Title

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Permalink

https://escholarship.org/uc/item/1kj0n42n

Journal

Biochemistry, 55(20)

ISSN

0006-2960

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Publication Date

2016-05-01

DOI

10.1021/acs.biochem.5b01339

Peer reviewed

Strict Regio-specificity of Human Epithelial 15-Lipoxygenase-2 Delineates its Transcellular Synthesis Potential

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FUNDING: This work was supported by the NIH NS081180 and GM56062.

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-6E,8Z,11Z,14Z-eicosatetraenoic acid; 5-HPETE, 5-hydro peroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 12-HPETE, 12-hydroperoxy-5Z,8Z,10E, 14Z-eicosatetraenoic acid; 15-HPETE, 15-hydroperoxy-5Z,8Z,10Z,13E-eicosatetraenoic acid; 5,15-HETE, 5S,15S-dihydroxy-6E,8Z,10Z,13E-eicosatetraenoic acid; 5,15-diHPETE, 5,15-dihydroperoxy-6E,8Z,10Z,13E-eicosatetraenoic acid; 5,6-diHETE, 5S,6R-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid; LTA_4, 5S-trans-5,6-oxido-7E,9E,11Z,14Z-eicosatetraenoic acid; LTB4, 5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid; $LipoxinA_4$ (LxA_4), 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid; $LipoxinB_4$ (LxB_4), 5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid.

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 regio-specificity catalyzing only the hydroperoxidation of AA's carbon 15. To determine the catalytic potential of h15-LOX-2 in transcellular syntheses 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 and despite the increased reactivity of 5,15-diHPETE. DFT calculations determined that the radical, after abstracting the C10 hydrogen atom from 5,15-diHPETE, was 5.4 kJ/mol more stable than 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 lipoxin A_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.

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 to 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], auto-immune 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, have 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 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 poly-unsaturated fatty acids (PUFAs), either individually or in concert transcellularly[17–22]. The lipoxygenase products 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 (<u>lip</u>oxygenase <u>in</u>teraction products) are tri-hydroyxlated 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-5Z,8Z,10Z,13E-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 5S,15S-dihydrperoxy-6E,8Z,10Z,13E-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 5S-trans-5,6-oxido-15S-hydroperoxy-7E,9E,11Z,14Z-eicosatetraenoic acid (15S-hydroperoxy-LTA$_4$) which can be hydrolyzed, by soluble epoxide hydrolase, and reduced, by glutathione peroxidase (GP), to form 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid (LipoxinA$_4$ (LxA$_4$))[21,26,34–36]. Due to the instability of the 5,6-epoxide on 15S-hydroperoxy-LTA$_4$, this intermediate can also be hydrolyzed non-enzymatically and reduced by GP to form 5S,6S,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid, or it can be hydrolyzed at carbon 14 to yield 5S, 14R(or S), 15S-trihydroxy-6E,8E,10E,12E-eicosatetraenoic acid (LxB_4 all-*trans* isomers)[26,30]. A similar lipoxin pathway, involving a 5,15-diHPETE intermediate, starts instead with 5-hydroperoxy-6E, 8Z,11Z,14Z-eicosatetraenoic acid (5-HPETE) from h5-LOX[21,26]. This 5-HPETE can then be reacted upon by 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 5S-hyroperoxy-15S-trans-14,15-oxido-6E,8Z,10E,12E-eicosatetraenoic acid, which can be hydrolyzed by soluble epoxide hydrolase and reduced by GP to form 5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid (LipoxinB_4 (LxB_4))[21,26,31,37,38]. Also, as with the 15S-hydroperoxy-LTA_4, the 14,15-epoxide is not stable and can be hydrolyzed non-enzymatically to form 5S,14S,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid or 5S, 6R(or S), 15S-trihydroxy-7E,9E,11E,13E-eicosatetraenoic acid (LxA_4 all-trans isomers)[31,37–39]. Yet another route is initiated by h5-LOX’s major product, 5S-trans-5,6-oxido-7E,9E,11Z,14Z-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 by epoxide hydrolase or non-enzymatically, and reduction by GP are needed to convert the resulting 15S-hydroperoxy-LTA_4 into the final product LxA_4[18,22,27,28]. Additionally, LTA_4 that is not hydrolyzed to its main product, 5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid (LTB_4), can also be converted to 5S,6R-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid (5,6-diHETE) by soluble epoxide hydrolase or non-enzymatically hydrolyzed to either 5S,6R-diHETE or 5S,6S-diHETE[18,29,40,41]. This 5S,6R-diHETE can then be reacted upon by h12-LOX to generate LxA_4, while the 5S,6S-diHETE will generate 6S-LxA_4[18]. Finally, lipoxins can be generated by the 6R-oxygenase and 14R-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 h15-LOX-2's potential to synthesize lipoxins. h15-LOX-2 was originally discovered as a distinct LOX isoform in epithelial cells and exhibited product regio-specificity 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). Based on its original expression patterns, h15-LOX-2 was not considered 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, in 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 5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid (LTB_4)[32]. Given this fresh perspective, h15-LOX-2's role in transcellular synthesis may be antagonistic to h5-LOX's leukotriene-mediated inflammatory pathways, 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 turnover AA secondary metabolites (e.g. mono- and di-hydroperoxy-eicosatetraenoic acids) into lipoxins[17,22,31,34]. Additionally in a study by Floyd Green, human keratinocytes containing 15-lipoxygenase activity were shown to turnover 5-HETE into 5,15-diHETE[20,44]. However, no study to date has demonstrated the potential of h15-LOX-2 to synthesize lipoxins or has determined its kinetics with AA secondary metabolites. In the current work, we have investigated both the kinetics and *in vitro* product profile of h15-LOX-2 with a variety of oxylipins and have defined the catalytic bounds of h15-LOX-2.

