Occurrence of Hepoxilins and Trioxilins in Psoriatic Lesions

Rosa Antón, Luís Puig,* Teresa Esgleyes,* Josep María de Moragas,* and Luís Vila

Laboratory of Inflammation Mediators, Institute of Research, Barcelona, Spain; *Dermatology Department of Santa Creu i Sant Pau Hospital, Barcelona, Spain

We recently found that normal human epidermis produces relatively high amounts of hepoxilins and trioxilins *in vitro*. Therefore, the aim of this study was to demonstrate the presence of these compounds in psoriatic lesions. Extracts from scales of patients with chronic stable plaque psoriasis were analyzed by a combination of high performance liquid chromatography and gas chromatography-mass spectrometry techniques. We found that the levels of hepoxilin B_3 were more than 16-fold higher in psoriatic scales than in normal epidermis (3.2 ± 2.3 and < 0.2 ng per mg, respectively), whereas hepoxilin A_3 was not detected in any sample. Trioxilins were semiquantitated and referred to 12-hydroxyeicosatetraenoic acid, ratios of trioxilins A_3 and B_3 12-hydroxyeicosatetraenoic acid in psoriatic lesions were 0.65 ± 0.23 and 0.32 ± 0.28, respectively, and they were not detected in normal epidermis. The presence of a great amount of trioxilin A_3 strongly suggests that hepoxilin A_3 was present in psoriatic lesions and it was totally degraded to trioxilin A_3 during the analysis procedure. Our results demonstrate that hepoxilins and trioxilins are produced by human skin *in vivo* and that the levels of these compounds are increased in psoriasis. The reported biologic activities of hepoxilins indicate that they could amplify and maintain the inflammatory response. Our results reinforce the idea that these compounds could play a role as mediators in the inflammatory response in skin, particularly in psoriasis. ***Key words: arachidonic acid/eicosanoid/psoriasis/12-lipoxygenase. J Invest Dermatol 110: 303–310, 1998***

Altered arachidonic acid (AA) metabolism is a typical feature of psoriasis (Hammarström *et al*, 1975; Camp *et al*, 1983; Kragballe *et al*, 1986; Vila *et al*, 1991), and AA derived compounds are involved in its pathophysiology and in other dermatoses (Soter *et al*, 1983; Chan *et al*, 1985; Ruzicka and Burg, 1987; Waldman *et al*, 1989). 12-Lipoxygenase (12-LO) is the major AA oxygenation enzyme in epidermal cells with total product formation generally exceeding cyclooxygenase activity (Holtzman *et al*, 1989; Solá *et al*, 1992). The most abundant eicosanoid found in psoriatic lesions and produced by preparations of fresh human epidermis is 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE) (Hammarström *et al*, 1975; Camp *et al*, 1983; Fogh *et al*, 1987; Solá *et al*, 1992). 12-HETE is formed by the 12-LO catalyzed peroxidation of AA at C12. Platelet type 12-LO has been found to be the predominant isoenzyme expressed in human and murine skin epidermis (Takahashi *et al*, 1993; Hussain *et al*, 1994; Krieg *et al*, 1995), and an "epidermal" type 12-LO that functionally resembles the platelet type 12-LO is also present in murine epidermis (Van Dijk *et al*, 1995; Funk *et al*, 1996; Kinzig *et al*, 1997). The initial product of any LO is a hydroperoxide with a predominant S configuration (Hamberg and Samuelsson, 1974; Nugteren, 1975). Hydroperoxide-eicosatetraenoic acids (HPETE) are reduced to the corresponding HETE by peroxidases. 12(S)-HPETE has been reported to undergo further types of transformations to form epoxy-hydroxy acids and trihydroxy acids in several cell types and tissues. It has been reported that 12-HPETE can be transformed into 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid and 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid, termed hepoxilin A_3 (HxA_3) and hepoxilin B_3 (HxB_3), respectively, which can be enzymatically and nonenzymatically transformed into 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid and 10,11,12-trihydroxy-5,8,14-eicosatrienoic acid, termed trioxilin A_3 ($TrXA_3$) and trioxilin B_3 ($TrXB_3$), respectively (reviewed in Pace-Asciak *et al*, 1995a, b).

We have recently reported that normal human epidermis produces – in addition to 12-HETE – HxA_3, HxB_3, $TrXA_3$, $TrXB_3$, 8,9,12-trihydroxyeicosatrienoic acid (8,9,12-THETrE), and 12-oxo-eicosatetraenoic acid from exogenous AA (Antón *et al*, 1995). Some of the reported biologic activities of these compounds suggest their role as inflammatory mediators in skin (Dho *et al*, 1990; Laneuville *et al*, 1991; Nigam *et al*, 1993; Reynaud *et al*, 1996; Wang *et al*, 1996). With the exception of 12-HETE, the presence of 12-LO derived eicosanoids under pathophysiologic situations has not been reported to date. All this prompted us to determine the presence of hepoxilins (HX) and trioxilins (TrX) in psoriatic lesions.

MATERIALS AND METHODS

Materials Hydroxyoctadecadienoic acid (HODE) and HETE were from Cayman (Ann Arbor, MI). Authentic 8(R,S)-hydroxy-11(S),12(S)-epoxy-5,9,14,-eicosatrienoic acid [(±)HxA_3] and 10(R,S)-hydroxy-11(S),12(S)-epoxy-5,8,14-eicosatrienoic acid [(±)HxB_3] were from Cascade Biochem (Berkshire, U.K.). Butyrylcholine esterase from horse serum was from Sigma (St. Louis,

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Reprint requests to: Dr. Luís Vila, Hospital de Sant Pau (Casa de Convalecencia), S. Antonio Mª Claret 167, 08025-Barcelona, Spain.

Abbreviations: AA, arachidonic acid; CN, carbon number; CTF, carboxyl terminal fragment; EI, electron impact; GC-MS, gas chromatography-mass spectrometry; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; HX, hepoxilins; HxA_3, 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid; (±)HxA_3, 8(R,S)-hydroxy-11(S),12(S)-epoxy-5,9,14,-eicosatrienoic acid; HxB_3, 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid; (±)HxB_3, 10(R,S)-hydroxy-11(S), 12(S)-epoxy-5,8,14-eicosatrienoic acid; LO, lipoxygenase; ME-H-TMS, hydrogenated methyl ester trimethylsilyl ether; ME-TMS, methyl ester trimethylsilyl ether; MTF, methyl terminal fragment; THETrE, trihydroxyeicosatrienoic acid; TMSOH, $(CH_3)_3$ SiOH; TrX, trioxilins; $TrXA_3$, 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid; $TrXB_3$, 10,11,12-trihydroxy-5,8,14-eicosatrienoic acid.

