Hepoxilin B_3 and its Enzymatically Formed Derivative Trioxilin B_3 are Incorporated into Phospholipids in Psoriatic Lesions

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In previous studies we observed that normal human epidermis forms 12-oxo-eicosatetraenoic acid (12-oxo-ETE) and hepoxilin B_3 (HxB_3) as major eicosanoids, both being elevated in psoriasis. We also observed that normal epidermis, in a reaction probably catalyzed by 12-lipoxygenase, only synthesize one of the two possible 10-hydroxy epimers of HxB_3. We have now extended these previous studies investigating further transformation of HxB_3 into trioxilin B_3 ($TrXB_3$) and esterification of both into phospholipids. Phospholipids were extracted from normal epidermis and from psoriatic scales. A combination of high performance liquid chromatography and gas chromatography–mass spectrometry analysis demonstrated the occurrence of HxB_3 and $TrXB_3$ in the phospholipids of psoriatic lesions. Alkaline- and phospholipase-A_2-mediated hydrolysis of the phospholipids yielded similar quantities of both HxB_3 and $TrXB_3$ indicating their preference for the *sn-2* position of glycerophospholipids. The thin layer chromatography analysis of the phospholipid classes after incubation of epidermal cells with [^{14}C]-labeled HxB_3, $TrXB_3$, 12-hydroxy-eicosatetraenoic acid (12-HETE), 12-oxo-ETE, or 15-HETE showed that 12-HETE was the most esterified (12-HETE > 15-HETE > $TrXB_3$ > 12-oxo-ETE > HxB_3). HxB_3 and $TrXB_3$ were mainly esterified in phosphatidyl-choline and phosphatidyl-ethanolamine. HxB_3 was also enzymatically converted into $TrXB_3$ *in vitro*. HxB_3 epoxide hydrolase-like activity was not observed when boiled tissue was incubated with [^{14}C]-HxB_3, this activity being located in the cytosol fraction (100,000 × *g* supernatant) of fresh tissue. These findings suggest that *in vivo* some part of HxB_3 is transformed into $TrXB_3$ and both compounds are partially incorporated into the phospholipids. ***Key words: human epidermis/12-lipoxygenase/arachidonic acid. J Invest Dermatol 118:139–146, 2002***

12-Lipoxygenase (12-LO) is the major arachidonic acid (AA) oxygenation pathway in epidermal cells with total product formation generally exceeding cyclooxygenase activity (Holtzman *et al*, 1989; Solá *et al*, 1992). 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). We previously reported that, in addition to 12-hydroxyeicosatetraenoic acid (12-HETE), normal human epidermis incubated with exogenous AA produces 12-oxo-eicosatetraenoic acid (12-oxo-ETE), hepoxilin A_3 (HxA_3), and hepoxilin B_3 (HxB_3) through the 12-LO pathway (Antón *et al*, 1995; Antón and Vila, 2000). Recently, we also observed increased levels of nonesterified hepoxilins and trioxilins in the psoriatic scales (Antón *et al*, 1998). Normal human epidermis synthesized only one of the two possible 10-hydroxy epimers of HxB_3 whose formation is probably catalyzed by 12-LO (Antón *et al*, 1995; Antón and Vila, 2000). Hepoxilins exert action on plasma permeability on skin (Laneuville *et al*, 1991; Wang *et al*, 1996, 1999a; 1999b), and induce a specific-receptor-dependent Ca^{2+} mobilization from endogenous sources (Dho *et al*, 1990; Laneuville *et al*, 1993) and the release of AA and diacylglycerol (Nigam *et al*, 1993). Interestingly, only the epimer 10(*R*)-HxB_3, which is probably the epimer synthesized by normal epidermis (Antón *et al*, 1995; Antón and Vila, 2000), stereospecifically enhances the vascular permeability evoked by intradermal injection of the platelet-activating factor (Wang *et al*, 1996; 1999a; 1999b).

On the other hand, less polar eicosanoids and octadecanoids, such as monohydroxy and epoxy derivatives of arachidonic and linoleic acids, have been found to esterify into cell phospholipids. Oxidized phospholipids have pro-inflammatory activities and are involved in atherogenesis, psoriasis, and other inflammatory diseases. In human epidermis, HxB_3 can be further converted into trioxilin B_3 ($TrXB_3$, 10,11,12-trihydroxy-5,8,14-eicosatrienoic acid) (Antón *et al*, 1995). As relevant biologic activity for

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Abbreviations: AA, arachidonic acid; HPETE, hydroperoxyeicosatetraenoic acid; HxA_3, hepoxilin A_3, 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid; HxB_3, hepoxilin B_3, 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid; LO, lipoxygenase; ME-H-TMS, hydrogenated methyl ester trimethylsilyl ether; ME-TMS, methyl ester trimethylsilyl ether; 12-oxo-ETE, 12-oxo-eicosatetraenoic acid; PhC, phosphatidyl-choline; PhE, phosphatidyl-ethanolamine; PhI, phosphatidyl-inositol; PhS, phosphatidyl-serine; RP-HPLC, reverse phase-high performance liquid chromatography; SPh, sphingomyelin; $TrXA_3$, trioxilin A_3, 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid; $TrXB_3$, trioxilin B_3, 10,11,12-trihydroxy-5,8,14-eicosatrienoic acid.

trioxilins has not been reported (Pace-Asciak *et al*, 1999) these compounds could represent a pathway for hepoxilin inactivation. Enzymatic transformation of hepoxilins into trihydroxy compound was originally reported in rat lung (Pace-Asciak *et al*, 1983). No data about the enzymatic metabolism of HxB_3 in human epidermis are at present available, however. This prompted us to extend our previous investigations (Antón *et al*, 1995; 1998; Antón and Vila, 2000) on the presence of HxB_3 in the phospholipid fraction of psoriatic lesions, on HxB_3 incorporation into the different phospholipid classes in human epidermal cells, and on the enzymatic pathways involved in the catabolism of HxB_3.

