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Strict Regiospecificity of Human Epithelial 15-Lipoxygenase-2 Delineates Its Transcellular Synthesis Potential

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Abstract

Lipoxins are an important class of lipid mediators that induce the resolution of inflammation and arise from transcellular exchange of arachidonic acid (AA)-derived lipoxygenase products. Human epithelial 15-lipoxygenase-2 (h15-LOX-2), the major lipoxygenase in macrophages, has exhibited strict regiospecificity, catalyzing only the hydroperoxidation of carbon 15 of AA. To determine the catalytic potential of h15-LOX-2 in transcellular synthesis events, we reacted it with the three lipoxygenase-derived monohydroperoxy-eicosatetraenoic acids (HPETE) in humans: 5-HPETE, 12-HPETE, and 15-HPETE. Only 5-HPETE was a substrate for h15-LOX-2, and the steady-state catalytic efficiency (k_{cat}/K_m) of this reaction was 31% of the k_{cat}/K_m of AA. The only major product of h15-LOX-2's reaction with 5-HPETE was the proposed lipoxin intermediate, 5,15-dihydroperoxy-eicosatetraenoic acid (5,15-diHPETE). However, h15-LOX-2 did not react further with 5,15-diHPETE to produce lipoxins. This result is consistent with the specificity of h15-LOX-2 despite the increased reactivity of 5,15-diHPETE. Density functional theory calculations determined that the radical, after abstracting the C10 hydrogen atom from 5,15-diHPETE, had an energy 5.4 kJ/mol lower than that of the same radical generated from AA, demonstrating the facility of 5,15-diHPETE to form lipoxins. Interestingly, h15-LOX-2 does react with 5*S*,6*R*-diHETE, forming LipoxinA_4, indicating the gemdiol does not prohibit h15-LOX-2 reactivity. Taken together, these results demonstrate the strict regiospecificity of h15-LOX-2 that circumscribes its role in transcellular synthesis.

Graphical abstract

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.5b01339. Supplemental Figures S1, S2, and S3 (PDF)

Notes

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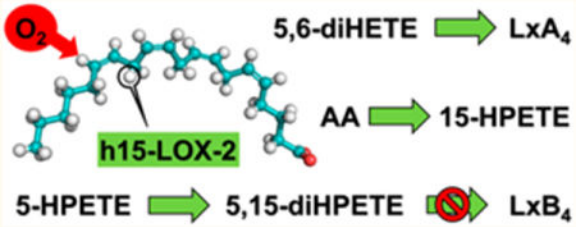

The acute inflammatory response is essential for host defense from pathogens or injury and is typically stimulated by lipid autacoids that recruit leukocytes to the affected area. The recruited leukocytes then release more lipid autacoids and other signaling molecules to either amplify the inflammatory response or resolve it.[1] The regulation of this response and its resolution are crucial for homeostasis, as uncontrolled or chronic inflammation can result in a number of diseases, including atherosclerosis,[2–4] diabetes,[5,6] periodontal disease,[7] autoimmune disorders,[8] and cancer.[9–13]

Although many factors contribute to the overall inflammatory response and resolution, an important class of regulatory molecules, the eicosanoids, has demonstrated the ability to recruit neutrophils and macrophages, induce thrombus formation, regulate vasodilation/constriction, and even contribute to apoptosis.[9,14–16] These eicosanoids are derived from arachidonic acid (AA) in leukocytes and other cells by cyclooxygenases or lipoxygenases. In humans, there are six known lipoxygenases (LOX): leukocyte 5-LOX (h5-LOX), platelet 12-LOX (h12-LOX), 12R-LOX, epidermal LOX-3, reticulocyte 15-LOX-1 (h15-LOX-1 or 12/15-LOX), and epithelial 15-LOX-2 (h15-LOX-2). Each LOX is classified by the carbon position of AA that is predominately oxygenated and the tissue in which each LOX was originally found.[14] Lipoxygenases are non-heme iron-containing dioxygenases that synthesize eicosanoids by sequential hydroperoxidation of polyunsaturated fatty acids (PUFAs), either individually or in concert transcellularly.[17–22] The lipoxygenase products that are of particular interest in this study are the lipoxins, which mediate inflammation catabasis via vasodilation/constriction, suppression of leukotriene-mediated inflammation, M2 macrophage recruitment, and effects on cytokine signaling.[8,23,24] These lipoxins (lipoxygenase interaction products) are trihydroxylated eicosatetraenoic acids that result from the transcellular exchange of lipoxygenase products.[21] Several transcellular exchange routes have been demonstrated to produce lipoxins in vivo and in vitro (Scheme 1).[18,21,22,25–31] The first route starts with 15-hydroperoxy-5*Z*,8*Z*,10*Z*,13*E*-eicosatetraenoic acid (15-HPETE), produced by either of the h15-LOXs in reticulocytes, macrophages, endothelial cells, etc.[17,21,26,32–34] This 15-HPETE can then be converted into 5*S*,15*S*-dihydroperoxy-6*E*,8*Z*,10*Z*,13*E*-eicosatetraenoic acid (5,15-diHPETE) by h5-LOX in neutrophils or other cells. The resulting 5,15-diHPETE can be further epoxidated by h5LOX to form 5*S*-*trans*-5,6-oxido-15*S*-hydroperoxy-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid (15*S*-hydroperoxy-LTA_4). 15*S*-hydroperoxy-LTA_4 can be hydrolyzed by soluble epoxide hydrolase and reduced by glutathione peroxidase (GP) to form 5*S*,6*R*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,13*E*-eicosatetraenoic acid (LipoxinA_4 (LxA_4)).[21,26,34–36] Due to the instability of the 5,6-epoxide on 15*S*-hydroperoxy-LTA_4, this intermediate can also be hydrolyzed nonenzymatically at carbon 6 and reduced by GP to form 5*S*,6*S*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,