MO). [^{18}O]-labeled water (95% ^{18}O) was from Cambridge Isotope Laboratories (Andover, MA). Sep-Pak C-18 silica cartridges were from Waters Millipore (Milford, MA). All high performance liquid chromotography (HPLC) solvents were supplied by Scharlau (Barcelona, Spain).

Recovery of normal human epidermis fragments and scales from psoriatic patients Epidermis was isolated from skin using the Liu and Karasek technique (Liu and Karasek, 1978). Fragments of epidermis, obtained as described previously (Antón *et al*, 1995), were stored in methanol at –80°C under a N_2 atmosphere until the analysis of eicosanoid and octadecanoid basal levels.

Scales from untreated patients with chronic stable plaque psoriasis involving more than 10% of body surface, were removed by scraping with a surgical blade and stored in methanol at –80°C under a N_2 atmosphere until analysis of eicosanoids and octadecanoids.

Preparation of internal standards Labeled internal standards were prepared as described by Lehman *et al* (1992) with small modifications. Briefly, ^{18}O-labeled HODE, HETE, and HxB_3 were prepared by addition of 50 μl [^{18}O]-H_2O containing butyrylcholine esterase (500 units per 225 μl [^{18}O]-H_2O) to 5 μl of a mixture containing 7 μg each of 9-HODE, 13-HODE, 12-HETE, 15-HETE, and 5-HETE, or to 13 μg of HxB_3, dissolved in ethanol. The reactions were allowed to proceed during 7 h at 37°C under continuous agitation and, afterwards, another 50 μl of [^{18}O]-H_2O containing butyrylcholine esterase were added and incubated for another 20 h. After incubation, the reaction mixtures were extracted three times with two volumes of ethyl acetate. The extracts were mixed, dried under a gentle N_2 stream, redissolved in acetonitrile, and stored at –80°C.

Standard curves Ten, 50, 200, 500, 1000, and 2000 ng of each standard were processed as described under work-up procedure with 350 ng of every [^{18}O]-labeled HODE and HETE, and 600 ng of [^{18}O]-HxB_3. After derivatization, calibration curves were made by plotting the relative response of each compound with respect to that of the corresponding [^{18}O]-labeled analog *versus* the amount injected. For 5-HETE, the *y*-axis was the ratio of the response of 5-HETE to that of the [^{18}O]-12-HETE, for reasons given in the *Results*.

Extraction of eicosanoids and octadecanoids from normal epidermis and psoriatic scales Normal human epidermal fragments and psoriatic scales (200 mg) were vortexed vigorously in the presence of 2 ml of methanol. Samples were centrifuged at 5000 × *g* for 5 min and the supernatant was recovered. Pellets were washed twice with 2 ml of methanol and the extracts were pooled. For quantitative analysis 350 ng of [^{18}O]-9-HODE, [^{18}O]-13-HODE, [^{18}O]-12-HETE, and [^{18}O]-15-HETE, and 600 ng of [^{18}O]-HxB_3 were added to the methanolic extracts before drying under a N_2 stream. They were added after methanol extraction to avoid the action of esterases present in biologic material. Afterwards, residues were redissolved in 10 ml sodium acetate 0.1 M (pH = 3.2) containing 5% ethanol and subjected to a solid phase extraction using C18-silica cartridges according to Powell (1982). HODE, HETE, HX, and TrX were finally eluted by flushing 20 ml of ethyl acetate and 20 ml of methanol through the cartridge consecutively. Both extracts were mixed, dried under N_2, and redissolved in reverse phase HPLC buffer.

Reverse phase HPLC separation Separation of TrX, HX, and HODE and HETE was accomplished by HPLC, which was performed isocratically with a mixture of methanol/water/trifluoroacetic acid/triethylamine 75:25:0.1:0.05 at 1 ml per min in a Ultrasphere-ODS column (5 μm diameter of particle, 4.6 × 250 mm, Beckman, San Ramón, CA). The selected retention times to collect the different class of compounds were 4–9 min for TrX, 14–20 min for HX, and 22–40 min for HODE and HETE (Solá *et al*, 1992; Godessart *et al*, 1994; Antón *et al*, 1995).

Liquid–liquid extraction In order to concentrate the HPLC fractions, acidic water (pH = 3.2, acidified with hydrochloric acid) was added to achieve a methanol:water ratio of 1:1. They were then extracted three times with half a volume of ether:hexane (1:1). Organic extracts were mixed, dried under a N_2 stream, and redissolved in acetonitrile until derivatization.

Derivatization procedure The methyl esters trimethylsilyl ethers (ME-TMS) and hydrogenated methyl esters trimethylsilyl ethers (ME-H-TMS) were obtained as described previously (Antón *et al*, 1995) with small modifications. To obtain the ME derivatives 1 ml of ethereal diazomethane (Fales *et al*, 1973) was added, and allowed to react for 20 min, and H_2 was bubbled for 50 s to obtain the hydrogenated derivatives. All samples were redissolved in 20 μl acetonitrile, with 2 μl being injected into the gas chromatograph.

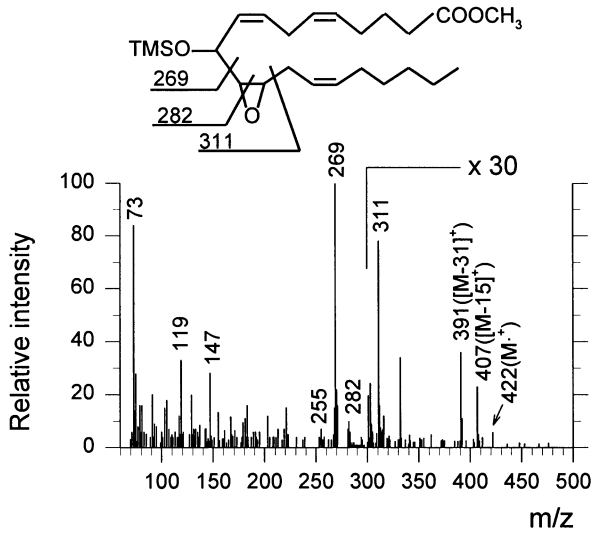

Figure 1. EI mass spectra of the ME-TMS derivative of the 14–20 min HPLC fraction from psoriatic scales was consistent with the structure of HxB_3. With monitoring at m/z 73, 269, 282, and 311 a single gas chromatography peak (CN 22.02) was obtained. The scheme of fragmentation shows the origin of the most characteristic fragments. The spectrum was essentially identical to that obtained from normal epidermis and authentic (±)HxB_3.