MATERIALS AND METHODS

Materials [1–^{14}C]-AA (55–58 mCi per mmol) was supplied by Amersham Ibérica (Madrid, Spain). Phospholipase A_2 from bee venom was supplied by Sigma-Aldrich Química (Madrid, Spain). Hydrated platinum (IV) oxide was purchased from ICN Biochemicals (Costa Mesa, CA). Hydrogen gas was purchased from Abelló Oxígeno-Linde (Barcelona, Spain). 1-Stearoyl-2-[1–^{14}C]-arachidonoyl-L-3-phosphatidylcholine was obtained from Amersham Ibérica. (±)-HxB_3 were from Cascade Biochem (Berkshire, U.K.). All high performance liquid chromatography (HPLC) solvents were supplied by Scharlau (Barcelona, Spain) and solvents for mass spectrometry analysis and N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) were purchased from Merck (Darmstadt, Germany).

Source of normal human epidermis fragments and epidermal cell suspensions Epidermis was isolated from normal skin, obtained from plastic surgery, using the Liu and Karasek technique (Liu and Karasek, 1978) with minor modifications (Solá *et al*, 1992). Briefly, narrow strips of skin were cut and rinsed twice in phosphate-buffered saline, pH 7.4, free of Ca^{2+} and Mg^{2+} (PBS*). The strips were then placed in PBS* containing 0.5% trypsin (wt/vol) (Difco Laboratories, Paisley, Scotland) and kept at 37°C for 50–60 min. When dermo-epidermal detachment occurred, epidermal strips were transferred to a culture medium [Dulbecco's modified Eagle's medium (DMEM) (Flow Laboratories, Irvine, Scotland) + 2 mM glutamine + 1 mM sodium pyruvate] containing 10% vol/vol fetal bovine serum (FBS) (Flow Laboratories). After peeling the epidermis from the dermis, the epidermal fragments were washed in DMEM. Epidermal fragments were isolated by filtration through a sterile gauze. The remaining sheets were divided into small fragments with a surgical blade (approximately 1 mm^2) and used for experiments without further manipulation. To obtain epidermal cell suspensions, after peeling the epidermis from the dermis, the epidermal fragments were then gently stirred for 10–15 min in DMEM supplemented with 2 mM glutamine, 1 mM sodium pyruvate, and 10% vol/vol FBS. The cellular suspension thus obtained was filtered through a sterile gauze to discard fragments of whole epidermis and then kept at 37°C until incubation. Only cell suspensions with viability greater than 95% were used. Fragments of fresh human epidermis and epidermal cell suspensions were used immediately.

Source of psoriatic scales 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 at –80°C under an N_2 atmosphere until analysis.

Isolation of phospholipid fraction from psoriatic scales Psoriatic scales (500–700 mg) were cut and mechanically homogenized with a Turrax T-8 in 3 ml of degassed distilled water containing 0.025‰ (wt/vol) BHT placed in an ice-water bath under an N_2 atmosphere. Next, 100,000 cpm of 1-stearoyl-2-([1–^{14}C]-arachidonyl)-phosphatidylcholine were added as internal standard. Immediately, total lipids were extracted as described previously (Bligh and Dyer, 1959). Extracts of $CHCl_3$ were dried under an N_2 stream and redissolved in 200 µl of $CHCl_3$.

Phospholipids were separated from free fatty acids and neutral lipids as described previously (Kaluzny *et al*, 1985) using solid phase extraction chromatography. All solvents were supplemented with 0.025‰ (wt/vol) BHT and the extraction was performed under an N_2 atmosphere.

Hydrolysis of ester bonds The phospholipid fraction was divided in two aliquots and phospholipids were hydrolyzed by following two different procedures: alkaline saponification and treatment with purified phospholipase A_2 (PLA_2). For alkaline saponification phospholipid extracts were dried under an N_2 stream, the residue was redissolved in 850 µl of MeOH/$CHCl_3$ 8:1, and 150 µl 40% wt/vol KOH were added. The mixture was allowed to react for 30 min at 60°C under an N_2 atmosphere (Kühn *et al*, 1994). The reaction was stopped by adding 700 µl of 50 mM phosphate buffer pH = 7.4 and acidifying until pH 2–3. Free fatty acids were then extracted twice with 2 ml diethyl ether/hexane 1:1. The reaction yield measuring release of [1–^{14}C]-AA from 1-stearoyl-2-[1–^{14}C]-arachidonoyl-L-3-phosphatidylcholine was about 94%.

For specific hydrolysis of the sn-2 position, phospholipid extracts were dried under an N_2 stream, and the residue was redissolved in 10 µl of $CHCl_3$ and 70 µl of 30 mM borate buffer (pH = 9.0) supplemented with 1.6 mM $CaCl_2$. The mixture was then shaken vigorously and $CHCl_3$ was removed under an N_2 stream. 850 U of bee venom PLA_2 in 85 µl of 30 mM borate buffer pH 9.0 were added and the mixture was allowed to react for 45 min at 37°C under an N_2 atmosphere with continuous agitation. Afterwards, another 850 U of PLA_2 were added and allowed to react for another 100 min (Smiley *et al*, 1991). Reaction was stopped by acidification until pH 2–3 and lipids were extracted as described by Bligh and Dyer (1959). After analysis of the free [^{14}C]-AA released from 1-stearoyl-2-[1–^{14}C]-arachidonoyl-L-3-phosphatidylcholine the yield of the reaction was about 96%.

Preparation of purified [14C]-labeled HxB3, 12-oxo-ETE, and 12-HETE Labeled 12-LO-derived eicosanoids were obtained from incubations of fragments of human epidermis, previously treated with 200 µM aspirin for 15 min, with 100 µM [^{14}C]-AA for 30 min at 37°C, and processed as described previously (Antón and Vila, 2000). Supernatants of several incubations were mixed and processed together. Supernatants of the incubations, which had a ratio MeOH:H_2O of 1:1 and a pH of 2–3, were extracted three times with half a volume of diethyl ether:hexane 1:1. Extracts were evaporated under an N_2 stream until dryness, redissolved in the eluent of reverse phase high performance liquid chromatography (RP-HPLC), and chromatographied as described later. Fractions containing hepoxilins (14–20 min) and 12-oxo-ETE plus 12-HETE (25–33 min) were collected and purified by straight-phase HPLC as reported previously (Antón *et al*, 1995). The specific activity of the labeled compounds was assumed to be the same as the [^{14}C]-AA batch used in their preparation as AA is labeled in the C of the carboxyl group, which is conserved in all these compounds.

