

Stereospecificity of the products of the fatty acid oxygenases derived from psoriatic scales

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Abstract The principal *in vivo* oxygenase products of arachidonic acid and linoleic acid in psoriatic skin scales are 12-hydroxyeicosatetraenoic acid (R/S ratio = 5.7), 13-hydroxyoctadecadienoic acid (S/R = 1.9), and 9-hydroxyoctadecadienoic acid (R/S = 2.4). Definition of the enzymatic origin of these fatty acid derivatives is an important step in assessing their possible role in the pathogenesis of psoriasis. Psoriatic skin scales were incubated with radiolabeled arachidonic acid and linoleic acid and the monohydroxylated derivatives produced *in vitro* were characterized. The products of incubation with [³H]arachidonic acid were an enantiopure 15(S)-[³H]hydroxyeicosatetraenoic acid and a nonracemic mixture of the 12-[³H]hydroxyeicosatetraenoic acid stereoisomers (R/S ratio = 4.5). An enantiopure 13(S)-[¹⁴C]hydroxyoctadecadienoic acid was produced from [¹⁴C]linoleic acid. No radiolabeled products were derived from incubations with heat-denatured scales. These results provide evidence for two distinct oxygenase activities that are preserved in psoriatic skin scales. One is that of an ω -6 oxygenase with strict (S) stereospecificity, consistent with the activity of a lipoxygenase. This enzyme activity appears to be similar to that of the 15-lipoxygenase which has been described in cultured human keratinocytes. The second activity is that of an arachidonic acid 12(R)-oxygenase that has not been observed in normal human epidermis but which appears to be expressed in psoriatic epidermis. — Baer, A. N., P. B. Costello, and F. A. Green. Stereospecificity of the products of the fatty acid oxygenases derived from psoriatic scales. *J. Lipid Res.* 1991. 32: 341–347.

Supplementary key words 15-lipoxygenase • psoriasis • 12-hydroxyeicosatetraenoic acid • arachidonic acid • 12(R)-oxygenase

The psoriatic epidermis contains monohydroxylated derivatives of linoleic and arachidonic acids, some of which are present in biologically active concentrations. The principal fatty acid oxygenase product in psoriatic epidermis is 13-HODD, an ω -6 oxygenase product of linoleic acid (1–3). The principal eicosanoid is 12-HETE. Smaller amounts of 9-HODD and 15-, 11-, 9-, and 8-HETEs have also been described in extracts of psoriatic skin scales (3) or in keratome specimens of psoriatic epidermis (2, 4).

The oxygenases responsible for the generation of these fatty acid derivatives have not been characterized, yet this is an important step in defining the role of the products in pathogenesis of psoriasis. The stereochemistry of the principal fatty acid oxygenase products provides an indication as to the nature of the oxygenase, since mammalian lipoxygenases have been shown thus far to have strict (S) stereospecificity (5–7). In psoriatic skin scales, 13-HODD, 9-HODD, and 12-HETE are each present as nonracemic mixtures of their stereoisomers (1, 8). In an earlier study, we documented the average S/R ratio of 13-HODD to be 1.9 and the average R/S ratios of 12-HETE and 9-HODD to be 5.7 and 2.4, respectively (1). These stereochemical findings have suggested a nonlipoxygenase origin for the oxygenase products in psoriatic skin scales.

In the current study, we report on the stereochemistry of the oxygenase products generated *in vitro* during incubations of psoriatic skin scales with radiolabeled arachidonic acid or linoleic acid. The survival of active oxygenase enzymes in psoriatic skin scales is surprising and potentially important in characterizing the enzymes involved.

MATERIALS AND METHODS

Reagents

[5,6,8,9,11,12,14,15-³H]Arachidonic acid (210 Ci/mmol), [1-¹⁴C]linoleic acid (54 mCi/mmol), and 12(S)[5,6,8,9,11,12,14,15-³H(N)]HETE (172 Ci/mmol) were purchased

Abbreviations: HODD, hydroxyoctadecadienoic acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography; LC, liquid chromatography; HEPES, N-2-hydroxyethylpiperazine-n'-2-ethanesulfonic acid; RT, retention time; SEM, standard error of the mean.

