Published in final edited form as:
Arch Biochem Biophys. 2006 November 15; 455(2): 188–196. doi:10.1016/j.abb.2006.09.002.

Human and mouse eLOX3 have distinct substrate specificities: implications for their linkage with lipoxygenases in skin

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Abstract

Genetic and biochemical evidence suggests a functional link between human 12*R*-lipoxygenase (12*R*-LOX) and epidermal lipoxygenase-3 (eLOX3) in normal differentiation of the epidermis; LOX-derived fatty acid hydroperoxide is isomerized by the atypical eLOX3 into a specific epoxyalcohol that is a potential mediator in the pathway. Mouse epidermis expresses a different complement of LOX enzymes, and therefore this metabolic linkage could differ. To test this concept, we compared the substrate specificities of recombinant mouse and human eLOX3 toward sixteen hydroperoxy stereoisomers of arachidonic and linoleic acids. Both enzymes metabolized *R*-hydroperoxides 2–3 times faster than the corresponding *S* enantiomers. Whereas 12*R*-hydroperoxyeicosatetraenoic acid (12*R*-HPETE) is the best substrate for human eLOX3 (2.4 sec^{-1}; at 30 μM substrate), mouse eLOX3 shows the highest turnover with 8*R*-HPETE (2.9 sec^{-1}) followed by 8*S*-HPETE (1.3 sec^{-1}). Novel product structures were characterized from reactions of mouse eLOX3 with 5*S*-, 8*R*-, and 8*S*-HPETEs. 8*S*-HPETE is converted specifically to a single epoxyalcohol, identified as 10*R*-hydroxy-8*S*,9*S*-epoxyeicosa-5*Z*,11*Z*,14*Z*-trienoic acid. The substrate preference of mouse eLOX3 and the unique occurrence of an 8*S*-LOX enzyme in mouse skin point to a potential LOX pathway for the production of epoxyalcohol in murine epidermal differentiation.

Keywords

hepoxilin; epoxyalcohol; HPETE; fatty acid hydroperoxide; epidermis; differentiation; ichthyosis

Introduction

Of the six lipoxygenases (LOX) encoded in the human genome, five show activity typical of this class of dioxygenase. They react polyunsaturated fatty acids with molecular oxygen and form specific fatty acid hydroperoxide products [1]. The atypical LOX, known as eLOX3 for epidermal lipoxygenase-3, is unusual in being incapable of oxygenating polyunsaturated fatty acids [2,3]. The issue of the enzymatic activity of eLOX3 assumed direct biomedical importance when it was reported that mutations in an inherited form of ichthyosis were pinpointed to the eLOX3 gene (*ALOXE3*) [4]. Of equal import was the finding that mutations in a second lipoxygenase, 12*R*-LOX, are similarly associated with ichthyosis exhibiting very similar details of phenotype to the eLOX3 mutations [4]. The authors of the genetics paper

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suggested that the two gene products may operate in the same metabolic pathway. The inherited form of ichthyosis associated with mutations in 12*R*-LOX and eLOX3, non-bullous congenital ichthyosiform erythroderma (NCIE), is rare [4,5]. Nonetheless, the implication of its occurrence in association with the LOX gene mutations suggests that the differentiation process in normal epidermis relies on the combined functioning of these two LOX proteins.

In testing the hypothesis that the 12*R*-LOX and eLOX3 enzymes function in the same pathway, we found that eLOX3 exhibits hydroperoxide isomerase activity towards fatty acid hydroperoxide substrates, and that the 12*R*-LOX primary product, 12*R*-hydroperoxy-eicosatetraenoic acid, is a preferred substrate over the other hydroperoxy fatty acids produced in human skin [3]. The products of the combined action of 12*R*-LOX and eLOX3 are an allylic epoxyalcohol, the hepoxilin isomer 8*R*-hydroxy-11*R*,12*R*-epoxyeicosatrienoic acid, and 12-ketoeicosatetraenoic acid, formed in approximately 3:2 ratio [3]. The fact that eLOX3 will efficiently metabolize 12*R*-HPETE provided some biochemical support for the concept that the two lipoxygenases normally work together in helping produce the epidermal water permeability barrier of normal skin. The implication is that these 12*R*-LOX/eLOX3 products, or their further derivatives, are significant mediators or signaling molecules in skin differentiation. Also consistent with this interpretation, the mutations found in the ichthyosis patients inactivate the enzymatic activities of 12*R*-LOX or eLOX3 [5,6], which is compatible with the usual products having a significant biological role.

Mouse and human show distinct species differences in their complement of LOX genes [7]. Several orthologs have altered catalytic activities across the two species. For example, the ortholog of human 15-LOX-2 has 8-LOX activity in the mouse [8,9]. And of particular relevance here, there are differences between mouse and human 12*R*-LOX. As its name implies, human 12*R*-LOX converts arachidonic acid to 12*R*-HPETE [10]. However, free arachidonic acid is not a substrate for mouse 12*R*-LOX [11]. The enzyme does convert arachidonate methyl ester to 12*R*-HPETE methyl ester, but no naturally occurring free fatty acid or ester has been identified as an acceptable substrate [11]. This raises questions about the equivalent LOX enzyme combinations that may perform similar functions in mouse epidermis as is postulated to occur in humans. The combination proposed in humans of 12*R*-LOX and eLOX3 would appear to be catalytically an incompatible combination in the mouse. Perhaps a different mouse LOX enzyme could provide a suitable substrate for mouse eLOX3? To examine the possibility, we have here determined the substrate specificities of mouse and human eLOX3 towards a complete range of hydroperoxides of linoleic and arachidonic acids.

MATERIALS AND METHODS

Preparation of hydroperoxides

HPETEs and HPODEs with specific positional and stereo configurations were prepared from arachidonate and linoleate methyl esters respectively by the following route: (i) autoxidation of 500 mg methyl ester (Nu-Chek Prep Inc, Elysian, MN) in the presence of 70 mg α-tocopherol (Sigma) for four days at 37°C under an atmosphere of oxygen [12]; (ii) for arachidonate methyl ester products, the sample was dissolved in 10 ml of 5% ethyl acetate in hexane (by volume); 2 ml aliquots were resolved by SP-HPLC using a Econosil Silica 10-μm column (2.2 × 25 cm). Mobile phases consisted of 5% ethyl acetate in hexane (solvent A), 15% ethyl acetate in hexane (solvent B) and 50% ethyl acetate in hexane (solvent C). A gradient system was adopted as follows: 0–5 min, 100% A; 5–50 min, linear gradient from 0 to 37.5% B; 50–60 min, 100% B; 60–80 min, 100% C. The flow rate was 7 ml/min. 15-, 12-, 11-, 8-, 9-, and 5-HPETE methyl esters were collected sequentially between 40 min and 60 min. For linoleate methyl ester products, aliquots of the 13- and 9-HPODE methyl esters were resolved by SP-HPLC using a Beckman Ultrasphere silica column (1 × 25 cm) and a solvent system of hexane/isopropanol (100:0.5, by volume). (iv) resolution of *R*- and *S*- HPETE and HPODE methyl esters using a

Chiralpak AD-RH column, eluted with a solvent of methanol/water 88:12 by volume, and a flow rate of 1 ml/min; for each of the HPETE methyl esters, the *R* enantiomer eluted before the *S* enantiomer; (v) preparation of the free acids by treatment for 30 min with 0.5 M KOH in water/methanol/dichloromethane (1:1:0.1) at room temperature, followed by acidification to pH 6.0 and extraction into dichloromethane; (vi) final purification of the free acids by SP-HPLC (Alltech Econosil Silica column, solvent system of hexane/isopropanol/acetic acid 100:1:0.1 by volume, flow rate of 2 ml/min). The HPETEs were quantified by uv spectroscopy using a molar extinction coefficient of 23,000 and stored in ethanol (1 mg/ml) at −20°C.