Preparation of purified [^{14}C]-labeled $TrXB_3$ Labeled $TrXB_3$ was obtained from incubations of fragments of human epidermis with 10 µM [^{14}C]-labeled HxB_3 for 30 min at 37°C. After the incubation period one volume of methanol and 1 M HCl to yield a pH of 2–3 were added. Supernatants of several incubations were mixed and processed together. These supernatants were then extracted three times with half a volume of diethyl ether:hexane 1:1. Extracts were evaporated under an N_2 stream until dryness, redissolved in the eluent of RP-HPLC, and chromatographied as described above. The fraction containing $TrXB_3$ (4–11 min) was collected and purified by straight-phase HPLC as reported previously (Antón *et al*, 1995). The specific activity of the labeled $TrXB_3$ was assumed to be the same as that of [^{14}C]-HxB_3.

Preparation of purified [^{14}C]-labeled 15-HETE [^{14}C]-15-hydroperoxyeicosatetraenoic acid (15-HPETE) was obtained by incubating [^{14}C]-AA with soybean LO and was purified by straight-phase HPLC, after reduction with $NaBH_4$, as previously described (Camacho *et al*, 1995). The specific activity of the labeled 15-HETE was assumed to be the same as that of the [^{14}C]-AA batch used in its preparation.

Cell fractionation and incubation of cell fractions Epidermis fragments (1–2 g) were homogenized and microsomal (100,000 × *g* pellet) and cytosolic (100,000 × *g* supernatant) fractions were obtained as described previously (Antón and Vila, 2000) and incubated with 10 µM [^{14}C]-HxB_3 or unlabeled HxB_3 (as required) at 37°C for 30 min. Eicosanoids were analyzed by RP-HPLC, and by gas chromatography–mass spectrometry (GC-MS) when necessary, as described below.

Esterification of eicosanoids into phospholipids Ten million epidermal cells in suspension were incubated in 0.5 ml of RPMI-1640 containing 10 µM of [^{14}C]-15-HETE, [^{14}C]-12-HETE, [^{14}C]-12-oxo-ETE, [^{14}C]-HxB_3, or [^{14}C]-$TrXB_3$ for 4 h at 37°C. Reactions were stopped by adding a volume of a cold solution of 2% acetic acid in methanol. Cells were then centrifuged and supernatants were stored at –80°C for further analysis. Pellets were suspended in 1.5 ml of 2% acetic acid in methanol followed by 1.5 ml of water. Lipids were extracted according to the method described by Bligh and Dyer (1959). Extracts were dried under an N_2 stream and the residues were redissolved in 35 µl of methanol:chloroform 1:2. Labeled phospholipids were analyzed as previously described (Godessart *et al*, 1996).

RP-HPLC analysis Chromatography was performed as previously described (Antón *et al*, 1998). Quantitative analysis of 12-LO-derived compounds was done by injecting the samples directly into the column without further manipulation. The column was then coupled on line with a radioactivity detector (Beckman-171) equipped with a liquid scintillation cell. Eluents were mixed with scintillation cocktail pumped at 3 ml per min. Data from the detector were processed with a System Gold Software Beckman in a PC computer.

When isolation of eluted material was required, fractions were collected either on exit from the radioactivity detector equipped with a solid scintillation cell, or from the column, depending on labeled or unlabeled samples. In this way we collected fractions of TrXs at 4–11 min, Hxs at 14–20 min, HODEs at 20–25 min, 12-oxo-ETE (and also HODEs and 15-HETE) at 25–27 min, and HETEs 27–40 min. These fractions were later concentrated by liquid–liquid extraction after adjusting the MeOH:H_2O ratio to 1:1. Dried extracts were then derivatized for GC-MS analysis. Moreover, [1–^{14}C]-AA from an internal standard was collected in order to assess quantitative ester hydrolysis and counted in a liquid scintillation counter. Both kinds of hydrolysis were quantitative.

Derivatization All fractions except that of 12-oxo-ETE were derivatized to their hydrogenated methyl ester trimethylsilyl ethers (ME-H-TMS) as described previously (Antón *et al*, 1998). 12-oxo-ETE was first transformed into its methyl ester trimethylsilyl ether (ME-TMS), to demonstrate it was not contaminated with 12-HETE, before transformation to its ME-H-TMS derivative. All samples were dried and redissolved in heptane/BSTFA.

GC-MS analysis Analysis by GC-MS was performed as previously described (Antón *et al*, 1995, 1998). In this case, the GC column was a TRB-1 fused silica capillary column (15 m length, 0.25 mm internal diameter, 0.25 μm film thickness, Tracer Analítica, Barcelona, Spain). The gas chromatograph and mass spectrometer were Hewlett-Packard 6890 series and 5973 model, respectively.

RESULTS

HxB_3 and TrXs are present in the phospholipid fraction of psoriatic lesions After isolation, the phospholipids were hydrolyzed by two procedures: treatment with purified PLA_2 or with KOH. Samples were then subjected to HPLC fractionation. Hx and TrX fractions were then derivatized and subjected to GC-MS analysis using the full scan mode. Monitoring suitable ions for HxB_3 (*m/z* 73, 269, 282, and 311), the ME-TMS derivatives of the Hxs fraction from psoriatic scales subjected to GC-MS analysis eluted as a single peak. The EI mass spectrum was consistent with the structure of 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid (HxB_3) and it was essentially identical to that obtained with authentic (±)HxB_3. (See our previous studies for details of mass spectra (Antón *et al*, 1995, 1998).) The fragments monitored in the hydrogenated samples were at *m/z* 73, 257, 273, and 287. Samples of phospholipids from psoriatic patients showed three peaks with CN of 22.65, 22.80, and 22.96, essentially with identical mass spectra consistent with the structure of hydrogenated HxB_3. The ME-H-TMS derivative of authentic (±)HxB_3 yielded only two peaks, at CN 22.80 and 22.96 (Antón *et al*, 1995; 1998). Based on these results, we concluded that not only the two 10-hydroxy epimers of HxB_3 were present in the phospholipids of psoriatic lesions but, as occurred with the free acid fraction (Antón *et al*, 1998), another isomer was also present. No HxB_3 was detected in the phospholipids of normal epidermis (n = 6) and heel callus from normal and psoriatic subjects (n = 3). As expected, HxA_3 was not detected in the phospholipid fraction of either psoriatic scales or normal epidermis as it was not stable under our work-up conditions.

