

# Stereoselective Biosynthesis of Hepoxilin B<sub>3</sub> in Human Epidermis

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We previously reported that normal human epidermis forms 12-oxo-eicosatetraenoic acid and hepxilin B<sub>3</sub> as major eicosanoids and that hepxilins and trioxilins are dramatically elevated in psoriatic lesions. We also observed that normal epidermis only synthesized one of the two possible 10-hydroxy-epimers of hepxilin B<sub>3</sub>, suggesting its enzymatic origin. This study investigated the enzymatic pathways involved in the formation of hepxilin B<sub>3</sub> in human epidermis. Human epidermal fragments or cell fractions were incubated with [<sup>14</sup>C]-arachidonic acid or authentic 12(*S*)-hydroperoxyeicosatetraenoic acid. Products were analyzed by high-performance liquid chromatography, gas chromatography-mass spectrometry or a combination of both techniques. Esculetin and nordihydroguaiaretic acid inhibited formation of hepxilin B<sub>3</sub>, 12-oxo-eicosatetraenoic acid, trioxilins, and 12-hydroxyeicosatetraenoic acid in a concentration-dependent manner. 12-Lipoxygenase activity was mainly located in the microsomal fraction (100,000 × *g* pellet) and 12-

hydroxyeicosatetraenoic acid, hepxilin B<sub>3</sub>, and 12-oxo-eicosatetraenoic acid were formed. The hepxilin B<sub>3</sub>-synthesizing activity was not observed in subcellular fractions incubated with authentic 12(*S*)-hydroperoxyeicosatetraenoic acid, although it was located at least in the microsomal fraction when incubated with arachidonic acid. Similar results were obtained using preparations of recombinant platelet-type 12-lipoxygenase that yielded 12-oxo-eicosatetraenoic acid and hepxilin B<sub>3</sub> in addition to 12-hydroxyeicosatetraenoic acid, when incubated with arachidonic acid but not when incubated with 12-hydroperoxyeicosatetraenoic acid. Nevertheless, recombinant 12-lipoxygenase produced a lower ratio of 12-oxo-eicosatetraenoic acid and hepxilin B<sub>3</sub>-12-hydroxyeicosatetraenoic acid than epidermis. Our results support the concept that 12-lipoxygenase catalyzes the formation of hepxilin B<sub>3</sub> and 12-oxo-eicosatetraenoic acid. **Key words:** arachidonic acid/epidermis/hepxilin/12-lipoxygenase. *J Invest Dermatol* 114:554-559, 2000

**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, which 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). 12-Hydroperoxide-eicosatetraenoic acid (12-HPETE) formed from AA by the action of 12-LO is reduced by peroxidases to the corresponding hydroxide, 12-hydroxy-5,8,10,14-eicosatetraenoic acid (12-HETE). Consistently, 12-HETE is the most abundant eicosanoid present in skin inflammatory lesions such as in psoriasis (Hammarström *et al*, 1975; Camp *et al*, 1983; Fogh *et al*, 1987).

We previously reported that in addition to 12-HETE, normal human epidermis incubated with exogenous AA produces 12-oxo-eicosatetraenoic acid (12-oxo-ETE), and 8-hydroxy-11,12-epoxy-5,9,14-eicosatrienoic acid and 10-hydroxy-11,12-epoxy-5,8,14-eicosatrienoic acid, termed hepxilin A<sub>3</sub> (HxA<sub>3</sub>) and hepxilin B<sub>3</sub> (HxB<sub>3</sub>), respectively, which can be further converted into 8,11,12-trihydroxy-5,9,14-eicosatrienoic acid [trioxilin A<sub>3</sub> (TrXA<sub>3</sub>)] and 8,9,12-trihydroxy-eicosatrienoic acid (8,9,12-THETrE), and 10,11,12-trihydroxy-5,8,14-eicosatrienoic acid [trioxilin B<sub>3</sub> (TrXB<sub>3</sub>)], respectively (Antón *et al*, 1995).

Hepoxilins exert action on plasma permeability on skin (Laneville *et al*, 1991; Wang *et al*, 1996), induce a specific receptor-dependent Ca<sup>2+</sup> mobilization from endogenous sources (Dho *et al*, 1990; Laneville *et al*, 1993) and release AA and diacylglycerol (Nigam *et al*, 1993). Recently, we also observed increased levels of hepxilins and trioxilins in the psoriatic scales (Antón *et al*, 1998).

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Abbreviations: GC-MS, gas chromatography-mass spectrometry; HODE, hydroxyoctadecadienoic acid; HPETE, hydroperoxyeicosatetraenoic acid; (±)HxA<sub>3</sub>, hepxilin A<sub>3</sub> (8(*R,S*)-hydroxy-11(*S*),12(*S*)-epoxy-5,9,14-eicosatrienoic acid); (±)HxB<sub>3</sub>, hepxilin B<sub>3</sub> (10(*R,S*)-hydroxy-11(*S*),12(*S*)-epoxy-5,8,14-eicosatrienoic acid); LO, lipoxygenase; 12-oxo-ETE, 12-oxo-eicosatetraenoic acid; RP-HPLC, reverse phase-high performance liquid chromatography; SP-HPLC, straight phase-high performance liquid chromatography; THETrE, trihydroxy-eicosatrienoic acid. TrXA<sub>3</sub>, trioxilin A<sub>3</sub> (8,11,12-trihydroxy-5,9,14-eicosatrienoic acid); TrXB<sub>3</sub>, trioxilin B<sub>3</sub> (10,11,12-trihydroxy-5,8,14-eicosatrienoic acid).

