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SELECTIVE EXPRESSION OF 15-LIPOXYGENASE ACTIVITY BY CULTURED HUMAN KERATINOCYTES

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Human keratinocytes isolated from neonatal skin express 15-lipoxygenase activity at a level far greater than that of any of the other pathways for lipoxygenation of arachidonic acid. The 10,000 x g supernatant of sonicates of 10^6 keratinocytes generates 15-hydroxy-eicosatetraenoic acid from 5 μg/ml of arachidonic acid at a mean maximum rate of 38 ng/30 min at 37°C, that is similar to the activity of the 15-lipoxygenase of human airway epithelial cells and greater than that of endothelial cells and leukocytes. The unique mediators derived from the 15-lipoxygenation of arachidonic acid, that stimulate secretion and exert hyperalgesic effects, may achieve a concentration in skin sufficient to regulate local cellular and neural functions.

The 5-lipoxygenation of arachidonic acid (AA)* in many types of leukocytes yields 5-hydroxy-eicosatetraenoic acid (5-HETE) and more complex products with additional polar substituents, that constititute a family of potent lipid mediators termed leukotrienes (1-3). The C6-peptide leukotrienes C_4, D_4, and E_4 are predominantly smooth muscle contractile and vasoactive factors, while the specific 5,12-di-HETE designated leukotriene B_4 stimulates PMN leukocyte migration and other functions, and inhibits T-lymphocyte activities (1-3). The 15-HETE and di-HETEs generated by the 15-lipoxygenation of arachidonic acid in eosinophils, neutrophils, endothelial cells, and pulmonary airway epithelial cells (4-7) lack most of the mediator activities of the 5-lipoxygenase products. The unique effector functions of the 15-lipoxygenase products are exemplified by the capacity of 15-HETE to stimulate secretion

* Abbreviations used are: AA, arachidonic acid; 5-HETE, 5-hydroxy-eicosatetraenoic acid; DME, Dulbecco's modified Eagle's medium (with 4.5 g of glucose/L); PBS, phosphate-buffered saline; FBS, fetal bovine serum; PMSF, phenylmethylsulfonyl fluoride; HPLC, high performance liquid chromatography; and EDTA, ethylenediaminetetraacetate.

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from epithelial cells (8) and the hyperalgesic effect of 8(R),15(S)-di-hydroxy-eicosa-5 cis-9,11,13 trans-tetraenoic acid on primary sensory neurons (9). The current finding that human keratinocytes express predominantly a 15-lipoxygenase pathway suggests that such products may have important roles in the regulation of cutaneous secretory and neural functions.

MATERIALS AND METHODS

Dulbecco's modified Eagle's medium with 4.5 g of glucose/L (DME) and Dulbecco's phosphate-buffered saline without calcium or magnesium (PBS) (Cell Culture Facility, UCSF); fetal bovine serum (FBS) (Whittaker M.A. Bioproducts, Inc., Walkersville, MD); mitomycin, hydrocortisone, cholera toxin, phenylmethylsulfonyl fluoride (PMSF), and indomethacin (Sigma Chemical Co., St. Louis, MO); trimethyl phosphite (Aldrich Chemical Co., Milwaukee, WI); prostaglandin B_2 (PGB_2) (The Upjohn Co., Kalamazoo, MI); amphotericin (fungizone), glutamine, trypsin, and collagenase (Grand Island Biological Co. Laboratories, Grand Island, NY); mouse submaxillary gland epidermal growth factor (Collaborative Research, Inc., Lexington, MA), arachidonic acid (AA) (Supelco, Inc., Bellefonte, PA); [^{3}H]arachidonic acid ([^{3}H]AA) (New England Nuclear, Inc., Boston, MA); kits for the radioimmunoassay of 15-HETE, 12-HETE, and 5-HETE (Seragen, Inc., Boston, MA); and high-performance liquid chromatography (HPLC)-grade organic solvents that had been redistilled from glass (Burdick and Jackson Laboratories, Inc., Muskegon, MI) were obtained from the designated suppliers. Synthetic 15-HETE and 5-HETE were a generous gift from Professor E.J. Corey (Harvard University, Cambridge, MA). 12-HETE was biosynthesized, purified, and characterized as described (10).