The ME-TMS derivatives of TrXs were analyzed monitoring *m/z* 213 (corresponding to the methyl terminal fragment from cleavage of the C11–C12 bond indicating the presence of a hydroxyl group at C12) and 243 (corresponding to the carboxyl terminal fragment from cleavage of the C8–C9 bond, which indicates the presence of a hydroxyl group at C8), for both trioxilin A_3 ($TrXA_3$) and 8,9,12-trihydroxyeicosatrienoic acid (8,9,12-THETrE), and at *m/z* 269 (corresponding to the carboxyl terminal fragment from cleavage of the C10–C11 bond indicating the presence of a hydroxyl group at C10) for $TrXB_3$. Three peaks monitoring at *m/z* 213 showed essentially identical mass spectra consistent with the structure of $TrXA_3$ (see our previous papers for details: Antón *et al*, 1995, 1998). As occurred with the free acid fraction of psoriatic scales (Antón *et al*, 1998), the detection of three GC peaks indicates the presence of at least three diastereoisomers of $TrXA_3$ in the phospholipid fraction of psoriatic scales. Monitoring at *m/z* 269, two GC peaks had similar mass spectra consistent with the $TrXB_3$ structure. This indicates that the $TrXB_3$ was present in psoriatic samples as at least two diastereoisomeric forms. Monitoring the 243 ion mass we observed two peaks consistent with the structure of 8,9,12-THETrE. The presence of TrXs was also confirmed by GC-MS analysis of the hydrogenated fractions. We were unable to detect TrXs in the phospholipids of normal epidermis (n = 6) and in heel callus from normal and psoriatic subjects (n = 3). Based on HPLC and GC-MS analysis we can conclude that HxB_3 and TrXs were present in the phospholipids of psoriatic lesions. For quantitative analysis we selected the ME-H-TMS derivatives of HXs, TrXs, HETEs, and HODEs as the most suitable due to their structure-specific fragmentation and their high relative intensity in the positive ion EI mode GC-MS (Antón *et al*, 1998). As authentic TrXs were not commercially available, TrXs were only semiquantitatively evaluated. Table I shows the quantitative data from the analysis of the phospholipid fraction of psoriatic scales. As expected, we found that 12-HETE, 15-HETE, 13-HODE, and 9-HODE were present in significant amounts in the phospholipids of psoriatic lesions, 13-HODE being the most abundant. HxB_3 was also present. Apparently, the most abundant triol in the phospholipids of psoriatic scales was $TrXA_3$ followed by $TrXB_3$, whereas 8,9,12-THETrE was present in small amounts. Results in Table I show that although the amount of all the eicosanoids and octadecanoids analyzed was slightly lower in the samples treated with PLA_2, differences with respect to those in which phospholipids were hydrolyzed with KOH failed to reach significance.

HxB_3 and $TrXB_3$ were mainly esterified in phosphatidyl-choline (PhC) and phosphatidyl-ethanolamine (PhE) To observe the esterification of the HxB_3 and $TrXB_3$ into the different classes of phospholipids in comparison with 12-HETE, 12-oxo-ETE, and 15-HETE, the radioactivity associated with each phospholipid class was evaluated after incubation of epidermal cell suspensions with 10 μM [^{14}C]-labeled substrates for 4 h. Results in Table II show that all eicosanoids tested were incorporated into cell phospholipids 12-HETE > 15-HETE > $TrXB_3$ > 12-oxo-ETE > HxB_3. Each eicosanoid exhibited a characteristic pattern of esterification, 12-HETE being the most esterified (PhC > PhE > phosphatidyl-serine (PhS) ≈ sphingomyelin (SPh) > phosphatidyl-inositol (PhI). PhC and PhE were the phospholipid classes in which the eicosanoids were in general mainly incorporated. In particular, esterification of HxB_3 and $TrXB_3$ were only detectable in PhC > PhE. Results concerning HxB_3 in Table II are thus approximate, as a part of the radioactivity associated with the phospholipids was probably due to $TrXB_3$. Whereas the amount of the 12-LO-derived compounds incorporated into PhI was relatively low, however, the relative amount of 15-HETE incorporated into PhI was remarkable. It was also notable that the amount of 12-oxo-ETE incorporated into PhS was the highest compared with the other compounds assayed (PhS > PhC > PhE ≈ PhI ≈ SPh).

HxB_3 was enzymatically transformed into $TrXB_3$ by epidermal cells Substrate concentration kinetics were performed by incubating fragments of human epidermis with a range of [^{14}C]-AA for 30 min and then analyzing by HPLC. Figure 1 shows a typical RP-HPLC chromatogram from samples of human epidermis incubated with 100 μM [^{14}C]-AA. As expected, four peaks corresponding to 12-LO-derived eicosanoids were observed, the identities of which were confirmed by GC-MS analysis: trioxilins ($TrXB_3$, $TrXA_3$, and 8,9,12-THETrE, which

Table I. HxB_3 was found esterified mainly in the sn-2 position of phospholipids in psoriatic lesions[a]

	PLA_2		Saponification	
Compound	ng per mg	$A/A_{12\text{-HETE}}$[b]	ng per mg	$A/A_{12\text{-HETE}}$
HxB_3	1.7 ± 0.1[c]	—	2.0 ± 0.1	—
$TrXB_3$	—	0.07 ± 0.05	—	0.08 ± 0.01
HxA_3	—	n.d.[d]	—	n.d.
$TrXA_3$	—	0.15 ± 0.13	—	0.18 ± 0.14
8,9,12-THETrE	—	0.03 ± 0.02	—	0.03 ± 0.01
12-HETE	8.8 ± 4.5	1	10.8 ± 4.6	1
15-HETE	3.2 ± 1.1	—	3.4 ± 1.8	—
9-HODE	18.3 ± 7.0	—	21.0 ± 2.6	—
13-HODE	38.1 ± 22.6	—	47.3 ± 19.8	—

[a]The internal standard was added, lipids were extracted, and phospholipids purified and hydrolized by alkaline saponification or by means of PLA_2. Free acids were extracted, separated by HPLC, and derivatized to their ME-H-TMS derivatives prior to GC-MS analysis. Quantification was performed taking into account the curves built with authentic standards.
[b]As standards were not available for TrXs, semiquantitative evaluation was accomplished by calculating the ratio of the area of the corresponding GC peak to that of 12-HETE.
[c]Mean ± SD, $n = 6$.
[d]n.d., not detected.