Hepoxilins are formed by an intramolecular rearrangement of 12-HPETE (Pace-Asciak, 1984). In a previous study (Antón *et al.*, 1995) we observed that normal human epidermis only synthesized one of the two possible 10-hydroxy-epimers of HxB<sub>3</sub>, which suggested an enzymatic origin. Interestingly, whereas (±)HxA<sub>3</sub> and (±)HxB<sub>3</sub> are both active in enhancing the bradykinin-evoked permeability in skin, only 10(R)-HxB<sub>3</sub>, which is probably 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). Enzymatic conversion of 12(S)-HPETE into 8(R,S)-HxA<sub>3</sub> has been found in the rat pineal gland (Reynaud *et al.*, 1994). Enzymatic biosynthesis of HxB<sub>3</sub>, however, has not been fully demonstrated since Pace-Asciak *et al.* (1993) observed that formation of a small quantity of a racemic mixture of HxB<sub>3</sub> from 12-HPETE by different rat tissues was abolished by tissue boiling. Nevertheless, further works performed by these authors led them to the conclusion that, in contrast with that which occurs with HxA<sub>3</sub>, HxB<sub>3</sub> is a nonenzymatic product (reviewed in Pace-Asciak *et al.*, 1995a, b). All this prompted us to extend our previous investigation (Antón *et al.*, 1995, 1998) on the enzymatic pathways involved in the formation and further transformations of HxB<sub>3</sub>.

#### MATERIALS AND METHODS

**Materials** 1-[<sup>14</sup>C]-arachidonic acid ([<sup>14</sup>C]-AA) 55–58 mCi per mmol was supplied by Amersham Ibérica (Madrid, Spain). Esculetin, nordihydroguaiaretic acid, metyrapone, bifonazole, and clotrimazole were from Sigma-Aldrich Química S.A. (Alcobendas, Spain). Unlabeled AA and authentic 12-HPETE were from Cayman (Ann Arbor, MI). Authentic (±)HxB<sub>3</sub> was from Cascade Biochem (Berkshire, U.K.). 9-anthryldiazomethane (ADAM) was from Serva (Heidelberg, Germany). Recombinant human platelet-type 12-LO (1300 U per mg protein) was from Oxford Biomedical Research (Oxford, MI). All high-performance liquid chromatography (HPLC) solvents were supplied by Scharlau S.A. (Barcelona, Spain).

**Preparation 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). Fragments of fresh human epidermis, obtained as described previously (Antón *et al.*, 1995) were used immediately.

**Incubation of human epidermal fragments with AA** Epidermal fragments were placed in an Eppendorf containing RPMI-1640 plus 1 mM CaCl<sub>2</sub> in a ratio medium/tissue of 450 µl per 100 mg. Afterwards, 20 µl of an ethanolic solution of [<sup>14</sup>C]-AA or unlabeled AA, as required, were added to yield the indicated substrate concentration. Incubation was performed at 37°C for the indicated period of time, after which 1 M HCl to yield pH 2–3 followed by half a volume of cold methanol were added. Incubation mixtures were centrifuged immediately at 15,000 × g for 5 min and the supernatant was recovered. The pellet was washed with another half a volume of cold methanol and the methanolic extract was added to the previous supernatant. Samples were kept at –80°C until analysis.

**Reverse phase (RP)-HPLC analysis** Chromatography for quantitative analysis of 12-LO derived compounds was performed by injecting the samples directly into the column without further manipulation. Chromatography was performed isocratically with a mixture of methanol/water/trifluoroacetic acid/triethylamine 75:25:0.1:0.05 pumped at 1 ml per min. The column (Ultrasphere-ODS, 5 µm diameter particle, 4.6 × 250 mm, Beckman, San Ramón, CA) was coupled on line with a radioactivity detector (Beckman-171) equipped with a liquid scintillation cell. Eluents were mixed with a scintillation cocktail pumped at 3 ml per min. When required, ultraviolet absorption was monitored by means of a diode array detector Beckman-168 coupled between the column and the radioactivity detector. Data from detectors were processed with a System Gold Software Beckman in a PC-computer. When collection of eluted material was required the radioactivity detector was equipped with a solid scintillation cell.