Expression and purification of human and mouse eLOX3

The cDNA for mouse eLOX3 was cloned by PCR using cDNA prepared from mouse keratinocytes. To prepare the eLOX3 protein with an N-terminal 6×His tag, the eLOX3 cDNA was subcloned into the pCW expression vector (a generous gift from Dr. Michael R. Waterman, Vanderbilt University, Nashville, TN) with the 5' sequence encoded as ATG CAT CAC CAT CAC CAT CAC *GCA*-, with the last codon representing the start of the wild type enzyme. The mouse eLOX3 was expressed in *E. coli* BL21 (DE3) cells (Novagen, Madison, WI) and the 6×His tagged protein was purified on Ni-NTA agarose (Qiagen, Valencia, CA) according to the manufacturer's instructions. Fractions of 0.5 ml were collected off the affinity column and assayed using SDS-PAGE. Fractions containing mouse eLOX3 were pooled and dialyzed against a buffer of 50 mM Tris (pH 7.5) containing 150 mM NaCl. Expression and purification of human eLOX3 were performed as described before [3].

eLOX3 activity assay

eLOX3 activity was measured by monitoring disappearance of the UV absorbance of the HPETE substrates at 235 nm in the time-drive mode. On account of the limited availability of most of the HPETE substrates, it was only practical to monitor reaction at a single substrate concentration. Based on previous results on the reactions of 12*R*-HPETE, 12*S*-HPETE, and 15*S*-HPETE with human eLOX3, a substrate concentration approximating an average K_m of the reaction of HPETE with human eLOX3 was selected. Incubations with the purified human or mouse eLOX3 were conducted in 500 μl incubation buffer (50 mM Tris, 150 mM NaCl, pH 6.0) in a 1 cm path length microcuvette at room temperature using an enzyme concentration of 0.01 – 0.1 μM. Reaction was initiated by addition of 30 μM HPETE in 10 μl ethanol and monitored at 235 nm for 10 min; reaction rate was calculated from the initial linear part of the curve and normalized to the enzyme concentration. Without addition of the eLOX3 enzyme, disappearance of the chromophore at 235 nm was negligible.

8-HPETE-derived epoxyalcohol standards

A mixture of epoxyalcohol derivaties were prepared by reacting 8-HPETE (500 μg) with hematin (125 μg) in 1 ml of K_2HPO_4 (0.1 M). Reaction was allowed to proceed for 15 min at room temperature and was then terminated by acidification (pH 4) with 1 N HCl and immediate extraction with 2 volumes of dichloromethane. The organic phase was washed with water and taken to dryness under a stream of nitrogen; the extract was redissolved in ethyl acetate (0.5 ml), diluted with hexane to 25% ethyl acetate and immediately applied to a 0.5 g silica Bond-Elut cartridge. After washing with 25% ethyl acetate in hexane (7 ml), the mixture of epoxyalcohol products was eluted with 6 ml of ethyl acetate; aliquots were analyzed subsequently by HPLC. The two saturated epoxyalcohol derivatives, *cis*-8,9-epoxy-*threo*-10-hydroxyeicosanoate methyl ester and *cis*-8,9-epoxy-*erythro*-10-hydroxyeicosanoate methyl ester standards were prepared by total chemical synthesis.

HPLC analysis

Products of the eLOX3 reactions with HPETE substrates were extracted using Waters Oasis® HLB 1cc (30 mg) extraction cartridges (Waters Corp., Milford, MA) according to the manufacturer's instructions and analyzed initially by RP-HPLC using a Waters Symmetry C18 5-μm column (0.46 × 25 cm) eluted at a flow rate of 1 ml/min with methanol/water/acetic acid (80:20:0.01 by volume), and UV detection at 205, 220, 235, and 270 nm using an Agilent 1100 series diode array detector. The main products were recovered from the reversed-phase solvent by addition of water and extraction with dichloromethane. Further purification was carried out by SP-HPLC using an Alltech Econosil Silica column (0.46 × 25 cm), a solvent system of hexane/isopropanol/acetic acid (100:2:0.1 by volume), and a flow rate of 1 ml/min.

Derivatization and GC-MS

Methyl esters of the products were prepared using ethereal diazomethane/methanol (5:1). Catalytic hydrogenations were performed in 100 μl of ethanol using about 1 mg of palladium on alumina and bubbling with hydrogen for 2 min at room temperature. Reactions were terminated by the addition of water and extraction with ethyl acetate. Trimethylsilyl ether derivatives were prepared by treatment overnight with *bis*(trimethylsilyl)trifluoracetamide (10 μl) and pyridine (5 μl) at room temperature. Subsequently, the reagents were evaporated under a stream of nitrogen and the samples were dissolved in hexane for GC-MS analysis.

Analysis of the methyl ester trimethylsilyl ether derivatives of the products was carried out in the positive ion electron impact mode (70 eV) using a Hewlett-Packard 5989A mass spectrometer coupled to a Hewlett-Packard 5890 gas chromatograph equipped with a RTX-1701 fused silica capillary column (6 m × 0.25 mm, internal diameter). Samples were injected at 150°C, and after 1 min the temperature was programmed to 300°C at 12 or 20°C/min.

LC-MS

A Thermo Finnigan LC Quantum system was used. Samples were introduced via a Waters Symmetry C18 3-μm column (0.2 × 10 cm) eluted with a water/acetonitrile gradient containing 10 mM ammonium acetate at a flow rate of 0.2 ml/min. The heated capillary ion lens was operated at 220°C. Nitrogen was used as a nebulization and desolvation gas. The electrospray potential was held at 4 kV. Mass spectra were acquired over the mass range *m/z* 200 to 750 at 2 sec/scan.

NMR

^{1}H NMR and 2D (H,H-COSY) NMR spectra were recorded on a Bruker DRX 400 MHz or 500 MHz spectrometer using deuterated benzene as solvent. The ppm values are reported relative to residual non-deuterated solvent (δ = 7.24 ppm for C_6H_6).

RESULTS

Specificity of human and mouse eLOX3 toward HPETE and HPODE substrates

To compare the substrate selectivity of human and mouse eLOX3, fixed concentrations of the purified human and mouse enzymes (typically 0.01–0.1 μM) were incubated with 30 μM hydroperoxy fatty acid (HPETEs or HPODEs) at room temperature in 500 μl incubation buffer (50 mM Tris, 150 mM NaCl, pH 7.5) in a 1-cm path length microcuvette. The reaction rates were measured by monitoring the disappearance of the signal at 235 nm in the time-drive mode and calculated from the initial slope of the curve. We reported previously that among the hydroperoxy fatty acids tested (12*R*-, 12*S*- and 15*S*-HPETE), the preferred substrate for human eLOX3 is 12*R*-HPETE [3]. Here we tested the complete series of *R*- and *S*-hydroperoxides

derived from arachidonic and linoleic acids, a total of twelve HPETE substrates and four HPODE substrates (Fig. 1). Using human eLOX3, we found that for each regioisomer, the *R* enantiomer reacts faster than the *S* enantiomer, typically at 2–3 times faster rate. The results also confirm that 12*R*-HPETE is the preferred substrate for human eLOX3 (Fig. 1A).