Gas chromatography-mass spectrometry (GC-MS) analysis To analyze all samples the electron impact (EI) mode was used as previously described (Antón *et al*, 1995). The gas chromatography column was a TRB-1 fused silica capillary column (15 m length, 0.25 mm i.d., 0.25 μm film thickness, Tracer analítica, Barcelona, Spain).

RESULTS

To investigate whether or not HX and TrX were present in normal epidermis and in psoriatic lesions, pooled fragments of normal epidermis and psoriatic scales were analyzed. First we processed the samples without the addition of ^{18}O-labeled internal standards. This was done in order to avoid detection of HX and TrX due to the presence of small amounts of unlabeled compounds in the samples coming from the internal standards.

Hepoxilins are present in psoriatic lesions HX were extracted from the HPLC fraction collected between 14 and 20 min and the extracts were divided into two parts to produce the ME-TMS and ME-H-TMS derivatives. The derivatives obtained were subjected to GC-MS analysis using the full scan mode.

The presence of HxA_3 in the skin samples was analyzed by monitoring at m/z 73, 243 [carboxyl terminal fragment (CTF) from cleavage of the C8–C9 bond], 281 [methyl terminal fragment (MTF) from cleavage of the C7–C8 bond], and 311 (CTF from cleavage of the C12–C13 bond). We did not find any peak with mass spectrum compatible with the structure of HxA_3 in the carbon number (CN) range 21.0–25.0 [CN of authentic (±)HxA_3 = 22.95], either in psoriasis or in normal epidermis pools. Even when authentic (±)HxA_3 was processed identically to the skin samples, no peak corresponding to the structure of HxA_3 was detected.

Monitoring suitable ions for HxB_3 (m/z 73, 269, 282, and 311), the ME-TMS derivatives of the HX fraction from both psoriatic and normal epidermis subjected to GC-MS analysis eluted as a single peak (CN 22.02). The EI mass spectrum had characteristic fragments at m/z 422 ($M^{\cdot+}$, not always present), 407 ($[M-15]^+$), 391 ($[M-31]^+$), 311 (CTF from cleavage of the C12–C13 bond), 282 (CTF from cleavage of the epoxide double ring at C11), 269 (CTF from cleavage of the C10–C11 bond), and 255 (MTF from cleavage of the C9–C10 bond, not always present). This fragmentation (**Fig 1**) was consistent with the structure of HxB_3 and it was essentially identical to the one obtained with authentic (±)HxB_3.

Because with the gas chromatography technique used it is possible

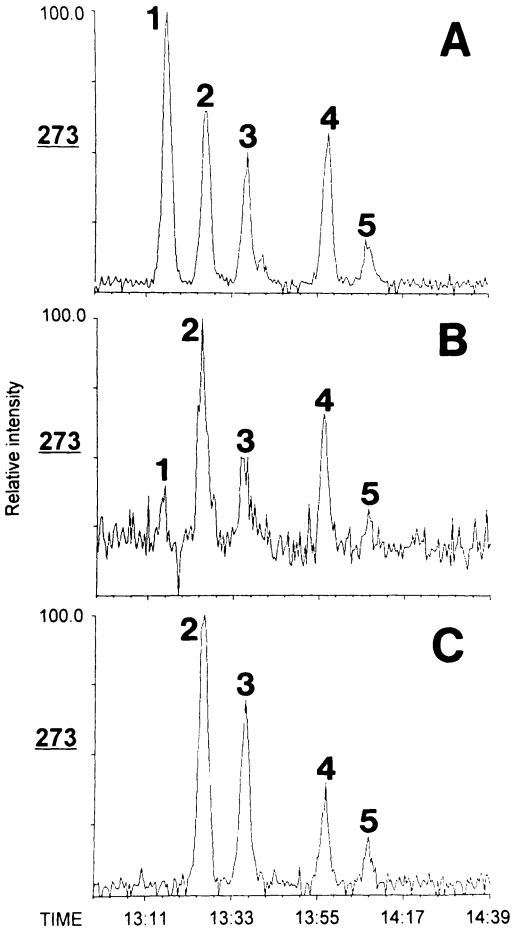

Figure 2. Samples from psoriatic scales showed an additional gas chromatography peak (peak 1), which was almost undetectable in the samples from normal epidermis, when compared with authentic (±)HxB_3. GC-MS selected ion, m/z 273, chromatograms of the ME-H-TMS derivatives of the 14–20 min HPLC fraction from psoriatic scales pool (*A*), and normal epidermis pool (*B*), and authentic (±)HxB_3 (*C*). The three samples were processed identically.

to resolve the two hydrogenated 10-hydroxy epimers of HxB_3 (Antón *et al*, 1995), the HX fraction was also catalytically hydrogenated before TMS derivatization.

To analyze HxB_3 in psoriatic lesions the fragments monitored in the hydrogenated samples were m/z 73, 257, 273, and 287. **Figure 2** shows typical m/z 273 selected ion gas chromatograms obtained from a psoriatic scales pool, a pool of normal epidermal fragments and authentic 10(R,S)-hydroxy-11(S),12(S)-epoxy-arachidic acid [hydrogenated (±)HxB_3]. Samples from psoriatic patients showed three peaks with CN of 22.65, 22.80, and 22.96 (peaks 1, 2, and 3 in **Fig 2**, respectively) that had essentially identical mass spectra consistent with a 10-hydroxy-11,12-epoxy-arachidic acid structure (**Fig 3**). Significant fragments were seen at m/z 428 ($M^{\cdot +}$, not always present), 413 [$(M-15)^+$], 287 (cleavage of the epoxide double ring), 273 (CTF of α-cleavage at C10), and 257 (MTF from cleavage of the C9–C10 bond). The ME-H-TMS derivative of authentic (±)HxB_3 yielded only two peaks with the same carbon numbers and mass spectra as peaks 2 and 3 of the psoriatic samples. Although in samples from psoriatic lesions peak 1 was the highest, this peak was almost undetectable in samples from normal epidermis (**Fig 2*B***). In addition, peaks corresponding to the hydrogenolysis products, 10,11-dihydroxy-arachidic acid, were also detected in psoriatic lesions, normal skin, and authentic (±)HxB_3 samples (peaks 4 and 5 in **Fig 2**). Based on these results we concluded that not only were the two 10-hydroxy epimers of HxB_3 (peaks 2 and 3 in **Fig 2**) present in psoriatic lesions, but another isomer was also present (peak 1 in **Fig 2**), the possible identity of which will be discussed later.