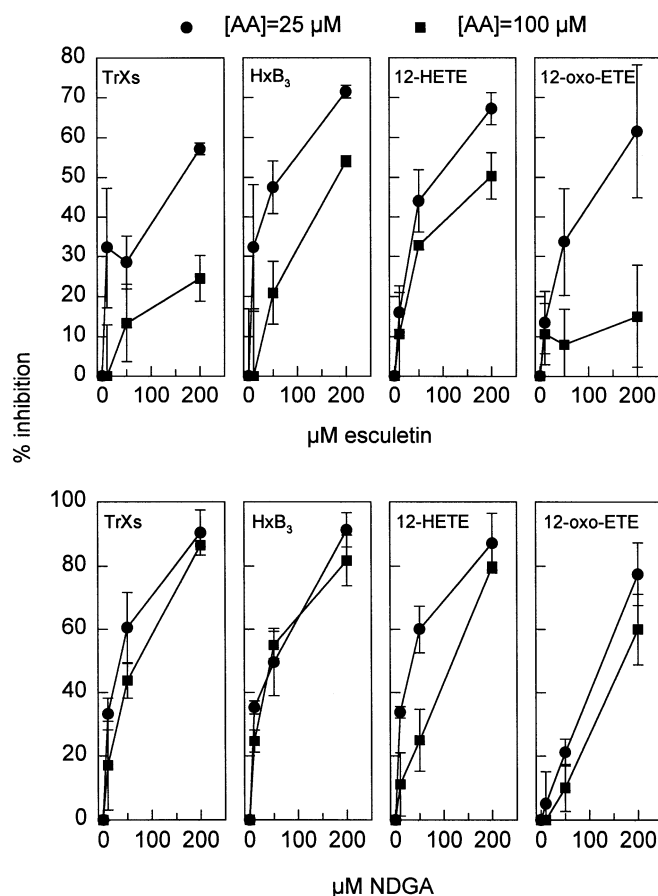
For the analysis of the HxB<sub>3</sub> epimers ADAM derivatives were obtained. Acidic water was added to the HPLC fractions containing HxB<sub>3</sub> (14–20 min) to achieve a ratio methanol/water 1:1 (pH 2–3) and were then extracted three times with half a volume of ether/hexane 1:1. Extracts were evaporated under a N<sub>2</sub> stream until dryness. Samples, dissolved in 30 µl of

MeOH, were mixed with 30 µl of 0.2% (wt/vol) ADAM in ethylacetate, and left in the dark stirring for 40 h at room temperature under N<sub>2</sub> atmosphere. The reaction mixture was evaporated and dissolved in 60 µl of CH<sub>3</sub>CN/H<sub>2</sub>O 72/28. A fraction was then injected into the chromatograph through a 20 µl loop and analyzed as previously described (Antón *et al.*, 1995).

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

**Cell fractionation** Epidermis fragments (1–2 g) were placed in 2 ml of 50 mM Tris–HCl, pH 7.4, containing protease inhibitors (10 µM pepstatin A, 10 µM leupeptin, 1 mM sodium metabisulfite, 1 mM benzamide, 1 mM phenylmethylsulfonyl fluoride, 1 mM ethylenediamine tetraacetic acid, and 1 mM ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid). Tissue was then disrupted mechanically with a Potter-Elvehjem placed in an ice-water bath. Non-lysed cells were eliminated by centrifugation at 600 × g for 15 min. The supernatant was immediately centrifuged at 8000 × g for 30 min, and the resulting supernatant at 100,000 × g for 90 min at 4°C. The 100,000 × g pellet, corresponding to the microsomal fraction, was suspended in 1.5 ml of the above-mentioned buffer. The protein content was measured by the method of Bradford (1976) using the Bio-Rad Protein Assay (Bio-Rad Laboratories S.A., Madrid, Spain).

Aliquots of 700 µl of cell fractions containing 5 mM CaCl<sub>2</sub> and 1 mM MgCl<sub>2</sub> were incubated with 100 µM [<sup>14</sup>C]-AA or 50 µM 12-HPETE at



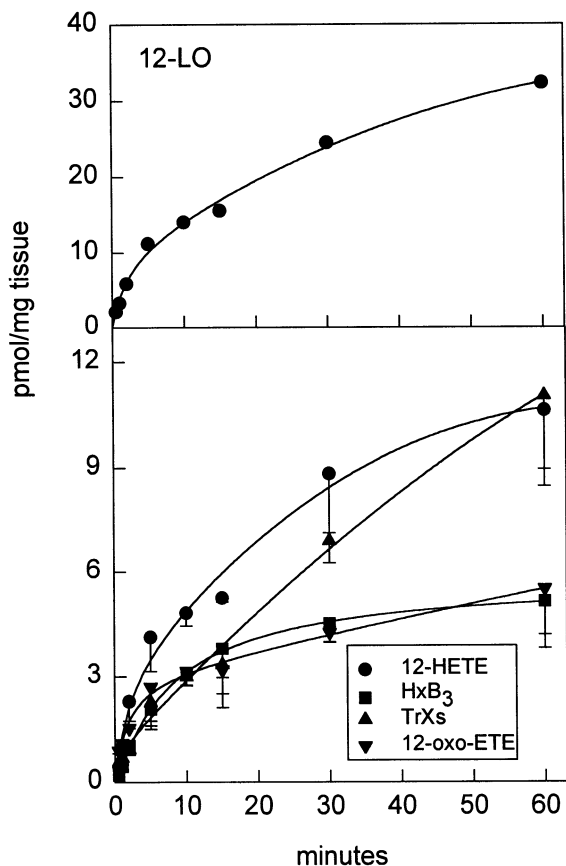
**Figure 1.** 12-LO inhibitor (esculetin) and a general LO inhibitor (nordihydroguaiaretic acid) inhibited the formation of trioxilins, HxB<sub>3</sub>, 12-oxo-EETE, and 12-HETE in a concentration-dependent manner. Fragments of human epidermis were incubated with the indicated concentration of [<sup>14</sup>C]-AA for 30 min at 37°C after preincubation for 5 min with the indicated concentration of drug. 12-LO derived eicosanoids were then analyzed by RP-HPLC. Points are the mean ± SD, n = 4.

