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Biochemistry

Evidence for a lipoxygenase mechanism in the biosynthesis of epoxide and dihydroxy leukotrienes from 15(S)-hydroperoxyicosatetraenoic acid by human platelets and porcine leukocytes*

(lipoxygenase/arachidonic acid/15-series leukotrienes/oxygenation/reaction mechanism)

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ABSTRACT **Leukocyte preparations convert the hydroperoxy icosatetraenoic acids 5(S)-HPETE and 15(S)-HPETE to the unstable leukotriene epoxides LTA_4 and 14,15-LTA_4. In several ways, the conversion of 5- or 15-HPETE to leukotriene epoxide bears a formal mechanistic resemblance to the reaction catalyzed by the 12-lipoxygenase in the conversion of arachidonic acid to 12(S)-HPETE. Points of similarity include enzymatic removal of a hydrogen at carbon 10, double bond isomerization, and formation of a new carbon-to-oxygen bond. In the case of 15(S)-HPETE, two 8,15- and an *erythro*-14,15-dihydroxy acid (8,15- and 14,15-DiHETEs), which result from incorporation of molecular oxygen into each hydroxyl group, are coproducts in the formation of 14,15-LTA_4. These facts prompted us to test the hypothesis that the biosynthesis of 14,15-LTA_4 and of 8,15- and 14,15-DiHETEs from 15(S)-HPETE occurs by a mechanism similar to that observed in lipoxygenase reactions. Based on the results presented here, we conclude that the biosynthesis of 14,15-LTA_4 and of 8,15- and 14,15-DiHETEs from 15(S)-HPETE occurs via a common intermediate and that, moreover, the formation of these metabolites from 15(S)-HPETE is catalyzed by an enzyme with many mechanistic features in common with the 12-lipoxygenase.**

Recently, we described several conjugated triene-containing 8, 15- and 14,15-dihydroxy acid metabolites of arachidonic acid, in porcine leukocytes, which were formed via an initial 15(S)-oxygenation (1). Experiments with $^{18}O_2$ and $H_2{}^{18}O$ indicated that two of the 8,15-dihydroxy acids, containing *all-trans* conjugated trienes and epimeric at C-8, incorporated molecular oxygen into the hydroxyl group at C-15 but water into that at C-8 (8,15-LTs). This established the existence of a 14,15-oxido leukotriene intermediate by virtue of straightforward mechanistic precedent from the formation of 5,12-dihydroxy acids from LTA_4. Two other 8,15-dihydroxy acids and two 14,15-dihydroxy acids were also identified. These four compounds incorporated ^{18}O from molecular oxygen, as opposed to water, into each hydroxyl group (8,15- and 14,15-DiHETEs). In view of the close structural similarity between these 14,15-dihydroxy compounds and the 14,15-oxido intermediate, we originally suggested that reaction of an activated molecular oxygen species such as superoxide anion with the epoxide might account for the existence of 14,15-DiHETE (see refs. 2 and 3 for reviews). This hypothesis was discounted when it was found that the products could be formed in the absence of a leukocyte respiratory burst—e.g., when only 6 μM arachidonic acid was added to cells.

More recently, additional scrutiny of certain key mechanistic features of lipoxygenase reactions brought to light another equally plausible mechanism that might account for the formation of the doubly oxygenated 14,15-diol, as well as of the 14,15-oxido leukotriene intermediate, from a 15(S)-oxygenated precursor. The essential feature of this new proposal is the conversion of 15(S)-HPETE to 14,15-LTA_4 or to 14,15-DiHETE by the common intermediate shown in Fig. 1, which in turn is formed by a free radical mechanism initiated by hydrogen atom abstraction at C-10 of 15(S)-HPETE. This is in contrast to the ionic mechanism by which 5(S)-HPETE and 15(S)-HPETE are converted chemically to LTA_4 and 14,15-LTA_4 in the biomimetic synthetic procedure, which presumably involves proton abstraction, as opposed to hydrogen atom abstraction, at C-10.