Experimental Procedures

Chemicals

The lipid mass spectrometry standards, 5S,15S-dihydroxy-6E,8Z,10Z, 13E-eicosatetraenoic acid (5,15-diHETE), 5S,6R-dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid (5,6-diHETE), 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid (LipoxinA$_4$ (LxA$_4$)), and 5S,14R,15S-trihydroxy-6E,8Z, 10E,12E-eicosatetraenoic acid (LipoxinB$_4$ (LxB$_4$)), were purchased from Cayman Chemical. Arachidonic acid (AA) was purchased from Nu Chek Prep, Inc. and used to synthesize 5(S)-hydroperoxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HPETE), 5(S)-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), 12(S)-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (12-HPETE), 15(S)-hydroperoxy-5Z,8Z,10E,14Z-eicosatetraenoic acid (15-HPETE), 13(S)-hydroxy-9Z,11E-octadecadienoic acid (13-HODE), 13(S)-hydroperoxy-9Z,11E-octadecadienoic acid (13-HPODE), and 5(S),15(S)-dihydroperoxy-6E,8Z,10Z,13E-eicosatetraenoic acid (5,15-diHPETE) as follows. Synthesis 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. 20 μM of 15-HPETE was reacted in 1 L of 50 mM HEPES, pH 7.5, 50 mM NaCl, 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. The 5,15-diHPETE was then purified via high performance liquid chromatography (HPLC) on a Higgins Haisil Semi-preparative (5μm, 250mm x 10mm) C18 column with an isocratic elution of 50:50 acetonitrile and water. Purity was assessed via liquid chromatography-mass spectrometry to be greater than 90%.

Expression and Purification of h15-LOX-2

Overexpression and purification of wild-type h15-LOX-2 was performed as previously described[48]. The purity of the enzyme was assessed by SDS gel to be greater than 85%. Metal content was assessed on a Finnigan inductively-coupled plasma-mass spectrometer (ICP-MS), via comparison with 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 of oxylipin (5-HETE, 5-HPETE, 12-HPETE, 15HPETE, 5,15-diHPETE, or 5,6-diHETE) for one hour, 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. Reactions were then reconstituted in 30 μL of methanol, further diluted with 60μL of 0.1% formic acid in water, and analyzed via LC-MS/MS. Control reactions without h15-LOX-2 were run to ensure that products formed were not a result of oxylipin degradation. Additional reactions were performed for 1 and 10 minutes to determine relative turnover rates of secondary substrates. Chromatographic separation was performed on a Dionex UltiMate 3000 UHPLC with a C_{18} column (Phenomenex Kinetex, 1.7μm, 150mm x 2.1mm). The autosampler was held at 4°C and injection volume was 20 μL. Mobile phase A consisted of water with 0.1% (v/v) formic acid and mobile phase B was acetonitrile with 0.1% formic acid. Flow rate was 0.350 mL/min. The initial condition (40% B) was ramped to 45% B over 19 minutes. Mobile phase B was then ramped to 75% over 19 more minutes, and returned to 40% to equilibrate for 10 minutes. 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, 35, 10, and 0 arbitrary units for sheath, auxiliary and sweep gas, respectively. The RF amplitude of the S-Lens was 52.5%, and the probe and capillary temperatures were 45°C 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 $\pm$ 0.1: 319.2, 335.2, 351.2, and 367.2. 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 are included in figures. Products were identified by matching retention times, UV spectra, and fragmentation patterns to known standards (Supplemental 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 0-29 μM, 5-HPETE concentrations were varied from 0-35 μM, and 5-HETE concentrations were varied from 0-32 μM. Higher concentrations were avoided to prevent the formation of micelles. Concentration of AA was determined by measuring the amount of 15-HPETE produced from complete reaction with soybean lipoxygenase-1 (sLO-1). Concentration of 5-HETE and 5-HPETE were determined by absorbance at 234 nm. Reactions were initiated by the addition of 0.2 pmol of h15-LOX-2 and were 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} = 25,000 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-H(P)ETE absorbance at 234 nm. The molar extinction coefficient for 5,15-diHPETE at 254 nm was derived semi-empirically from the literature value (ε_{247nm} =33,500 M^{-1} cm^{-1} in methanol)[49] and standard dilutions to be 24,300 ± 30 M^{-1} cm^{-1} in methanol and 21,900 ± 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

DFT calculations were performed using the Gaussian 09 software package[50], using the 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 confirmed by frequency calculation (no imaginary frequencies). Low energy conformers have been obtained through manual adjustment of dihedral angles with subsequent re-optimization. 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 mono-hydro(pero)xyeicosatetraenoic acids

In previous studies, h15-LOX-2 has demonstrated complete regio-specificity 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 have 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 (Supplemental 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 (Supplemental S3B). This is not surprising, given 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 non-traditional 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 has been suggested to be an intermediate in lipoxin biosynthesis[17,20].