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from Amersham (Arlington Heights, IL). 15(S)[5,6,8,9,11,12,14,15-³H(N)]HETE (187 Ci/mmol) was purchased from New England Nuclear Research Products (Dupont Company, Boston, MA). Synthetic 15(S)-HETE, 13(S)-HODD, 12- and 15-ketoeicosatetraenoic acids, and racemic 9-HODD were obtained from Biomol Research Laboratories (Plymouth Meeting, PA). Racemic 12-HETE and 15-HETE were prepared by reduction of the corresponding ketoeicosatetraenoic acid using sodium borohydride. All solvents were of HPLC grade.

Subjects

Skin scales were obtained from patients with chronic plaque psoriasis by gentle abrasion of their lesional skin with a scalpel blade. The scales were stored at -20°C until use. The sample of heel stratum corneum was obtained from a normal male volunteer by gentle abrasion with a file.

Oxygenase assay

Psoriatic skin scales, 26–108 mg, were hydrated in 1–2 ml medium 199 with 40 mM HEPES buffer (pH 7.6) prior to the incubations. The scales were incubated with either [³H]arachidonic acid (2–4 μCi; 9.5 nM) or [¹⁴C]linoleic acid (0.2–1.0 μCi; 3.7–18 μM) in the presence of 4.8% ethanol for 1 h at 37°C. The incubation was terminated by freezing the reaction vial in an acetone-dry ice bath. Control experiments were performed in which all conditions were kept the same except for the substitution of scales heat-denatured by immersion in boiling water for 20 min. In order to test for inhibition of the oxygenase activity by exposure to carbon monoxide, the scales were suspended in 250 μl air-saturated medium to which was added 750 μl medium gassed for 15 min with carbon monoxide or with nitrogen for the control. The incubations were then carried out in an atmosphere of room air. The molar gas ratios were calculated using the solubilities of the gasses in water. The effect of specific drugs on oxygenase activity was assessed by pre-incubating the hydrated scales with the drug for 15 min prior to adding [³H]arachidonic acid. Tests for inhibition of oxygenase activity were each performed using weighed, equal aliquots of individual samples of psoriatic scales. The concentration of in vivo-produced 13-HODD present in each skin scale aliquot served as an internal standard for product recovery.

Extraction of oxygenation products

The sample was lyophilized and then treated by the anhydrous methanolic sodium hydroxide method of Kates (9) to quantitatively transesterify all esterified fatty acids and their derivatives to their respective methyl esters while preserving free fatty acids and their derivatives intact. A single modification was made in order to partition nonesterified fatty acids and their hydroxylated deriva-

tives into the organic layer: after the addition of water to separate the phases, 100 μl of 2 M ammonium formate buffer, pH 3.2, was added. After washing with methanol-water 10:9 saturated with chloroform, the lower phase was reconstituted into mobile phase consisting of methanol-water-acetic acid 80:20:0.1.

Reversed-phase high performance liquid chromatography

Reversed-phase HPLC was performed isocratically on a Hewlett-Packard 1090 liquid chromatograph using Hewlett-Packard ODS-Hypersil columns, 20 cm in length × 4.6 mm, at a flow rate of 0.4 ml/min. This instrument is equipped with a diode array spectrophotometer and a Hewlett-Packard Chem-Station computer for on-line display and storage of UV spectra, and thus precise measurement of the λ_{max} of each peak could be performed after each run was completed. The LC runs were monitored at a wavelength of 236 nm. A Radiomatic Flo-One A-140 (Radiomatic Instruments and Chemical Co., Inc., Tampa, FL) radioactivity detector was used for concurrent measurement of ³H or ¹⁴C in the outflow from the HPLC spectrophotometer. An interface between the radioactivity detector and the Hewlett-Packard Chem-Station computer permitted storage of the radioactivity data for subsequent analysis. Radioactivity and UV absorbance peaks were quantified by the use of existing software programs in the Hewlett-Packard Chem-Station computer for peak integration. All chromatograms were reproduced directly from the HPLC computer plots.

