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IN VIVO ACTIVATION OF AN ω-6 OXYGENASE IN HUMAN SKIN

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To test the hypothesis that an epidermal fatty acid oxygenase is activated *in vivo* under physiologic conditions, surface lipids from normal human skin were analyzed for oxygenase products. With high-performance liquid chromatography on reversed-phase and straight-phase chiral columns and gas-liquid chromatography/mass spectrometry, these lipids were found to contain free 13-hydroxyoctadeca-9Z,11E-dienoic acid and 9-hydroxyoctadeca-10E,12Z-dienoic acid. The 13-hydroxyoctadecadienoic acid was present as a stereoisomeric mixture, with an average S/R ratio of 2.2, and exceeded the concentration of 9-hydroxyoctadecadienoic acid by a factor of 2. These observations and others indicate that the 13-hydroxyoctadecadienoic acid was derived mostly from an ω-6 oxygenase (probably 15-lipoxygenase) which is activated *in vivo* in normal skin.

Lipoxygenase activation, particularly of inflammatory cells, is thought to represent a predominantly pathological event. The only demonstration of a physiological role for lipoxygenase activation has been in the erythrocyte, where a 15-lipoxygenase may be essential for maturation of the reticulocyte (1,2). Cultured human keratinocytes contain 15-lipoxygenase (*i.e.* ω-6 oxygenase), but a physiological role for this enzyme in these cells has yet to be determined (3-5). Human epidermal cells demonstrate fatty acid oxygenase activity when studied *ex vivo* (6,7). It is not known whether these oxygenases are normally activated *in vivo* or whether their activation *ex vivo* is a consequence of the tissue removal procedure or the subsequent *in vitro* handling (8). To date, there has been no evidence that lipoxygenases are activated in the normal epidermis. In this paper, solid evidence for the activation of an ω-6 oxygenase in normal human epidermis is provided by the demonstration of the presence of enzymatically-derived 13-HODE in surface lipids isolated from normal human skin. Further evidence suggests that this ω-6 oxygenase activity is that of a 15-lipoxygenase, as has been demonstrated in cultured human keratinocytes.

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ABBREVIATIONS

13-HODE (13-hydroxyoctadeca-9Z, 11E-dienoic acid); 9-HODE (9-hydroxyoctadeca-10E, 12Z-dienoic acid); HPLC (high performance liquid chromatography); LC (liquid chromatography); GLC/MS (gas-liquid chromatography/mass spectrometry); RT (retention time).

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MATERIALS AND METHODS

Reagents: 13(S)-hydroxyoctadeca-9Z,11E-dienoic acid was obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Racemic 13-hydroxyoctadeca-9Z,11E-dienoic acid, racemic 9-hydroxyoctadeca-10E,12Z-dienoic acid, and 9(S)-hydroxyoctadeca-10E,12Z-dienoic acid were obtained from Cayman Chemical Company (Ann Arbor, MI). The methyl esters of these linoleic acid derivatives were prepared using ethereal diazomethane. All solvents were of HPLC grade. Lipid standards (cholesterol, cholesteryl linoleate, squalene, tripalmitin, ceramide, and palmitic acid palmityl ester) for thin-layer chromatography were obtained from Sigma Chemical Co. (St. Louis, MO).

Source material: Stratum corneum powder was obtained from the heels of 5 normal adult volunteers, three males and two females. The heels were first wiped with 70% isopropyl alcohol and then gently abraded using a rasp in order to produce a fine powder. Beard hair was collected from 3 adult males. Sebum was collected from cosmetic-free foreheads of male and female volunteers with a cotton pledget saturated with either 70% isopropyl alcohol or ether. Sebum pooled from 4-25 individuals was used for each analysis.