13*E*-eicosate-traenoic acid or at carbon 14 to yield 5*S*,14*R*(or *S*),15*S*-trihydroxy-6*E*,8*E*,10*E*,12*E*-eicosatetraenoic acid (LxB_4, all *trans* isomers).[26,30] A similar lipoxin pathway involving a 5,15-diHPETE intermediate starts instead with 5-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-HPETE) from h5-LOX.[21,26] This 5-HPETE can then react with h15-LOX-1 to produce 5,15-diHPETE. This 5,15-diHPETE can then be epoxidated by h12-LOX or h15-LOX-1 to form 5*S*-hyroperoxy-15*S*-*trans*-14,15-oxido-6*E*,8*Z*,10*E*,12*E*-eicosatetraenoic acid. This product can be hydrolyzed by soluble epoxide hydrolase and reduced by GP to form 5*S*,14*R*,15*S*-trihydroxy-6*E*,8*Z*,10*E*,12*E*-eicosatetraenoic acid (LipoxinB_4(LxB_4)).[21,26,31,37,38] Also, as with 15*S*-hydroperoxy-LTA_4, the 14,15-epoxide is not stable and can be hydrolyzed non-enzymatically to form 5*S*,14*S*,15*S*-trihydroxy-6*E*,8*Z*,10*E*,12*E*-eicosatetraenoic acid or 5*S*,6*R*(or *S*),15*S*-trihydroxy-7*E*,9*E*,11*E*,13*E*-eicosatetraenoic acid (LxA_4, all *trans* isomers).[31,37–39] Yet another route can be initiated by h5-LOX's major product, 5*S*-*trans*-5,6-oxido-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid (LTA_4), which can then be converted into LxA_4 via hydroperoxidation by either h15-LOX-1 in reticulocytes or h12-LOX in platelets; hydrolysis of the epoxide nonenzymatically or by epoxide hydrolase and reduction by GP are needed to convert the resulting 15*S*-hydroperoxy-LTA_4 into the final product LxA_4.[18,22,27,28] Additionally, LTA_4 compounds that are not hydrolyzed to their main product (5*S*,12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid (LTB_4)) can also be converted to 5*S*,6*R*-dihydroxy-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid (5,6-diHETE) by soluble epoxide hydrolase or non-enzymatic hydrolysis to either 5*S*,6*R*-diHETE or 5*S*,6*S*-diHETE.[18,29,40,41] 5*S*,6*R*-diHETE can then react with h12-LOX to generate LxA_4, while 5*S*,6*S*-diHETE will generate 6*S*-LxA_4.[18] Finally, lipoxins can be generated by the 6*R*-oxygenase and 14*R*-oxygenase activities of h5-LOX and h12-LOX, which are not illustrated here.[31,40] Thus, as their name implies, lipoxins arise from the many possible exchanges of lipoxygenase AA products.

In this study, we aim to elucidate the potential of h15-LOX-2 to synthesize lipoxins. h15-LOX-2 was originally discovered as a distinct LOX isoform in epithelial cells and exhibited product regiospecificity that set it apart from h15-LOX-1.[42] Principally, h15-LOX-2 converts AA into 15-HPETE exclusively, producing none of the 12-HPETE product seen in the h15-LOX-1 reaction with AA (Scheme 2). On the basis of its original expression patterns, h15-LOX-2 was not considered as a key player in atherosclerosis and other vascular diseases, but recent studies have revealed that h15-LOX-2 is the major lipoxygenase expressed in macrophages, is found in high abundance in atherosclerotic plaques, and is induced by hypoxia and other inflammation factors.[2,4,43] Additionally, using macrophages from cystic fibrosis patients, the Urbach lab established a correlation between the mRNA levels of h15-LOX-2 and the ratio of two key opposing lipid mediators: LxA_4 and LTB_4.[32] Given this fresh perspective, the role of h15-LOX-2 in transcellular synthesis may be antagonistic to the leukotriene-mediated inflammatory pathways of h5-LOX, but its direct biosynthetic potential has not been evaluated.

Previous work has demonstrated the abilities of porcine 5-LOX, porcine 15-LOX-1, and h12-LOX to turn over AA secondary metabolites (e.g., mono- and dihydroperoxy-eicosatetraenoic acids) into lipoxins.[17,22,31,34] Additionally, in studies by Green[20] and Zhao et al.,[44] human keratinocytes containing 15-lipoxygenase activity were shown to turn over 5-HETE into 5,15-diHETE. However, no study to date has demonstrated the potential of h15-

LOX-2 to synthesize lipoxins or determined its kinetics with AA secondary metabolites. In this work, we investigated both the kinetics and the in vitro product profile of h15-LOX-2 with a variety of oxylipins and defined the catalytic bounds of h15-LOX-2.

EXPERIMENTAL PROCEDURES

Chemicals

The lipid mass spectrometry standards for 5,15-diHETE, 5,6-diHETE, LxA_4, and LxB_4 were purchased from Cayman Chemical. AA was purchased from Nu Chek Prep, Inc., and used to synthesize 5-HPETE, 5-HETE, 12-HPETE, 15-HPETE, 13*S*-hydroxy-9*Z*,11*E*-octadecadienoic acid (13-HODE), 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid (13-HPODE), and 5*S*,5,15-diHPETE as follows. Syntheses of 5-HPETE, 5-HETE, 12-HPETE, 15-HPETE, 13-HODE, and 13-HPODE were performed as previously described.[45–47] 5,15-diHPETE was synthesized from 15-HPETE as follows. Twenty micromolar 15-HPETE was reacted in 1 L of 50 mM HEPES (pH 7.5), 50 mM NaCl, and 100 μM EDTA (buffer A) with 200 μM ATP and 1 g of h5-LOX ammonium sulfate precipitate, prepared as previously described.[45] This reaction was monitored at 254 nm to completion, quenched with 0.5% (v/v) glacial acetic acid, extracted with 1 L of DCM, and evaporated to dryness. 5,15-diHPETE was then purified via high-performance liquid chromatography (HPLC) on a Higgins Haisil semipreparative (5 μm, 250 mm × 10 mm) C18 column with an isocratic elution of 50/50 acetonitrile/water. Purity was assessed via liquid chromatography–mass spectrometry to be greater than 90%.