The oxygenation of arachidonic acid catalyzed by the 12-lipoxygenase has been shown (4) to involve, as an initial step, removal of the pro-(S) hydrogen at C-10, isomerization of the $\Delta^{11,12}$-*cis* double bond to $\Delta^{10,11}$-*trans* with concomitant migration of the radical to C-12, and antarafacial addition of oxygen and release of 12(S)-HPETE as product. Because of the presence of a conjugated Δ^{13}-*trans* double bond, a similar mechanistic process operating on 15(S)-HPETE would reasonably be expected to lead to [1,5] migration of the radical to C-14, instead of [1,3] migration to C-12. A radical centered on C-14 could thence proceed to react with the carbon-bound hydroperoxy oxygen with homolytic scission of the weak oxygen-oxygen single bond to yield 14,15-LTA_4 and a hydroxyl radical. Alternatively, a lipoxygenase could be envisioned as catalyzing an antarafacial addition of oxygen at C-14 which, after reduction, would give an *erythro*-14,15-dihydroxy compound showing incorporation of molecular oxygen into both hydroxyl groups. Moreover, [1,3] migration of a radical initially centered on C-10 in the opposite direction along the 15(S)-HPETE molecule, a process with mechanistic precedent in other lipoxygenases, could be envisaged to give antarafacial addition of oxygen at C-8 and hence, after reduction, one of the bis-molecular oxygen-labeled 8,15-DiHETE compounds we previously described.

The present studies were conducted to test the hypothesis

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Abbreviations: 14,15-LTA_4, 14(S),15(S)-*trans*-oxido-5,8-Z-10,12-*E*-icosatetraenoic acid; 8(*R*),15(*S*)- and 8(*S*),15(*S*)-LTs, 8(*R*),15(*S*)- and 8(*S*),15(*S*)-dihydroxy-5-Z-9,11,13-*E*-icosatetraenoic acids; *erythro*- and *threo*-14,15-LTs, *erythro*-14(*R*),15(*S*)- and *threo*-14(*S*),15(*S*)-dihydroxy-5,8-Z-10,12-*E*-icosatetraenoic acids; LT, compounds formed by hydrolysis of the epoxide 14,15-LTA_4; 8(*R*),15(*S*)- and 8(*S*),15(*S*)-DiHETEs, 8(*R*),15(*S*)- and 8(*S*),15(*S*)-dihydroxy-5,11-Z-9,13-*E*-icosatetraenoic acids; 14,15-DiHETE, *erythro*-14(*R*),15(*S*)-dihydroxy-5,8-Z-10,12-*E*-icosatetraenoic acid; DiHETE, compounds formed by incorporation of molecular oxygen into each hydroxyl group (i.e., by double oxygenation).

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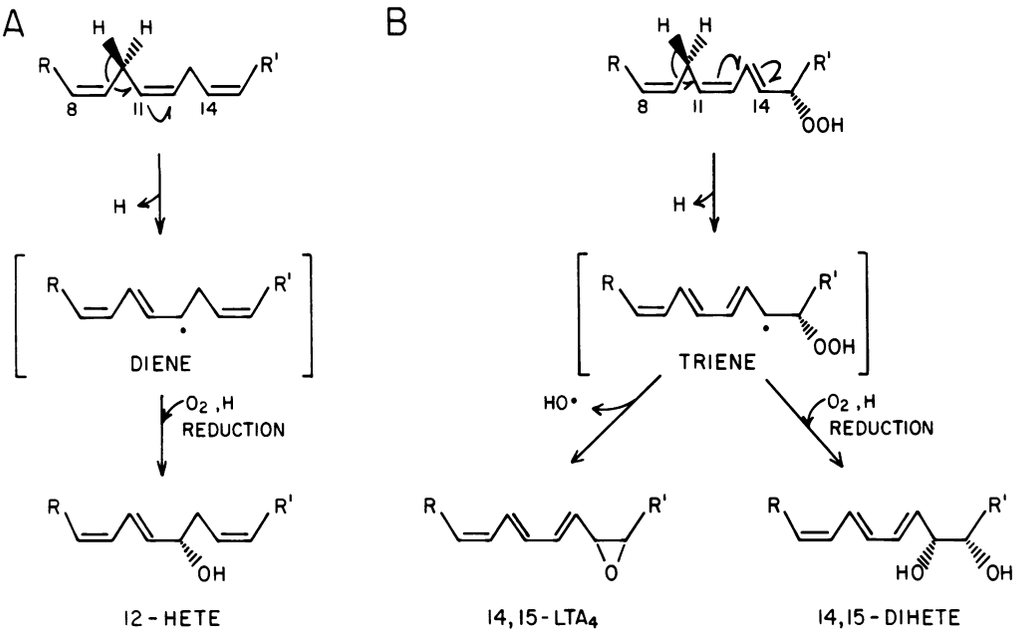