Steady-state kinetics of 5-H(P)ETE 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, the catalytic efficiency of this turnover was determined. 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 $0.64 \pm 0.02\ s^{-1}$ and $0.16 \pm 0.02\ \mu M^{-1}\ s^{-1}$, respectively, which corroborates previously reported values[47]. Kinetic k_{cat} and k_{cat}/K_m parameters of the secondary metabolites are reported as relative to AA. As reported in Table 1, h15-LOX-2 displayed a greater k_{cat} for 5-HETE and 5-HPETE than it did for AA, but the K_m (Michaelis constant) for these secondary metabolites was 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 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 on 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, employing Density Functional Theory (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)**, which 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 be 3.0 kcal/mol more stable (64.2 kcal/mol) than 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, the model **m3** yielded a fully conjugated radical (nine carbon centers) upon hydrogen atom abstraction, and the bond dissociation energy is lowered by an additional 2.5 kcal/mol (61.8 kcal/mol). As noted in the methods section, 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. But 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 di-hydro(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 allowed 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 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 to not be LxB_4 or LxA_4 due to its longer retention time and its UV-Visible absorbance spectrum. (Supplemental S4). As a positive control, h15-LOX-1 was reacted with 5,15-diHPETE and shown to be able to form LxB_4[17,26]. Considering that h15-LOX-2 has an allosteric site for 13S-hydroxy-9Z,11E-octadecadienoic acid (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 regio-specifity for the C13 hydrogen abstraction and C15 oxygen attack, even with the lowered abstraction energy for 5,15-diHPETE.

This regio-specificity 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 non-enzymatically or by epoxide hydrolase[18,29]. This is a minor pathway in the cell, however, with the main hydrolysis product being LTB_4 (5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid) formed by LTA_4 hydrolase[63,64]. We had demonstrated that h15-LOX-2 reacts with 5-H(P)ETE; and considering that the location of the hydroperoxy/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 either 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 (~2.2μM/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 5S,6R-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 since h15-LOX-2 generates LxA_4 from 5S,6R-diHETE, then it most likely could convert LTA_4 to LxA_4, since a 5,6-diHETE is bulkier and more polar than a 5,6-epoxide.

Implications of findings: h15-LOX-2's role in transcellular synthesis

With its expression in macrophages, endothelial, and epithelial tissues, h15-LOX-2 has the potential to play a role in many inflammatory events involving transcellular syntheses. But, as observed in this and previous studies, h15-LOX-2 has strict regio-specificity 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 increasing 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 anti-tumorigenic 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 it could be converted 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: directly demonstrating 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.

Supplemental information:

The supplemental figures that are referenced in this article (S1, S2, and S3) along with their corresponding legends are available online. These materials are supplied free of charge at http://pubs.acs.org.

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Table 1: Steady State Parameters for h15-LOX-2 hydroperoxidation of 5-HETE and 5-HPETE

	Relative k_{cat}*	K_m (μM)	Relative k_{cat}/ K_m*
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