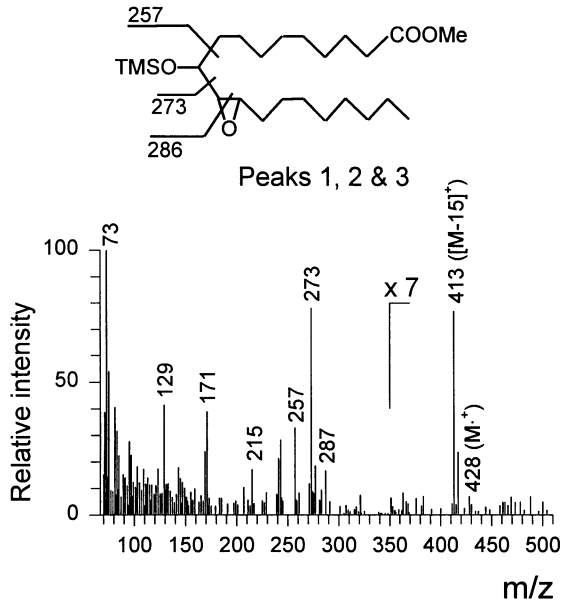

Figure 3. Mass spectra of the compounds corresponding to peaks 1, 2, and 3 in Fig 2 are consistent with the structure of hydrogenated HxB_3. The figure shows the EI mass spectrum of peak 2 from psoriatic scales; spectra of peaks 1 and 3 being essentially identical. Peaks 3 and 4 corresponded to the hydrogenolysis products of HxB_3: 10,11-dihydroxy-arachidic acid.

Trioxilins are present in psoriatic lesions To explore the presence of TrX in skin samples, they were extracted from the 4–9 min HPLC fractions from normal epidermis and psoriatic scales and the extracts were divided into two parts to synthesize the ME-TMS and ME-H-TMS derivatives of TrX. Then, the TrX fraction derivatives were subjected to GC-MS analysis, performed using the full scan mode.

The ME-TMS derivatives of trioxilins would come out at m/z 213 (corresponding to the MTF from cleavage of the C11–C12 bond, which indicates the presence of a hydroxyl group at C12) and 243 (corresponding to the CTF from cleavage of the C8–C9 bond, which indicates the presence of a hydroxyl group at C8), for both $TrXA_3$ and 8,9,12-THETrE, and at m/z 269 (corresponding to the CTF from cleavage of the C10–C11 bond, which indicates the presence of a hydroxyl group at C10) for $TrXB_3$. Single ion chromatograms from psoriatic samples are represented in **Fig 4**, and the chromatographic and MS data are summarized in **Table I**. The three peaks monitoring at m/z 213 (peaks 1, 3, and 6 in **Fig 4**) showed essentially identical mass spectra, with significant fragments at m/z 569 ($[M-15]^+$), 479 [loss of TMS as $(CH_3)_3$ SiOH (TMSOH) from the fragment at m/z (569)], 473 (CTF from cleavage of the C12–C13 bond), 463 (loss of TMSOH from the fragment $[M-31]^+$), 444 (443 + H, MTF from cleavage of the C7–C8 bond), 383 (loss of TMSOH from the fragment at m/z 473), 371 (CTF corresponding to cleavage of the C11–C12 bond), 353 (443-TMSOH), 315 (MTF corresponding to cleavage of the C10–C11 bond), 281 (371-TMSOH), 243 (second most significative abundant fragment), and 213 (base peak). This fragmentation was consistent with the structure of $TrXA_3$ (Jones *et al*, 1978; Bryant and Bailey, 1979; German and Kinsella, 1986) (**Fig 5**). The detection of three gas chromatography peaks indicates the presence of at least three diastereoisomers of $TrXA_3$ in the samples from psoriatic samples. The two gas chromatography peaks monitoring at m/z 269 (peaks 2 and 4 of **Fig 4**) had similar mass spectra, with ions at m/z 569 ($[M-15]^+$), 479, 473, 463, 383, 371 (CTF corresponding to cleavage of the C11–C12 bond), 315 (MTF from cleavage of the C10–C11 bond), 281, 269 (base peak), 225 (the second most abundant ion that likely arises from the loss of TMSOH of fragment m/z 315), and 213. This fragmentation was consistent with the $TrXB_3$ structure (German and Kinsella, 1986) present in psoriatic samples at least as two diastereoisomeric forms (**Fig 5**). Peak 5 showed significant ions at m/z 569, 479, 473, 463, 444, 383, 353, 341 (MTF corresponding to cleavage of the C8–C9 bond), 243 (base peak), and 213 (second most

signified abundant fragment). This fragmentation was consistent with the structure of 8,9,12-THETrE (**Fig 5**). The mass spectrum of peak 2 showed signified additional fragments at m/z 353 and 341 and a relative abundant ion at m/z 243, indicating that its identity actually consisted of a mixture of $TrXB_3$ and 8,9,12-THETrE (**Table II**). No trioxilins were detected in normal epidermal fragments.

For additional support of the structural assignment of the triols, the TrX fraction was subjected to catalytic hydrogenation and the ME-H-TMS derivatives were analyzed by full scan GC-MS and monitoring specific ions for the different expected hydroxyl group positions. The unresolved ME-H-TMS derivatives of trihydroxy acids in the total ion chromatogram were deconvoluted by monitoring at m/z 255 (specific for hydrogenated 8,9,12-THETrE), m/z 273 (specific for hydrogenated $TrXB_3$), and m/z 285 (base ion of hydrogenated $TrXA_3$). Chromatograms monitoring at m/z 255 and 285 ions of samples corresponding to the TrX fraction from psoriatic scales showed two peaks with CN of 24.47 and 24.67 (peaks 1 and 2 in **Fig 6**, respectively) and essentially identical mass spectra. Significant fragments were observed at m/z 375 (CTF from C11–C12 bond cleavage), 317 (MTF from C10–C11 bond cleavage), 285 (base peak, loss of TMSOH from the m/z 375 fragment), 245 (CTF from C8–C9 bond cleavage), and 215 (MTF from C11–C12 bond cleavage). This fragmentation was consistent with the structure of the ME-H-TMS of $TrXA_3$ and similar to those obtained by other authors (Jones *et al*, 1978). These two peaks contained additional ions at m/z 345 (MTF from C8–C9 bond cleavage) and 255 (loss of TMSOH from the m/z 345 fragment) that are consistent with the structure of the ME-H-TMS derivative of the 8,9,12-THETrE (Antón *et al*, 1995). The spectra and the fragmentation schemes of the aforementioned compounds are depicted in **Fig 7**. These results indicated that peaks 1 and 2 in **Fig 6** were a mixture of the ME-H-TMS derivatives of the $TrXA_3$ and 8,9,12-THETrE. The relative intensity data apparently indicated that the former was the major compound.