Extraction procedure: Stratum corneum powder (218-1422 mg), beard hair (200-1189 mg), and 4-25 forehead swabs were extracted for each analysis with methanol/chloroform (2:1, vol:vol) at 37°C. The extracted lipids were then subjected to a two-phase extraction with methanol:chloroform (2-4:1, vol:vol) followed by water (0.9 vol) and 2.0 M ammonium formate, pH 3.2 (0.1 vol). The lower organic layer was washed twice with pure upper phase solvents and then dried under vacuum. The quantity of extracted lipids was determined gravimetrically. The extracted lipids were reconstituted into a mobile phase consisting of methanol:water:acetic acid, 80:20:0.1, for the separation of the monohydroxylated derivatives of arachidonic acid and linoleic acid.

Reversed-phase HPLC was performed isocratically on a Hewlett-Packard 1090 liquid chromatograph (LC) using Hewlett-Packard ODS-Hypersil columns 20 cm in length x 4.6 mm at a flow rate of 0.4 ml/min. This instrument is equipped with a diode array spectrophotometer and a computer for on-line display and storage of ultraviolet spectra, and thus precise measurement of the λ_{max} of each peak can be performed after each run is completed. The LC runs were monitored at a wavelength of 236 nm. All chromatograms shown in the figures are reproduced directly from the LC computer plots. Software programs were used to calculate the quantities of lipoxygenase products with conjugated diene structures by measurement of the area beneath the peak height at a given λ_{max}. A molar extinction coefficient of 23,000 $M^{-1}cm^{-1}$ for both 13- and 9-HODE was used in these calculations (9). The average recovery for each chromatographic step was 80%.

Stereochemical analyses: The LC eluate at the retention time of 13/9-HODE was collected during the LC runs, methylated with ethereal diazomethane, and then rechromatographed on the same LC columns (delayed retention time of approximately 13 min). The methylated material was collected during the LC run and then rechromatographed on two 25 cm Bakerbond chiral phase LC columns in series (dinitrobenzoyl phenylglycine coupled ionically with aminopropyl residues; J. T. Baker Inc., Phillipsburg, NJ). The mobile phase for this straight-phase HPLC consisted of hexane:isopropanol, 1000:15. Mixing experiments with synthetic standards were carried out for each product to confirm co-elution in the chiral phase studies.

Gas-liquid chromatography/mass spectrometry was carried out on a gas chromatograph (Hewlett-Packard, model 5890, Series II) interfaced with a mass selective detector (Hewlett-Packard, model 5971A). The mass spectrometer was operated in the electron impact mode with an ionization voltage of 70 eV. The trimethylsilyl ether derivatives of hydroxylated fatty acid methyl esters purified from their biologic source with reversed-phase and straight-phase liquid chromatography were prepared by the addition of 20 µl of pyridine and 40 µl of bis(trimethylsilyl)-trifluoroacetamide (Aldrich Chemical Co., Milwaukee, WI).

Thin-layer chromatography: The lipids extracted from hair and from heel stratum corneum were analyzed by one-dimensional thin-layer chromatography using silica gel plates (Whatman LabSales, Hillsboro, OR). The plates were developed using benzene:hexane, 1:1, followed by hexane:diethyl ether:glacial acetic acid, 70:30:0.1, as described by Imokawa et al (10). The lipid bands were visualized with iodine vapor. These chromatographic conditions resolve lipids characteristic of sebum, *e. g.* squalene and wax esters, from those which are common to both stratum corneum and sebaceous lipids (10).

RESULTS

When equal amounts of lipids extracted from stratum corneum and from hair were analyzed by thin layer chromatography, bands corresponding to the relative mobilities of squalene and palmityl wax ester were evident only in the lanes applied with the lipids extracted from hair. This observation supported the distinction between two types of skin surface lipids that were analyzed: sebaceous lipids collected from foreheads of normal individuals or extracted from hair, and stratum corneum lipids extracted from material obtained from the heels of normal volunteers.