FIG. 1. (*A*) Formation of 12(*S*)-HETE by (*i*) initial removal of the 10-L_S-hydrogen, (*ii*) [1,3] radical migration with Δ^{11}-*cis* (*Z*) to Δ^{10}-*trans* (*E*) isomerization of the double bond, and (*iii*) antarafacial addition of oxygen to give the 12(*S*)-hydroperoxide (see ref. 4). (*B*) Formation of 14,15-LTA_4 and 14,15-DiHETE from 15(*S*)-HPETE by an analogous reaction involving [1,5] radical migration to C-14 after initial C-10 hydrogen atom abstraction. Reaction of the radical intramolecularly with the C-15 hydroperoxide group affords 14,15-LTA_4; intermolecular reaction with oxygen affords 14,15-DiHETE. The details of conjugated triene geometry, absolute configuration of hydroxyls, and stereochemistry of hydrogen removal at C-10 were established in the present investigation and are correct as shown. The choice of a radical process is arbitrary in both cases.

that biosynthesis of 14,15-DiHETE and of 14,15-LTA_4 from 15(*S*)-HPETE proceeds by a lipoxygenase-catalyzed mechanism that is similar mechanistically to the reaction that occurs when arachidonic acid is converted to 12(*S*)-HPETE by the 12-lipoxygenase.

MATERIALS AND METHODS

Preparation of Washed Human Platelets, Porcine Leukocytes, and Incubation Conditions. Washed human platelets were prepared from 3.2% citrated plasma by washing and resuspension at $1.5 \times 10^6/\mu l$ in 150 mM NaCl/50 mM Tris/1 mM EDTA, pH 7.4. Porcine leukocytes were prepared as before. Incubation procedures, experiments with $H_2{}^{18}O$ and $^{18}O_2$, and product isolation and analysis were as described (1).

HPLC, Derivatizations, Steric Analyses, and GC/MS. Analytical procedures were performed as described (1).

Preparation of Reference Compounds. [$^3H_8$]- and [^{14}C]arachidonic acid were purchased from New England Nuclear. 15(*S*)-HPETE was prepared by incubation of arachidonic acid with soybean lipoxygenase; 15(*S*)-HETE was prepared by $NaBH_4$ reduction; methyl esters were prepared by reaction with diazomethane. 5(*S*)-HPETE was prepared from incubation of arachidonic acid with a lipoxygenase preparation obtained from fresh New Red potatoes (5). 14,15-LTA_4, 14,15-LTA_3, and their corresponding Δ^{10}-*cis* analogs were prepared by synthetic procedures outlined elsewhere (6, 7). 14,15-LTA_3 was prepared from 15(*S*)-hydroperoxy-8,11-*Z*-13-*E*-icosatrienoic acid methyl ester. All compounds were purified by reverse-phase or straight phase HPLC prior to use. Leukotriene epoxides were characterized by ^1H NMR, UV absorbance, hydrolytic conversions, and capacity for facile [1,7] hydride rearrangement (8). For addition to cells, 14,15-LTA_4 methyl ester was converted to the free acid by treatment with dimethoxyethane/0.23 M LiOH, 1:1 (vol/vol), at ~1 mg/ml under argon at room temperature for 30 min and used directly in concert with appropriate vehicle controls. The synthetic scheme and experimental procedures for preparation of [3-^{14}C,10-D_R-^3H]- and [3-^{14}C,10-L_S-^3H]arachidonic acids and 15(*S*)-HPETEs have been outlined (9) and will be published in detail separately.