Table II. 15-HETE, 12-HETE, 12-oxo-ETE, HxB_3, and $TrXB_3$ were esterified in the different phospholipid classes

	15-HETE[a]	12-HETE	12-oxo-ETE	HxB_3(+ $TrXB_3$)[c]	$TrXB_3$
PhC	1.55 ± 1.22[b]	4.81 ± 2.73	0.29 ± 0.25	0.58 ± 0.30	1.79 ± 0.63
PhE	0.40 ± 0.32	1.46 ± 0.92	0.14 ± 0.10	0.31 ± 0.16	0.59 ± 0.27
PhI	0.40 ± 0.27	0.02 ± 0.02	0.17 ± 0.12	n.d.[d]	n.d.
PhS	0.11 ± 0.08	0.35 ± 0.29	0.95 ± 0.49	n.d.	n.d.
SPh	0.04 ± 0.03	0.20 ± 0.07	0.08 ± 0.05	n.d.	n.d.

[a]0.5 ml of epidermal cell suspensions (20×10^6 cells per ml) were incubated with 10 μM of [^{14}C]-labeled eicosanoids for 4 h and the radioactivity associated with the indicated phospholipid classes was evaluated as previously described (Godessart *et al*, 1996).
[b]Results are expressed as pmoles of [^{14}C]-labeled eicosanoid incorporated in 4 h by 10^6 cells, $n = 4$, mean ± SD.
[c]These results are approximate as part of the radioactivity associated with the phospholipids was due to $TrXB_3$.
[d]Not detected.

migrated unresolved), HxB_3, 12-oxo-ETE, and 12-HETE. Results in Fig 2 showed that 12-LO activity was almost linear in the range of substrate concentrations assayed (5–100 μM) indicating that the range assayed was far from that which would yield the apparent maximum velocity. Results expressed as a ratio of each 12-LO-derived eicosanoid to total 12-HPETE showed that 12-HETE decreased as substrate concentration increased, whereas 12-oxo-ETE and TrXs increased with substrate concentration. The relative amount of HxB_3 was similar in all AA concentrations tested, suggesting that the rate of further transformations of HxB_3 increased with the substrate concentration.

To observe further transformations of HxB_3, suspensions of human epidermal cells (20×10^6 cell per ml) were incubated with 10 μM of [^{14}C]-HxB_3, at 37°C for 4 h. Compounds in the supernatant were analyzed. As Fig 3 shows, part of the HxB_3 was found transformed into $TrXB_3$ (47.3% ± 11.7%, mean ± SD, $n = 4$). The identity of the $TrXB_3$ HPLC peak (Fig 4) was confirmed by GC-MS analysis of parallel incubations with nonlabeled material. We performed a full scan GC-MS of ME-TMS derivatives of the collected peak corresponding to $TrXB_3$. Specific ion monitoring at m/z 269 was performed and two peaks were observed (Fig 4). The two GC peaks had essentially identical mass spectra consistent with the $TrXB_3$ structure (Antón *et al*, 1995; 1998), which was present in at least two stereoisomeric forms. For additional support for the presence of two isomers of $TrXB_3$ in the incubates, the HPLC fraction was subjected to catalytical hydrogenation and was analyzed by full scan GC-MS, monitoring the specific ion m/z 273. Two GC peaks (Fig 4) with a fragmentation

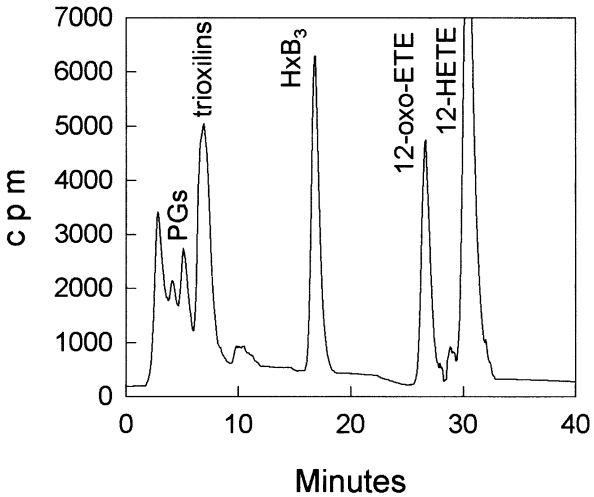

Figure 1. Human epidermis produced trioxilins HxB_3, 12-oxo-ETE, and 12-HETE as the main 12-LO-derived eicosanoids. Typical RP-HPLC chromatogram from samples of human epidermis incubated with 100 μM [^{14}C]-AA. Fragments of human epidermis were incubated with 100 μM of [^{14}C]-AA at 37°C for 30 min. Chromatography was performed isocratically in a Ultrasphere-ODS column (Beckman) with methanol:water:trifluoroacetic acid:triethylamine 75:25:0.1:0.05 pumped at 1 ml per min. The identity of the peaks was confirmed by GC-MS.