Stereochemical analysis

During reversed-phase HPLC of the psoriatic skin scale extracts, the HPLC eluate was collected during the elution of 13-HODD, 15-HETE, or 12-HETE. The compounds within appropriate peaks were subsequently methylated with ethereal diazomethane before rechromatographing on the same LC columns (delayed retention time of approximately 15 min). The methylated fatty acids were collected during the LC run and then chromatographed on two 25-cm Bakerbond chiral phase LC columns in series (dinitrobenzoylphenyl glycine coupled ionically over aminopropyl residues; J. T. Baker Research Products, Philipsburg, NJ) at a flow rate of 0.4 ml/min and with a mobile phase mixture that consisted of hexane-isopropanol 1000:15. The eluate from the chiral phase HPLC columns was monitored for both radioactivity and UV absorbance. Retention times become gradually longer in repeated HPLC analyses with these chiral phase columns. Therefore, the identity and chirality of specific compounds were confirmed in almost all cases by performing mixing experiments in which synthetic methyl ester standards were added to the derivatized material originally obtained from the extracts of the psoriatic scale incubations.

RESULTS

The principal monohydroxylated fatty acid derivatives in the extracts of psoriatic skin scales, representing *in vivo*-produced compounds, were 13-HODD and 9-HODD, co-eluting at 22.5 min, and 12-HETE, eluting at 26 min (Fig. 1). The methyl esters of 13-HODD and 9-HODD were routinely found (RT = 37.5 min) in the analyses and represented material esterified *in vivo* that had been released by transesterification. In order to determine the possible presence of 15-HETE (which has a retention time of 23 min under these HPLC conditions), the HPLC eluate between 22 and 25 min was collected, methylated, and rechromatographed under straight phase conditions that would resolve 13-HODD, 9-HODD, and 15-HETE. No 15-HETE was detected in this manner. The chromatographic separation of extracts from incubations of psoriatic skin scales with [³H]arachidonic acid each yielded two major radioactive peaks. One eluted at 23.5 min and had a retention time identical to that of authentic 15-HETE standard and the second eluted at 26 min and had a retention time identical to that of authentic 12-HETE standard (Fig. 2). These two peaks were not observed in radiochromatograms of the extracts derived from incubations of [³H]arachidonic acid with heat-denatured scales (Fig. 2). The production of radioactive 15-[³H]HETE usually exceeded that of 12-[³H]HETE; the ratio of radioactive 15-HETE to 12-HETE in nine samples from eight patients averaged 1.8 ± 0.3 (mean \pm SEM) with a range of 0.7 to 3.1. When the extracts of psoriatic skin scale incubations with [³H]arachidonic acid were treated with ethereal diazomethane prior to HPLC, the retention times of the two major radioactive peaks were each delayed by approximately 15 min, consistent with their conversion to fatty acid methyl esters.

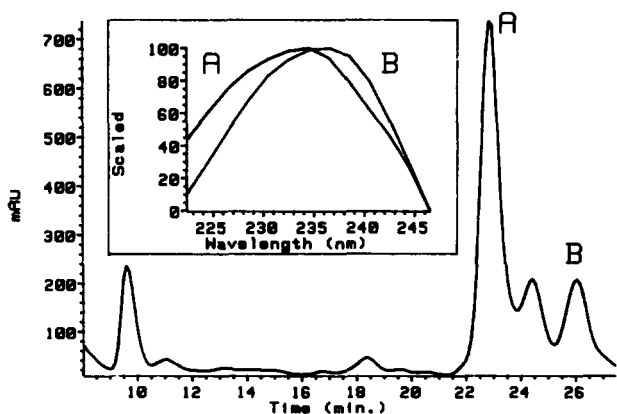


Fig. 1. Reversed-phase HPLC chromatogram of the monohydroxylated fatty acids extracted from psoriatic skin scales. Peaks A and B with RT of 22.5 and 26 min had UV spectra with λ_{max} of 234 nm and 236 nm (inset). These values correspond to RT and UV spectra of 13- and 9-HODD (peak A) and 12-HETE (peak B). No other HETEs were evident. The peak with the retention time of 24.5 min had a UV spectrum with a λ_{max} of 230 nm.