37°C for 30 min. Incubations with AA were stopped as described for epidermal fragments, and those with 12(S)-HPETE were stopped with half a volume of MeOH containing 5 mg SnCl<sub>2</sub> per ml (final pH 2.5–3.0). 12-LO derived eicosanoids were analyzed as described.

**Incubations with human recombinant platelet-type 12-LO** To observe the formation of 12-oxo-EETE and HxB<sub>3</sub> catalyzed by 12-LO, 50 U of human recombinant platelet-type 12-LO in 250 µl of Tris-HCl 50 mM pH 8 were incubated with 200 µM [<sup>14</sup>C]-AA or 50 µM 12(S)-HPETE at 37°C for 5 min. Incubations with AA were stopped with one volume of cold methanol, and those with 12(S)-HPETE were stopped with one volume of MeOH containing 5 mg SnCl<sub>2</sub> per ml (final pH 2.5–3.0). 12-LO derived eicosanoids were analyzed by RP-HPLC and the corresponding HxB<sub>3</sub> fraction was collected, ADAM-derivatized and analyzed for epimer characterization as described above.

## RESULTS

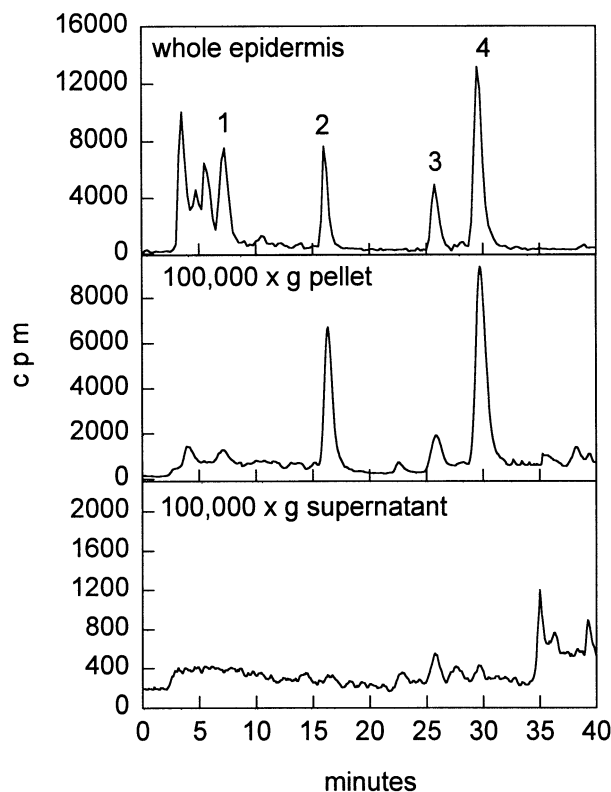
Four peaks corresponding to 12-LO derived eicosanoids were observed after RP-HPLC analysis when human epidermis was incubated with 100 µM [<sup>14</sup>C]-AA, the identity of which was confirmed by GC-MS analysis: trioxilins, HxB<sub>3</sub>, 12-oxo-EETE, and 12-HETE were detected. The 12-LO origin of these eicosanoids was demonstrated by the concentration dependent inhibition of their formation by nordihydroguaiaretic acid, an unspecific LO inhibitor, and esculetin, a 12-LO inhibitor (Fig 1). No inhibition of any of these compounds by metyrapone, bifonazole, or clotrimazole, P450 inhibitors, was observed (not shown). The progression curves of formation of these compounds, obtained by incubating fragments of human epidermis with 100 µM [<sup>14</sup>C]-AA for several periods of time, showed that whereas the levels of HxB<sub>3</sub>



**Figure 2.** Trioxilins, HxB<sub>3</sub>, 12-oxo-EETE, and 12-HETE were formed in a time-dependent manner. Fragments of human epidermis were incubated with 100 µM [<sup>14</sup>C]-AA at 37°C for distinct periods of time and the radioactive 12-LO derived compounds were analyzed by RP-HPLC. The total activity of 12-LO expressed as the sum of all 12-LO derived peaks is also depicted. Points are the mean ± SD, n = 4.

and 12-oxo-EETE achieve a plateau after 10 min of substrate addition, a continuous increase in the formation of 12-HETE and trioxilins was observed (Fig 2).