Expression and Purification of h15-LOX-2

Over-expression and purification of wild-type h15-LOX-2 was performed as previously described.[48] The purity of the enzyme was assessed by SDS gel electrophoresis to be greater than 85%. Metal content was assessed on a Finnigan inductively coupled plasma-mass spectrometer (ICP-MS) via comparison with an iron standard solution. Cobalt-EDTA was used as an internal standard.

Analysis of h15-LOX-2 Products from 5-HETE, 5-HPETE, 12-HPETE, 15-HPETE, 5,6-diHETE, and 5,15-diHPETE

h15-LOX-2 (0.6 pmol) was reacted in 6 mL of 25 mM HEPES (pH 7.5) at ambient temperature with 10 μM oxylipin (5-HETE, 5-HPETE, 12-HPETE, 15-HPETE, 5,15-diHPETE, or 5,6-diHETE) for 1 h, quenched with 0.5% glacial acetic acid, extracted with 6 mL of DCM, reduced with trimethylphosphite, and evaporated under a stream of N_2 to dryness. The reaction mixtures were then reconstituted in 30 μL of methanol, further diluted with 60 μL of 0.1% formic acid in water, and analyzed via LC-MS/MS. Control reactions without h15-LOX-2 were run to ensure that the products formed were not a result of oxylipin degradation. Additional reactions were performed for 1 and 10 min to determine relative turnover rates of the secondary substrates. Chromatographic separation was performed on a Dionex UltiMate 3000 UHPLC with a C_{18} column (Phenomenex Kinetex, 1.7 μm, 150 mm × 2.1 mm). The autosampler was held at 4 °C, and the injection volume was 20 μL. Mobile phase A consisted of water with 0.1% (v/v) formic acid, and mobile phase B consisted of acetonitrile with 0.1% formic acid. The flow rate was 0.350 mL/min.

The initial condition (40% mobile phase B) was ramped up to 45% over 19 min. Mobile phase B was then ramped up to 75% over an additional 19 min and returned to 40% to equilibrate for 10 min. The chromatography system was coupled to a Velos Pro linear ion trap (Thermo Scientific) for mass analysis. Analytes were ionized via heated electrospray ionization with −4.0 kV spray voltage and 35, 10, and 0 arbitrary units for sheath, auxiliary, and sweep gas, respectively. The radiofrequency amplitude of the S-Lens was 52.5%, and the probe and capillary temperatures were 45 and 350 °C, respectively. All analyses were performed in negative ionization mode at the normal resolution setting. MS^2 was performed at 35% normalized collision energy in a targeted manner with a mass list containing the following m/z ratios ±0.1: 319.2, 335.2, 351.2, and 367.2. The UV detectors used were Thermo PDA Plus and Dionex Ultimate-3000 DAD. The DAD is slightly blue-shifted and not as sensitive as the Thermo PDA Plus, while the PDA Plus can lose small spectral features by its spectrum averaging. Both detectors were used for identification in all cases, but the most informative spectrum for each is included in the figures. We identified the products by matching retention times, UV spectra, and fragmentation patterns to those of known standards (Figure S1).

Steady-State Kinetics of h15-LOX-2 with 5-HETE and 5-HPETE

Reactions were performed at ambient temperature in a quartz cuvette containing 2 mL of buffer A with substrate (AA, 5-HPETE, or 5-HETE). AA concentrations were varied from 0 to 29 μM; 5-HPETE concentrations were varied from 0 to 35 μM, and 5-HETE concentrations were varied from 0 to 32 μM. Higher concentrations were avoided to prevent the formation of micelles. We determined the concentration of AA by measuring the amount of 15-HPETE produced from complete reaction with soybean lipoxygenase-1 (sLO-1). The concentrations of 5-HETE and 5-HPETE were determined from the absorbance at 234 nm. Reactions were initiated by the addition of 0.2 pmol of h15-LOX-2 and monitored on a Hewlitt-Packard 8453 UV–vis spectrophotometer. Product formation was determined by the change in absorbance at 234 nm for 15-HPETE (ε_{234nm} = 25000 M^{-1} cm^{-1}) and 254 nm for 5,15-diHPETE. The absorbance maximum for 5,15-diHPETE (247 nm) was not used due to the overlap from the decaying 5-HPETE absorbance at 234 nm. The molar extinction coefficient for 5,15-diHPETE at 254 nm was derived semiempirically from the literature value (ε_{247nm} = 33500 M^{-1} cm^{-1} in methanol)[49] and standard dilutions to be 24300 ± 30 M^{-1} cm^{-1} in methanol and 21900 ± 700 M^{-1} cm^{-1} in buffer A. KaleidaGraph (Synergy) was used to fit initial rates (at less than 20% turnover) as well as the second order derivatives (k_{cat}/K_m) to the Michaelis–Menten equation for the calculation of kinetic parameters.

Computational Methods

Density functional theory (DFT) calculations were performed using the Gaussian 09 software package[50] with a combination of the Becke exchange functional (B)[51] and the Lee–Yang–Parr correlation functional (LYP).[52,53] The 6-311G(d,p) basis set was employed to describe the system.[54] No symmetry constraints were applied during the geometry optimization, and the obtained minimum was confirmed by frequency calculation (no imaginary frequencies). Low-energy conformers were obtained through manual adjustment of dihedral angles with subsequent reoptimization. Higher energy conformers (typically 4–5 kcal/mol higher in energy) are not discussed.