Mouse eLOX3, by contrast, shows a different substrate selectivity (Fig. 1B). The preferred substrate for mouse eLOX3 is 8*R*-HPETE, which reacts at about 4 times the rate of 12*R*-HPETE. The product of mouse 8-LOX, 8*S*-HPETE, is the second best substrate for mouse eLOX3. Similar to human eLOX3, mouse eLOX3 shows a consistent preference for *R* over *S* enantiomers, a sole exception being the 5*R*- and 5*S*-HPETEs that react at very similar rates (Fig. 1B). In previous work we have shown that human eLOX3 transforms 12*R*-HPETE, 12*S*-HPETE, and 15*S*-HPETE into specific hepoxilin-type epoxyalcohols, or a mixture of two hepoxilins in the case of 12*S*-HPETE [3]. Since several of the different hydroperoxides tested in the present study are naturally occurring products in mouse and human epidermis, we have analyzed the structures of the eLOX3-derived products of the substrates 8*S*-HPETE, 5*S*-HPETE, and 8*R*-HPETE. (Mouse eLOX3 converts 12*R*-HPETE to the same main hepoxilin product as human eLOX3, data not shown).

Different reactions of mouse eLOX3 with 8S-HPETE and 8R-HPETE

Comparison of the product profiles of the reaction of mouse eLOX3 with its preferred substrates 8*S*-HPETE and 8*R*-HPETE using RP-HPLC showed differences in the relative abundance of epoxyalcohol versus the 8-KETE products (Fig. 2A and 2B; the data for the structural identification of the products is presented below). The natural isomer 8*S*-HPETE is transformed by mouse eLOX3 to one major epoxyalcohol product with a minor abundance of 8-KETE while, in contrast, its enantiomer 8*R*-HPETE was converted to the keto compound as the major product with less abundant formation of a mixture of several epoxyalcohol products.

The RP-HPLC chromatogram from incubation of 8*S*-HPETE with mouse eLOX3 (Fig. 2A) showed a main product with retention time of 12 min with only end absorbance in the UV and a small peak with the UV spectrum of a conjugated dienone with λ_{max} at 285 nm that eluted near 20 min. Several minor products that eluted between 6 and 8 min showed a UV spectrum typical of a conjugated triene double bond system. The two triene peaks were designated T1 and T2 in Fig. 2A, and their identification will be described below.

Identification of the epoxyalcohol product formed from 8S-HPETE

To prepare sufficient material for structural identification of the main product at 12 min retention time, 1 mg of 8*S*-HPETE was incubated in 25 ml of buffer with 0.1 μM mouse eLOX3. The product was isolated using RP-HPLC and treated with ethereal diazomethane. The methyl ester derivative was further purified using SP-HPLC. GC-MS analysis (electron impact mode) gave a mass spectrum of the methyl ester trimethylsilyl ether derivative with structurally significant ions at *m/z* 407 $[M\text{-}CH_3]^+$ (<1% relative abundance), 391 (M-31, <1%), 281 (C8 – C20, $[C_2H_2OCHOSi(CH_3)_3\ C_{10}H_{17}]^+$) and 239 (C10 – C20, $[CHOSi(CH_3)_3\ C_{10}H_{17}]^+$), indicating a C-10 hydroxyl, and a base peak at *m/z* 73 (100%) (Fig. 3A). After hydrogenation the M-15 ion peak increased by 6 mass units (*m/z* 413) and the α-cleavage ion adjacent to C-10 appeared at 243 (239 + 4) (Fig. 3B). The predicted molecular weight of 422 is compatible with a C20 fatty acid methyl ester containing a C-10 hydroxyl, an epoxide moiety, and three double bonds.

^{1}H-NMR (400 MHz, in deuterated benzene) with assignment of all proton signals by H,H-COSY analysis defined the complete covalent structure of the product as a single diastereomer of 10-hydroxy-8,9-epoxyeicosa-5,11,14-trienoic acid (Fig. 4). The configuration of the 8,9-epoxide was assigned as *trans* based on the coupling constant ($J_{8,9}$ = 2.2 Hz) in comparison to

the known coupling constants for *cis* and *trans* epoxides (about 4 and 2 Hz, respectively). Since the proposed mechanism of formation of the epoxide preserves the original 8*S* configuration [3], the absolute configuration of the epoxide is 8*S*,9*S*. The coupling constant between H9 and H10 ($J_{9,10}$ = 4.9 Hz) indicates the *threo* diastereomer of 10-hydroxy-8*S*,9*S*-epoxy, i.e., 10*R*-hydroxy-8*S*,9*S*-epoxy. The three *cis* double bonds do not participate in the transformation of 8*S*-HPETE to the epoxyalcohol product and therefore retain the original *cis* configurations. Overall, the main product from the reaction of mouse eLOX3 with 8*S*-HPETE was identified as 10*R*-hydroxy-8*S*,9*S*-epoxyeicosa-5*Z*,11*Z*,14*Z*-trienoic acid (Fig. 4A).

The signals obtained in the ^{1}H NMR analysis of 10*R*-hydroxy-8*S*,9*S*-epoxyeicosa-5*Z*,11*Z*,14*Z*-trienoic acid methyl ester were the following (C_6D_6, 400 MHz): δ 0.97 (t, *J* = 3.1 Hz, 3H, H20); 1.26–1.45 (m, 6H, H17, H18, H19); 1.61–1.67 (m, 2H, H3); 1.95 (q, *J* = 6.6 Hz, 2H, H4); 2.07 (q, *J* = 7.0 Hz, 2H, H16); 2.14 (t, *J* = 7.3 Hz, 2H, H2); 2.18 (m, 1H, H7a); 2.27 (m, 1H, H7b); 2.82 (dd, *J* = 2.2 Hz/4.8 Hz, 1H, H9); 2.85–2.90 (m, 2H, H13); 2.94 (dt, *J* = 2.2 Hz/5.4 Hz, 1H, H8); 3.42 (s, 3H, -OC$\underline{H}_3$); 4.35 (m, 1H, H10); 5.37–5.53 (m, 4H, H5, H6, H14, H15); 5.54–5.59 (m, 2H, H11, H12).

Treatment of the dienone product eluting at 20 min retention time with $NaBH_4$ yielded 8-HETE, as confirmed by the identical HPLC retention time and UV spectrum with authentic 8-HETE, indicating that the original enzymatic product was 8-ketoeicosa-5*Z*,9*E*,11*Z*,14*Z*-tetraenoic acid (8-KETE).

Formation of conjugated triene products from the reaction of mouse eLOX3 with 8S-HPETE

Initial RP-HPLC analysis of the reaction of 8*S*-HPETE with mouse eLOX3 showed two peaks eluting between 6 and 8 min with a UV absorbance typical of a conjugated triene double bond system (peaks T1 and T2, Fig. 2A). After T1 and T2 were collected separately and treated with triphenylphosphine, each resolved into two products on RP-HPLC. The first peak on each chromatogram (designated as products I and III) exhibited a UV spectrum indicative of a *trans*,*trans*,*trans* conjugated triene [13] and their RP-HPLC retention characteristics were unaffected by TPP treatment. The later eluting products II and IV had shifted retention time upon TPP reduction and showed the characteristic UV of a *trans*,*cis*,*trans* conjugated triene [14,15]. GC-MS analysis (electron impact mode) of the methyl ester trimethylsilyl ether derivatives of the four products gave very similar mass spectra, with structurally significant ions at *m/z* 404 (M-90), 173 (C15 – C20), 353 (C8 – C20), 263 (353-90), and for the hydrogenated products at *m/z* 487 (M-15), 431 (C1 – C15), 359 (C8 – C20), 341 (431-90), 269 (359-90), 245 (C1 – C8) and 173 (C15 – C20), indicating that the four compounds I–IV are isomeric 8,15-dihydroxy-5,9,11,13-eicosatetraenoic acids.