Primary cultures of human keratinocytes were initiated with cells dispersed from foreskins by physical disaggregation and treatment with trypsin and collagenase as described (11). Suspensions of the keratinocytes were plated on feeder layers of one-third the number of mitomycin-treated 3T3 mouse fibroblasts, that had been established 48 hr previously, in 100 mm diameter plastic wells and incubated in DME containing 10% (v:v) FBS, 0.4 µg/ml of hydrocortisone, 1 nM cholera toxin, 20 ng/ml of epidermal growth factor, 1 µg/ml of glutamine, and 2.5 µg/ml of fungizone at 37°C under 5% CO_2:95% air. When cell density reached 2.0 x 10^7/well, the adherent keratinocytes were washed twice with PBS, scraped up into 4 ml of PBS/well, centrifuged, and resuspended in 4 ml/well of 50 mM Tris-HCl (pH 7.0) containing 1 mM disodium ethylenediaminetetraacetate (EDTA), 100 µM PMSF, and 40 µM indomethacin. The suspensions were sonified at 150W for 2 min at 4°C to break up all keratinocytes, the sonicates were centrifuged at 10,000 x g for 30 min at 4°C, and the supernatants were either used immediately or frozen at -70°C. For most studies, 950 µl of keratinocyte supernatant, 45 µl of 100 mM $CaCl_2$, and 5 µg of AA or 0.1 µCi of [^{3}H]AA (specific activity = 87.2 Ci/mmol) in 5 µl of ethanol were incubated for 30 min at 37°C and the reaction stopped by the addition of 1 ml of methanol at 4°C, 175 µg of trimethyl phosphite, and 200 ng of PGB_2. After centrifugation at 2,000 x g for 10 min at 4°C, each supernatant was acidified with 0.5 M acetic acid to pH 4.0 and applied to a prewashed Sep-Pak column (Waters Associates, Inc., Milford, MA), from which the products of AA were eluted in methanol.

The products of AA and [^{3}H]AA were resolved by HPLC on a 4.6 mm x 150 mm column of 5 µm octadecylsilane (Altex-Beckman Instruments, Inc., Mountain-view, CA) that was developed isocratically with methanol:0.1% glacial acetic acid in water (77:23, v:v) at a flow rate of 1 ml/min in a Beckman Instruments, Inc. system with two metered 100 A pumps and a diode-array detector controlled by an HP-85B personal computer and disc storage module

(Hewlett Packard, Inc., Palo Alto, CA), that analyzed the ultraviolet light absorption spectrum of each product in the eluate. Portions of the eluate also were analyzed for 15-HETE, 12-HETE, and 5-HETE by radioimmunoassays of greater than 99% specificity and 10 pg sensitivity (12). The identity of the 15-HETE purified from keratinocyte products was confirmed by gas chromatography-mass spectrometry in the laboratories of Dr. Frank Sun (The Upjohn Co., Kalamazoo, MI) (5,10).

RESULTS AND DISCUSSION

The incubation of [^{3}H]AA with the 10,000 x g supernatants of sonicates of cultured human keratinocytes resulted in time-dependent generation of [^{3}H]15-HETE, as assessed by HPLC, that accounted for a mean of 40% of the initial radioactivity by 45 min and more than 95% of all of the lipoxygenase products recovered at each of the times examined (Fig. 1). The rate of conversion of [^{3}H]AA to [^{3}H]15-HETE was markedly diminished at 4°C relative to 37°C, with a 94% mean reduction in the formation of [^{3}H]15-HETE at 45 min. Similarly, boiling of replicate portions of the supernatants prior to the introduction of [^{3}H]AA suppressed the generation of [^{3}H]15-HETE by a mean of 95%. The omission of EDTA or PMSF during sonification of the keratinocytes led to loss of 15-lipoxygenase activity in the supernatants, as reflected in a more than 25% mean reduction in the generation of 15-HETE at 30 min. In contrast, the generation of 15-HETE was not altered significantly by the elimination of indomethacin from the incubation buffer.