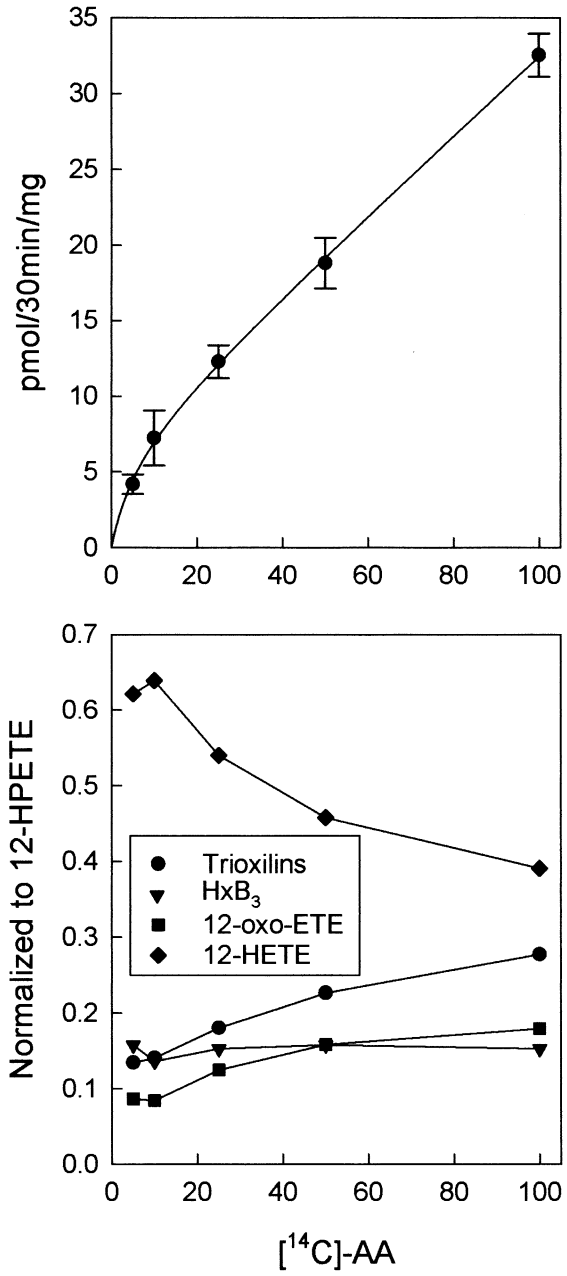

Figure 2. Human epidermis produced trioxilins, HxB_3, 12-oxo-ETE, and 12-HETE in a concentration-dependent manner and their relative amounts varied with the substrate concentration. Epidermal fragments were incubated with 5, 10, 25, 50, 100 μM concentration of $[^{14}C]$-AA at 37°C for 30 min. Reactions were stopped with one volume of cool methanol and samples were analyzed by RP-HPLC. Chromatography was performed as described in **Fig 1.** Results in the *upper panel* show the total 12-LO activity (evaluated as the sum of all 12-LO-derived peaks). The *bottom panel* shows the relative amount of each eicosanoid with respect to the total amount of 12-HPETE produced. Mean ± SD, $n = 4$.

consistent with a 10,11,12-trihydroxy-arachidic acid structure were observed (Antón *et al*, 1995, 1998).

The "HxB_3 epoxide hydrolase" activity was located in the cytosolic fraction To locate the "HxB_3 epoxide hydrolase" activity, the supernatant and pellet, obtained after centrifugation of homogenate epidermis at 100,000 × *g*, were incubated with 10 μM $[^{14}C]$-HxB_3 and labeled eicosanoids were analyzed. The enzymatic origin of $TrXB_3$ was supported by the fact that boiled

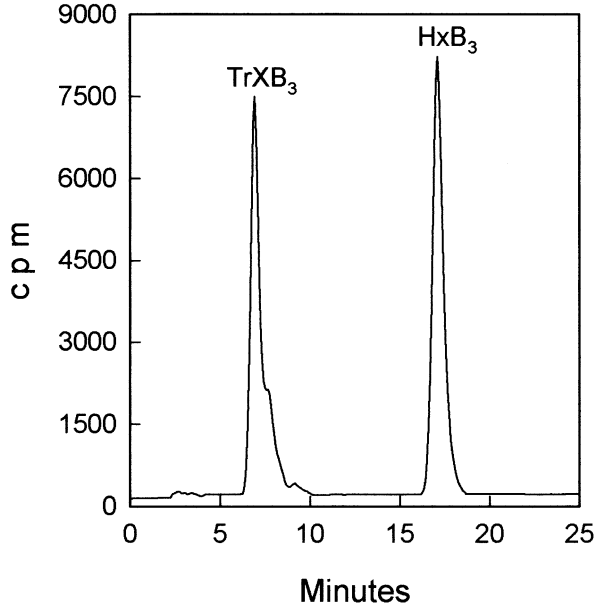

Figure 3. HxB_3 was slowly transformed into $TrXB_3$ by epidermal cells. Chromatogram obtained from a sample of the supernatant of human epidermal cell suspension (20×10^6 cell per ml) incubated with 10 μM $[^{14}C]$-HxB_3 for 4 h at 37°C. One volume of cool methanol was added to the supernatants and samples were analyzed by RP-HPLC. Chromatography was performed as described in **Fig 1**. Part of the HxB_3 was found transformed into $TrXB_3$ (47.3% ± 11.7%, mean ± SD, $n = 4$). The identity of the $TrXB_3$ peak was confirmed by GC-MS analysis of parallel incubations with nonlabeled material.

tissue did not produce $TrXB_3$ when incubated with $[^{14}C]$-HxB_3 (Fig 5). The 100,000 × *g* pellet did not transform HxB_3 into $TrXB_3$; in contrast, the supernatant efficiently converted HxB_3 into $TrXB_3$ (90.3 ± 36.0 pmol per 30 min per 100 μg protein, mean ± SD, $n = 3$).

DISCUSSION

Chromatographic behavior of HxB_3 shows that it is a hydrophobic compound but less so than monohydroxy acids. We expected that, as occurred with other hydrophobic eicosanoids and octadecanoids, HxB_3 could remain associated to the lipid fractions and would be retained in the inflammatory lesions. This concept is consistent with the fact that nonesterified HxB_3 is notably elevated in psoriatic lesions (Antón *et al*, 1998). Nonpolar eicosanoids are not only present in the inflammatory lesions as a free form but also tend to esterify in cell phospholipids, which results in large amounts of monohydroxy fatty acids esterified into cell phospholipids in inflammatory lesions (Baer *et al*, 1991; Kühn *et al*, 1992). Indeed, among the eicosanoids and octadecanoids only the hydroxy and epoxy derivatives have been reported to be incorporated into cellular phospholipids (Stenson and Parker, 1979; Schade *et al*, 1987; Brezinski and Serhan, 1990; Wang *et al*, 1990; Brinkman *et al*, 1991; Bernstrom *et al*, 1992). Here we report that HxB_3 is also present in psoriatic lesions esterified in the phospholipids in an HxB_3:12-HETE ratio of 0.19 after both alkaline saponification and PLA_2-catalyzed hydrolysis. This ratio is similar to that found on analyzing free acids (0.15, from Antón *et al*, 1998). The fact that there were no significant differences between alkaline- and PLA_2-catalyzed hydrolysis regarding HxB_3 indicated that in psoriatic lesions HxB_3 is mainly esterified in the sn-2 position of glycerophospholipids. Esterified HxB_3 and $TrXB_3$ were not detected either in normal epidermis or heel callus from normal and psoriatic subjects, indicating that they were not formed in significant amounts in places other than psoriatic lesions. Taking into account that these compounds were also undetectable in the