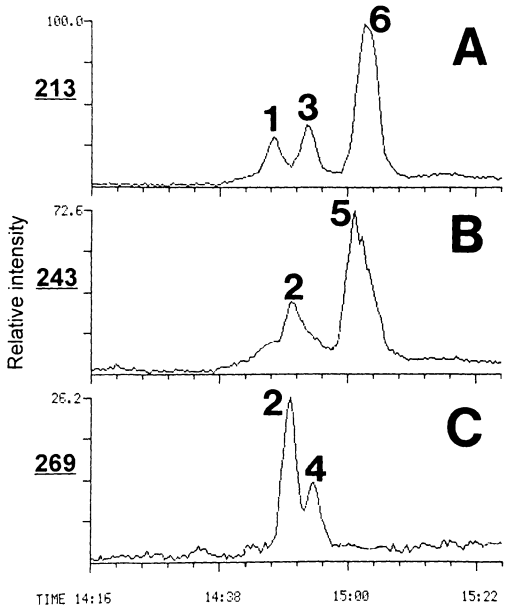

Figure 4. Different isomers of $TrXA_3$, $TrXB_3$, and 8,9,12-THETrE are present in psoriatic scales. GC-MS selected ion chromatograms of ions characteristic of the ME-TMS derivatives of the HPLC fraction 4–9 min (TrX-fraction) from psoriatic scales. Monitoring at m/z 213, 269 and 243 was performed to visualize the isomers of $TrXA_3$ (*A*), $TrXB_3$ (*B*), and 8,9,12-THETrE (*C*), respectively. Gas chromatography and mass spectra data are shown in **Table I**.

The chromatogram of monitoring at m/z 273 from the TrX fraction of psoriatic scales showed two gas chromatography peaks with CN of 24.58 and 24.70 (peaks 3 and 4 in **Fig 6**, respectively) with essentially identical mass spectra. Significant fragments were observed at m/z 375 (CTF from the C11–C12 bond cleavage), 317, 273 (CTF from the C10–C11 bond cleavage), and 215 (base ion). This fragmentation was consistent with the structure of the ME-H-TMS derivative of $TrXB_3$ (**Fig 7**). We were unable to detect TrX in the hydrogenated extract from the normal epidermis pool.

Based on HPLC and gas chromatography retention times, and on the MS analysis, we can conclude that psoriatic lesions contain HX and TrX.

Hepoxilins and trioxilins are elevated in psoriatic lesions In order to compare levels of HX and TrX in psoriatic lesions with those in normal epidermis, quantitative or semiquantitative analysis of HX, TrX, HETE, and HODE were carried out, analyzing individual samples instead of pools. For quantitative analysis we selected the ME-H-TMS derivatives of HX, TrX, HETE, and HODE as the most suitable due to their structure specific fragmentation and their high relative intensity in the positive ion EI mode GC-MS (Lehmann *et al*, 1992; Guido *et al*, 1993; Antón *et al*, 1995). Although single ion monitoring would have enabled more sensitivity, we preferred the total scan mode to assure identification. Quantitative evaluation was achieved by measuring intensity of the specific major ion in the EI mass spectrum of each compound (**Table II**). The ions in **Table II** chosen for analysis of mono-hydroxy compounds and HxB_3, formed by cleavage of the C–C bond adjacent to the TMS ether group, contain the carboxyl moiety and are therefore specific for the different positional isomers. For TrX the fragment ions selected were specific and the major ones originated from the cleavage of two adjacent carbons carrying TMS

Table I. Gas chromatography and mass spectral data for the ME-TMS derivatives of the trihydroxy compounds present in the 4–9 min HPLC fraction are consistent with the presence of TrX in psoriatic scales[a]

Compound	CN[b]	Diagnostic mass spectral ions (m/z)	Peak number in **Fig 4**
$TrXA_3$ ($TrXB_3$)[c]	23.611	569, 479, 473, 463, 444, 383, 371, 353, 315, 281, (269), **243**,[d] **213**[e]	1
$TrXB_3$ (8,9,12-THETrE)	23.643	569, 479, 473, 463, (444), 383, 371, (353), 2 (341), 315, 281, **269**, (**243**), **225**, 213	
$TrXA_3$ ($TrXB_3$)	23.702	569, 479, 473, 463, 444, 383, 371, 353, 315, 281, (269), **243**, **213**	3
$TrXB_3$ (8,9,12-THETrE)	23.715	569, 479, 473, 463, (444), 383, 371, (353), (341), 315, 281, **269**, (243), **225**, 213	4
8,9,12-THETrE ($TrXA_3$)	23.805	569, 479, 473, 463, 444, 383, 353, 341, (315), **243**, **213**	5
$TrXA_3$	23.850	569, 479, 473, 463, 444, 383, 371, 353, 315, 281, **243**, **213**	6

[a]Extracts of a pool of scales were chromatographied by reverse phase HPLC. Then, the fraction corresponding to elution of TrX was collected, derivatized, and analyzed by GC-MS in the EI mode. Gas chromatography chromatograms and fragmentation schemes are shown in **Figs 4** and **5**, respectively.
[b]CN, carbon number.
[c]Compounds in parentheses are those whose specific ions (also in parentheses) are present in minor abundance in the mass spectrum.
[d]The most abundant ions are highlighted.
[e]Base ions are underlined.

ether groups. In the case of $TrXA_3$ and $TrXB_3$ the selected ions also contain the carboxyl moiety. Whereas, for 8,9,12-THETrE, the selected ion contains the methyl moiety, because the fragment containing the carboxyl moiety is common to 8,9,12-THTrE and $TrXA_3$ ME-H-TMS derivatives that coeluted in our gas chromatography system.

$^{18}O_2$-carboxyl labeled internal standards of HxB_3 and the major monohydroxy acids (9-HODE, 13-HODE, 12-HETE, 15-HETE) were synthesized. As only 3.6% of authentic (±)HxB_3 remained unlabeled, this percentage was not enough to interfere with the quantitative analysis. The yield of labeling for 9-HODE, 13-HODE, 12-HETE, and 15-HETE was high ($\%^{18}O_0 < 1$). In addition, the nonlabeled fraction from the internal standards was intrinsically taken into account in the calibration curves. A low yield was obtained with the ^{18}O labeling of 5-HETE ($\%^{18}O_0 > 12$) and during the manipulation of the samples the percentage of ^{18}O decreased notably, probably due to the formation of the lactone intermediate between the hydroxyl at C5 position and the carboxyl group. Therefore, 5-HETE was evaluated using the $^{18}O_2$ 12-HETE as internal standard instead of the $^{18}O_2$ 5-HETE. A linear response was obtained in the range tested (1–200 ng injected) for each compound with correlation coefficients varying between 0.989 and 1.

^{18}O labeling of authentic TrX was not possible because they were not commercially available. Therefore minor HETE and TrX were only semiquantitatively evaluated.