RESULTS AND DISCUSSION

Product Profiles of h15-LOX-2 with AA and Monohydro(pero)xy-eicosatetraenoic Acids

In previous studies, h15-LOX-2 has demonstrated complete regiospecificity in contrast to h15-LOX-1.[42,55,56] In particular, h15-LOX-1 can produce both 15-HPETE and 12-HPETE from AA by hydrogen abstraction at the C13 and C10 positions, respectively[55,56] (Scheme 2). On the other hand, h15-LOX-2 has been shown to produce only 15-HPETE from AA, selectively abstracting only the C13 hydrogen atom.[42] In our experiments, we observed this selectivity as well. Reactions of h15-LOX-2 with AA produced only 15-HPETE (data not shown), and no further reaction was seen with 15-HPETE (Figure S3A). Despite the availability of a C13 hydrogen atom to abstract and a C15 to oxygenate, h15-LOX-2 did not react with 12-HPETE (Figure S3B). This is not surprising given that the C13 hydrogen atoms are no longer bisallylic due to the location of the C12 hydroperoxy moiety. Additionally, this hydroxyl may also block the proper insertion of 12-HPETE into the active site. Despite h15-LOX-2's lack of reactivity with 12-HPETE and 15-HPETE, we tested these HPETEs to ensure that no reactions were available via nontraditional binding modes. However, when h15-LOX-2 was reacted with 5-HPETE, it produced 5,15-diHETE (Figure 1). 5,15-diHETE has been observed in previous reactions with keratinocytes containing 15-lipoxygenase activity[20,44] and was suggested to be an intermediate in lipoxin biosynthesis.[17,20]

Steady-State Kinetics of 5-HPETE and Formation of the Lipoxin Intermediate 5,15-diHPETE

In addition to demonstrating h15-LOX-2's ability to convert 5-H(P)ETE to 5,15-diH(P)ETE, we determined the catalytic efficiency of this turnover. The steady-state kinetic parameters of h15-LOX-2 were obtained for AA, 5-HETE, and 5-HPETE (Table 1). The absolute k_{cat} (catalytic rate) and k_{cat}/K_m (catalytic efficiency) for h15-LOX-2 with AA (normalized to metal content) were 0.64 ± 0.02 s^{-1} and 0.16 ± 0.02 μM^{-1} s^{-1}, respectively, which corroborates previously reported values.[47] Kinetic k_{cat} and k_{cat}/K_m parameters of the secondary metabolites are reported relative to AA. As reported in Table 1, h15-LOX-2 displayed k_{cat} values for 5-HETE and 5-HPETE greater than that for AA, but the K_m (Michaelis constant) values for these secondary metabolites were much higher than that for AA. The net result was a lower catalytic efficiency (k_{cat}/K_m) relative to AA for 5-HETE and 5-HPETE (25% and 31%, respectively). These relative k_{cat}/K_m values are large, considering that the relative k_{cat}/K_m value of h5-LOX with 5-HPETE is 2% of its catalytic efficiency with AA.[45] These results can be explained by the fact that h15-LOX-2 is abstracting its "native" hydrogen atom from 5-HETE (C13), while h5-LOX abstracts the less-preferred hydrogen (C10 versus C7 for AA). Nonetheless, the fact that the catalytic efficiency of h15-LOX-2 with 5-HETE is large indicates the minor effect the hydroxyl of C5 has on catalysis.

Energetics of C10 Abstraction from 5,15-diHPETE

Considering the potential of 5,15-diHPETE as a lipoxin intermediate, we modeled its reactivity through homolytic CH bond cleavage by employing DFT methods. We calculated the energy required for C10 hydrogen abstraction and the resulting radical stabilization. DFT calculations were performed on model compounds (Figure 2, **m1**–**m3**) that correspond to the conjugated systems of AA (**m1**), 5(or 15)-HPETE (**m2**), and 5,15-diHPETE (**m3**). The

simplest case, model **m1**, contains four alkene moieties that are separated with methylene spacers and terminated with methyl groups. The two conceivable hydrogen abstraction pathways, denoted A (C10 abstraction) and B (C7 or C13 abstraction), both yield radical systems that can delocalize over five carbon atoms and are uphill by 67.2 kcal/mol, independent of position. In model **m2**, the abstraction pathway A leads to a radical that can delocalize over seven carbon centers, and the intermediate is calculated to have an energy 3.0 kcal/mol (64.2 kcal/mol) lower than that of the intermediate generated through pathway B. The latter is practically identical in stability to the radical intermediates computed for model **m1** (67.1 kcal/mol). Finally, model **m3** yielded a fully conjugated radical (nine carbon centers) upon hydrogen atom abstraction, and the bond dissociation energy was lowered by an additional 2.5 kcal/mol (61.8 kcal/mol). As noted in Computational Methods, these energies correspond to the lowest energy conformers in each case, and the conformations to which these molecules would be constrained in the active site may differ. Even so, the ability of the C10 radical to extend over nine carbon centers instead of seven or five lends to the ease of hydrogen atom abstraction and subsequent epoxidation in which the 5,15-diHETE intermediate could be converted into lipoxins.

Product Profiles of h15-LOX-2 with Dihydro(pero)xy-eicosatetraenoic acids

In view of this lowered energy for C10 hydrogen atom abstraction, we reasoned that h15-LOX-2 may be capable of lipoxin formation from 5,15-diHPETE. Thus, we reacted h15-LOX-2 with 5,15-diHPETE to determine if this lowered bond energy allows for an exception to h15-LOX-2's stringent regiospecificity; however, h15-LOX-2 continued to demonstrate strict C13 hydrogen atom abstraction. h15-LOX-2 did not react with 5,15-diHPETE (Figure 3) to form either of the lipoxins. Although a small peak with the same retention time and parent mass as LxA_4 was observed in the h15-LOX-2 reaction, the MS^2 spectra did not match those of LxA_4. There was, however, a trace amount of an unknown product produced by h15-LOX-2 in both its reaction with 5-HPETE and 5,15-diHPETE, which is consistent with a previous report on 15-lipoxygenase activity.[20] This unidentified product was determined not to be LxB_4 or LxA_4 due to its longer retention time and UV–vis absorbance spectrum (Figure S4). As a positive control, h15-LOX-1 was reacted with 5,15-diHPETE and was able to form LxB_4.[17,26] With the consideration that h15-LOX-2 has an allosteric site for 13-HODE and is readily activated by 13-HPODE,[57,58] h15-LOX-2 was reacted with 5,15-diHPETE in the presence of 10 μM 13-HPODE. However, even with excess 13-HPODE, h15-LOX-2 was unable to generate lipoxins from this 5,15-diHPETE intermediate (data not shown). In other words, h15-LOX-2 continues to demonstrate regiospecificity for the C13 hydrogen abstraction and C15 oxygen attack even with the lowered abstraction energy for 5,15-diHPETE.