As illustrated in Figure 5 there are two conceivable pathways to form conjugated triene products from 8*S*-HPETE. Both pathways are initiated by a bis-allylic hydrogen abstraction from C-13 of 8*S*-HPETE. One pathway involves a LTA_4-like epoxide intermediate followed by its hydrolysis with water to give diastereomeric 8*S*,15*R*/*S*-dihydroxy-5*Z*,9*E*,11*E*,13*E*-tetraenoic acids (with *trans*,*trans*,*trans* configuration of the conjugated triene) (Fig. 5, right side). Treatment with TPP will not affect the retention time of the dihydroxy products on RP-HPLC. The second possible pathway is through oxygenation of 8*S*-HPETE at carbon 15 in a LOX-type reaction, which will lead to formation of diastereomeric 8*S*,15*R*/*S*-dihydroperoxy-5*Z*,9*E*,11*Z*,13*E*-tetraenoic acids (the hydroperoxy precursors of products II and IV)). Via this route, the resulting conjugated triene has *trans*,*cis*,*trans* configuration of the three double bonds. Following reduction of the diHPETEs with TPP, the resulting diHETEs will show a diagnostic shift in retention time on RP-HPLC. Based on these considerations, I and III were identified as diastereomeric 8*S*,15*R*,*S*-dihydroxy-5*Z*,9*E*,11*E*,13*E*-eicosatetraenoic acids while II and IV are derived from the reduction of diastereomeric 8*S*,15*R*,*S*-dihydroperoxy-5*Z*,9*E*,11*Z*,13*E*-eicosatetraenoic acid precursors (Fig. 5). Since both reactions are catalyzed

simultaneously and both diastereomers are formed at C-15, it appears that eLOX3 exerts little control over these transformations. The presence of the 9*E*,11*Z*-conjugated diene on one side of the CH_2 at C-13 of 8*S*-HPETE makes the hydrogens more easily abstracted, and can rationalize why eLOX3 facilitates this reaction on a HPETE substrate, but not on a typical 1,4 *cis*,*cis* pentadiene.

Identification of the products of mouse eLOX3 with 8R-HPETE

The products of mouse eLOX3 with 8*R*-HPETE were extracted and analyzed using RP-HPLC with diode array detection (see Fig. 2B). The main product with retention time of 20.2 min has the UV spectrum of a conjugated dienone with λ_{max} at 285 nm and was identified as 8-KETE as described above. The incubation of 8*R*-HPETE with mouse eLOX3 also produces a mixture of peaks eluting between 9 and 12.5 min (Fig. 2B). These peaks display only end absorbance in the UV and a molecular weight of 336 as measured by LC-MS, compatible with their being a mixture of epoxyalcohol products. The most prominent peak (10.8 min in RP-HPLC) was isolated and gave a single peak when further purified using SP-HPLC. Comparison of its SP-HPLC retention to that of epoxyalcohols prepared by the hematin-catalyzed transformation of 8-HPETE indicated that it resolved from the six major hematin-derived epoxyalcohols (*viz.* two 8,9-*trans*-epoxy-12-hydroxy epoxyalcohol derivatives, two 8,9-*cis*-epoxy-12-hydroxy derivatives, and two 8,9-*trans*-epoxy-10-hydroxy epoxyalcohols (data not shown)). This left two 8,9-*cis*-epoxy-10-hydroxyeicosatrienoic acids as possible candidates. We had available from another study samples of two saturated 8,9-*cis*-epoxy-10-hydroxyeicosanoates, and we used these to compare with the saturated eLOX3 product on GC-MS. Analysis of the hydrogenated eLOX3-derived epoxyalcohol product as the methyl ester trimethylsilyl ether derivative gave a major peak corresponding in GC and MS characteristics to 8,9-*cis*-epoxy-*threo*-10-hydroxyeicosanoate, with a minor peak of the *erythro* isomer (5:1, *threo:erythro*). The structures of the other minor epoxyalcohols were not further analyzed.

Identification of the epoxyalcohol product formed from 5S-HPETE

Both human and mouse eLOX3 transform 5*S*-HPETE to give the same main product eluting at about 12.5 min on RP-HPLC (Fig. 6). The product formed in the reaction with human eLOX3 was further purified using SP-HPLC. One- and two-dimensional ^{1}H-NMR analysis was used to define the covalent structure and to provide key details of the stereochemistry. The product contains a 7-hydroxy-5,6-epoxy moiety which was indicated in the H,H-COSY spectrum. The coupling constant between H5 and H6 (J = 2.2 Hz) indicates the 5,6-*trans* epoxide configuration, i.e. 5*S*,6*S*-epoxy, considering retention of the original 5*S* configuration. The configuration of the 7-hydroxy group was assigned from the coupling constant between H6 and H7 ($J_{6,7}$ = 4.8 Hz) , which indicates the *threo* arrangement [16], i.e., 7*R*-hydroxy-5*S*,6*S*-epoxy. Assuming that the double bonds not involved in the reaction retain their original *cis* configuration, we assigned the structure of the epoxyalcohol product formed in the reaction of 5*S*-HPETE with human and mouse eLOX3 as 7*R*-hydroxy-5*S*,6*S*-epoxyeicosa-8*Z*,11*Z*,14*Z*-trienoic acid.

The signals obtained in the ^{1}H NMR analysis of 7*R*-hydroxy-5*S*,6*S*-epoxyeicosa-8*Z*,11*Z*,14*Z*-trienoic acid were the following (C_6D_6, 500 MHz): δ 1.0 (t, J = 7.0 Hz, 3H, H20); 1.3–1.5 (m, 8H, H4,H17, H18, H19); 1.68–1.78 (m, 2H, H3); 2.16 (m, 2H, H16); 2.26 (t, J = 7.3 Hz, 2H, H2); 2.77 (dd, J = 2.2 Hz/4.8 Hz, 1H, H6); 2.87 (dt, J = 2.2 Hz/6.0 Hz, 1H, H5); 2.91–2.98 (m, 4H, H10, H13); 4.38 (dd, J = 5.1 Hz/7.5 Hz, 1H, H7); 5.45–5.51 (m, 2H, H11, H12); 5.54–5.64 (m, 4H, H8, H9, H14, H15).

DISCUSSION

As noted in the Introduction, genetic evidence on the occurrence of ichthyosis associated with mutations in 12*R*-LOX or eLOX3 has implicated a functional link between these two human enzymes in differentiation of the water impermeable barrier of the epidermis [4] and biochemical findings suggest that they may function together in formation of a specific epoxyalcohol/hepoxilin [3]. What is the corresponding state of affairs in the mouse? In the following we deal with both the catalytic activities of the epidermal LOX proteins, and as a final point, on the possibility that they have bioactivities unrelated to catalysis.

Similar to its human counterpart, mouse eLOX3 transforms different regioisomeric HPETE substrates into both epoxyalcohol and keto products. A general rule for both enzymes is the 2–3 times faster rate of reaction with *R* configuration hydroperoxides compared to the *S* configuration counterparts. The epoxyalcohols formed by mouse eLOX3 from the substrates 8*S*-HPETE and 5*S*-HPETE are of the hepoxilin B-type and therefore structurally different from the hepoxilin A-type product formed by human eLOX3 in the transformation of 12*R*-HPETE [3]. (Mouse eLOX3 converts 12*R*-HPETE to the same hepoxilin A-type product, data not shown). Although there is a substantial literature on the production of hepoxilin-type epoxyalcohols [17], there are few instances in mammalian systems in which a single specific structural isomer is formed, one being in incubations of human keratinocytes [18]. Two examples from enzymological studies are the conversion of 12*R*-HPETE to a single hepoxilin A-type product by human eLOX3 [3], and, as shown here, the conversion of 8*S*-HPETE to a single hepoxilin B-type product by mouse eLOX3.