To observe the location of the “HxB<sub>3</sub>-synthase” activity, supernatant and pellet obtained after centrifugation of homogenate epidermis at 100,000 × g were incubated with 100 µM [<sup>14</sup>C]-AA, and labeled 12-LO-derived eicosanoids were analyzed. The pellet of the 100,000 × g centrifugation produced 12-HETE, HxB<sub>3</sub>, and 12-oxo-EETE, and trioxilins were almost undetectable (Fig 3). No eicosanoids were detected in the supernatant of the 100,000 × g, indicating that 12-LO activity was associated to the microsomal fraction and that “HxB<sub>3</sub>-synthase” activity was at least in this subfraction. Fragments of fresh human epidermis produced only one of the two possible 10-hydroxy-epimers of HxB<sub>3</sub> when incubated with AA (Fig 4A), whereas boiled epidermis did not produce an appreciable amount of HxB<sub>3</sub> (Fig 4B). When 100,000 × g pellet and supernatant were incubated with authentic 12(S)-HPETE (n = 4) we failed to find appreciable amounts of the “epidermis epimer” of HxB<sub>3</sub>. A small amount of racemic HxB<sub>3</sub> was occasionally observed, but was also observed in the incubation with buffer alone, indicating its nonenzymatic origin (not shown).



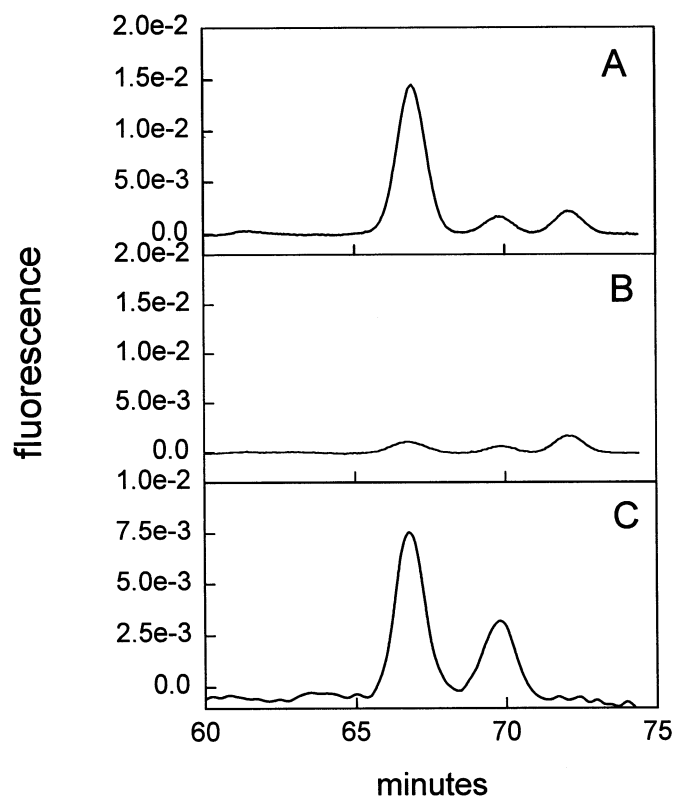
**Figure 3.** Human epidermis forms trioxilins, HxB<sub>3</sub>, 12-oxo-EETE, and 12-HETE as main eicosanoids; HxB<sub>3</sub>, 12-oxo-EETE, and 12-HETE being formed in the microsomal fraction. Representative RP-HPLC radio-chromatograms from samples of whole epidermis, and epidermal cell fractions incubated with [<sup>14</sup>C]-AA. Fragments of human epidermis were incubated with 100 µM [<sup>14</sup>C]-AA at 37°C for 30 min. Human epidermis was also homogenized, and the 100,000 × g supernatant and pellet were incubated with 100 µM [<sup>14</sup>C]-AA at 37°C for 30 min. The identity of the numbered peaks was confirmed by GC-MS. (1) Mixture of triols (TrXA<sub>3</sub>, TrXB<sub>3</sub>, and 8,9,12-THETrE, the TrXA<sub>3</sub> being the most abundant); (2) HxB<sub>3</sub>; (3) 12-oxo-EETE; and (4) 12-HETE. Total 12-LO activity were 0.56 ± 0.1 and 0.15 pmol per mg of protein for 100,000 × g pellet and supernatant, respectively. For the pellet, values are the mean ± SD, n = 4. 12-LO activity in the 100,000 × g supernatant was only detected in one experiment and the 12-LO activity value shown corresponds to this experiment. Chromatography was performed isocratically with methanol/water/trifluoroacetic acid/triethylamine 75:25:0.1:0.05 pumped at 1 ml per min.

To explore the possibility that 12-LO catalyzes the transformation of AA into HxB<sub>3</sub> and 12-oxo-EETE, recombinant human platelet-type 12-LO was incubated with [<sup>14</sup>C]-AA. **Figure 5** shows that both 12-oxo-EETE and HxB<sub>3</sub> were formed by recombinant 12-LO, but to a lesser proportion than epidermis when compared with 12-HETE. Nevertheless, we failed to obtain any product other than 12-HETE when 12-LO was incubated with 12(S)-HPETE.