Assignments of Conjugated Triene Geometry and Absolute Configuration in Epoxides and Vicinal Diols. ^1H NMR spectra of methyl esters of 14,15-dihydroxy icosatetraenoic and icosatrienoic acids obtained from 14,15-LTA_4 and 14,15-LTA_3 were recorded at 90 MHz in C^2HCl_3 at ambient temperature on a JEOL FX 90Q FT-NMR instrument using a 1.8-mm-diameter probe. To improve spectral dispersion at 90 MHz, the compounds were titrated with the lanthanide shift reagent Eu(fod)$_3$ (Aldrich). Homonuclear decoupling experiments were performed to permit measurement of vinyl coupling constants and assignment of ethylenic geometry. For assignment of absolute configuration in the *erythro* diol 14,15-DiHETE, standards were prepared from 14,15-LTA_4 by conversion to methyl *threo*-14(*S*),15(*S*)- and *erythro*-14(*R*),15(*S*)-dihydroxy icosatetraenoate, derivatization as the bismenthoxycarbonate, ozonolysis, and esterification. The bismenthoxycarbonation proceeded quantitatively with 1.0 M (−)-menthoxy carbonyl chloroformate in toluene/pyridine, 9:1 (vol/vol), with 1 mM dimethylaminopyridine at 100°C under argon for 4 hr, followed by use of reverse-phase HPLC for purification. A (±)-*erythro* standard was prepared from *trans*-2-octenoic acid (Aldrich) via the sequence: (*i*) esterification with diazomethane, (*ii*) epoxidation with trifluoroperacetic acid, (*iii*) hydrolysis of the glycidate with sulfuric acid, and (*iv*) bismenthoxycarbonation. The resulting standards were fully characterized by TLC and GC/MS.

RESULTS

Conversions of Eicosanoid Substrates. *Arachidonic and icosatrienoic acids.* Falardeau *et al.* (10) observed that, when incubated with 8,11,14-icosatrienoic acid, human platelets produced an 8,15- and a 14,15-dihydroxy acid. In order to determine whether or not these metabolites originated from 14,15-LTA_3, we defined their precise structures. Incubation of washed

human platelets with 8,11,14-icosatrienoic acid led to the formation of a single main 14,15-dihydroxy conjugated triene and a single main 8,15-dihydroxy conjugated triene. The 14,15-dihydroxy compound was identified as *erythro*-14(*R*),15(*S*)-dihydroxy-8-Z-10,12-*E*-icosatrienoic acid—i.e., the three-double-bond analog of 14,15-DiHETE. The 8,15-dihydroxy acid was identified as 8(*R*),15(*S*)-dihydroxy-9,13-Z-11-*E*-icosatrienoic acid—i.e., the three-double-bond analog of 8(*R*),15(*S*)-DiHETE. Only trace amounts of 14,15-LTA_3 derived metabolites could be detected. When arachidonic acid was incubated with washed human platelets under the same conditions, the major dihydroxy acid formed was 14,15-DiHETE (Table 1). Much smaller amounts of 8(*R*),15(*S*)-DiHETE and of the two 8,15(*S*)-dihydroxy acids derived from 14,15-LTA_4 were detected. The formation of 14,15-DiHETE in the relative absence of 14,15-LTA_4 formation argues against the view that the 14,15-DiHETE originates from oxygenation of 14,15-LTA_4 and implies that another mechanism accounts for the formation of this metabolite. The percentage conversion to these metabolites was relatively low, ranging from 0.2% to 1.4%, compared to ≈20% for 12(*S*)-HETE. Porcine leukocytes formed the same spectrum of products with the amount of 14,15-LTA_4 more nearly equal to the amount of 14,15-DiHETE.

15(S)-HPETE and 15(S)-HPETE (20:3). 15(*S*)-HPETE was converted by both platelets and leukocytes to 14,15-DiHETE and to 14,15-LTA_4 hydrolysis products (Fig. 2). The *erythro*-14,15-DiHETE and the *erythro*-14,15-diol originating from hydrolysis of 14,15-LTA_4 were shown to be identical in chemical structure (see below). Based on ^{18}O experiments and comparison of the relative amounts of *erythro*- and *threo*-14,15-diols by HPLC, it could be determined that up to 90% of the *erythro*-14(*R*),15(*S*)-dihydroxy metabolite typically derives from bis-oxygenation and 10%, by hydrolysis of 14,15-LTA_4. Control incubations using boiled platelets or buffer plus 15(*S*)-HPETE showed no appreciable formation of any of these products.

15(S)-HETE and 15(S)-HETE (20:3). Addition of 15(*S*)-HETE to platelet and leukocyte preparations under conditions identical to those used for 15(*S*)-HPETE led to smaller but definite production of 14,15-DiHETE. As expected, there was no formation of 14,15-LTA_4, as judged by the absence of its hydrolysis products. Comparable results were obtained with the 8,11,14 double bond analog of 15(*S*)-HETE. All conversions were documented by using 1-^{14}C-labeled substrates.

14,15-LTA_4. When incubated with 14,15-[1-^{14}C]LTA_4 with or without ionophore A23187, both porcine leukocytes and human platelets gave a radioactive product pattern indistinguishable from that obtained when 14,15-LTA_4 was added to buffer (Fig. 2). Thus, no evidence could be obtained to support the conversion of 14,15-LTA_4 to 14,15-DiHETE.