Among the fatty acid hydroperoxides we tested, mouse eLOX3 exhibited the fastest rate of reaction with 8*R*-HPETE. The 8*R*-HPETE was converted mainly to the 8-keto derivative, and to a lesser extent to a mixture of several epoxyalcohol products. Concerning the natural occurrence of 8*R*-HPETE, it is perhaps not well recognized, but there is a documented and so far unexplained occurrence of 8*R*-HETE in mouse skin; it was detected in papillomas induced by multiple topical treatments with phorbol ester in 8*R*:8*S* ratios of ~70:30 to ~20:80 [19]. Although 8*R*-LOX is common in invertebrates, there is no known mammalian enzyme that could account for the specific synthesis of 8*R*-H(P)ETE [20] and possibly in this instance the 8*R*-HETE product arises partly through non-enzymic processes or isomerization catalyzed by microbial contaminants. Furthermore, it is the hydroxy product, not the hydroperoxide that is found in the papillomas, and hydroxy fatty acids such as 8-HETE are not suitable substrates for conversion to hepoxilins by eLOX3.

Besides 8*R*-HPETE, 8*S*-HPETE was the most efficient substrate for mouse eLOX3, and from what is known of murine arachidonic acid metabolism one would anticipate that the enzyme is more likely to encounter the 8*S* hydroperoxide as a substrate in the physiological context of mouse skin. This follows from the well-recognized expression of 8*S*-LOX in mouse epidermis [21,22]. It is detected by immuno-histochemistry in the granular layer of the epidermis, corresponding to the terminal differentiation of keratinocytes [8]. In contrast to the reaction of mouse eLOX3 with the 8*R*-HPETE enantiomer, 8*S*-HPETE was converted to one main epoxyalcohol product. Thus, there is at least the biochemical possibility that 8-LOX and eLOX3 are a coupled pair of LOX enzymes in a pathway forming a specific epoxyalcohol as is postulated in human skin where eLOX3 preferentially metabolizes the 12*R*-LOX-derived 12*R*-HPETE. Although human 12*R*-LOX and mouse 8-LOX are not orthologous genes, there is other circumstantial evidence that they may act as functional homologs. For example, in a murine model of psoriasis there is upregulation of 8-LOX [23], while psoriasis in humans is characterized by accumulation of the 12*R*-LOX product, 12*R*-HETE [24,25]. Both enzymes, human 12*R*-LOX and mouse 8-LOX, were proposed to participate in the biosynthesis of lipid mediators that play a role in modulating the differentiation process of the rapidly proliferating

keratinocytes [23]. If this biochemical catalytic activity is related to a functional role of eLOX3 in differentiation of the water impermeable barrier in skin (as implicated through the human genetic studies), then the mouse eLOX3 is best suited to utilize 8-HPETE as substrate for production of an epoxyalcohol product.

While the foregoing conclusions on the catalytic activities of human and mouse lipoxygenases are reasonable based on the biochemical evidence reported here, other potential explanations should be considered. Notably, the role of 8-LOX in murine epidermal differentiation can at least partly be explained by the biological activity of 8*S*-HETE, which can account for some of the pro-differentiating effects observed upon 8*S*-LOX overexpression [26,27]. An equivalent biological activity of the hepoxilin products remains to be demonstrated. Moreover, the possibility exists that the biological actions of the epidermal LOX proteins are not tied directly to their catalytic activities and the oxygenated fatty acids they can produce. A precedent for this is the report that a catalytically inactive splice variant of human 15-LOX-2 exhibits similar bioactivity to the native 15-LOX-2 enzyme in arresting the proliferative cell cycle of prostate epithelial cells [28]. The issue has also been raised with regard to possible transcriptional activity of 15-LOX-2 in which a direct association of 15-LOX-2 with PPARγ was detected using yeast and mammalian two-hybrid assays, implicating the possible functioning of 15-LOX-2 as a nuclear receptor coregulator [29]. The data regarding this concept are not all pointing in this direction, for the catalytic activities of mouse 8-LOX or its human homologue 15-LOX-2 were found essential for their anti-proliferative effects in premalignant epithelial cells [30], but it certainly stands as a debatable point. The question is especially prompted by the lack of oxygenase activity of murine 12*R*-LOX using free fatty acids as substrate. If this type of product-independent activity holds up for mouse 12*R*-LOX it could be involved in mouse epidermal differentiation without having catalytic activity and without being functionally linked to eLOX3. Evaluation of the physiological significance of these epidermal LOX enzymes and their catalytic activities will await clarification of a number of issues related to the role of LOX enzymes in forming the water impermeable barrier of the epidermis.

Abbreviations

LOX, lipoxygenase
eLOX3, epidermal lipoxygenase 3
NCIE, non-bullous congenital ichthyosiform erythroderma
H(P)ETE, hydro(pero)xyeicosatetraenoic acid
H(P)ODE, hydro(pero)xyoctadecadienoic acid
HPLC, high performance liquid chromatography
SP-HPLC, straight phase HPLC
RP-HPLC, reversed phase HPLC
KETE, ketoeicosatetraenoic acid
LTA_4, leukotriene A_4
GC, gas chromatography
LC, liquid chromatography
MS, mass spectrometry
NMR, nuclear magnetic resonance
TPP, triphenylphosphine
TMS, trimethylsilyl

Acknowledgment

This work was supported by NIH grant AR051968 (to A.R.B.) and the Vanderbilt Skin Diseases Research Center Grant P30 AR-41943.

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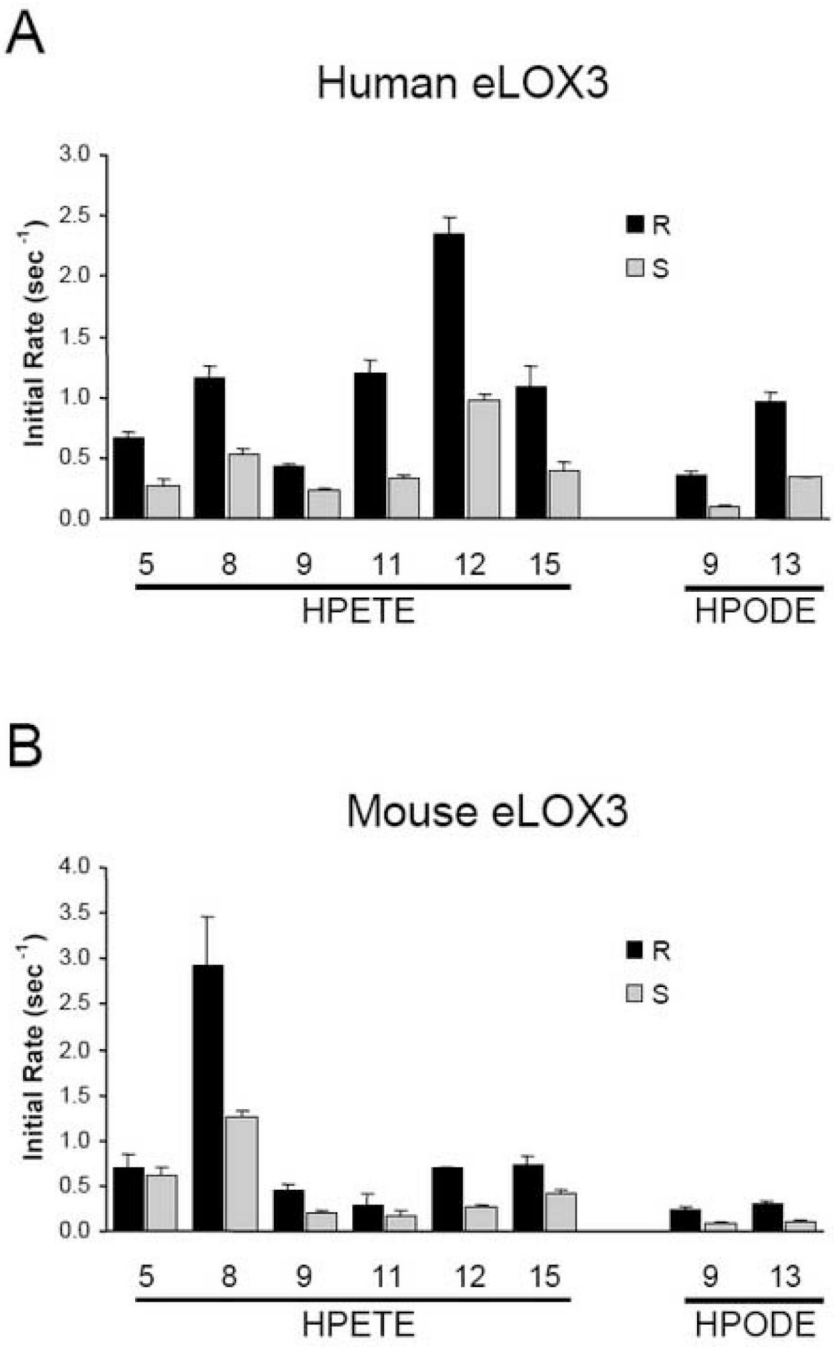