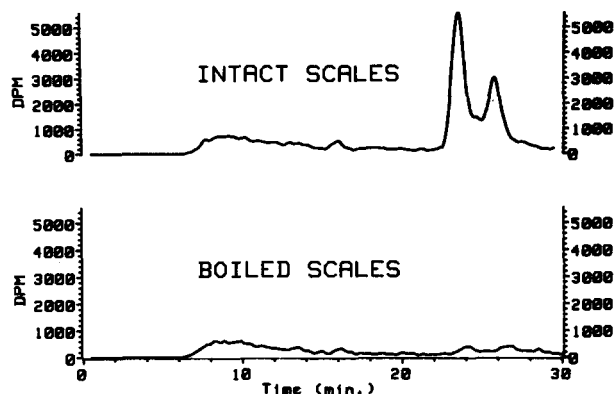


Fig. 2. Reversed-phase HPLC radiochromatograms of the radiolabeled products of the incubation of [³H]arachidonic acid with intact (upper chromatogram) or heat-denatured (lower chromatogram) psoriatic skin scales. Two major peaks of radioactivity were observed in the chromatogram of the products generated by scales which had not been heat-denatured and co-eluted with authentic 15-HETE (23.5 min) and 12-HETE (26 min) standards. No significant radiolabeled products were generated by the heat-denatured scales.

The chromatographic separation of extracts from incubations of psoriatic skin scales with [¹⁴C]linoleic acid yielded two major radioactive peaks (Fig. 3). A peak eluted at 22–25 min and co-eluted with the endogenous 13-HODD (RT = 22.5 min). The second peak which eluted at 27 min was totally absent in some runs and has not been characterized further. There was no corresponding ultraviolet peak at 27 min. When the radioactive material in the first peak was collected, methylated, and chromatographed under straight-phase conditions with the chiral phase columns, a radioactive peak was seen which co-eluted with the *in vivo*-produced 13-HODD. No radioactive products were recovered by HPLC analysis in an incubation of normal heel stratum corneum (92 mg)

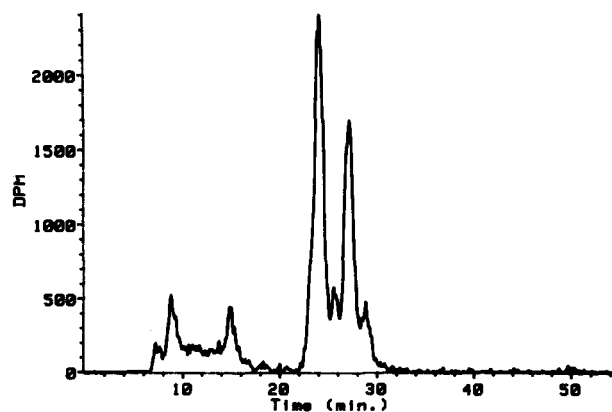


Fig. 3. Reversed-phase HPLC radiochromatogram of the radiolabeled products of the incubation of [¹⁴C]linoleic acid with psoriatic skin scales. Two major peaks of radioactivity were observed, the one at 22–25 min was shown on subsequent straight phase HPLC analysis to include 13-[¹⁴C]HODD and 9-[¹⁴C]HODD. The second peak, eluting at 27 min, was totally absent in some analyses and has not been characterized further.

with [^3H]arachidonic acid. There were no monohydroxylated derivatives of either arachidonic acid or linoleic acid identified by HPLC in this or in a previously reported analysis of normal heel stratum corneum (1).

The results of experiments evaluating the effect of various inhibitors on the oxygenase activities in the scales are summarized in **Table 1**. The most notable effect was that produced by nordihydroguaiaretic acid (30 μM) which inhibited 15- ^3H]HETE and 12- ^3H]HETE production by 58% and 40%, respectively. This effect was confirmed in three additional experiments (data not shown). Indomethacin (7 μM) had no inhibitory effect. The oxygenase activities of the scales were comparable when incubated either in medium saturated with carbon monoxide or in medium saturated with nitrogen. SKF-525A (500 μM) showed some inhibitory activity, but the degree of inhibition was small (18–26%) and affected both 15- ^3H]HETE and 12- ^3H]HETE production.

The stereospecificity of the 15-HETE, 12-HETE, and 13-HODD generated during the incubations of the psoriatic skin scales with [^3H]arachidonic or [^{14}C]linoleic acid was analyzed using chiral HPLC columns under straight phase conditions. For these analyses, the radioactive compounds were collected during reversed-phase chromatography, treated with ethereal diazomethane to produce the methyl ester, rechromatographed on reversed phase columns, and the derivatized compound was then chromatographed on straight phase chiral columns. Mixing experiments were performed as illustrated in Figs. 4 and 5 to confirm the identity of each methyl ester and to differentiate the stereoisomers. Using synthetic standards of racemic 15-HETE and 15(S)- ^3H]HETE or racemic 12-HETE and 12(S)- ^3H]HETE, it was found that the stereoisomer labeled with tritium had a retention time delayed relative to that of the corresponding unlabeled stereoisomer, consistent with an isotope effect. The stereoisomers of unlabeled racemic 15-HETE and those of racemic 12-HETE were each clearly resolved on these columns ($\Delta\text{RT} = 1.0\text{--}1.5$ min) (7).