Table III shows the quantitative data from the analysis of psoriatic scales and normal epidermis. As expected, we found that HETE and HODE were present in significantly higher amounts in psoriatic lesions

Table II. Ions selected to quantitate the indicated compounds by EI GC-MS analysis were the specific ones

Species	$^{18}O_0$	$^{18}O_1$	$^{18}O_2$
9-HODE	259[a]	261	263
13-HODE	315	317	319
5-HETE	203	205	207
8-HETE	245	–	–
9-HETE	259	–	–
10-HETE	273	–	–
11-HETE	287	–	–
12-HETE	301	303	305
15-HETE	343	345	347
HxB_3	273	275	277
$TrXA_3$	285	–	–
8,9,12[b]	255	–	–
$TrXB_3$	273	–	–

[a]Ions correspond to α-cleavage to a hydroxyl group of the ME-H-TMS derivatives. These derivatives were the most suitable due to their structure specific fragmentation and their high relative intensity in the EI mode GC-MS.
[b]8,9,12-THETrE.

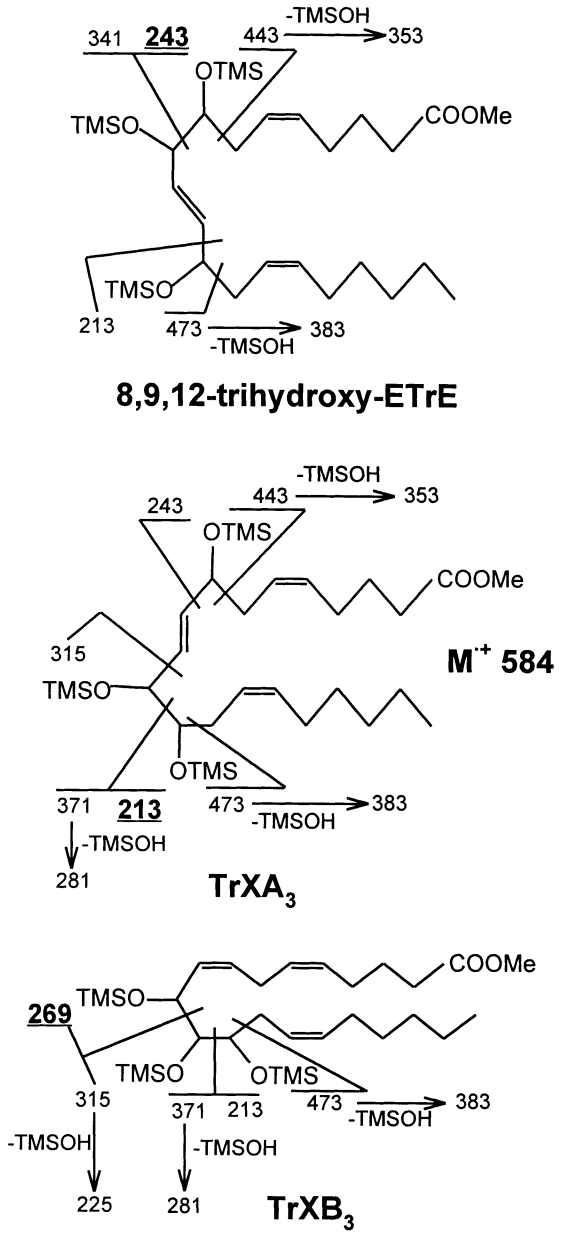

Figure 5. Mass spectra of gas chromatography peaks of the samples from psoriatic scales are consistent with the structures of $TrXA_3$, $TrXB_3$, and 8,9,12-THETrE. Fragmentation schemes for the ME-TMS derivatives of the triols present in the TrX-HPLC fraction from psoriatic scales (**Fig 4**, **Table I**). The major diagnostic ions (numbers underlined) correspond to those underlined as base ions in **Table I**. No TrX were detected in normal epidermis.

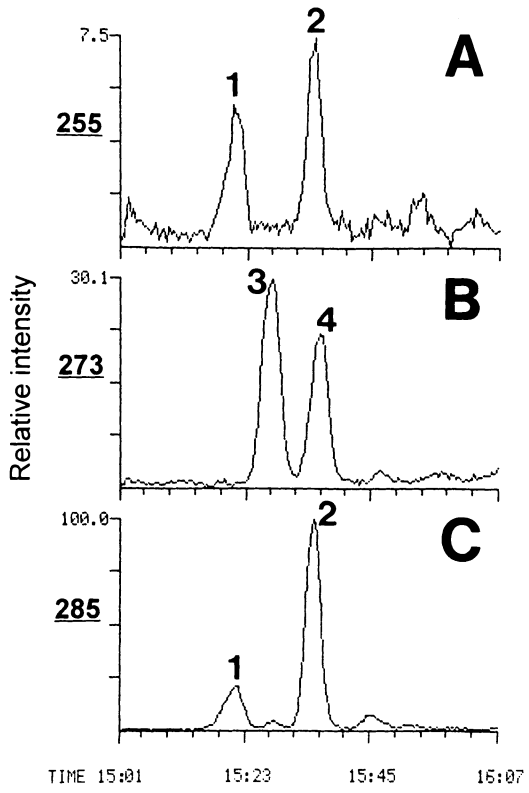

Figure 6. GC-MS analysis of the hydrogenated 4–9 min HPLC fraction confirmed the presence of different triols in psoriatic scales. EI GC-MS selected ion chromatograms of the ME-H-TMS derivatives of the TrX HPLC fraction from the psoriatic scale pool. The selected ions were m/z 255 for the 8,9,12-trihydroxy derivatives (*A*), 273 for $TrXB_3$ (*B*), and 285 for $TrXA_3$, (*C*). Mass spectra and fragmentation schemes are depicted in **Fig 7**.