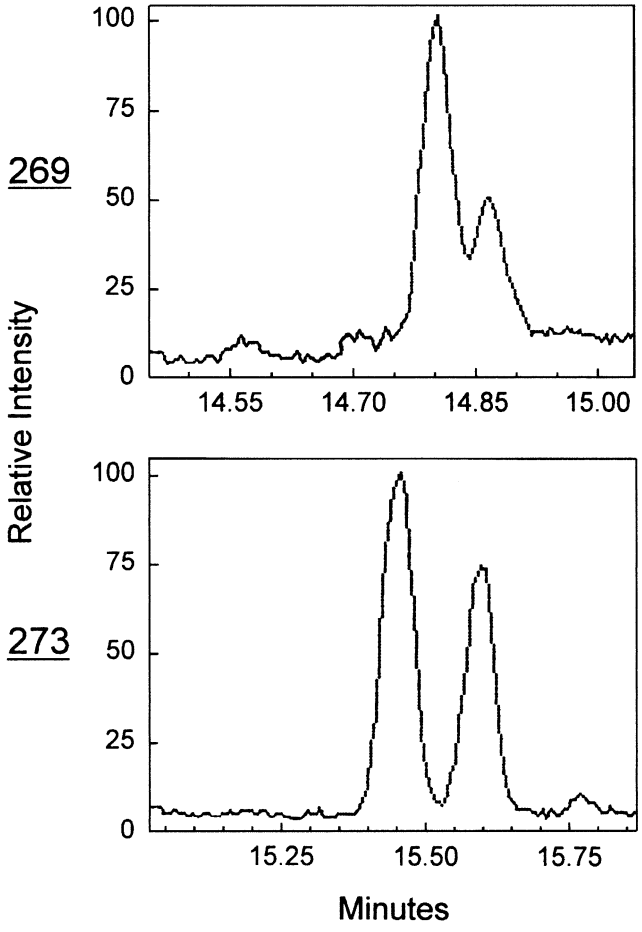

Figure 4. HxB_3 was transformed into two isomers of $TrXB_3$. GC-MS selected ion chromatograms of ions characteristic of the ME-TMS derivatives of the native (*upper panel*) and the hydrogenated ME-TMS derivative of $TrXB_3$ (*bottom panel*). Human epidermal cell suspensions (20×10^6 cell per ml) were incubated with 10 μM [^{14}C]-HxB_3 for 4 h at 37°C. One volume of cool methanol was added to the supernatants and samples were subjected to RP-HPLC as described in **Fig 1**. RP-HPLC peak corresponding to $TrXB_3$ was collected and derivatized. Full scan GC-MS of ME-TMS derivatives of native and hydrogenated $TrXB_3$ fraction were performed as described in *Methods*.

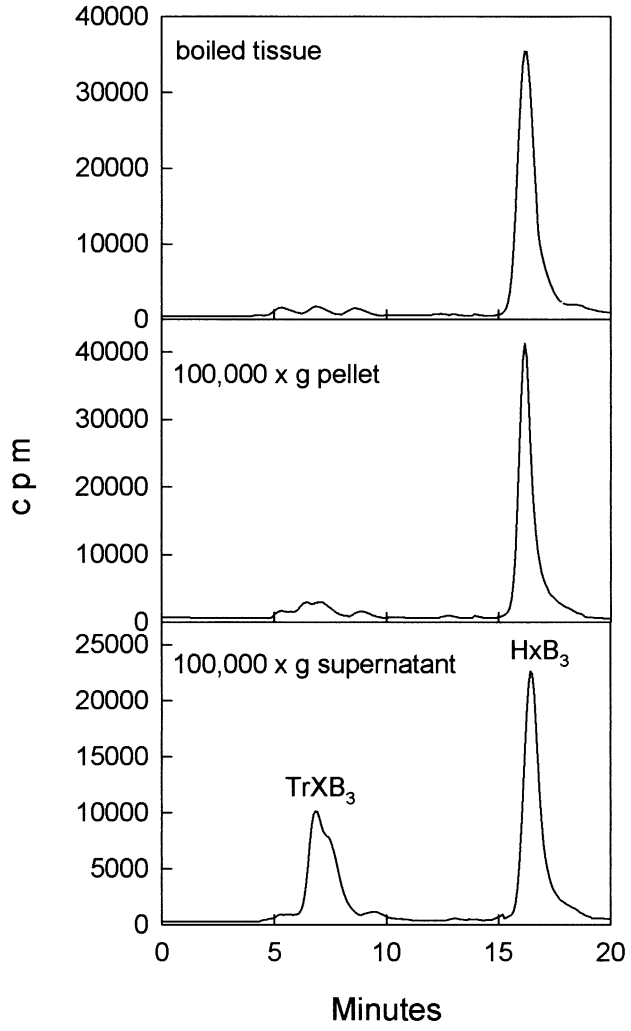

Figure 5. Formation of $TrXB_3$ from HxB_3 was enzymatic and the HxB_3 epoxide hydrolase-like activity was located in the cytosolic fraction. Representative chromatograms of boiled epidermis and cytosolic and microsomal fractions incubated with [^{14}C]-HxB_3 are shown. Human epidermis was mechanically homogenized and centrifuged at 100,000 × *g* for 90 min at 4°C. Protein equivalent amounts (250 μg) of boiled homogenate and 100,000 × *g* supernatant and pellet were incubated with 10 μM [^{14}C]-HxB_3 at 37°C for 30 min. Reactions were stopped with one volume of cool methanol and samples were analyzed by RP-HPLC. Chromatography was performed as described in **Fig 1**. 100,000 × *g* supernatant efficiently converted HxB_3 into $TrXB_3$ (90.3 ± 36.0 pmol per 30 min per 100 μg protein, mean ± SD, $n = 3$).

free form in normal epidermis (Antón *et al*, 1998), the presence of HxB_3 and $TrXB_3$ seems to be a characteristic feature of the psoriatic lesion.

