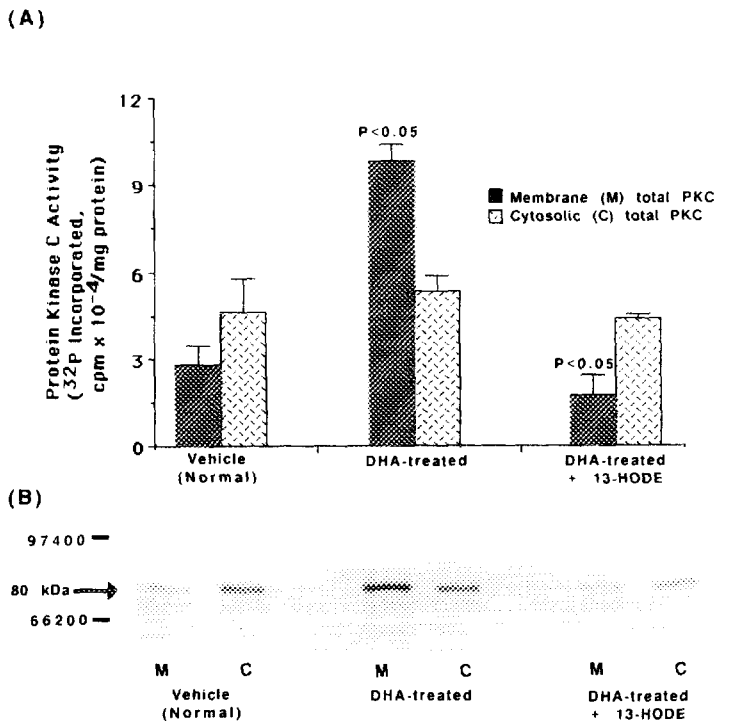

Figure 3. **Subcellular distribution of total PKC activities in vehicle treated (normal), DHA-treated (hyperproliferation) and DHA-treated plus 13-HODE (reversal) Guinea Pig Epidermis.**

Membrane-associated and cytosolic total PKC were prepared and assayed as described in Methods. **(A)** Represents total PKC-activity in the membrane and cytosolic fractions of the three groups of animals. **(B)** To confirm the subcellular distribution of total PKC, 30 μg protein of partially purified membrane-bound and cytosolic total PKC from each treated epidermis was subjected to separation on SDS-PAGE (10% gel) followed by Western-blot assay with specific PKC antibody as described under Methods. Values represent Means ± SEM (n-12) from three separate experiments.

in DHA-treated epidermis when compared to normal control epidermis. Topical application of 13HODE to the DHA-treated epidermis revealed dimished expression of the 80 KD membrane-associated PKC. Interestingly, the expression of 80 KD protein in Fig. 3B corresponded with the elevated PKC activity shown in Fig. 3A.

Altered PKC Isozyme activities in DHA-treated and 13-HODE-treated Epidermis: To determine whether or not the altered effects of total PKC activity was general or selective on the specific PKC-isozymes, the pooled eluent fractions from each treated epidermis were first fractionated on hydroxyapatite as reported previously and then subjected to SDS-PAGE/Western Blot. The PKC

isozymes in each collected fraction was ascertained with specific monoclonal PKC isozyme antibodies. Data from these assays revealed two major PKC-isozymes (PKC-α and PKC-β) in the G.P. epidermis (data not shown). The PKC activity of each fraction revealed that the activity of PKC-β was markedly elevated in DHA-treated epidermis (approximately 257% over control) when compared to vehicle-treated epidermis as shown in Fig. 4. Similarly, the activity of PKC-α was also moderately elevated (approximately 136%). Interestingly, topical application of 13-HODE to the DHA-treated epidermis selectively suppressed the elevated PKC-β activity while exerting negligible effect on PKC-α.

DISCUSSION

The data from these studies demonstrate that the topical application of DHA on guinea pig skin induced marked hyperplasia and hyperproliferation of the epidermis. The hyperproliferation paralleled decreased tissue levels of free 13-HODE and 13HODE-DAG. These decreases paralleled the marked increase of membrane-associated total PKC activity (Fig. 3A). Topical treatment with 13-HODE resulted in tissue replenishment of 13HODE-DAG and the suppression of elevated

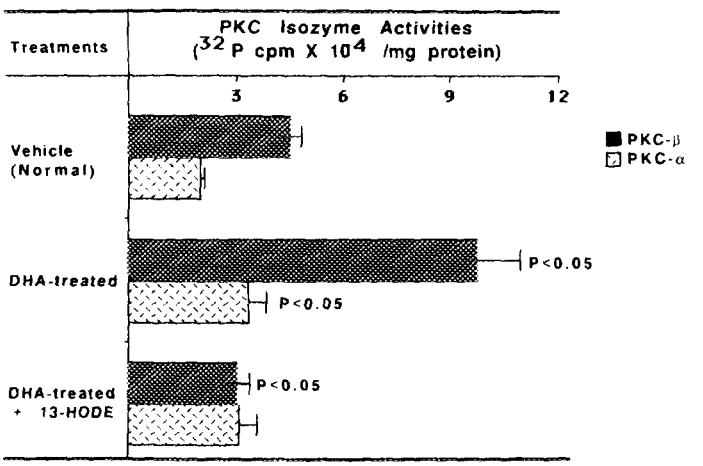

Figure 4. **Activities of PKC isozymes (β and α) in vehicle treated (normal), DHA- treated (hyperproliferative) and DHA-treated plus 13-HODE (reversal) guinea pig epidermis.**

Epidermal PKC isozymes were prepared and PKC activity in each treated group was assayed as described under Methods. Values represent Means ± SEM (n=12) from three separate experiments.

membrane-associated total PKC activity. These findings suggest that suppression of endogenous 13HODE-DAG in the epidermis could explain at least in part, the abnormal epidermal proliferation induced by DHA. These findings are consistent with reports that alterations of second messengers: inositol 1,4,5-trisphosphate and diacylglycerol may be associated with epidermal hyperproliferation (17,18).

The diversity of PKC in distinct tissues have revealed multiple isotypes with differential activities. For example, PKC-β isozyme was reported to be selectively required for the proliferation of human erythroleukemia cells whereas PKC-α is involved in differentiation (16). Data from our studies reveal that PKC-β isozyme activity is markedly elevated in the model of DHA-induced hyperproliferative epidermis (Fig. 4) and was significantly suppressed after treatment with free 13-HODE. In contrast, PKC-α activity although was elevated in DHA-treated was not altered after 13-HODE treatment. These studies, therefore, underscore the selective response of PKC-β to natural 13HODE-DAG in modulating epidermal hyperproliferation, suggesting that the activities of other PKC-isoforms may be regulated by other selective inhibitors.

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