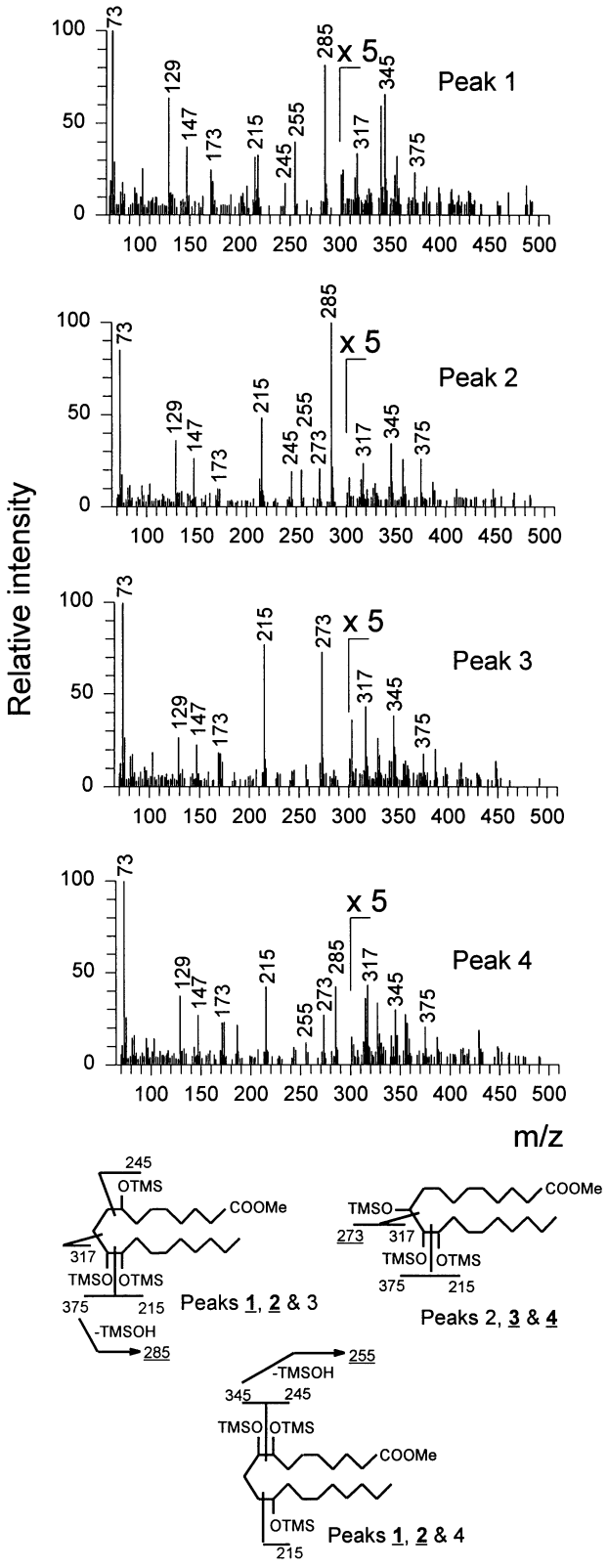

Figure 7. Mass spectra of gas chromatography peaks from hydrogenated 4–9 min HPLC fraction from psoriatic scale samples were consistent with the structures of hydrogenated $TrXA_3$, $TrXB_3$, and 8,9,12-THETrE. Mass spectra and fragmentation schemes of compounds corresponding to the peaks numbered in **Fig 6**. The major diagnostic ions numbers are underlined. The peaks in which the compounds are present are also indicated.

Table III. Levels of HX, TrX, HETE, and HODE are elevated in psoriatic scales[a]

	Normal epidermis		Psoriatic scales	
Compound	ng/mg	$A/A_{12\text{-HETE}}$[b]	ng/mg	$A/A_{12\text{-HETE}}$
HxA_3	–	nd[c]	–	nd
HxB_3	<0.2[d]	–	3.2 ± 2.3[e]	–
$TrXA_3$	–	nd	–	0.65 ± 0.23
8,9,12-THETrE	–	nd	–	0.05 ± 0.02
$TrXB_3$	–	nd	–	0.32 ± 0.28
12-HETE	0.8 ± 0.3	1	21.3 ± 12.4	1
15-HETE	<0.2[d]	–	13.2 ± 5.1	–
5-HETE	<0.2[df]	–	1.5 ± 0.3	–
8-HETE	–	<0.04	–	0.32 ± 0.17
9-HETE	–	nd	–	0.11 ± 0.06
10-HETE	–	nd	–	0.07 ± 0.03
11-HETE	–	<0.03	–	0.11 ± 0.09
9-HODE	0.5 ± 0.2	–	46.6 ± 41.5	–
13-HODE	0.4 ± 0.2	–	98.0 ± 88.9	–

[a]Samples were extracted with MeOH and the internal standards were added. The extracts were then processed by solid phase extraction, purified by HPLC, and derivatized to their ME-H-TMS derivatives previous to GC-MS analysis. Quantitation was performed taking into account the linearity curves built with respect to the internal standards. When standards were not available for a compound, semiquantitative evaluation of TrX, 8-HETE, 9-HETE, 10-HETE, and 11-HETE levels was accomplished by calculating the ratio of the areas of the corresponding gas chromatography peak to that of 12-HETE.

[b]These data are semiquantitative values in terms of peak area relative to 12-HETE.

[c]nd, not detected.

[d]HxB_3 was only detected on two samples of normal epidermis.

[e]Mean ± SD, n = 5 in all cases.

[f]5-HETE was quantitated using $^{18}O_2$ 12-HETE as internal standard.

than in normal epidermis. HxB_3 was also present and was more than 16-fold higher in psoriatic lesions than in normal epidermis ($p < 0.05$, t test). TrX were not detected in normal epidermis. Apparently the most abundant triol in the psoriatic scales was $TrXA_3$ followed by $TrXB_3$, whereas 8,9,12-THETrE was present in small amounts. As expected, 12-HETE was the most abundant HETE found in psoriatic scales. We observed that the amount of 12-HETE was not well correlated with those of HxB_3 and TrX (data not shown).

DISCUSSION

To date, the formation of HX and TrX has been observed in several animal and human tissues only *in vitro* (reviewed in Pace-Asciak *et al*, 1995a, b), or *in vivo* after a bolus injection of AA (Pace-Asciak *et al*, 1987). Here, we demonstrate the existence of HX and TrX in human tissue under a pathophysiologic situation.

The fact that untransformed HxA_3 was not found in the psoriatic samples or in normal epidermis was an expected result because HxA_3 is very unstable in the acidic conditions of our work-up, whereas HxB_3 is more stable (Nair and Jahnke, 1987; Pace-Asciak, 1994). $TrXA_3$ is formed from HxA_3 nonenzymatically or enzymatically by means of an epoxide hydrolase (Pace-Asciak *et al*, 1981; Pace-Asciak, 1988; Pace-Asciak and Lee, 1989). Formation of high amounts of HxA_3 in psoriatic lesions was supported by the fact that a great amount of $TrXA_3$ was found.