#### DISCUSSION

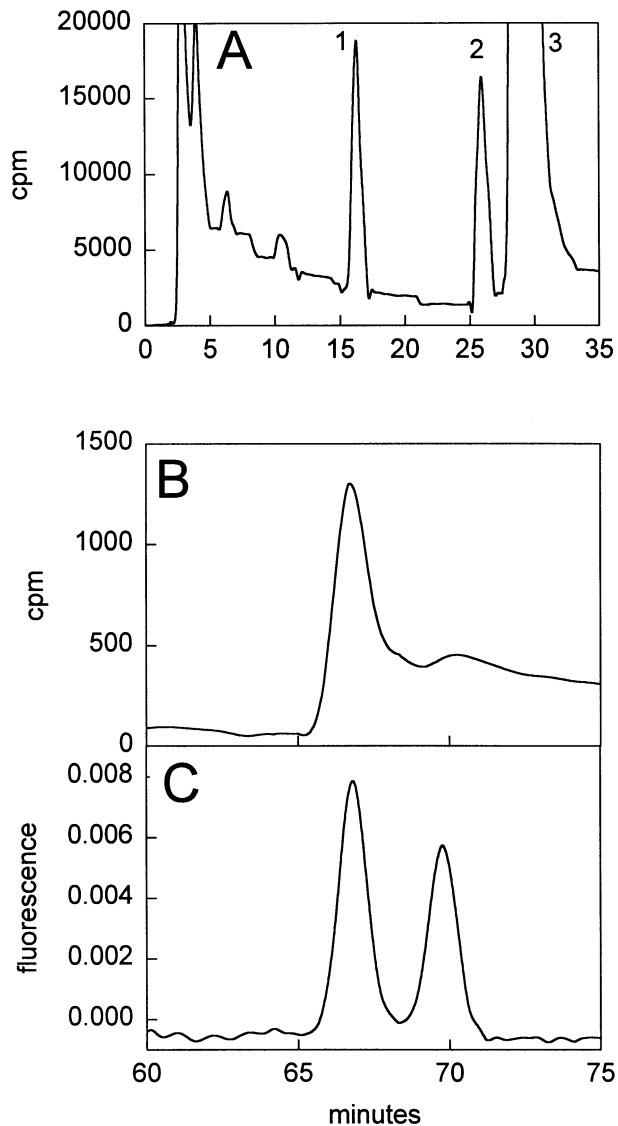
Metabolism of AA in human epidermis through the 12-LO pathway results in the formation of hepxilins, 12-oxo-EETE, and several triols in addition to 12-HETE (Antón *et al.*, 1995). A high amount of HxB<sub>3</sub> is produced by whole human epidermis. Unlike the hemin-catalyzed formation of HxB<sub>3</sub>, human epidermis only produced one of the two possible 10-hydroxy epimers. Whereas recent reports indicate that HxA<sub>3</sub> can be synthesized enzymatically from 12(S)-HPETE and not from the enantiomer *R* (Reynaud *et al.*, 1994), it has been stated that HxB<sub>3</sub> synthesis from 12-HPETE involves a nonenzymatic process (Pace-Asciak *et al.*, 1995a, b). Our results provide new evidence that the synthesis of HxB<sub>3</sub> is enzymatically catalyzed in human epidermis. This concept is supported by the following experimental evidence: (i) progression curves showed that HxB<sub>3</sub> levels reached a plateau; (ii) HxB<sub>3</sub> synthesis was product stereoselective; and (iii) despite the fact that HxB<sub>3</sub> was not formed by subcellular fractions from 12(S)-HPETE and only 12-HETE was detected, it was formed from AA by the microsomal fraction.

A radical mechanism for LO catalysis involving iron is generally accepted (reviewed in Yamamoto, 1991). Most work investigating the nature of the iron in LO has been done on soybean LO.



**Figure 4. Human epidermis enzymatically produced one of the two possible 10-hydroxy-epimers of HxB<sub>3</sub> when incubated with AA.** Representative chromatograms of the ADAM-derivative of HxB<sub>3</sub> from: (A) a sample of human epidermis incubated with exogenous 100 μM AA; (B) boiled epidermis incubated with 100 μM AA; (C) authentic (±)HxB<sub>3</sub>. These experiments were repeated three times with essentially identical results. Chromatography was performed isocratically with acetonitrile/water 72:28 pumped at 1 ml per min.

Mossbauer spectral analysis of soybean 15-LO enriched in <sup>57</sup>Fe revealed that the iron appears to cycle between Fe<sup>2+</sup> and Fe<sup>3+</sup> (Funk and Carrol, 1990). Hydrogen abstraction in the substrate is probably mediated by Fe<sup>3+</sup>-enzyme that undergoes reduction to Fe<sup>2+</sup>-enzyme, yielding the substrate radical (AA·). The abstraction of a hydrogen atom from a double allylic methylene group takes place in the molecule of substrate yielding a carbon centered radical, which tends to be stabilized by a molecular rearrangement to form conjugate dienes. A further reaction of the lipid radical with molecular oxygen gives a peroxy radical (AAOO·). This is an enzyme-bound intermediate in the formation of the corresponding hydroperoxide. The peroxy radical can either be transformed into a hydroperoxide (AAOOH) by picking up a hydrogen atom, or it