Reversed-phase liquid chromatography of the lipids extracted from hair, heel stratum corneum, and forehead sebum uniformly demonstrated a peak at 20.5 minutes which shared the retention time of authentic 13-hydroxyoctadeca-9Z,11E-dienoic (13-HODE) and 9-hydroxy-octadeca-10E,12Z-dienoic (9-HODE) acid standards (data not shown). These peaks demonstrated an ultraviolet absorption maximum of 234 nm. No peaks with UV absorption maxima of 236 nm were seen at the retention times of 15, 12, and 5-hydroxyeicosatetraenoic acids. When the material eluting at 20.5 minutes was collected, treated with ethereal diazomethane and re-chromatographed on the same LC columns, the retention time of the compound was delayed to 33.5 minutes and had a UV absorption maximum of 234 nm and a retention time identical with the methyl esters of the authentic standards, 13-HODE and 9-HODE (data not shown). This material was again collected, and then chromatographed on straight-phase chiral columns. Two separate pairs of peaks were seen at the retention times of authentic 13-HODE and 9-HODE methyl ester standards (Figures 1 and 2). Mixing experiments were performed as illustrated in Figures 1 and 2 to confirm the identity of the two compounds as the methyl ester derivatives of 13-HODE and 9-HODE. On the straight-phase chiral chromatograms, the amount of 13-HODE methyl ester exceeded that of 9-HODE by a factor of 2 (Table I). Additional confirmation of the structural identity of these two

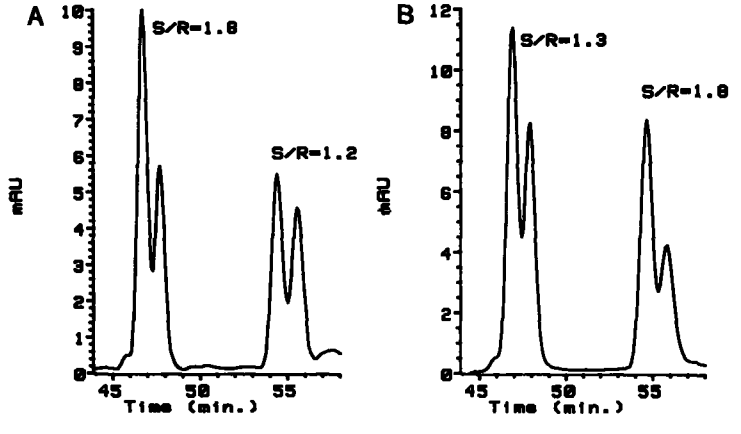


Figure 1. Representative straight-phase chiral HPLC chromatograms of the methyl esters of 13-HODE (RT=46-49) and 9-HODE (RT=53.5-56.5). The chromatogram in panel A is that of free 13-HODE and 9-HODE which was isolated from heel stratum corneum of a normal individual and then methylated *in vitro* prior to the straight-phase chiral chromatography shown here. The chromatogram in panel B is that of the same material to which the methyl esters of racemic 13-HODE and 9(S)-HODE have been added. The S/R ratios of the original mixtures showed the expected changes after the addition of the authentic methyl ester standards.

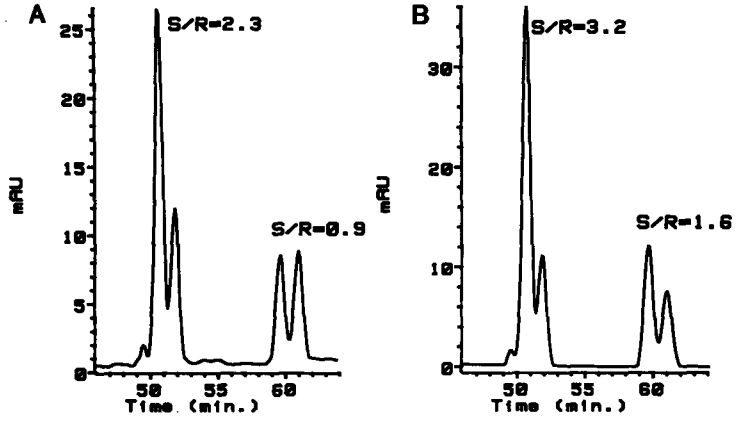


Figure 2. Same as Figure 1 except that the lipid source was beard hair. The mixing experiments utilized the methyl esters of 13(S)-HODE and 9(S)-HODE. The S/R ratios of the original mixtures increased after the addition of the authentic methyl ester standards.