This regiospecificity does not, however, preclude h15-LOX-2 from the formation of lipoxins in light of the h5-LOX products available (Scheme 1).[18,22] Given cellular conditions, the second major product of human and porcine h5-LOX is LTA_4,[59–62] which is unstable in water and easily hydrolyzed into various 5,6-diHETE epimers nonenzymatically or by epoxide hydrolase.[18,29] This is a minor pathway in the cell, however, with the main hydrolysis product being LTB_4 formed by LTA_4 hydrolase.[63,64] We demonstrated that h15-LOX-2 reacts with 5-HPETE, and considering that the locations of the hydroperoxy and

epoxy moieties were far from the methyl end of 5,6-diHETE and LTA_4, respectively, we suspected that h15-LOX-2 could generate LxA_4 from 5,6-diHETE and/or LTA_4. Unfortunately, we could not test whether h15-LOX-2 could generate LxA_4 from LTA_4 because h15-LOX-2's in vitro catalytic efficiency is slower than LTA_4's in vitro hydrolysis rate in pH 7.4 buffer (~2.2 μM/s).[65] Currently, no in vitro technique exists that can stabilize LTA_4 and trap 15-hydroxy-LTA_4, which makes the existence of 15-hydroxy-LTA_4 in lipoxin biosynthesis speculative. However, we were able to demonstrate that h15-LOX-2 does hydroperoxidate C15 of 5*S*,6*R*-diHETE to generate LxA_4 (Figure 4), and the relative rate of this reaction was 99.5% of h15-LOX-2's rate with 5-HPETE. Thus, the addition of a hydroxyl at C6 had little effect on the rate. With respect to LTA_4 reactivity, we speculate that because h15-LOX-2 generates LxA_4 from 5*S*,6*R*-diHETE, it most likely could convert LTA_4 to LxA_4 as 5,6-diHETE is bulkier and more polar than a 5,6-epoxide.

Implications of the Findings: h15-LOX-2's Role in Transcellular Synthesis

With its expression in macrophages and endothelial and epithelial tissues, h15-LOX-2 has the potential to play a role in many inflammatory events involving transcellular syntheses. However, as observed in this study and previous studies, h15-LOX-2 has strict regiospecificity for C15 hydroperoxidation, which constrains its role in these transcellular syntheses. In particular, we found that h15-LOX-2 will not react with its own AA product (15-HPETE) or h12-LOX's AA product (12-HPETE). Additionally, h15-LOX-2 cannot generate LxB_4 from 5-HPETE or 5,15-diHPETE due to its inability to abstract a C10 hydrogen atom. However, h15-LOX-2 does generate 15-HPETE from AA, which can then be exchanged with h5-LOX to form lipoxins.[26,30,31,39] In addition, this study demonstrates that h15-LOX-2 can convert h5-LOX-derived pro-inflammatory mediators (5-HETE and 5,6-diHETE) into anti-inflammatory precursors (i.e., LxA_4). This antagonism between h15-LOX-2 and h5-LOX may be an important switch between pro- and anti-inflammatory mediators in vivo, as demonstrated in studies of prostate cancer cells[10–12] and cystic fibrosis macrophages.[32] For example, 5-HETE has been implicated in the proliferation of prostate cancer,[13,66,67] and an increase in h15-LOX-2 expression results in decreased proliferation of prostate cancer cells.[10–12] Perhaps h15-LOX-2's efficient reaction with 5-HETE lends to the reduction of the cellular level of this compound and promotes antitumorigenic effects. In a similar vein, a more specific correlation was seen in macrophages of cystic fibrosis patients in which the ratio of LxA_4 to LTB_4 correlated directly with h15-LOX-2 mRNA levels.[32] This correlation lends credence to the "class-switching" ability of h15-LOX-2 in that LTA_4 can be converted to the potent inflammatory LTB_4 via LTA_4 hydrolase or to the potent anti-inflammatory LxA_4 via h15-LOX-2. In addition, h15-LOX-2 could convert 5,6-diHETE, which has demonstrated some inflammatory effects,[68,69] into LxA_4. This directly demonstrates h15-LOX-2's ability to efficiently switch h5-LOX products into their anti-inflammatory counterparts. Altogether, the role that h15-LOX-2 plays in the complex network of pro-inflammatory and pro-resolving lipid mediators has yet to be fully understood, but we have demonstrated, at the enzymatic level, h15-LOX-2's ability to efficiently "class-switch" h5-LOX pro-inflammatory mediators into anti-inflammatory intermediates and delineated its potential to participate in transcellular syntheses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

LOX	lipoxygenase
h15-LOX-2	human epithelial 15-lipoxygenase-2
h15-LOX-1	human reticulocyte 15-lipoxygenase-1
sLO-1	soybean lipoxygenase-1
5-LOX	leukocyte 5-lipoxygenase
12-LOX	human platelet 12-lipoxygenase
GP	glutathione peroxidase
AA	arachidonic acid
HETE	hydoxy-eicosatetraenoic acid
HPETE	hydroperoxy-eicosatetraenoic acid
diHETEs	dihydroxy-eicosatetraenoic acids
5-HETE	5-hydroxy- 6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
5-HPETE	5-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid
12-HPETE	12-hydroperoxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid
15-HPETE	15-hydroperoxy-5*Z*,8*Z*,10*Z*,13*E*-eicosatetraenoic acid
5,15-HETE	5*S*,15*S*-dihydroxy-6*E*,8*Z*,10*Z*,13*E*-eicosatetraenoic acid
5,15-diHPETE	5,15-dihydroperoxy-6*E*,8*Z*,10*Z*,13*E*-eicosatetraenoic acid
5,6-diHETE	5*S*,6*R*-dihydroxy-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid
LTA_4	5*S*-*trans*-5,6-oxido-7*E*,9*E*,11*Z*,14*Z*-eicosatetraenoic acid
LTB_4	5*S*,12*R*-dihydroxy-6*Z*,8*E*,10*E*,14*Z*-eicosatetraenoic acid
LipoxinA_4 (LxA_4)	5*S*,6*R*,15*S*-trihydroxy-7*E*,9*E*,11*Z*,13*E*-eicosatetraenoic acid