Metabolism of AA in human epidermis through the 12-LO pathway results in the formation of HxB_3, 12-oxo-ETE, and several triols in addition to 12-HETE (Antón *et al*, 1995). In particular, high amounts of HxB_3 and 12-oxo-ETE are produced by whole human epidermis. Unlike hemin-catalyzed formation of HxB_3, normal human epidermis only produced one of the two possible 10-hydroxy epimers (Antón *et al*, 1995; Antón and Vila, 2000). Samples from authentic hydrogenated racemic HxB_3 yielded two GC peaks, which correspond to the two epimeric forms at C10 (Antón *et al*, 1995; Antón and Vila, 2000). As occurred regarding free acids (Antón *et al*, 1998), in addition to these two peaks phospholipids of psoriatic samples yielded a major additional peak with an MS spectrum also consistent with the structure of the hydrogenated HxB_3. As previously discussed (Antón *et al*, 1998), it is possible that other isomers of HxB_3 corresponding to 10-hydroxy-*cis*-epoxides may be formed by rearrangement of an AA-peroxide radical intermediate as a result of an autocatalytic process induced by free radicals.

Cellular phospholipids containing oxidized forms of AA or linoleic acid could be originated in two ways: (i) direct oxidation of the fatty acid substituents at the sn-1 or sn-2 positions of glycerophospholipids and diacylglycerol and (ii) re-esterification of oxidized acids with lysoglycero-phospholipids. Enzymatic oxidation of polyunsaturated lipids within glycerophosphatide substrates has been demonstrated under certain conditions, particularly in cell-free systems using exogenously supplied 15-LO (Brash *et al*, 1987; Kühn *et al*, 1990). Consistently, all compounds tested were esterified *in vitro* into phospholipids when epidermal cells were incubated with labeled compounds. Our *in vitro* data and data elsewhere (Brezinski and Serhan, 1990; Karara *et al*, 1991; Legrand *et al*, 1991), argue in favor of remodeling as a significative origin of oxidized phospholipids in psoriatic lesions. We report herein the generation of a new class of oxidized phospholipids by esterification of oxo, epoxy-hydroxy, and trihydroxy eicosanoids in cell phospholipids, but to a lesser extent than hydroxy compounds. Nevertheless, whereas HxB_3 was found in the phospholipid

fraction of psoriatic scales, 12-oxo-ETE was not detected in the samples from psoriatic patients although it was esterified when incubated *in vitro* with epidermal cells. Indeed, neither was 12-oxo-ETE found in the free form in psoriatic lesions (unpublished results). This may be due to a further transformation of 12-oxo-ETE *in vivo*; our results are not conclusive with respect to this point.

Like 12-HETE, HxB_3 and $TrXB_3$ were mainly esterified in PhC and PhE. 12-oxo-ETE was esterified into PhI, although, consistently with data reported by others (Brezinski and Serhan, 1990; Legrand *et al*, 1991; Girton *et al*, 1994), 15-HETE exhibited the highest preference for PhI when compared with the other compounds tested. The generation of oxidized phospholipids may be of relevance, as these phospholipids interfere with PhC and PhI signaling pathways by the generation of altered phosphatidic acids and diacylglycerols (Brezinski and Serhan, 1990; Legrand *et al*, 1991, 1996; Cho and Ziboh, 1994a, b; Wallukat *et al*, 1994). Accumulation of HxB_3 in the sn-2 position of glycerophospholipids could also serve as a storage pool for agonist-mediated release of HxB_3 from PhC and PhE.

Further conversion of HxB_3 in human epidermis into $TrXB_3$ could represent a pathway for hepoxilin inactivation. HxB_3, which was stable under our work-up conditions, was only enzymatically transformed to $TrXB_3$, as the conversion of HxB_3 into $TrXB_3$ was abolished by tissue boiling. As normal epidermis exclusively produces the 10(*R*)-hydroxy isomer of HxB_3, the fact that we detected two stereoisomers of $TrXB_3$ suggests that they came from the opening of the epoxide ring in C11–C12, rather than from the two epimeric positions of the hydroxyl group at C10 in HxB_3.

The "HxB_3 epoxide hydrolase" activity was located almost exclusively in the cytosol of the epidermal cells, as we only detected $TrXB_3$ in the 100,000 × *g* supernatant fraction when incubated with purified [^{14}C]-HxB_3. The cytosolic location of "HxB_3 epoxide hydrolase" in human epidermis is consistent with that reported by Pace-Asciak and Lee (1989) who found "hepoxilin epoxide hydrolase" activity in the 100,000 × *g* supernatant of rat liver homogenates, although the authors did not provide data about the activity in the microsomal fraction. Nevertheless, the same authors found hepoxilin epoxide hydrolase-like activity located in the membrane fraction in platelets (Pace-Asciak *et al*, 1986). This suggests that several hepoxilin epoxide hydrolase isoenzymes could be expressed in different tissues.

$TrXA_3$ was also present in the sn-2 position of glycerophospholipids, strongly suggesting that HxA_3 and/or $TrXA_3$ were also esterified in the phospholipids. Nevertheless, we could not evaluate the quantity of HxA_3 present in the phospholipid fraction as HxA_3 was not stable under our work-up conditions.

Support for the potential role of hepoxilins in the pathogenesis of inflammatory skin diseases includes their potent action on plasma permeability when injected subcutaneously (Laneuville *et al*, 1991; Wang *et al*, 1996, 1999a, b) and the detection of a considerable amount in psoriatic lesions in free form (Antón *et al*, 1998) and also esterified. Hepoxilins could play an autocrine role as intracellular messengers and a paracrine role modulating leukocyte activation (reviewed in Pace-Asciak *et al*, 1999). The biologic role of these compounds on dermatoses is at present under research in our laboratory.

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