5(S)-HPETE. To determine if 5(*S*)-HPETE also was a substrate for conversion to leukotriene epoxide, 5(*S*)-HPETE was added to washed human platelets under the same incubation conditions as 15(*S*)-HPETE. In contrast to the results with 15(*S*)-HPETE, incubation of washed human platelets with 5(*S*)-HPETE led to no detectable formation of LTA_4 or any of its further metabolites. The main dihydroxy acid metabolite identified in these incubations was 5(*S*),12(*S*)-dihydroxy-6,10-*E*-8,14-*Z* icosatetraenoic acid [5(*S*),12(*S*)-DiHETE]. These results indicate that, although human platelets contain an enzyme that can convert 15(*S*)-HPETE to 14,15-LTA_4, the analogous conversion of 5(*S*)-HPETE to LTA_4 does not occur.

Table 1. Conversion of different substrates to 14,15-LTA_4 and 14,15-DiHETE by human platelets

Substrate	Product, nmol/μmol substrate	
	14,15-LTA_4*	14,15-DiHETE
Arachidonic acid	2	8
15(*S*)-HPETE	40	58
15(*S*)-HETE	ND	4

Conditions: 100 μM substrate, 1.5×10^6 platelets per μl, 37°C, 5 min. Conversions are based on ^{14}C radioactivity after reverse-phase HPLC. Results represent means of two or three experiments. Leukocytes were excluded as a source of products. Controls included substrate plus boiled platelets or plus buffer alone. ND, not detected.
* Measured as the sum of its hydrolysis products.

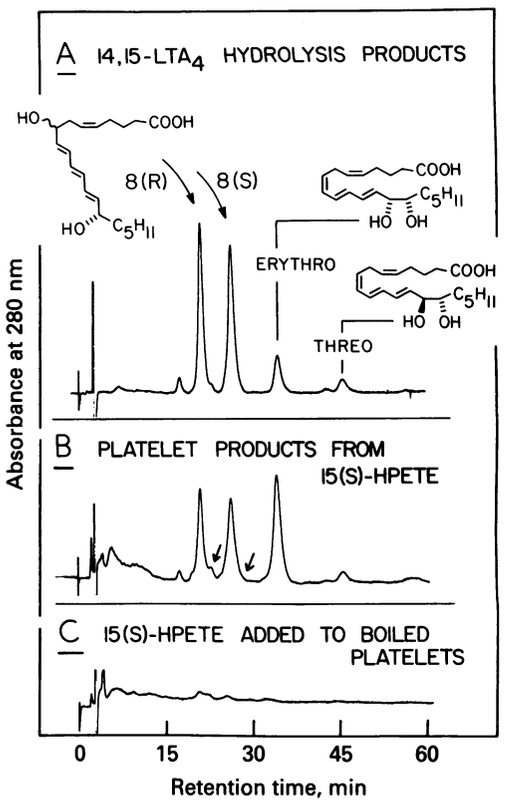


FIG. 2. Typical reverse-phase HPLC profiles showing separation of 14,15-LTA_4 hydrolysis products and DiHETEs after incubation of: (*A*) human platelets plus 14,15-LTA_4 (indistinguishable from hydrolysis of the epoxide in buffer or in boiled platelets, not shown); (*B*) human platelets plus 15(*S*)-HPETE; and (*C*) boiled human platelets plus 15(*S*)-HPETE. Structures of the four main hydrolysis products of the epoxide are shown (two 8,15-LTs and *erythro*- and *threo*-14,15-LTs). These four products are also formed from 15(*S*)-HPETE (via enzymatic formation of 14,15-LTA_4 followed by hydrolysis) and, in addition, products formed by double oxygenation are present: 8(*S*)15(*R*)-DiHETE and 8(*R*),15(*S*)-DiHETE (*B*, arrows; these minor products are well resolved from 8,15-LTs on straight phase HPLC) and *erythro*-14,15-DiHETE. The last compound is identical in structure to *erythro*-14,15-LT. Its formation by double oxygenation can only be deduced by isotopic labeling (e.g., with $^{18}O_2$). HPLC conditions: Bio-Rad ODS 5S column, 250 × 4 mm, eluted with 65% methanol/35% water/0.01% acetic acid at 1.0 ml/min.