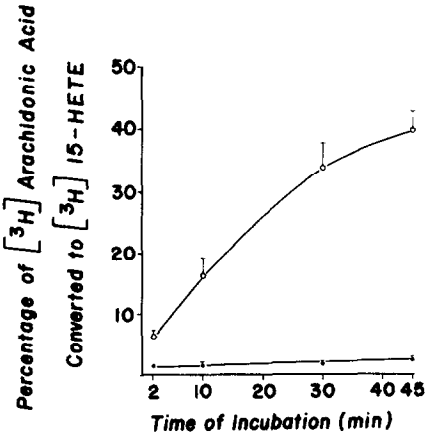

Fig. 1: Time-course of generation of [^{3}H]15-HETE from [^{3}H]AA by cultured human keratinocytes.
Each point and bracket is the mean and standard deviation of the results of three-four replicate determinations of the percentage conversion at 37°C (o) and 4°C (•) in three experiments with different preparations of keratinocytes.

The possibility that the feeder layer of 3T3 mouse fibroblasts contributed to the 15-lipoxygenation of AA was evaluated by determining the rates of generation of 15-HETE by supernatants of sonicates of replicate portions of 3.5×10^6 3T3 mouse fibroblasts alone, that represent approximately the number of feeder cells found in two wells of keratinocytes. The mean rate of generation of 15-HETE by the feeder 3T3 mouse fibroblast preparations was less than 5% of the concurrent rate for preparations from mixtures of keratinocytes and feeder cells. In addition, the incubation of AA with supernatants from replicate lysates of 2×10^6 human foreskin fibroblasts, that were cultured and sonified by identical techniques, yielded a mean of less than 5% of the quantity of [^{3}H]15-HETE produced by supernatants from the same number of keratinocytes.

Supernatants of sonicates of 5×10^6 keratinocytes were incubated with 5 µg/ml of AA for 30 min at 37°C in order to generate quantities of 15-HETE sufficient for definitive identification. HPLC resolved a quantitatively predominant peak of optical density at 235 nm, that co-eluted with the synthetic 15-HETE standard, from much lesser amounts of 12-HETE and 5-HETE, as well as from more polar compounds that eluted within the first 16 min (Fig. 2). The ultraviolet light absorption spectrum of the 15-HETE peak was that of a mono-HETE. The peak of 15-HETE immunoreactivity in the eluate coincided in time with that of the optical density at 235 nm and with the time of elution of synthetic 15-HETE. The total quantity of immunoreactive 15-HETE in the eluate from HPLC was within 20% of the quantity of 15-HETE determined by calculation from the integrated value of total optical density in the same peak of the eluate in this and another chromatogram. The quantities of immunoreactive 12-HETE and 5-HETE in the eluate corresponding to the time of appearance of the respective standards were less than 10% of that of 15-HETE. Analysis by gas chromatography-mass spectrometry of the keratinocyte-derived 15-HETE purified by HPLC revealed a fragmentation pattern identical to that of synthetic 15(S)-HETE (4).