HxB_3 formed from 12(S)-HPETE has a *trans*-configuration of the epoxide group, determined by the configuration of the epoxide carbons 11(S),12(S) (Pace-Asciak, 1994; Reynaud *et al*, 1994). The hydroxyl group is located in a chiral center at C10, hence HxB_3 exists in two diastereoisomeric forms. We reported that hydrogenated HxB_3 from normal epidermis incubated with exogenous AA yielded only one gas chromatography peak with a MS spectrum corresponding to one of the two possible 10-hydroxy epimers of HxB_3, whereas samples from authentic hydrogenated racemic HxB_3 yielded two gas chromatography peaks that correspond to the two epimeric forms at C10 (Antón *et al*, 1995). Unexpectedly, in addition to these two peaks, psoriatic samples yielded a major peak with a MS spectrum also consistent with the structure of the hydrogenated HxB_3. One can speculate that, in psoriatic lesions, the enantiomer 10(S,R)-hydroxy-11(R),12(R)-epoxy is also present. This could be possible because the R enantiomer of

12-HETE is more abundant than 12(S)-HETE in psoriatic lesions (Woollard, 1986; Baer *et al*, 1991). In contrast, 12(S)-HETE is the predominant enantiomer in human cell suspensions (S:R enantiomer ratio varied between 2:1 and 8:1) (Holtzman *et al*, 1989), epidermal homogenates, or fragments (S:R enantiomer ratio was ≈3:1) (unpublished results). Part of the 12(R)-HETE could originate from 12(R)-HPETE; however, 10(S,R)-hydroxy-11(S),12(S)-epoxy and 10(S,R)-hydroxy-11(R),12(R)-epoxy configurations are enantiomers, which coelute when a nonchiral stationary phase is used, resulting in only two peaks corresponding to the two epimers at C10. Hence, the most probable explanation for the additional peak found in psoriatic scales would be the presence of the diastereomers 10(S,R)-hydroxy-11(R),12(S)-epoxy or/and 10(S,R)-hydroxy-11(S),12(R)-epoxy compounds, which are *cis*-epoxides, in the psoriatic samples. Monooxygenases produce *cis*-epoxides (Capdevila *et al*, 1984, 1990), in contrast to *trans*-epoxides formed by lipoxygenases (Garssen *et al*, 1976). Nevertheless, monooxygenases produce epoxy-eicosatrienoic acids instead of hydroxy-epoxy acids (Carrol *et al*, 1988). So, it is possible that 10-hydroxy-*cis*-epoxides were formed by rearrangement of an AA peroxide radical intermediate as a result of an auto-catalytic process induced by free radicals, e.g., $OH\cdot$, or by $ONOO^-$ formed by the reaction of $NO\cdot$, with $O_2\cdot$, (Rubbo *et al*, 1994; Laskey and Mathews, 1996). In fact, overexpression of inducible NO synthase has been reported in psoriatic skin (Kolb-Bachofen *et al*, 1994; Sirsjö *et al*, 1996).

Human heel callus is only composed by stratum corneum and is not able to metabolize AA (Heinnecke-von Zepelin *et al*, 1991). In this aspect, the stratum corneum is different from psoriatic scales, which apparently still have active enzymes able to transform AA into HETE (Baer *et al*, 1991). So, for the quantitative study we preferred to compare psoriatic scales with normal human epidermal fragments formed by the corneal and granular layers instead of human heel callus.

Consistent with data reported by other authors (Camp *et al*, 1983), we found that the major products analyzed were octadecanoids derived from linoleic acid, 13-HODE, and 9-HODE, through the action of 15-LO and cyclooxygenase, most likely cyclooxygenase-2 (Burrall *et al*, 1988; Camacho *et al*, 1995; Godessart *et al*, 1996). As expected, 12-HETE was the major eicosanoid. We found levels of HETE and HODE in psoriatic scales in the same order of magnitude but higher than those reported by others (Camp *et al*, 1983; Fogh *et al*, 1989). These differences may arise from the different analytical procedures used, such as peak integration by optical density performed by Fogh *et al* (1989) or the semiquantitation by GC-MS without taking into account the recovery of the process as Camp *et al* (1983) did. We corrected for the recovery and uncontrollable instrument variations by adding a ^{18}O labeled internal standard. Another reason for the differences with respect to data reported by others could be the great variation between patients, even though our results ranged in the limits observed by Fogh *et al* (1987). These authors demonstrated differences in the content of lipoxygenase products between different layers of the lesions in the same patient. In this sense, we worked with scales from untreated chronic plaques, from different body areas containing only suprapapillary epidermis.

We found that the amount of HxB_3 was more than 16-fold higher in psoriatic lesions than in normal epidermis, and the ratio of HxB_3 12-HETE levels (0.15) was quite significant. It is worthwhile to emphasize that HxB_3 was present in psoriatic scales at concentrations able to exert biologic effects (assuming a density of 1 g per ml, the concentration of HxB_3 and 12-HETE in psoriatic epidermis was 10 μM and 66 μM, respectively) (Pace-Asciak, 1994). Apparently, the most abundant TrX in the psoriatic scales was $TrXA_3$ followed by $TrXB_3$, although 8,9,12-THETrE was present in small amounts. This could be explained by the conversion of HxA_3 into $TrXA_3$ in the tissue and/or during the analytical procedure. In fact, HxA_3 was converted into $TrXA_3$ because we did not detect it. So, the $TrXA_3$ measured was actually the sum of the HxA_3 and $TrXA_3$ present in the samples. An interesting observation is that the total amount of triols in psoriatic lesions was similar to that of 12-HETE in terms of peak area.

Support for the potential role of HX in the pathogenesis of inflammatory skin diseases, in particular in psoriasis, includes their potent action on plasma permeability when injected subcutaneously (Laneuville *et al*, 1991; Wang *et al*, 1996), a specific receptor dependent (Nigam *et al*, 1993; Reynaud *et al*, 1996) induction of Ca^{2+} mobilization from endogenous sources (Dho *et al*, 1990; Laneuville *et al*, 1993), release of AA and diacylglycerol (Nigam *et al*, 1993) in human neutrophils, and the detection of considerable amounts in psoriatic lesions. Interestingly, whereas (±)HxA_3 and (±)HxB_3 are both active in enhancing the bradykinin evoked permeability in skin, only 10(*R*)-HxB_3 (which probably is the epimer synthesized by normal epidermis; Antón *et al*, 1995) stereospecifically enhances the vascular permeability evoked by intradermal injection of platelet activating factor (Wang *et al*, 1996).

As a result, Ca^{2+} dependent phospholipase A2 could be activated and the translocation of 5-LO would lead to the release of other mediators such as leukotrienes. It has also been observed that a Ca^{2+} pulse, elicited by IL-8, promotes epidermal cell proliferation (Tuschil *et al*, 1992). The effect of HxA_3 on Ca^{2+} mobilization has been demonstrated in neutrophils and whether or not this effect also occurs in epidermal cells should be the subject of further investigations.

In summary, the presence of biologically active amounts of HX and TrX in psoriatic lesions suggests that these compounds could play a role as modulators of the inflammatory response on skin. More research focused on the biologic role on dermatoses and the biochemical pathways involved in the biosynthesis of HX in human epidermis is under progress in our laboratory.

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