Fig. 1. Substrate specificity of human and mouse eLOX3

(A) human and (B) mouse recombinant eLOX3 (0.01–0.1 μM) were incubated with different HPETE and HPODE substrates (30 μM each) at room temperature in 500 μl incubation buffer (50 mM Tris, 150 mM NaCl, pH 7.5) in a 1-cm path length microcuvette. Rates were measured by monitoring disappearance of the signal at 235 nm in the time-drive mode and calculated from the initial linear part of the curve. Rates are given as turnovers per s. *R* configuration hydroperoxides are shown in black bars, *S* configuration hydroperoxides in grey bars.

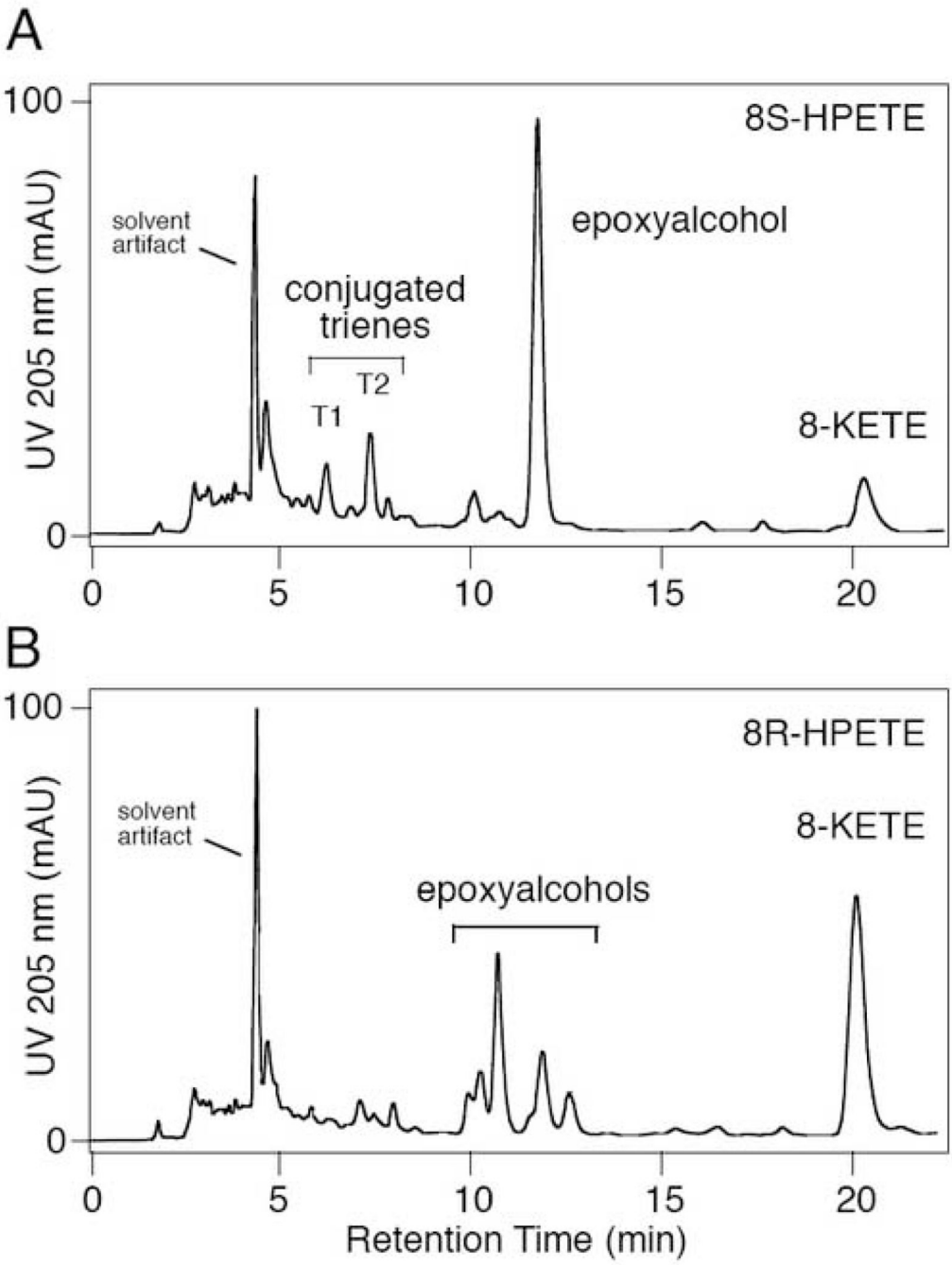

Fig. 2. RP-HPLC and UV analysis of the reaction of mouse eLOX3 with 8*S*-HPETE and 8*R*-HPETE

(A) The products derived from transformation of 8*S*-HPETE were analyzed by RP-HPLC using a Waters Symmetry C18 5-μm column (0.46 × 25 cm) eluted with methanol/water/acetic acid (80:20:0.01 by volume) at a flow rate of 1 ml/min and with UV detection at 205 nm. T1 and T2 are eLOX3-derived products with a UV chromophore typical of conjugated trienes. (B) Analysis of the transformation products from 8*R*-HPETE.