Incubations with Arachidonic Acid and 15(*S*)-HPETE Stereospecifically Labeled with ^3H at C-10. Stereoselective hydrogen atom abstraction from a methylene-interrupted position β to the site of oxygenation is a characteristic feature of lipoxygenase reactions. Experiments were therefore conducted to determine whether or not removal of this hydrogen at C-10 in the biosynthesis of 8,15- and 14,15-DiHETEs and 14,15-LTA_4 was stereoselective and also to ascertain how the stereochemistry of removal compared to that previously observed for 12(S)HETE (4) and LTA_4 (9). Incubation of platelets or leukocytes with [3-^{14}C]arachidonic acid or 15(*S*)-HPETE, mixed with either 10-L_S-^3H- or 10-D_R-^3H-labeled substrate, demonstrated that biosynthesis of both 14,15-DiHETE and 14,15-LTA_4 and also of 12(*S*)-

Table 2. Stereochemistry of H removal at C-10 in biosynthesis of 14,15-DiHETE and 14,15-LTA$_4$ in platelets and leukocytes

	$^3H/^{14}C$, % of original substrate			
	10-D_R-^3H		10-L_S-^3H	
Compound	Platelet	Leukocyte	Platelet	Leukocyte
8(*R*),15(*S*)LT*	94	89	2	2
8(*S*),15(*S*)LT*	95	86	1	4
14,15-DiHETE*	92	92	2	2
12(*S*)-HETE†	89	—	1	—

Conditions as described (9). Products in column 1 were purified by reverse-phase HPLC; counting error, <5%. All other products were purified to homogeneity by reverse-phase and straight phase HPLC; counting error, <2%. 12-Hydroxyheptadecatrienoic acid (HHT) retained <0.5% ^3H after incubations with either isomer. 8(*R*),15(*S*)-DiHETE in platelets retained the majority of the 10-D_R-^3H label. The 10-D_R and 10-L_S nomenclature used in the headings refers to the configuration in the parent arachidonic acid. The purity of each of the stereochemically labeled substrates was demonstrated to be >99% (9).
* After incubation with stereospecifically labeled 15(*S*)-HPETE (=100%).
† After incubation with stereospecifically labeled arachidonate (=100%).

HETE was accompanied by ≈98% stereoselective removal of the arachidonate 10-L_S H (Table 2). Although a modest ^3H enrichment of 15-HETE was observed after incubation of human platelets with 15(*S*)-HPETE derived from [10-L_S-^3H]arachidonic acid, the low percentage conversion to products and the large excess of substrate made it difficult to ascribe this to a primary isotope effect. However, a secondary isotope effect was observed in the form of an apparent decrease in $^3H/^{14}C$ ratio after incubation with 10-D_R-H-labeled substrate, whereas incubation with the other isomer was accompanied by essentially quantitative loss of ^3H. A similar effect has been observed in 12-HETE and LTA$_4$ biosynthesis (9).

Assignment of Conjugated Triene Geometry of 14,15-DiHETE and 14,15-LTA$_4$, and Absolute Stereochemistry of 14,15-DiHETE. The mechanism proposed in Fig. 1 implies that, if both 14,15-DiHETE and 14,15-LTA$_4$ are formed from the same intermediate, then certain details of their structures should also be similar. Spectroscopic and chromatographic analysis of the main *erythro*-14,15-dihydroxy icosatetraenoate hydrolysis products from pure, synthetic 14,15-LTA$_4$ and Δ^{10}-*Z*-14,15-LTA$_4$ indicated that these compounds had the 8*Z*,10*E*,12*E* and 8*Z*,10*Z*,12*E* configurations respectively. These results indicate that, as a general principle, the major vicinal diol acid hydrolysis products retain the same conjugated triene geometry of their respective parent leukotriene epoxides. Because an *erythro*-14,15-dihydroxy hydrolysis product from 14,15-LTA$_4$ with the 8*Z*,10*E*,12*E* structure can be identified in leukocyte incubations, it can be inferred that the precise structure of the conjugated triene of 14,15-LTA$_4$ in cells is also 8*Z*,10*E*,12*E*. Native 14,15-DiHETE was identical to the synthetic *erythro* isomer having the 8*Z*,10*E*,12*E* geometry by UV absorbance, reverse-phase and straight phase HPLC (four derivatives), and GC/MS and therefore was assigned this triene geometry.