compounds was achieved by gas-liquid chromatography/mass spectrometry (GLC/MS) of the trimethylsilyl ether derivatives of the material eluting from the straight-phase chiral columns. On GLC/MS, the derivative of 13-HODE methyl ester eluted as a single peak at 4.2 minutes and had a mass spectrum with major fragment ions at m/z 382 (M^+), 311 (M^+-71), 225 (M^+-157), and 130 (M^+-252) (Figure 3). There was a preponderance of the fragment at m/z 311 over that at m/z 225 (ratio=1.8), a finding characteristic of the trimethylsilyl derivative of 13-HODE methyl ester (11). The derivative of 9-HODE methyl ester eluted at the same retention time as 13-HODE methyl ester and had the same major fragment ions except that the relative abundance of the fragment at m/z 225 was greater than that at m/z 311 (Figure 4).

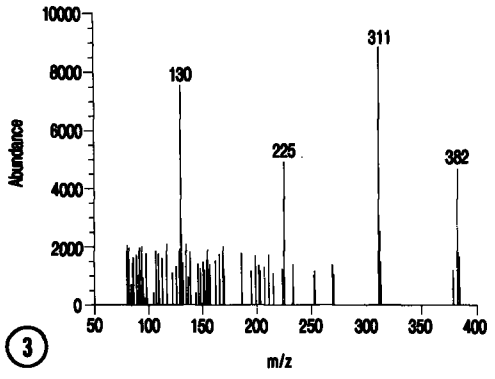
The S/R ratios of the 13-HODE and 9-HODE extracted from stratum corneum of the heel and finger, hair, and forehead swabs are shown in Table I. In one experiment, lipids were pooled from the foreheads from 4 volunteers both before and three hours after cleansing the forehead with a pledget saturated in ether. The S/R ratios of the 13-HODE and the 9-HODE did not differ between the two resulting samples.

The amount of the 13-HODE was determined by quantitating the area of the peaks of the stereoisomers of this material resolved on straight-phase chiral chromatography. The resultant figure was then corrected for losses occurring during the initial extraction and subsequent chromatographic analyses which preceded the final straight-phase chiral chromatographic runs. The concentration of 13-HODE in heel stratum corneum averaged 17 ± 6 ng/mg extracted lipid

Table I. Characteristics of the skin surface lipid 13-HODE and 9-HODE as demonstrated by straight-phase chiral chromatography

Lipid source	n	Product ratios	S/R ratios of the stereoisomers	
		13-HODE/9-HODE	13-HODE	9-HODE
Stratum corneum (heel)	5	2.0 ± 0.2*	2.0 ± 0.2	1.0 ± 0.1
Hair	3	1.8 ± 0.2	1.8 ± 0.3	0.9 ± 0.04
Sebum (forehead)	3	2.1 ± 0.1	2.7 ± 0.3	1.1 ± 0.2

*Data are presented as the mean ± SEM.



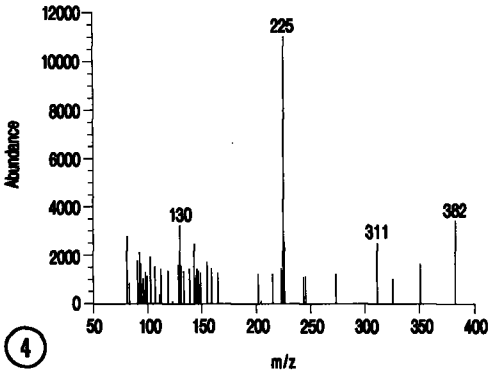


Figure 3. Mass spectrum of the trimethylsilyl derivative of the methyl ester of 13-HODE. The original material, free 13-HODE, was isolated from pooled human sebum and methylated *in vitro*. The 13-HODE methyl ester eluting from the straight-phase chiral columns was collected and derivatized prior to the GLC/MS shown here.