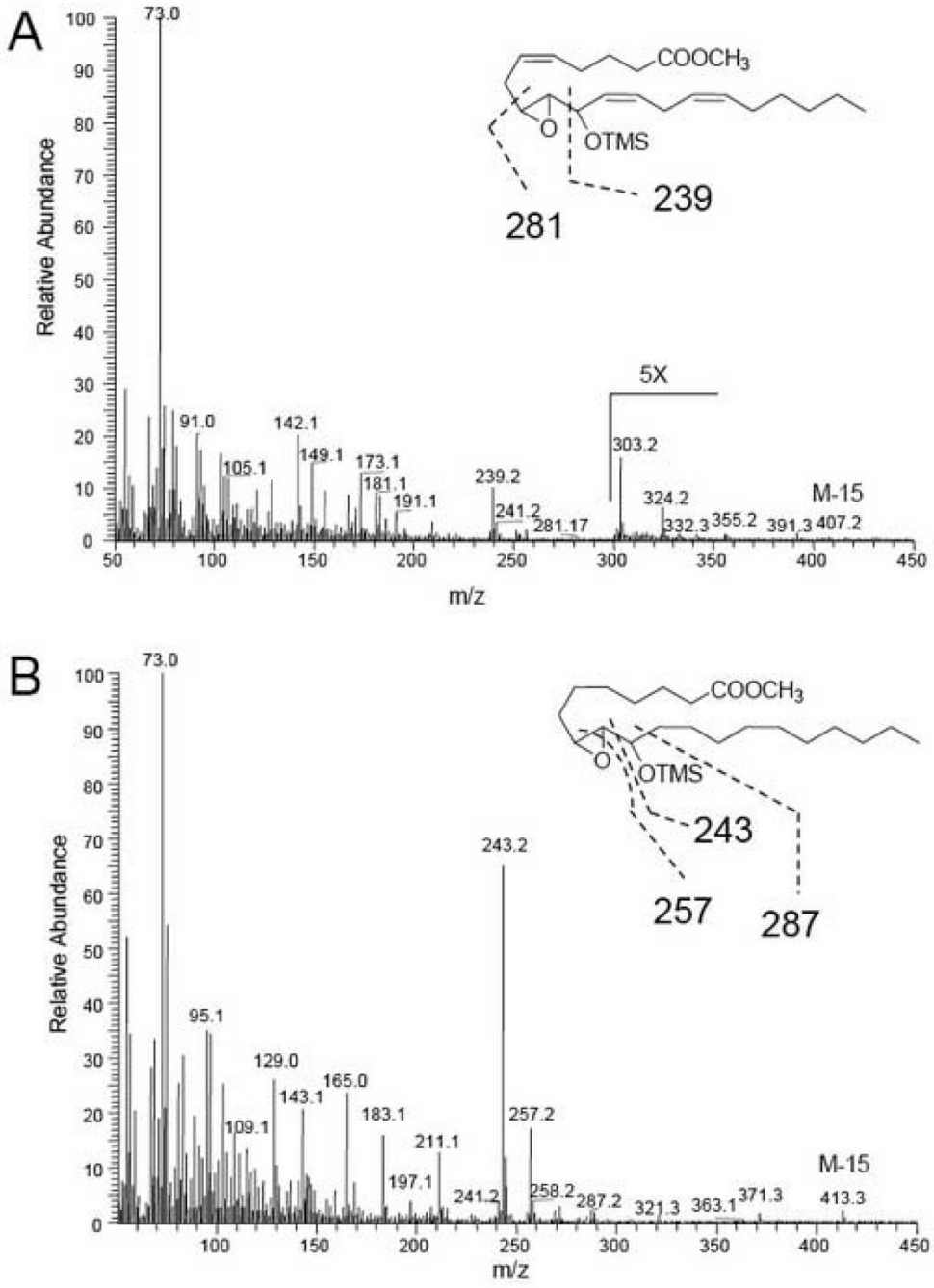

Fig. 3. GC-MS analysis of the epoxyalcohol product of mouse eLOX3 reacted with 8*S*-HPETE

(A) EI-mass spectrum (70 eV) of the methyl ester TMS ether derivative of the major epoxyalcohol product (Fig. 2A, ~12 min retention time). (B) EI-mass spectrum of hydrogenated methyl ester TMS ether derivative.

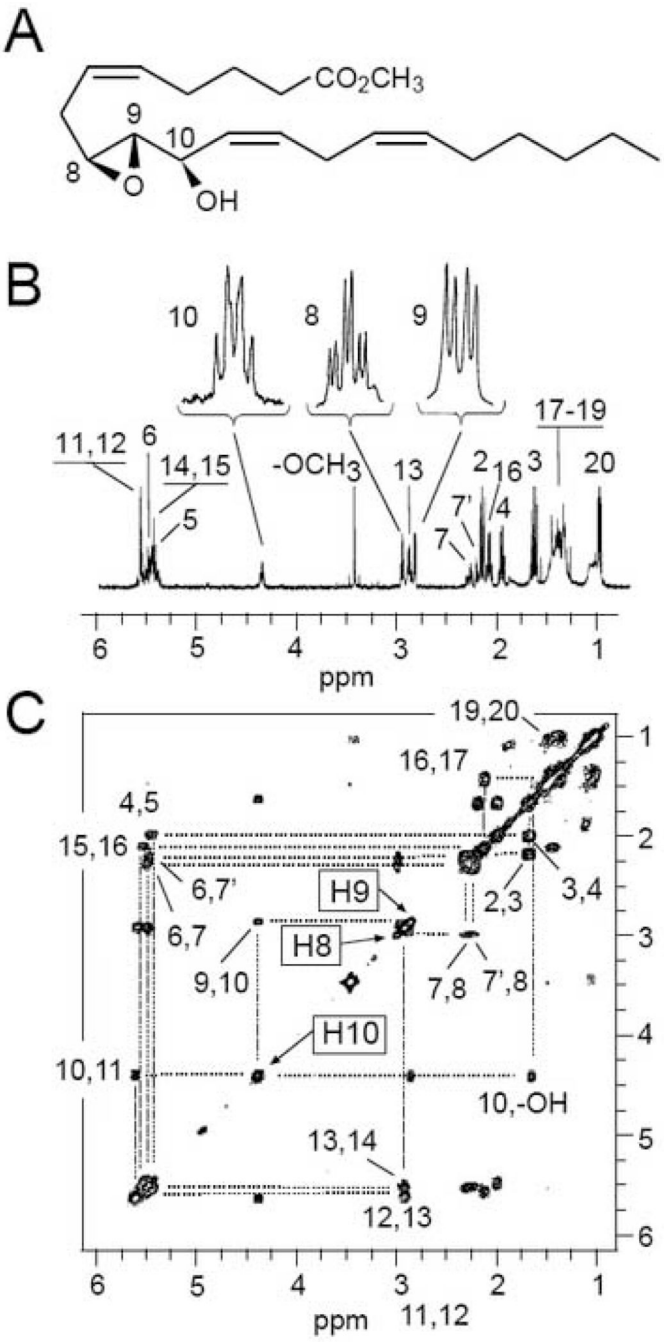

Fig. 4. NMR analysis of the epoxyalcohol product of mouse eLOX3 reacted with 8*S*-HPETE

(A) Structure of the product, 10*R*-hydroxy-8*S*,9*S*-epoxyeicosa-5*Z*,11*Z*,14*Z*-trienoic acid methyl ester. (B) ^{1}H NMR spectrum (400 MHz, C_6D_6) with an expanded view of the geminal hydroxyl (H10) and the epoxide protons (H8 and H9). (C) H,H-COSY spectrum with the main couplings indicated.