LipoxinB$_4$ (LxB$_4$)	5*S*,14*R*,15*S*-trihydroxy-6*E*,8*Z*,10*E*,12*E*-eicosatetraenoic acid

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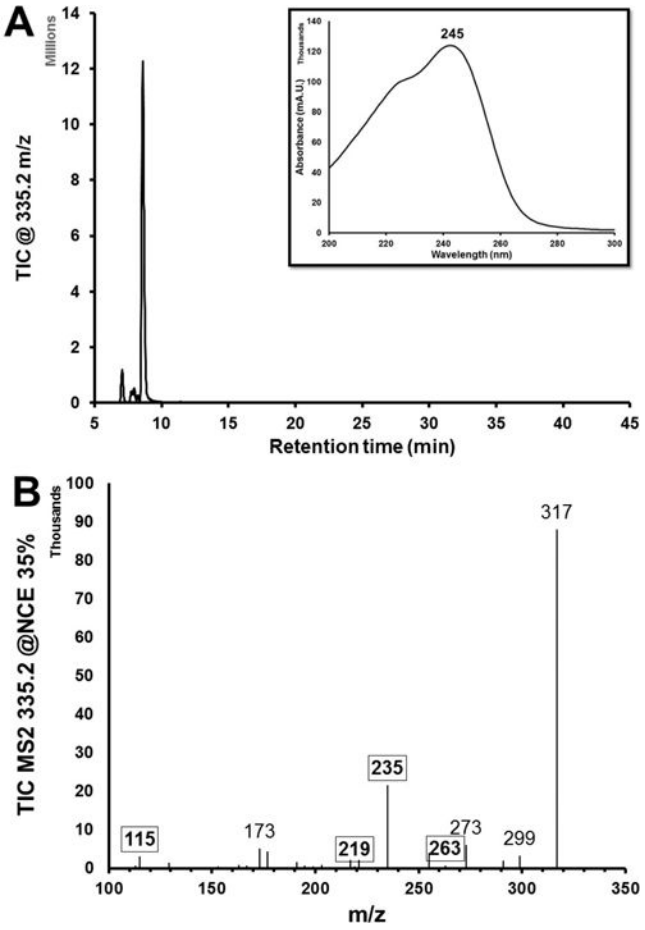


Figure 1.

h15-LOX-2 converts 5-HPETE to 5,15-diHPETE. (A) Total ion count (TIC) chromatogram of h15-LOX-2's reaction with 5-HPETE, which displays ions with a parent *m*/*z* of 335.2. The large peak has a retention time of 8.9 min and a λ_{max} value of 245 nm, as seen in the UV–vis spectra of the peak (inset), which matches the 5,15-diHETE standard. (B) Mass spectrum of the peak at 8.9 min. The diagnostic peaks for 5,15-diHETE are bolded and boxed.

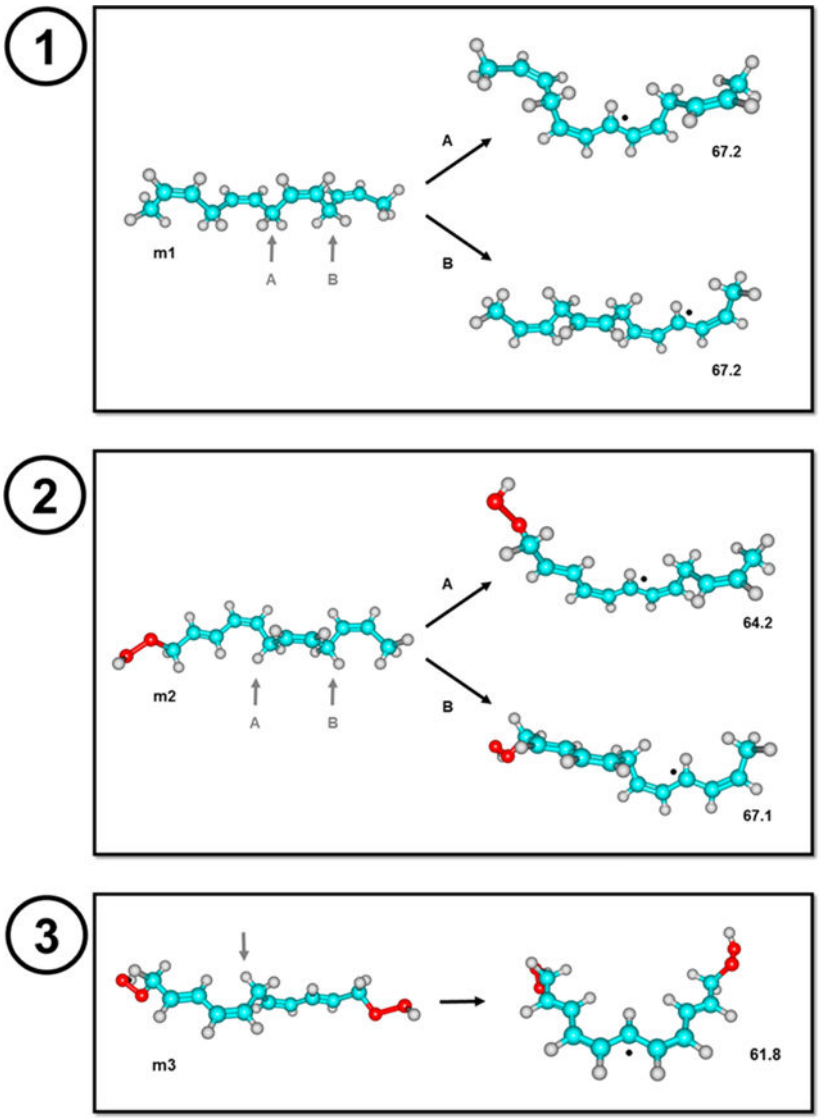


Figure 2.