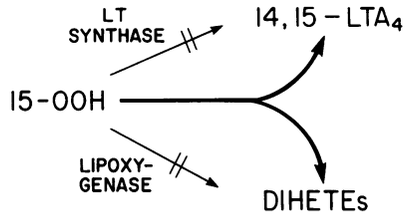
This contrasts with our earlier statement (1) that the hydrolysis-derived 14,15-diol and 14,15-DiHETE could be chemically distinguished and is attributable to contamination of our earlier 14,15-LTA$_4$ preparation with the corresponding Δ^{10}-*cis* isomer. The essential conclusion, based on $^{18}O_2$ experiments, remains unchanged: the majority of *erythro*-14(*R*),15(*S*)-dihydroxy-5,8-*Z*,10,12-*E*-icosatetraenoate originates from introduction of molecular oxygen into both hydroxyl groups and not by hydrolysis of 14,15-LTA$_4$. Further experiments with pure 14,15-LTA$_4$ showed that acid-catalyzed hydrolysis also affords small amounts of *erythro*- and *threo*-dihydroxy-5*Z*,8*E*,10*E*,12*E*-icosatetraenoates in addition to the major 14,15-diols. Corresponding amounts of these 14,15-diols were identified in incubations with both platelets and leukocytes.

Representative analytical data for three isomeric methyl *erythro*-14(*R*),15(*S*)-dihydroxy-8,10,12-icosatrienoates are as follows (**1**, 8*Z*,10*E*,12*E*; **2**, 8*Z*,10*Z*,12*E*; **3**, 8*E*,10*E*,12*E*). Reverse-phase HPLC (Waters C_{18} column, 7.8 mm × 30 cm; 70% methanol/30% water at 2.0 ml/min) R_v: **1**, 13.9; **2**, 13.0; **3**, 15.2. UV(λ_{max}^{MeOH}): **1**, 272.5 nm with well-defined shoulders at 263 and 283 nm; **2**, 272.5 nm with poorly defined shoulders at 263 and 283 nm; **3**, 270 nm with well-defined shoulders at 261 and 281 nm. IR (CH_2Cl_2, capillary): **1**, 995 cm^{-1}, 965 cm^{-1} (E_{995}/E_{965} ≈ 10); **3**, 1,000 cm^{-1}(*s*). GC (91.5 cm, 3% SP2100,C-value): **1**, 24.0; **2**, 24.5; **3**, 25.0. ^1H NMR (90 MHz, C^2HCl_3, tetramethylsilane): **1**, $J_{8,9}$ = 10.5 Hz, $J_{12,13}$ = 14.6 Hz; **3**, $J_{8,9}$ = 15.1 Hz (0.1–0.3 equiv Eu(fod)$_3$), $J_{12,13}$ = 14.9 Hz.

After oxidative ozonolysis and esterification of 50,000 dpm of purified bismenthoxycarbonate methyl ester of 14,15-[$^3H_8$]-DiHETE, isolated from leukocytes after incubation with [$^3H_8$]-arachidonic acid, thin-layer chromatography indicated the absolute configuration of native *erythro*-14,15-DiHETE to be ~2% 14(*S*),15(*R*) and ~98% 14(*R*),15(*S*) (ΔR_f = 0.05), the configuration depicted in Figs. 1 and 2.

DISCUSSION

The present investigation concerns the mode of formation of a series of conjugated triene-containing arachidonic acid metabolites originating from an initial 15(*S*) oxygenation. The key feature of this proposal is the conversion of 15(*S*)-HPETE to 8,15-DiHETEs and to 14,15-DiHETE by a radical mechanism which is initiated at C-10 and which is the common initiating step in the biosynthesis of both the epoxide and the doubly oxygenated



products. Several lines of reasoning suggested the plausibility of this idea. The biosynthetic reactions involved in the formation of all these metabolites involve hydrogen removal from C-10 of 15(*S*)-HPETE, a reaction already demonstrated to be the first step in the biosynthesis of 12(*S*)-HPETE from arachidonic acid by the 12-lipoxygenase. An important corollary that follows from the mechanism shown in Fig. 1 is that it also explains the formation of 14,15-LTA$_4$ via intramolecular reaction of a radical situated at C-14 with the C-15 hydroperoxy group, as opposed to intermolecular reaction oxygen which would afford 14,15-DiHETE. This concept, which is the major subject of this report, thus provides a unifying explanation for the associated formation of 14,15-LTA$_4$ and of 8,15- and 14,15-DiHETEs in leukocytes and platelets. It should be noted that in porcine leukocytes, the 12-lipoxygenase inhibitor 5,8,11,14-icosatetraynoic acid (ETYA) proved to be less efficient in inhibiting the conversion of 15(*S*)-HPETE to the DiHETEs and 14,15-LTA$_4$ derived products described here (IC_{50} = 5 μM) than in inhibiting the conversion of arachidonic acid to 12-HETE (IC_{50} < 1 μM). With few other exceptions, however, the experimental data presented herein prove remarkably consistent with this hypothesis.