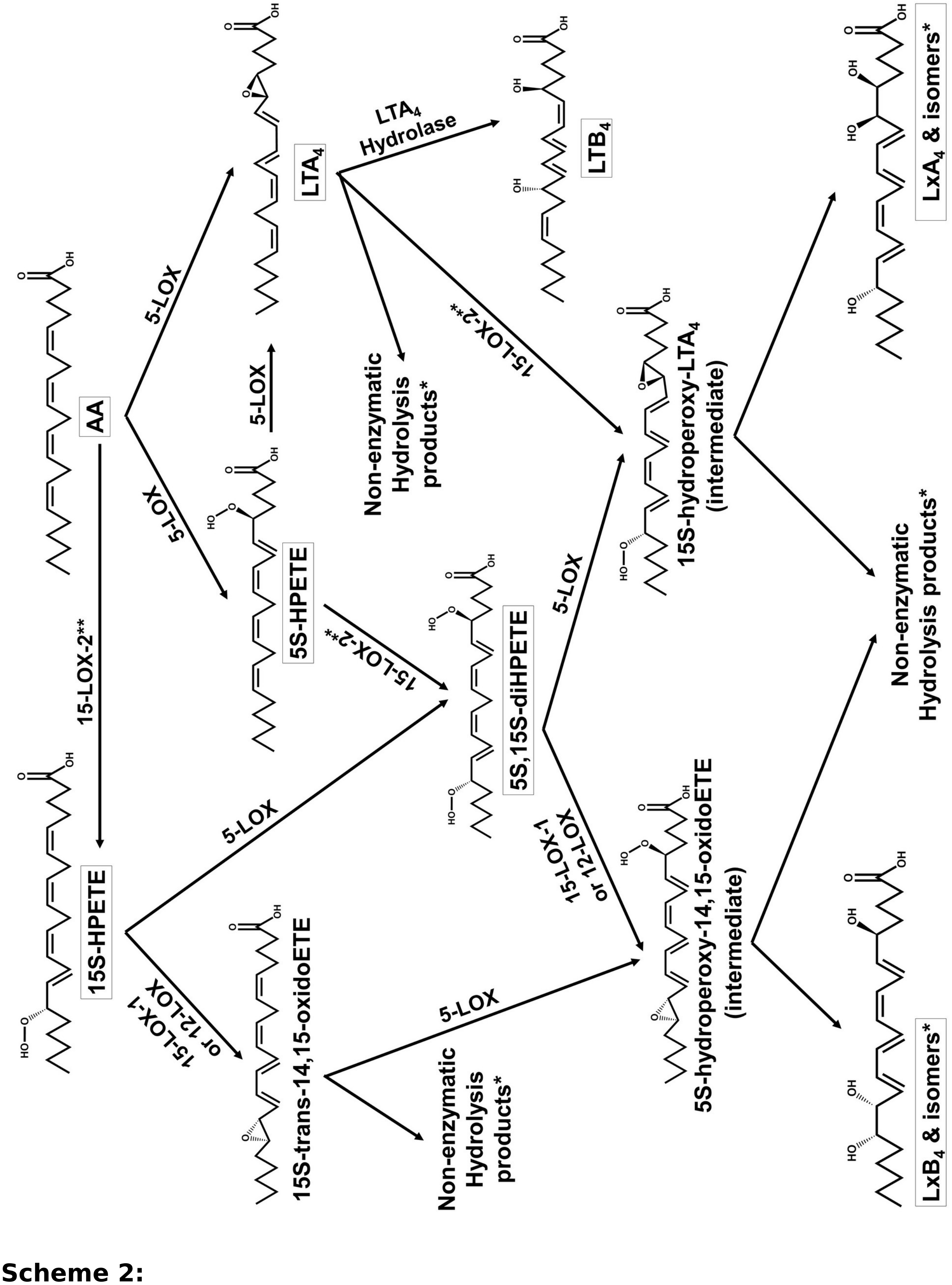
* All k_{cat} and k_{cat}/K_m values are relative to k_{cat} and k_{cat}/K_m of AA, which were 0.64 ± 0.02s^{-1} and 0.16 ± 0.02μM^{-1} s^{-1}, respectively.

Schemes

Scheme 1: Biosynthetic routes to lipoxins. LipoxinA_4 and LipoxinB_4 and their 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 reactions performed. The arrows with no enzyme listed are hydrolysis reactions that are either catalyzed by soluble epoxide hydrolase or are non-enzymatic, as indicated by the products. The numerous products that are observed and arise from non-enzymatic hydrolysis**,** indicated by a single asterisk **(*),** are listed in Supplemental S2. **Reactions that h15-LOX-2 can perform are indicated, but 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 is typically performed by glutathione peroxidase. These reactions, along with the 14R-oxygenase and 6R-oxygenase activities of h12-LOX and h5-LOX, have been excluded to increase clarity.

Scheme 2: Positional Specificity of 15-lipoxygenases. (A) h15-LOX-2 has only demonstrated the ability to abstract the hydrogen atom at carbon 13 and to allow oxygen attack at carbon 15 (solid arrows) of arachidonic acid. **(B)** h15-LOX-1 can abstract a hydrogen atom from C13 and oxygenate at C15 as well, but h15-LOX-1 also abstracts the C10 hydrogen atom and facilitates oxygen attack at the C12 position (dashed arrows).

Scheme 1:


AA
15-LOX-2**
5-LOX
5-LOX
15S-HPETE
5S-HPETE
LTA$_4$
5-LOX
LTA$_4$ Hydrolase
LTB$_4$
Non-enzymatic Hydrolysis products*
15-LOX-2**
15-LOX-2**
5-LOX
15-LOX-1 or 12-LOX
5S,15S-diHPETE
15S-trans-14,15-oxidoETE
5-LOX
Non-enzymatic Hydrolysis products*
5-LOX
15-LOX-1 or 12-LOX
15S-hydroperoxy-LTA$_4$ (intermediate)
5S-hydroperoxy-14,15-oxidoETE (intermediate)
Non-enzymatic Hydrolysis products*
LxA$_4$ & isomers*
LxB$_4$ & isomers*
OH
O
HO
HO—O

Scheme 2:

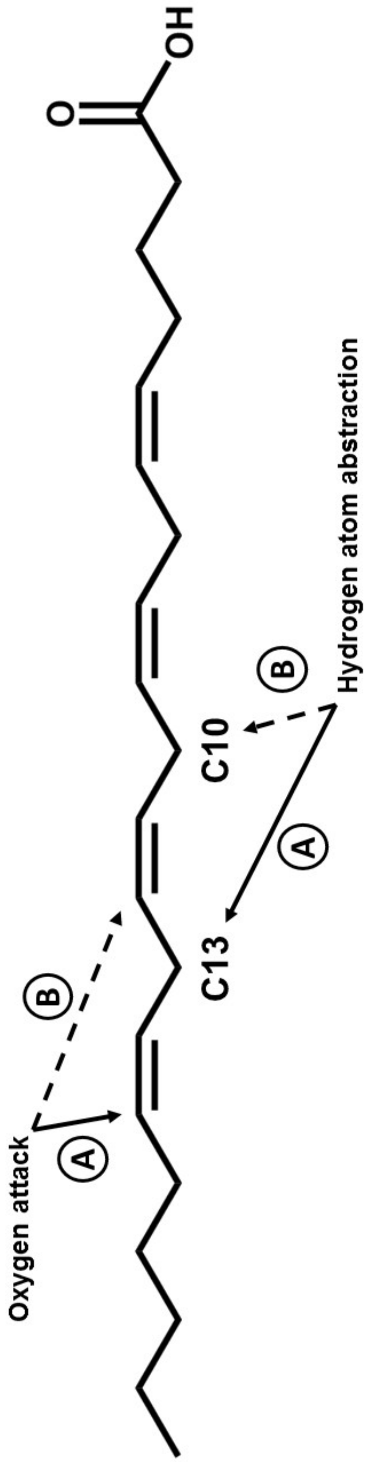


FIGURE LEGENDS

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, displaying ions with parent m/z of 335.2. Large peak has retention time of 8.9 minutes and λ_{max} of 247nm as seen in UV-Visible spectra of peak (inset), which match 5,15-diHETE standard. **(B)** Mass spectrum of 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 representing arachidonic acid's conjugated system and the two hydrogen atoms abstractions A and B that result in two radical structures with similar energies. **(2)** Model m2 representing 5-HPETE or 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 representing 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 5,15-diHPETE intermediate. Total ion count (TIC) chromatogram of h15-LOX-2's reaction (solid line) and h15-LOX-1's reaction (dashed line) with 5,15-diHPETE, displaying ions with parent m/z of 351.2. In the h15-LOX-1 reactions, Lipoxin B_4 and A_4 peaks were confirmed with retention times, UV-Visible spectra, and MS spectra as compared to 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 5S,6R-diHETE to Lipoxin A_4. (A) Total ion count (TIC) chromatogram of h15-LOX-2's reaction with 5S,6R-diHETE, displaying ions with parent m/z of 351.2. Large peak has retention time of 3.9 minutes and λ_{max} of 302nm as seen in UV-Visible spectra of peak (inset), which match LipoxinA_4 standard. **(B)** Mass spectrum of peak at 3.9 min. The diagnostic peaks for LipoxinA_4 are bolded and boxed.

Figure 1:

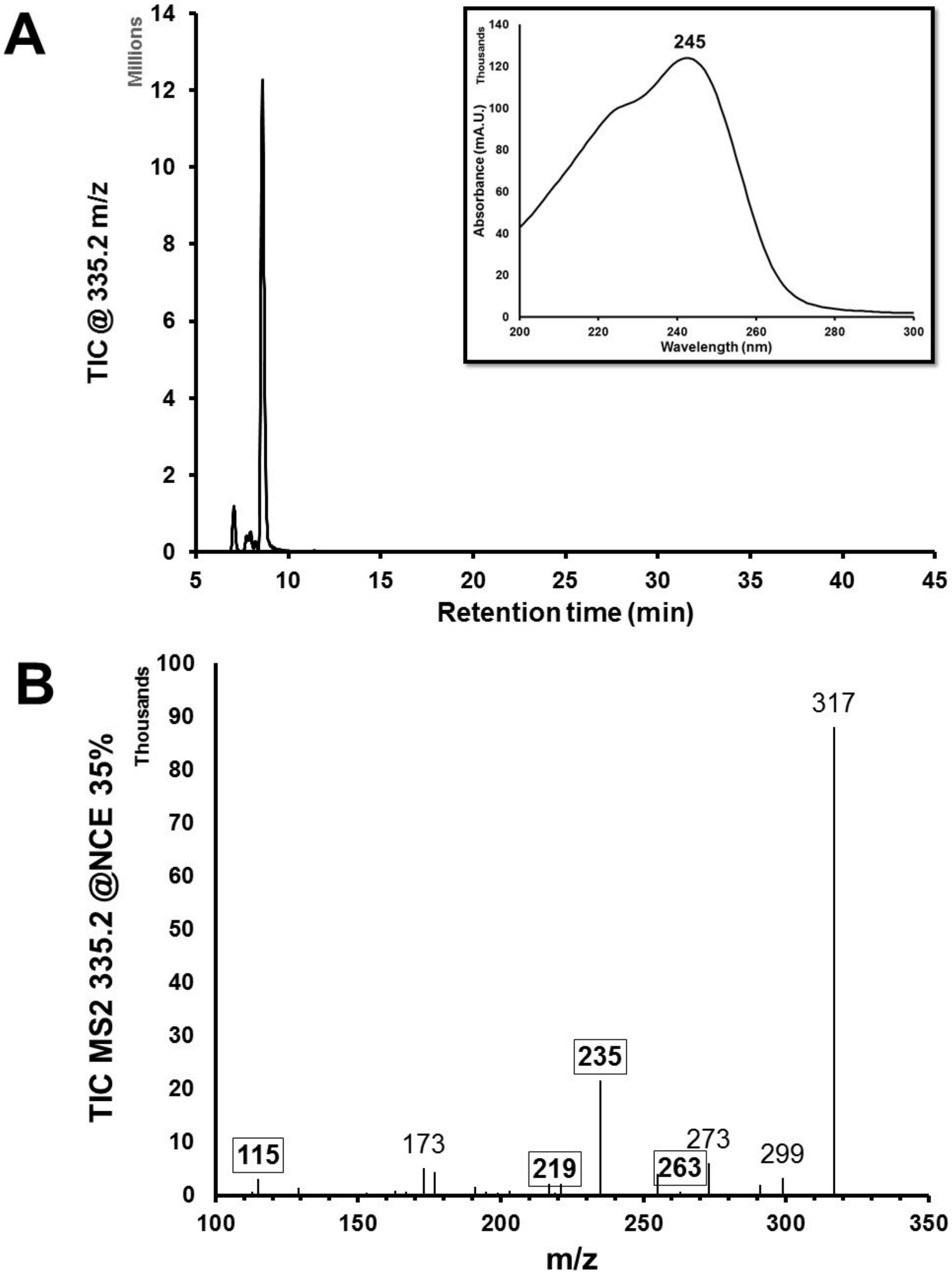

A
Millions
TIC @ 335.2 m/z
0
2
4
6
8
10
12
14
5
10
15
20
25
30
35
40
45
Retention time (min)
245
Thousands
Absorbance (mA.U.)
0
20
40
60
80
100
120
140
200
220
240
260
280
300
Wavelength (nm)
B
Thousands
TIC MS2 335.2 @NCE 35%
0
10
20
30
40
50
60
70
80
90
100
115
173
219
235
263
273
299
317
100
150
200
250
300
350
m/z

Figure 2:

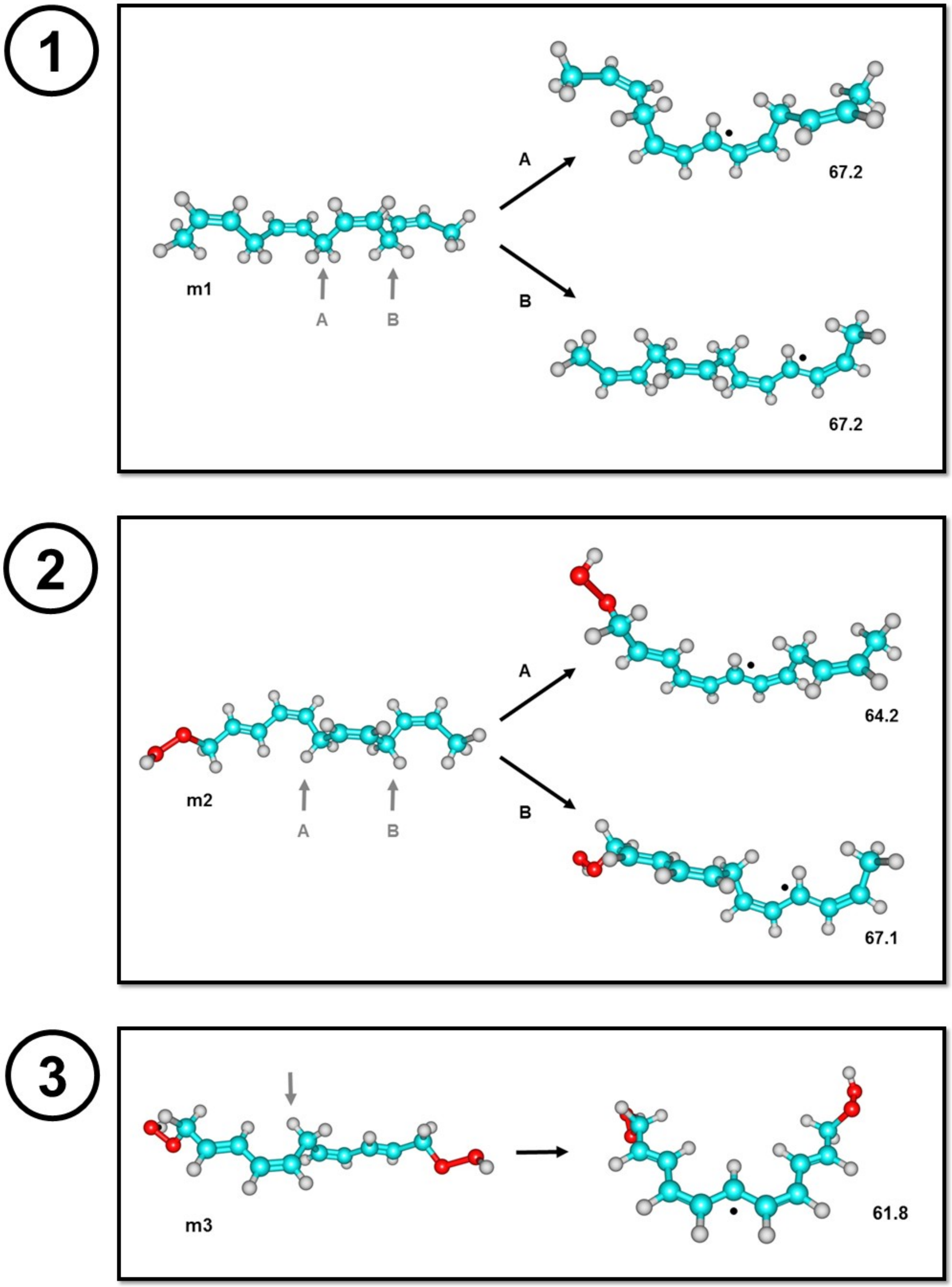


Figure 3:

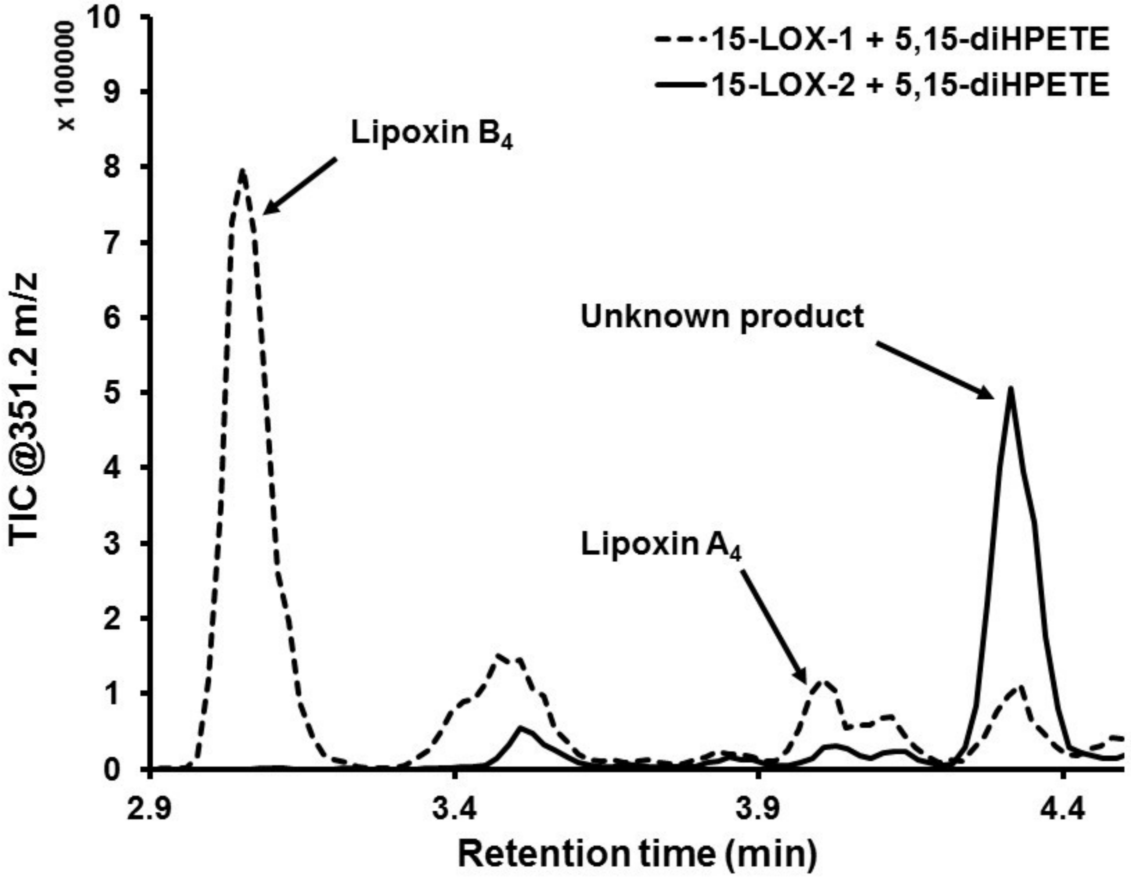
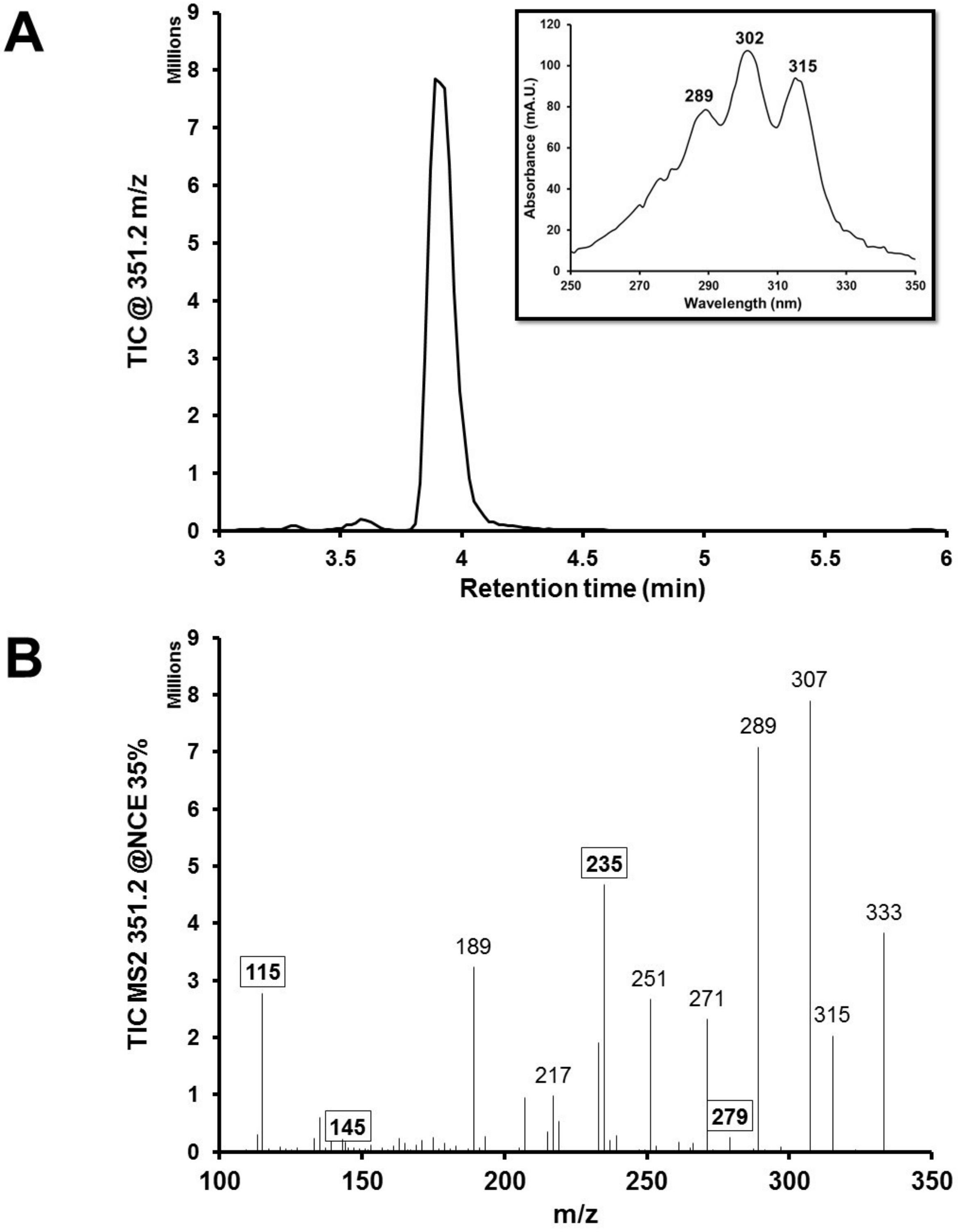
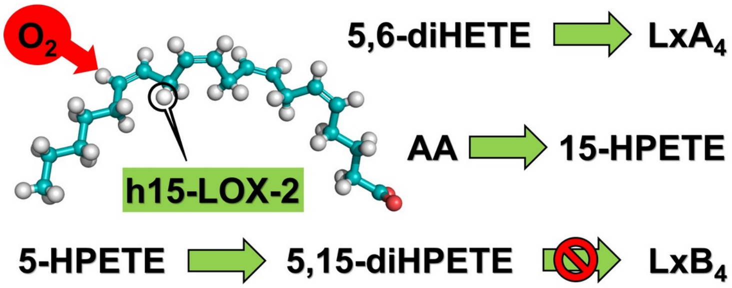

15-LOX-1 + 5,15-diHPETE
15-LOX-2 + 5,15-diHPETE
Lipoxin B_4
Unknown product
Lipoxin A_4
TIC @351.2 m/z
x 100000
0
1
2
3
4
5
6
7
8
9
10
2.9
3.4
3.9
4.4
Retention time (min)

Figure 4:



For Table of Contents Use Only


O2
5,6-diHETE → LxA4
AA → 15-HPETE
h15-LOX-2
5-HPETE → 5,15-diHPETE → LxB4