In each of three separate determinations, the in vitro-generated 15- ^3H]HETE eluted as a single peak when chromatographed on chiral phase HPLC columns in the form of its methyl ester. In mixing experiments, the scale-derived 15- ^3H]HETE eluted with authentic 15(S)- ^3H]HETE standard as a single peak (**Fig. 4**). The in vitro-produced 12- ^3H]HETE, converted to its methyl ester, eluted from the chiral phase columns as a large peak with a shoulder on its ascending limb (**Fig. 5**). In mixing experiments, authentic 12(S)- ^3H]HETE standard was added to an aliquot of the in vitro-produced 12- ^3H]HETE equal to that injected on the column in the initial run. The mixture chromatographed as a double peak with the 12(S)- ^3H]HETE standard eluting first (on the shoulder of the original peak) as indicated by the equivalence in amount of radioactivity in the 12(R)- ^3H]HETE peak between the two consecutive runs. The order of elution of the two stereoisomers was also that which was expected with these chiral phase columns (7). These results thus indicate that the principal 12- ^3H]HETE stereoisomer produced in vitro had (R) stereospecificity (**Fig. 5**). The R/S ratio of the in vitro-produced 12- ^3H]HETE averaged 4.5 ± 1.5 (mean \pm SEM, $n = 3$). The average R/S ratio of the in vivo-produced 12-HETE present in five scale extracts was 5.4 ± 0.3 .

The 13- ^{14}C]HODD, produced in vitro and converted to its methyl ester, eluted with a RT consistent with 13(S)-HODD ($n = 2$) (**Fig. 6**).

DISCUSSION

The preservation of fatty acid oxygenase activity in psoriatic skin scales was first observed by Nugteren and Kivits (10). The current results confirm and extend their observations and indicate that psoriatic skin scales in vitro synthesize 15-HETE and 12-HETE from exogenous arachidonic acid and 13-HODD from exogenous linoleic acid. The stereospecificity of the products of these ox-

TABLE 1. Effects of inhibitors on oxygenase activities in psoriatic skin scales

Experiment	Inhibitor	Radioactive Product Formation	
		15- ^3H]HETE	12- ^3H]HETE
1	Exogenous substrate alone	0.62 ± 0.10	0.43 ± 0.08
	+ Indomethacin (7 μM)	0.64 ± 0.02	0.48 ± 0.01
	+ Nordihydroguaiaretic acid (30 μM)	0.27 ± 0.02	0.29 ± 0.01
2	Nitrogen-saturated buffer (control)	1.96 ± 0.02	1.27 ± 0.07
	Carbon monoxide-saturated buffer	2.24 ± 0.05	1.44 ± 0.11
3	Exogenous substrate alone	2.29 ± 0.44	1.57 ± 0.03
	+ SKF 525A (500 μM)	1.87 ± 0.20	1.16 ± 0.06

Values shown are the means \pm SEM of duplicate determinations. Product formation is expressed as femtomoles of product formed in a 1-h incubation per mg scales. The $\text{N}_2:\text{O}_2$ molar ratio was 14:1 and the $\text{CO}:\text{O}_2$ molar ratio was 11:1.