Structures of *in silico* models and dehydrogenation mechanisms. (1) Model **m1** represents arachidonic acid's conjugated system and the two hydrogen atom abstractions A and B that result in two radical structures with similar energies. (2) Model **m2** represents 5-HPETE and 15-HPETE's conjugated system and the two hydrogen atom abstractions A and B that result in two radical structures with different energies. (3) Model **m3** represents 5,15-diHPETE and the resulting low energy radical structure from a C10 hydrogen atom abstraction.

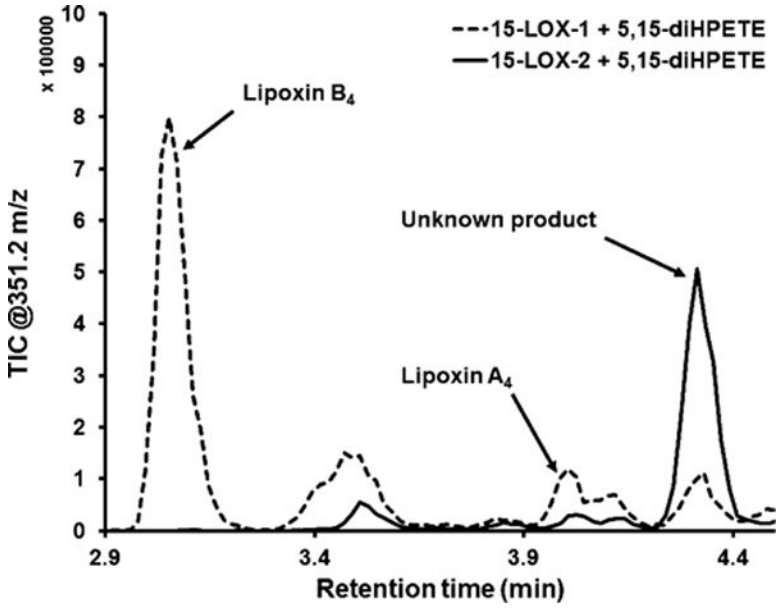


Figure 3.

h15-LOX-2 cannot synthesize lipoxins from the 5,15-diHPETE intermediate. A TIC chromatogram of the reactions of h15-LOX-2 (solid line) and h15-LOX-1 (dashed line) with 5,15-diHPETE, displaying ions with a parent *m*/*z* of 351.2. In the h15-LOX-1 reactions, LipoxinB_4 and -A_4 peaks were confirmed with retention times, UV–vis spectra, and MS spectra as compared to the standards. The tiny peak at the retention time of LxA_4 in the h15-LOX-2 reaction was not LxA_4, as determined by its MS^2 spectra. Thus, no lipoxin peaks were seen for h15-LOX-2.

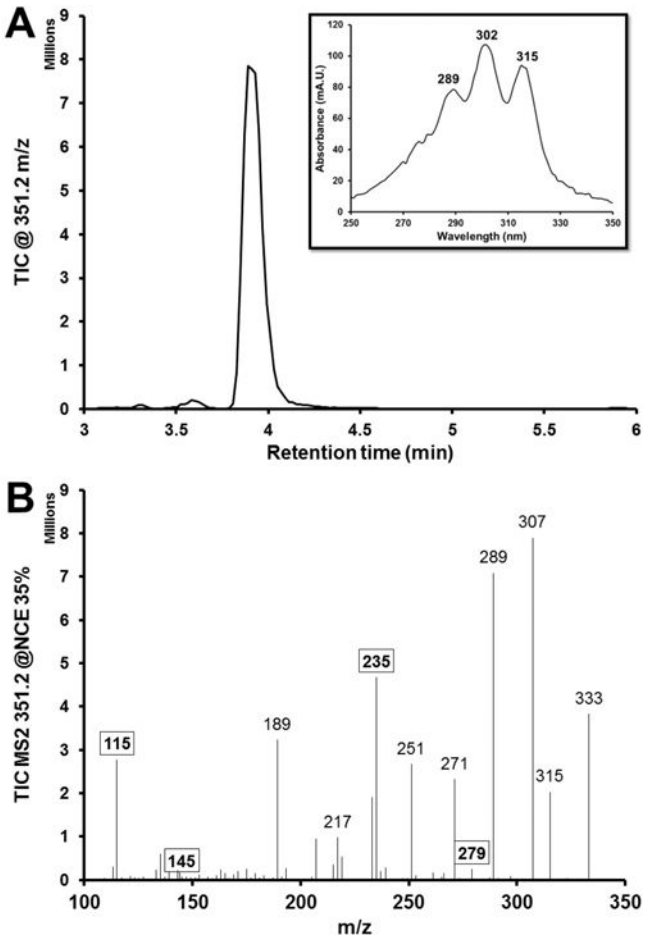
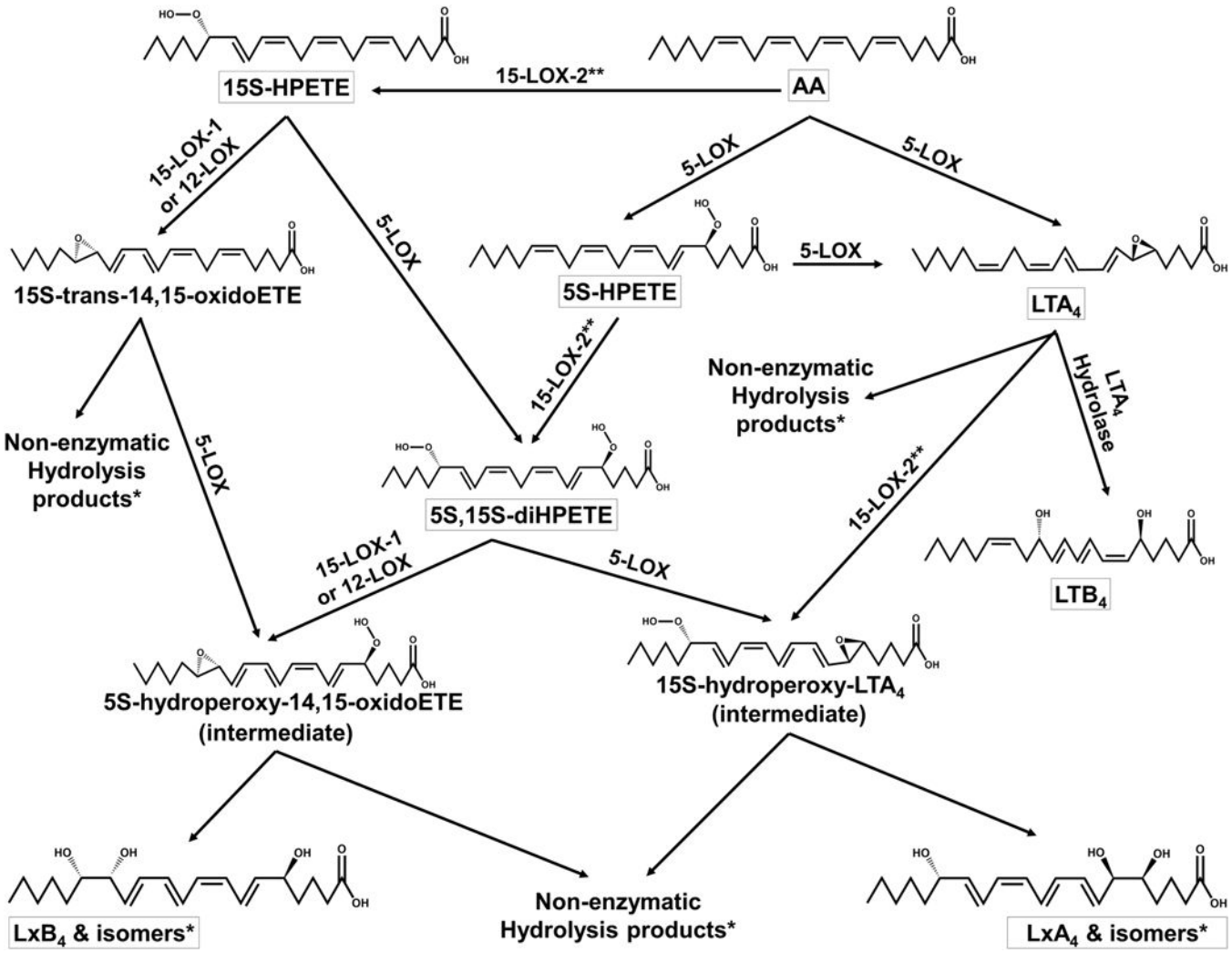