**Figure 5. Recombinant human platelet-type 12-LO forms 12-oxo-EETE and HxB<sub>3</sub>.** (A) a representative RP-HPLC radio-chromatogram from a sample of platelet-type 12-LO incubated with 200 μM [<sup>14</sup>C]-AA at 37°C for 5 min; chromatography was performed isocratically with a mixture of methanol/water/trifluoroacetic acid/triethylamine 75:25:0.1:0.05 pumped at 1 ml per min. (1) HxB<sub>3</sub>; (2) 12-oxo-EETE; and (3) 12-HETE. (B) Representative chromatograms of the ADAM derivative of HxB<sub>3</sub> collected from (A), and (C) authentic (±)HxB<sub>3</sub>. Chromatography was performed isocratically with acetonitrile/water 72:28 pumped at 1 ml per min. These experiments were repeated four times with essentially identical results; the production was 1.01 ± 0.19, 0.58 ± 0.07, and 22.94 ± 4.13 nmol per 5 min per 50 U 12-LO for 12-oxo-EETE, HxB<sub>3</sub>, and 12-HETE, respectively, mean ± SD.

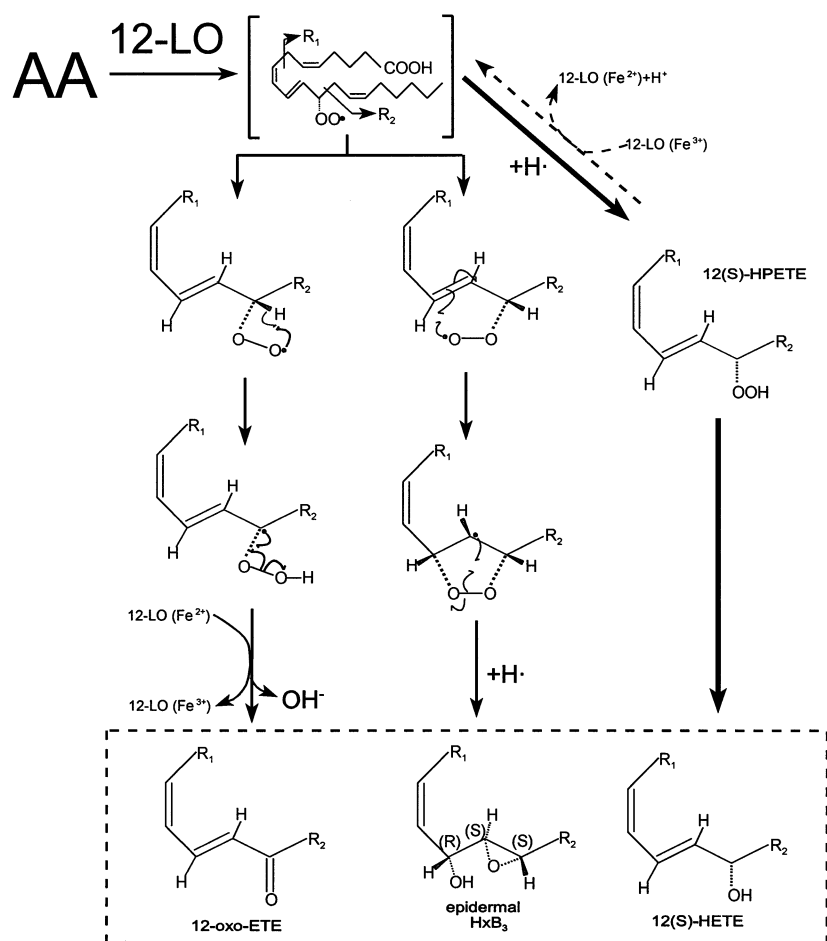
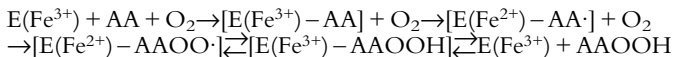


Figure 6. 10(R)-hydroxy epimer of HxB<sub>3</sub> and 12-oxo-EETE probably are formed from the 12-LO-bound 12(S)-peroxyl radical: a plausible mechanism for the 12-LO catalysis

can be added to a double bond to form cyclic peroxides, as occurs in the prostaglandin-endoperoxides. The 12-LO-catalyzed reaction with AA as a substrate could be represented as:



The equilibrium  $[\text{E}(\text{Fe}^{2+}) - \text{AAOO}\cdot] \rightleftharpoons [\text{E}(\text{Fe}^{3+}) - \text{AAOOH}]$  would usually tend to the right side due to the peroxidase-mediated or spontaneous conversion of 12-HPETE into 12-HETE. Nevertheless, if peroxidase activity is limited or 12-HPETE is in some way protected, 12-HPETE could accumulate and  $[\text{E}(\text{Fe}^{2+}) - \text{AAOO}\cdot]$  could be present in a high enough concentration to allow AAOO· to undergo alternative rearrangements.

The exact configuration of C10 in the HxB<sub>3</sub> produced by human epidermis was not directly determined, but chromatographic data of these compounds reported by other authors (Vasiljeva *et al*, 1993; Demin *et al*, 1994; Reynaud *et al*, 1994) show that 10(R)- is more polar than the 10(S)-epimer. GC-MS and HPLC data strongly suggested that human epidermis produces exclusively 10(R)-hydroxy-11(S),12(S)-epoxy-isomer of HxB<sub>3</sub>. **Figure 6** depicts the proposed putative mechanism of the 10(R) epimer of HxB<sub>3</sub> based on the 12(S) configuration of the peroxyl radical. We postulate the formation of cyclic 10,12-peroxide through the addition of the 12-peroxyl radical, oriented in the pro-R side, to the double bond at C10 leading to the formation of another free radical centered at C11. A further rearrangement that involves the homolytic fission of O-O bond of this intermediate and the pick up of an atom of hydrogen would exclusively yield the 10(R) epimer of HxB<sub>3</sub>.