One prediction of this hypothesis is that systems with 12-lipoxygenase activity might be effective in converting 15(*S*)-HPETE to 14,15-LTA$_4$ and 14,15-DiHETE and also in converting 15(*S*)-HETE to 14,15-DiHETE. Human platelets, rich

in 12-lipoxygenase, were found to convert 15(*S*)-HPETE to 14,15-LTA_4 and 14,15-DiHETE and to convert 15(*S*)-HETE to 14,15-DiHETE. In contrast, 5(*S*)-HPETE was converted only to 5,12-DiHETE and not to LTA_4. This result is consistent with the fact that the platelet 12-lipoxygenase selectively removes the pro-*S* hydrogen at C-10 of arachidonic acid (4) which is also the chirality of hydrogen removal in 14,15-LTA_4 and 14,15-DiHETE formation, whereas LTA_4 biosynthesis requires stereoselective removal of the pro-*R* hydrogen at C-10 (9). 15(*S*)-HPETE was converted in substantial quantities to 14,15-LTA_4 and 14,15-DiHETE by human platelets and porcine leukocytes, but the conversion of 15(*S*)-HETE to 14,15-DiHETE was considerably less. The reason for the large discrepancy in amounts of 14,15-DiHETE formed after incubation with 15(*S*)-HPETE and with 15(*S*)-HETE is not clear. However, the conversion of 15(*S*)-HETE to 14,15-DiHETE by both platelets and leukocytes indicates that 14,15-DiHETE can be synthesized by a mechanism that does not involve epoxide formation.

Perhaps the most compelling evidence favoring the involvement of a lipoxygenase mechanism in the biosynthesis of 15-series leukotrienes relates to specific details of product structure and reaction mechanism. The absolute configuration of the vicinal diol in *erythro*-14,15-DiHETE was established as 14(*R*),15(*S*). This indicates that both the C-14 and C-15 hydroxyl groups reside on the same face of the conjugated triene when the conformation is specified as in Fig. 1. Thus, in the conformation shown, the C-14 hydroxyl group, and therefore presumably oxygenation at C-14, occurs antarafacially with respect to removal of the L_S-hydrogen at C-10. Antarafacial addition of oxygen with respect to hydrogen atom removal has previously been shown to be a characteristic feature of lipoxygenase reactions (4). It should also be pointed out that one prediction of a lipoxygenase-catalyzed [1,5] radical migration for 14,15-DiHETE biosynthesis would be that the Δ^8 double bond, which remains in its original location, should retain its original *cis* (Z) configuration, whereas the newly formed Δ^{10} and Δ^{12} double bonds, by analogy to HPETE formation, should be *trans* (*E*). Based on ^1H NMR studies of synthetic material and detailed comparison with native material, the triene geometries of 14,15-DiHETE and 14,15-LTA_4 were found to be as expected. It is of great interest to note that precisely the same stereochemical relationship between hydrogen removal from C-10 and the configuration of the hydroperoxy group obtains in both LTA_4 (9) and 14,15-LTA_4 biosynthesis. The hydrogen which is removed is antarafacial with respect to the C-5 or C-15 hydroperoxy group in the conformation shown in Fig. 1.

Because no enzyme purification was undertaken in the present investigation, no definite conclusions can be made as to whether the same enzyme that forms 12(*S*)-HPETE in leukocytes also forms 14,15-LTA_4 and 14,15-DiHETE and 8,15-DiHETEs from 15(*S*)-HPETE. For instance, the existence of closely related enzyme forms catalyzing similar but different reactions would seem to be a distinct possibility. However, the data presented here suggest that the possibility of formation of 14,15-LTA_4 and of 8,15- and 14,15-DiHETEs from 15(*S*)-HPETE by a 12-lipoxygenase is not unreasonable.

Note Added in Proof. For a discussion of the nonenzymatic formation of these compounds, see ref. 11.

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