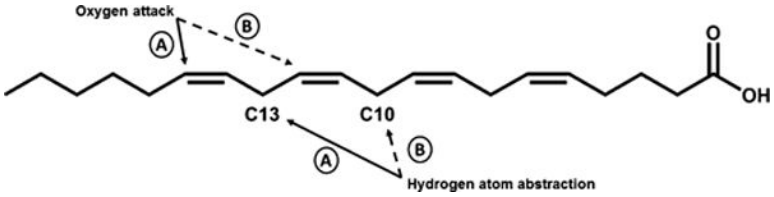
Figure 4.

h15-LOX-2 converts 5*S*,6*R*-diHETE to LipoxinA$_4$. (A) A TIC chromatogram of h15-LOX-2's reaction with 5*S*,6*R*-diHETE, displaying ions with a parent *m*/*z* of 351.2. The large peak has a retention time of 3.9 min and a λ_{max} value of 302 nm, as seen in the UV–vis spectrum of the peak (inset), which matches the LipoxinA$_4$ standard. (B) Mass spectrum of the peak at 3.9 min. The diagnostic peaks for LipoxinA$_4$ are bolded and boxed.



Scheme 1. Biosynthetic Routes to Lipoxins[a]

[a]LipoxinA$_4$, LipoxinB$_4$, and their respective isomers can arise from arachidonic acid (AA) through the pathways laid out in this scheme. The names of major products are boxed, such as LTA$_4$ and LxB$_4$. Enzymes are listed above the arrows of the reactions performed. The arrows with no enzyme listed are hydrolysis reactions that either are catalyzed by soluble epoxide hydrolase or are nonenzymatic, as indicated by the products. The numerous products observed that arise from nonenzymatic hydrolysis, indicated by a single asterisk, are listed in Figure S2. Reactions that h15-LOX-2 can perform are indicated by two asterisks; however, these reactions can also be performed by h15-LOX-1. Please note that this scheme has been simplified. For example, the required reductions of the hydroperoxy moieties to hydroxyl moieties are typically performed by glutathione peroxidase. These reactions, along with the 14*R*-oxygenase and 6*R*-oxygenase activities of h12-LOX and h5-LOX, have been excluded for clarity.



Scheme 2. Positional Specificity of 15-Lipoxygenases[a]

[a](A) h15-LOX-2 has demonstrated the ability to only abstract the hydrogen atom at C13 and allow oxygen attack at C15 (solid arrows) of arachidonic acid. (B) h15-LOX-1 can abstract a hydrogen atom from C13 and oxygenate at C15 as well but also abstracts the C10 hydrogen atom and facilitates oxygen attack at the C12 position (dashed arrows).

Table 1

Steady-State Parameters for h15-LOX-2 Hydroperoxidation of 5-HETE and 5-HPETE

	relative k_{cat}[a]	K_m (μM)	relative k_{cat}/K_m[a]
AA	1.00 ± 0.04	4.0 ± 0.6	1.0 ± 0.1
5-HETE	2.1 ± 0.2	33 ± 5	0.25 ± 0.01
5-HPETE	1.5 ± 0.1	19 ± 2	0.31 ± 0.02

[a]All k_{cat} and k_{cat}/K_m values are relative to the k_{cat} and k_{cat}/K_m values of AA, which are 0.64 ± 0.02 s^{-1} and 0.16 ± 0.02 μM^{-1} s^{-1}, respectively.