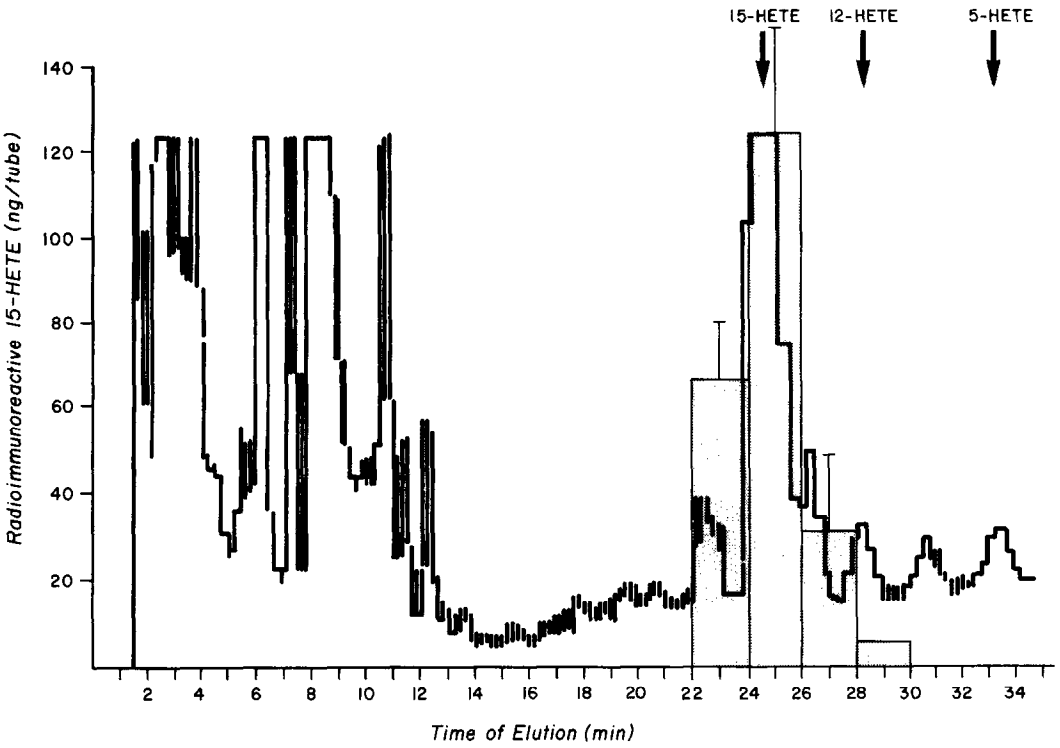

Fig. 2: High-performance liquid chromatography (HPLC) of the products of oxygenation of arachidonic acid by cultured human keratinocytes. The products generated by incubating the 10,000 x g supernatant of sonicates of 5 x 10^6 keratinocytes with 5 μg of AA for 30 min at 37°C were extracted, separated from unreacted AA, and resolved by HPLC. The continuous line tracing depicts the optical density in the eluate at 269 nm from 0-16 min and at 235 nm from 16-32 min. Each bar and bracket represents the mean and standard deviation of the results of four determinations of the quantity of immunoreactive 15-HETE. No immunoreactivity was found in other portions of the eluate.

15-HETE was known to be a major product of the lipoxygenation of AA in human airway epithelial cells (7) and endothelial cells (6), as well as eosinophils (4) and neutrophils (5) of several species. The 15-HETE generated by human skin tissue was attributed totally to the 15-lipoxygenase activity of human dermal fibroblasts (13,14). Neonatal mouse and human isolated keratinocytes converted [^{14}C]AA to metabolites resembling [^{14}C]15-HETE and [^{14}C]12-HETE, by chromatographic criteria, but the purported 15-HETE product was neither quantified chemically nor identified definitively (15,16). In contrast to the findings with adult human keratinocytes (17), the neonatal keratinocytes exhibited minimal 5-lipoxygenase activity. The present results demonstrate now that neonatal human keratinocytes exhibit predominantly a 15-lipoxygenase pathway that generates large quantities of authentic 15-HETE

(Fig. 1,2), with only barely detectable 12- and 5-lipoxygenase activities (Fig. 2). The amounts of 15-HETE produced are similar in magnitude to those generated from 5 μg/ml of AA by human airway epithelial cells (7), with maximal levels of 40-100 ng/10^6 cells/30 min at 37°C that far exceed the output from endothelial cells and leukocytes. The capacity of 15-HETE to inhibit the activity of 5-lipoxygenases (18) suggests that keratinocyte products may suppress the generation of pro-inflammatory leukotrienes by infiltrating leukocytes characteristic of psoriasis and other neutrophilic dermatitides. Future studies will be designed to elucidate the biochemical characteristics and cellular requirements for expression of the 15-lipoxygenase pathway of human keratinocytes and to examine the roles of the 15-HETE and di-HETEs in normal physiological responses and disease states of human skin.

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