The "hydroperoxidase" activity of 12-LO has also been proposed as a mechanism for the formation of 12-oxododeca-5,8,10-trienoic acid from 12-HPETE by porcine leukocytes

(Yamamoto, 1991; Glasgow *et al*, 1986). In addition, the formation of the homolog of 10(R)-epimer of HxB<sub>3</sub> from 13-hydroperoxide of linoleic acid by soybean LO has been reported (Garssen *et al*, 1976). Nevertheless, we did not observe products other than 12-HETE when epidermis fractions or recombinant platelet-type 12-LO were incubated with 12-HPETE. In contrast, hematin and hemoglobin catalyze the formation of both 10(S) and 10(R) epimers of HxB<sub>3</sub> from 12-HPETE (Pace-Asciak, 1984) and the corresponding homologs from 13-hydroperoxy-linoleate (Hamberg, 1975; Dix and Marnett, 1983). This supports the hypothesis that epidermal HxB<sub>3</sub> could be formed stereoselectively by rearrangement of the intermediate peroxyl radical at C12 in the bulk of 12-LO. 12-peroxyl radical could also pick up the hydrogen atom at C12 to form hydroperoxide and a C12 centered radical followed by the homolytic fission of O-O bond catalyzed by 12-LO by a "Fenton-like" reaction to yield 12-oxo-EETE. The putative mechanism that would yield 12-oxo-EETE by 12-LO is also depicted in **Fig 6**.

Whether or not 12-peroxyl radical could be formed from 12-HPETE is not clear from our results. Apparently, peroxyl radicals were not generated in a sufficient amount to form 12-oxo-EETE and HxB<sub>3</sub> from 12-HPETE when added exogenously to either cell fractions or recombinant platelet-type 12-LO. In contrast, when incubations of 100,000 × g or recombinant 12-LO were performed with exogenous AA, 12-HPETE was formed *in situ* in the bulk of 12-LO yielding HxB<sub>3</sub> and 12-oxo-EETE in addition to 12-HETE. This concept is also supported by the fact that recombinant platelet-type 12-LO produced both 12-oxo-EETE and the epidermis epimer of HxB<sub>3</sub> from AA. Differentiated and cornified epidermis are rich in proteins (keratins) and lipids (ceramides, phosphoglycerides, etc.). The enzyme environment in epidermis fragments differed from that in recombinant human platelet-type 12-LO preparations,

as in the latter case the reaction occurred in an almost homogeneous aqueous phase, whereas in the former case the reaction occurred in a lipid-rich phase. Moreover, when we performed incubations of AA with epidermal cell suspensions formation of HxB<sub>3</sub> and 12-oxo-ETE was very low and the ratio of HxB<sub>3</sub> and 12-oxo-ETE to 12-HETE resembled that obtained from the recombinant human platelet-type 12-LO experiments rather than that obtained from the experiments using fragments of epidermis (not shown). The rate of transformation of 12-peroxyl radical into HxB<sub>3</sub> and 12-oxo-ETE instead of 12-H(p)ETE was higher when keratinocytes (hence the enzyme) were "included" in a hydrated lipid-protein phase than when they were surrounded by water. This reasoning could be particularly relevant to explain results when 12-HPETE was added exogenously to preparations of recombinant human platelet-type 12-LO or cell fractions. In these incubations 12(S)-HPETE could be rapidly transformed into 12-HETE without the opportunity to reach the active site to generate 12-peroxyl radicals that further yield HxB<sub>3</sub> and 12-oxo-ETE (see Fig 6).

Support for the potential role of hepxoxilins in the pathogenesis of inflammatory skin diseases, includes their potent action on plasma permeability when injected subcutaneously (Laneville *et al*, 1991; Wang *et al*, 1996) and the detection of a considerable amount in psoriatic lesions (Antón *et al*, 1998). The effect of HxA<sub>3</sub> on Ca<sup>2+</sup> mobilization has been demonstrated in neutrophils and whether or not this effect also occurs in epidermal cells should be the subject of further investigation. Hepoxilins could play an autocrine part as intracellular messengers and a paracrine role modulating leukocyte activation (reviewed in Pace-Asciak, 1994).

The literature available on the physiologic role of HxB<sub>3</sub> is limited. HxB<sub>3</sub>, however, is elevated in psoriatic lesions (Antón *et al*, 1998) and Wang *et al* (1996) have observed that whereas HxA<sub>3</sub> and HxB<sub>3</sub> are both active in enhancing the bradykinin-evoked permeability in skin, only 10(R)-HxB<sub>3</sub> stereospecifically enhances the vascular permeability evoked by intradermal injection of platelet-activating factor. The biologic role of these compounds on dermatoses is presently under research in our laboratory.

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