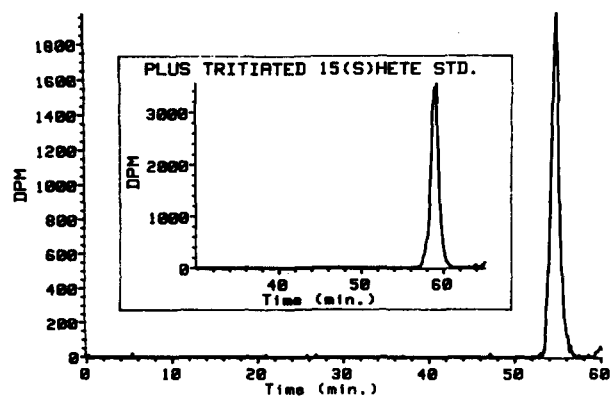


Fig. 4. Representative straight phase chiral HPLC radiochromatograms of the methyl ester of the radiolabeled 15- ^3H]HETE produced in the incubation of ^3H]arachidonic acid with psoriatic skin scales. The outer chromatogram is that of the material in the peak at 23.5 min (Fig. 2) which was then esterified. The chromatogram shown in the inset is that of an equal aliquot of the same material, to which authentic 15(S)- ^3H]HETE methyl ester standard had been added. The elution of this mixture of radioactive material as a single peak demonstrates that the 15-HETE produced by the psoriatic skin scales is strictly the (S) stereoisomer.

genases has not been previously analyzed. The oxygenase activities were clearly enzymatic in origin since they were absent in heat-denatured scales, were inhibited by nordihydroguaiaretic acid, and demonstrated stereospecificity in product formation. The relative production of 15-HETE and 12-HETE varied considerably between patient samples, indicating that these compounds were products of two separate enzyme activities, one being that of an ω -6 oxygenase and the other that of an arachidonic

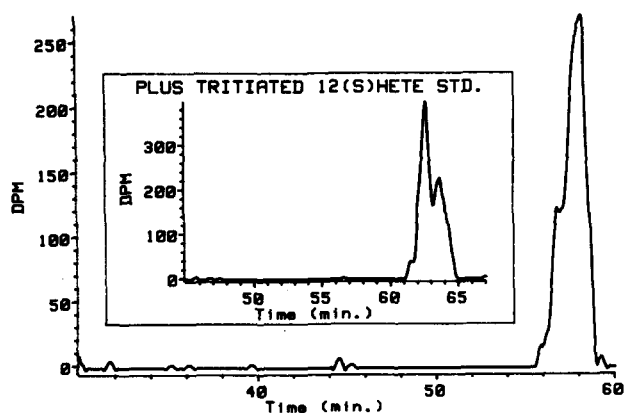


Fig. 5. Representative straight phase chiral HPLC radiochromatograms of the methyl esters of the radiolabeled 12- ^3H]HETE produced in the incubation of ^3H]arachidonic acid with psoriatic skin scales. The outer chromatogram is that of the material in the peak at 26 min (Fig. 2) which was then esterified. The chromatogram shown in the inset is that of an equal aliquot of the same material, to which authentic 12(S)- ^3H]HETE methyl ester standard had been added. The elution of this mixture of radioactive material as a double peak demonstrates that the majority of the 12-HETE produced by the psoriatic skin scales consists of the (R) stereoisomer. Note the equivalence in height of the radioactive peaks representing 12(R)-HETE in the two consecutive runs.

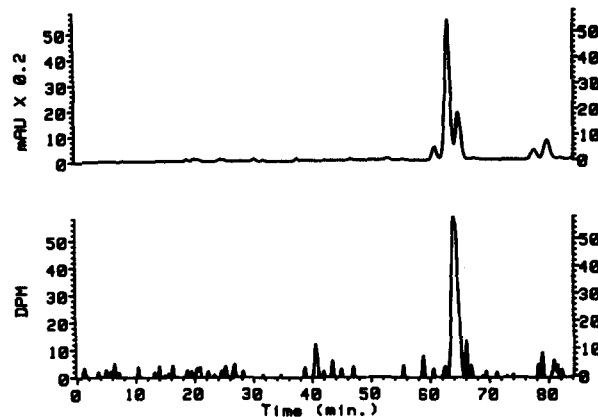


Fig. 6. Representative concurrent straight phase chiral HPLC chromatogram (upper panel) and radiochromatogram (lower panel) of the methyl ester of the radiolabeled 13- ^{14}C]HODD produced in the incubation of ^{14}C]linoleic acid with psoriatic skin scales. The retention times were 13(S)-HODD (62–64 min), 13(R)-HODD (64–66 min), 9(S)-HODD (76–78 min), and 9(R)-HODD (78–80 min). The 13- ^{14}C]HODD eluted as a single peak (lower chromatogram) at a retention time delayed by 1 min relative to that of the in vitro-produced 13(S)-HODD stereoisomer due to its passage through the radioactive detector.

acid 12-oxygenase. This observation was further supported by the difference in the stereospecificity of these two oxygenase activities.