Figure 4. Mass spectrum of the trimethylsilyl derivative of the methyl ester of 9-HODE. The original material, free 9-HODE, was isolated from pooled human sebum and methylated *in vitro*. The 9-HODE methyl ester eluting from the straight-phase chiral columns was collected and derivatized prior to the GLC/MS shown here.

(mean ± SEM, n=5). In the sebaceous lipids collected from hair and from the forehead, the concentration of 13-HODE averaged 14 ± 8 ng/mg extracted or pooled lipid (n=5).

DISCUSSION

In the current study, it is demonstrated that both stratum corneum and sebaceous lipids contained two monohydroxylated derivatives of linoleic acid, 13-HODE and 9-HODE, which were both present in a non-esterified (free) form. The 13-HODE was present in concentrations which exceeded that of 9-HODE by a factor of 2. The 13-HODE was consistently found to be a non-racemic mixture of its stereoisomers, with average S/R ratios which ranged from 1.8 to 2.7. These two observations provide proof that the 13-HODE was derived at least in part from the action of an ω-6 oxygenase. If the 13-HODE had been derived from non-enzymatic (auto)oxidation, then the amount of 13-HODE would have equalled that of 9-HODE and the 13-HODE would have been racemic. In contrast to the 13-HODE, the 9-HODE isolated from stratum corneum and sebaceous lipids was racemic and thus a distinction between an enzymatic *vs.* non-enzymatic origin for this compound cannot be made. While lipase activity is found in the bacteria which colonize the skin surface (12), fatty acid ω-6 oxygenase activity has not been reported in any type of microorganism (13).

There are several lines of evidence to suggest that the ω-6 oxygenase responsible for the production of 13-HODE in normal skin is 15-lipoxygenase. First, the observed regiospecificity with the predominant production of 13-HODE over that of the 9-HODE regioisomer is characteristic of 15-lipoxygenase activity (14). In contrast, the preferred regiospecificity of cyclooxygenase is the ω-10 position, resulting in the predominant production of 9-HODE as compared to 13-HODE when linoleic acid is the substrate (15-17). Second, incubations of human epidermal cell suspensions with arachidonic acid *in vitro* leads to the generation of 15(S)-HETE, a

product whose stereospecificity is characteristic of mammalian lipoxygenases (6,18). Third, cultured human keratinocytes have been shown to have a 15-lipoxygenase (*i.e.* ω-6 oxygenase) which has been partially characterized (3-5). The 15-lipoxygenase of cultured keratinocytes produces almost exclusively 13(S)-HODE and <1% racemic 9-HODE from linoleic acid (5 and unpublished studies). The present chirality data, which contrast with that derived from *in vitro* enzymatic studies, most likely reflect some contribution of autooxidation to the product pool in skin surface lipids.

The 13-HODE in the stratum corneum and sebaceous lipids was not enantiopure as would be expected for lipoxygenase action alone. We have previously documented similar findings in psoriatic skin scales, where the 13-HODE is present as a stereoisomeric mixture with a S/R ratio which averages 1.9:1 (19). Incubation of psoriatic skin scales *in vitro* with radiolabelled arachidonic acid or linoleic acid leads to the respective generation of 15(S)-HETE and 13(S)-HODE (20). This latter observation supports the conclusion that the 13(S, R)-HODE extracted from psoriatic skin scales represents an admixture of a lipoxygenase-derived 13(S)-HODE with racemic material derived from autooxidation. A similar mechanism most likely accounts for the lack of enantiopurity of the 13-HODE in lipids derived from normal human skin.

The role of 15-lipoxygenase in the epidermis needs to be defined. Nugteren *et al* (21,22) have suggested that skin lipoxygenases are essential to the maintenance of the epidermal barrier to water loss. A 15-lipoxygenase plays a role in the degradation of mitochondria and possibly other intracellular organelles in reticulocytes, a process which may be essential to the maturation of the erythrocyte (1,2). It is possible that a 15-lipoxygenase may play a role in the terminal differentiation of keratinocytes and sebocytes, processes which involve disintegration of subcellular membranes, akin to that which occurs in the reticulocyte.

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