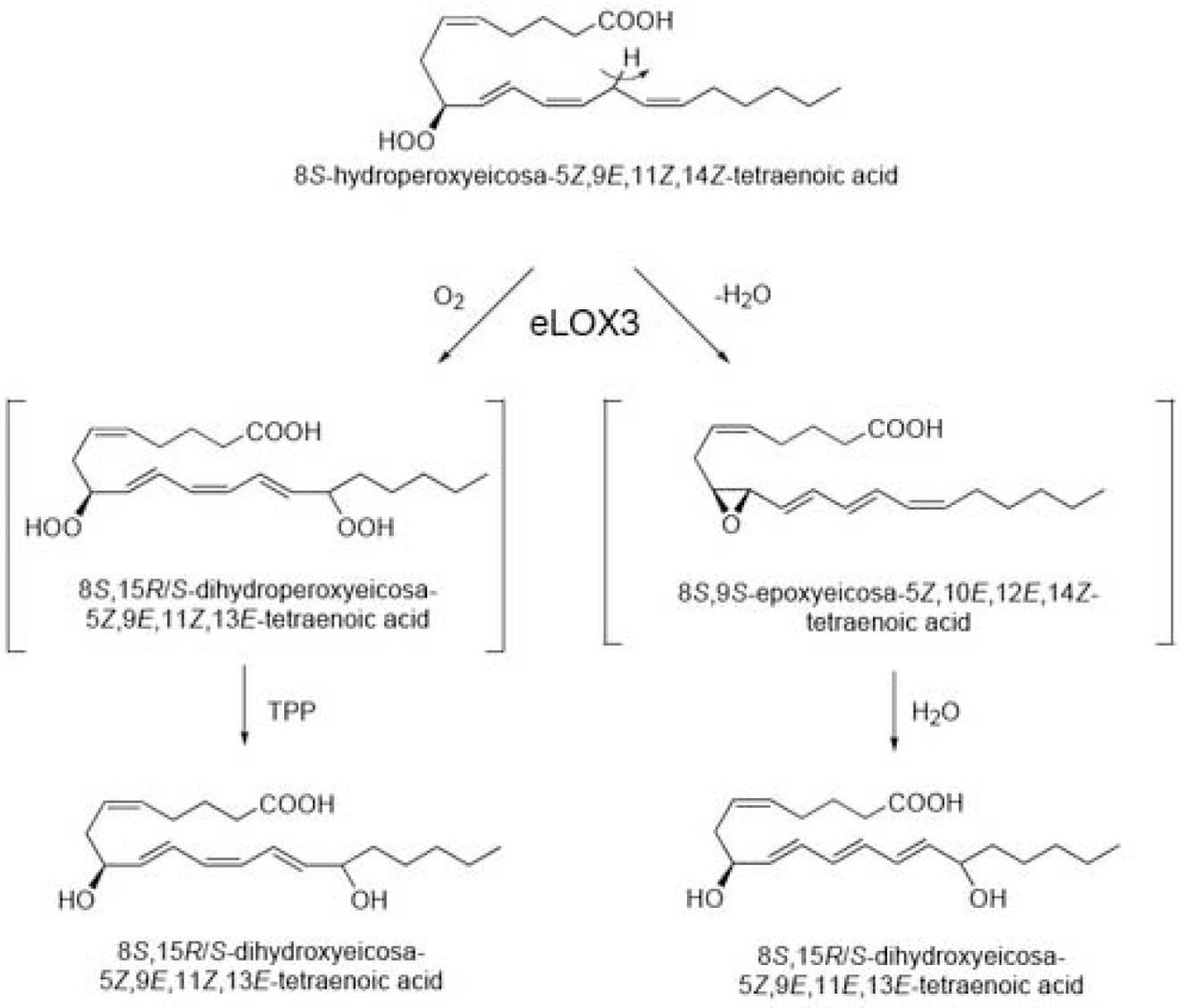

Fig. 5. Two possible pathways for the formation of conjugated triene products from 8*S*-HPETE

Following hydrogen abstraction at C-13 of 8*S*-HPETE, eLOX3 catalyzes the addition of oxygen at C-15 (left panel) or the dehydration of the 8-hydroperoxide to an 8,9-epoxide (right panel). The dihydroperoxide or epoxide intermediates (in square brackets) have not been isolated in this study.

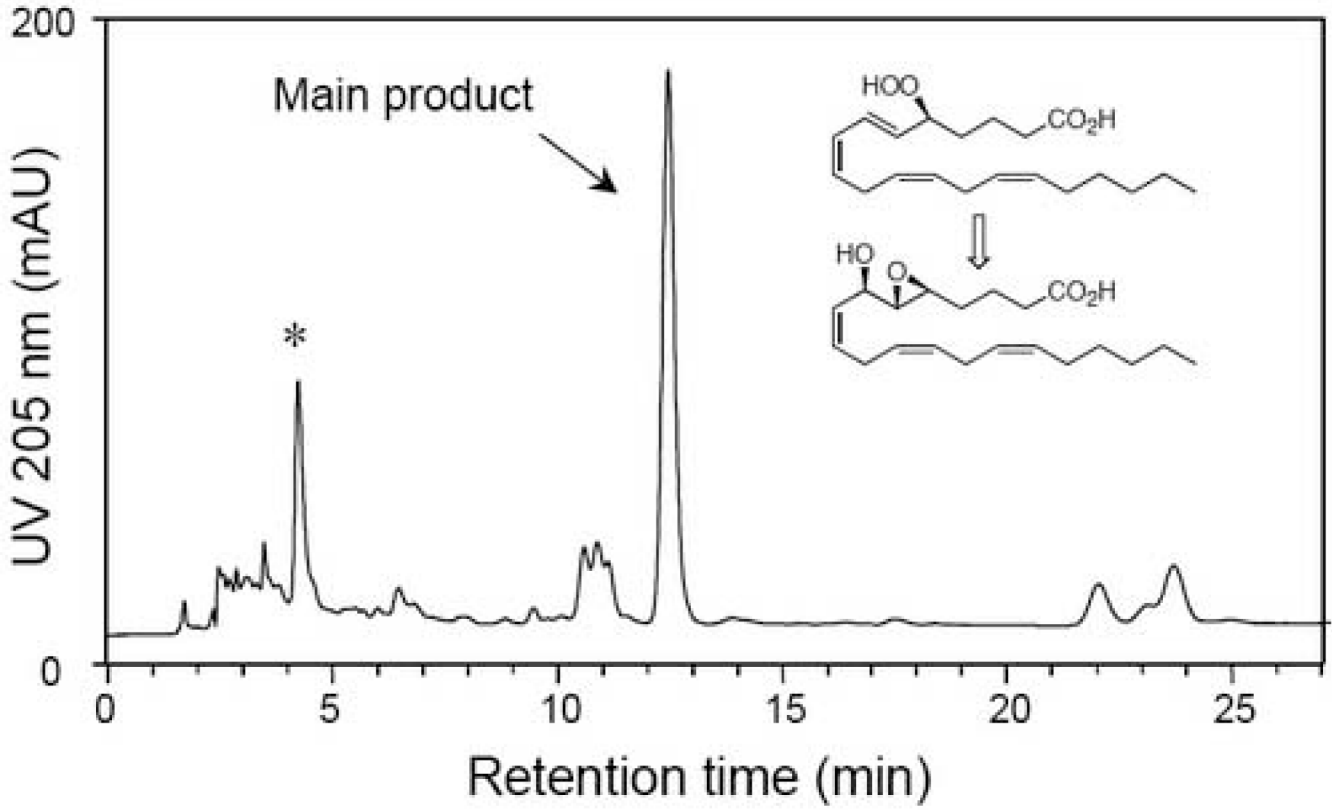

Fig. 6. RP-HPLC analysis of the products of human eLOX3 with 5*S*-HPETE

The products of the reaction of 5*S*-HPETE with human eLOX3 were analyzed by RP-HPLC using a Waters Symmetry C18 5-μm column (0.46 × 25 cm) eluted with methanol/water/acetic acid (80:20:0.01 by volume) at a flow rate of 1 ml/min and UV detection at 205 nm. The asterisk marks a solvent artifact. The insert shows the transformation of 5*S*-HPETE to the main epoxyalcohol product, 7*R*-hydroxy-5*S*,6*S*-epoxyeicosa-8*Z*,11*Z*,14*Z*-trienoic acid.