When the in vitro ω -6 oxygenase products of the scales, 15- ^3H]HETE and 13- ^{14}C]HODD, were analyzed by chiral phase HPLC, it was found that both compounds had strict (S) stereospecificity. This stereochemical finding constitutes strong evidence that the ω -6 oxygenase activity is that of a lipoxygenase that is still active in psoriatic scales. Strict (S) stereospecificity would not be expected with a cyclooxygenase or a cytochrome P450 monooxygenase (11, 12). A 15-lipoxygenase has also been demonstrated in cultured human keratinocytes (13, 14) and is "activated" in vitro by cell membrane damage (14).

The present data resolve the contradiction between the expected finding of the (S) stereoisomer from keratinocyte 15-lipoxygenase activation and the actual finding of 13(R,S)-HODD which we recently described (1). Both the free and esterified 13-HODD produced in vivo and recovered from extracts of psoriatic skin scales are not enantiopure, but rather are mixtures of the stereoisomers. The S/R ratio averaged 1.9 for the free compound in our previous study (1). The discrepancy between the pure (S) stereospecificity of the in vitro-produced 13-HODD (and 15-HETE) and nonracemic (S,R) stereospecificity of the in vivo compound suggests that there is autooxidation of both free and esterified linoleic acid in the psoriatic epidermis, resulting in a mixture of nonenzymatically derived racemic 13-HODD and enzymatically produced 13(S)-HODD. The present data also imply that a peroxidase survives in psoriatic skin scales that can convert in vitro-produced hydroperoxides to their respective hydrox-

ylated fatty acids. It is possible in vivo that the enzymatically produced hydroperoxide acts to stimulate autooxidation of the remaining linoleic acid.

Woollard (8) first reported that the 12-HETE in extracts of psoriatic skin scales, representing in vivo-produced material, was primarily the (R) stereoisomer. In the current study, the (R) stereoisomer of 12-HETE was the principal one produced during incubations of the psoriatic skin scales with arachidonic acid. In contrast, cell suspensions or homogenates derived from normal human epidermis and incubated with arachidonic acid produce primarily 12(S)-HETE (15–17). These results suggest that a unique oxygenase activity is expressed in the psoriatic epidermis with arachidonic acid 12(R)-specificity. Neither the nature of this oxygenase activity nor its cellular source is known at this time, but psoriatic scales are clearly a useful source for enzyme purification. The production of 12(R)-HETE is a characteristic of cytochrome P450 monooxygenases present in hepatic (R/S = 4) and corneal microsomes (11, 18). The data are conflicting as to whether the 12(S)-HETE producing oxygenase in normal human epidermis is a cytochrome P450 monooxygenase (16, 17). The inhibitors evaluated in the current study had effects that do not clearly define the nature of the 12(R)-oxygenase activity in psoriatic skin scales.

The observation that 13-HODD is present in psoriatic skin scales in both a free and esterified form may be an indication that the 15-lipoxygenase activity is expressed early in keratinocyte differentiation (1). Green (14, 19) has observed that terminally differentiated epithelial cells freshly isolated from the buccal cavity produce 12-HETE and 15-HETE from exogenous arachidonic acid but are unable to esterify these products. In contrast, cultured human keratinocytes, which are relatively undifferentiated, express 15-lipoxygenase activity only after membrane damage (by freeze-thawing). These cultured keratinocytes are able to esterify exogenous HETEs in the intact state but not after membrane damage (14). It needs to be determined whether esterification of fatty acids is strictly a function of the relatively undifferentiated keratinocytes in vivo. The observation in the current study that the 15-lipoxygenase activity and the 12(R) arachidonic acid oxygenase activity are preserved during keratinization may imply a significant role for these oxygenase activities in the pathogenesis of psoriasis. ■

This work was supported by the Veterans Administration, NIH Grant HL24009, the Western New York Chapter of the Arthritis Foundation, and the Jules and Gwen Knapp Charitable Foundation. The authors gratefully acknowledge the technical assistance of Norine Yuskiw and the secretarial assistance of Denise Jurewicz.

Manuscript received 25 September 1990 and in revised form 24 October 1990.

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