

5S,15S-Dihydroperoxyeicosatetraenoic acid (5,15-diHpETE) as a Lipoxin Intermediate: Reactivity and Kinetics with Human Leukocyte 5-Lipoxygenase, Platelet 12-Lipoxygenase, and Reticulocyte 15-Lipoxygenase-1

Abigail R. Green, Cody J. Freedman, Jennyfer Tena, Benjamin E. Tourdot, Benjamin Liu, Michael Holinstat, and Theodore Russell Holman

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4 **Kinetics with Human Leukocyte 5-Lipoxygenase, Platelet 12-Lipoxygenase, and Reticulocyte 15-**
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6 **Lipoxygenase-1**
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9 *Abigail R. Green¹, Cody Freedman¹, Jennyfer Tena¹, Benjamin E. Tourdo², Benjamin Liu¹, Michael Holinstar²,*
10
11 *Theodore R. Holman¹**
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13 ¹Department of Chemistry and Biochemistry, University of California Santa Cruz, 1156 High Street, Santa Cruz CA
14
15 95064, USA ²Department of Pharmacology, University of Michigan, 500 South State Street, Ann Arbor, MI 48109,
16
17 USA
18

19
20 *Corresponding author: Tel: 831-459-5884. Email: holman@ucsc.edu
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2 Abbreviations: LOX, lipoxygenase; 15-LOX-1, human reticulocyte 15-lipoxygenase-1; sLO-1, soybean lipoxygenase-
3 1; 5-LOX, leukocyte 5-lipoxygenase; 12-LOX, human platelet 12-lipoxygenase; GP, glutathione peroxidase; AA,
4 arachidonic acid; HETE, hydroxy-eicosatetraenoic acid; HpETE, hydroperoxy-eicosatetraenoic acid; diHETEs,
5 dihydroxy-eicosatetraenoic acids; 5-HETE, 5-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 5-HpETE, 5-hydro
6 peroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid; 12-HpETE, 12-hydroperoxy-5Z,8Z,11E,14Z-eicosatetraenoic acid; 15-
7 HpETE, 15-hydroperoxy-5Z,8Z,11Z,13E-eicosatetraenoic acid; 5,15-HETE, 5S,15S-dihydroxy-6E,8Z,11Z,13E-
8 eicosatetraenoic acid; 5,15-diHpETE, 5,15-dihydroperoxy-6E,8Z,11Z,13E-eicosatetraenoic acid; 5,6-diHETE, 5S,6R-
9 dihydroxy-7E,9E,11Z,14Z-eicosatetraenoic acid; LTA₄, 5S-trans-5,6-oxido-7E,9E,11Z,14Z-eicosatetraenoic acid;
10 LTB₄, 5S,12R-dihydroxy-6Z,8E,10E,14Z-eicosatetraenoic acid; LipoxinA₄ (LXA₄), 5S,6R,15S-trihydroxy-7E,9E,
11 11Z,13E-eicosatetraenoic acid; LipoxinB₄ (LXB₄), 5S,14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid.
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Abstract

The reaction of 5,15-diHpETE with human 5-lipoxygenase (LOX), human platelet 12-LOX and human reticulocyte 15-LOX-1 was investigated to determine the reactivity and relative rates of producing lipoxins (LXs). 5-LOX does not react with 5,15-diHpETE, although it can produce LXA₄ when 15-HpETE is the substrate. In contrast, both 12-LOX and 15-LOX-1 react with 5,15-diHpETE, forming specifically LXB₄. For 12-LOX and 5,15-diHpETE, the kinetic parameters are $k_{\text{cat}} = 0.17 \text{ s}^{-1}$ and $k_{\text{cat}}/K_{\text{M}} = 0.011 \text{ }\mu\text{M}^{-1}\text{s}^{-1}$ (106-fold and 1600-fold lower than for 12-LOX oxygenation of AA, respectively). On the other hand, for 15-LOX-1 the equivalent parameters are $k_{\text{cat}} = 4.6 \text{ s}^{-1}$ and $k_{\text{cat}}/K_{\text{M}} = 0.21 \text{ }\mu\text{M}^{-1}\text{s}^{-1}$ (3-fold higher and similar to that for 12-HpETE formation by 15-LOX-1 from AA, respectively). This contrasts with the complete lack of reaction of 15-LOX-2 with 5,15-diHpETE (*Biochemistry* 55, 2832-2840, 2016). Our data indicate that 12-LOX is markedly inferior to 15-LOX-1 in catalyzing the production of LXB₄ from 5,15-diHpETE. Platelet aggregation was inhibited by the addition of 5,15-diHpETE, with an IC₅₀ of 1.3 μM , however, LXB₄ did not significantly inhibit collagen-mediated platelet activation up to 10 μM . In summary, LXB₄ is the primary product of 12-LOX and 15-LOX-1 catalysis if 5,15-diHpETE is the substrate, with 15-LOX-1 being 20-fold more efficient than 12-LOX. LXA₄ is the primary product with 5-LOX, but only if 15-HpETE is the substrate. Approximately equal proportions of LXA₄ and LXB₄ are produced by 12-LOX, but only if LTA₄ is the substrate, as described previously (*Biochimica et Biophysica Acta* 1133, 223-234, 1992).

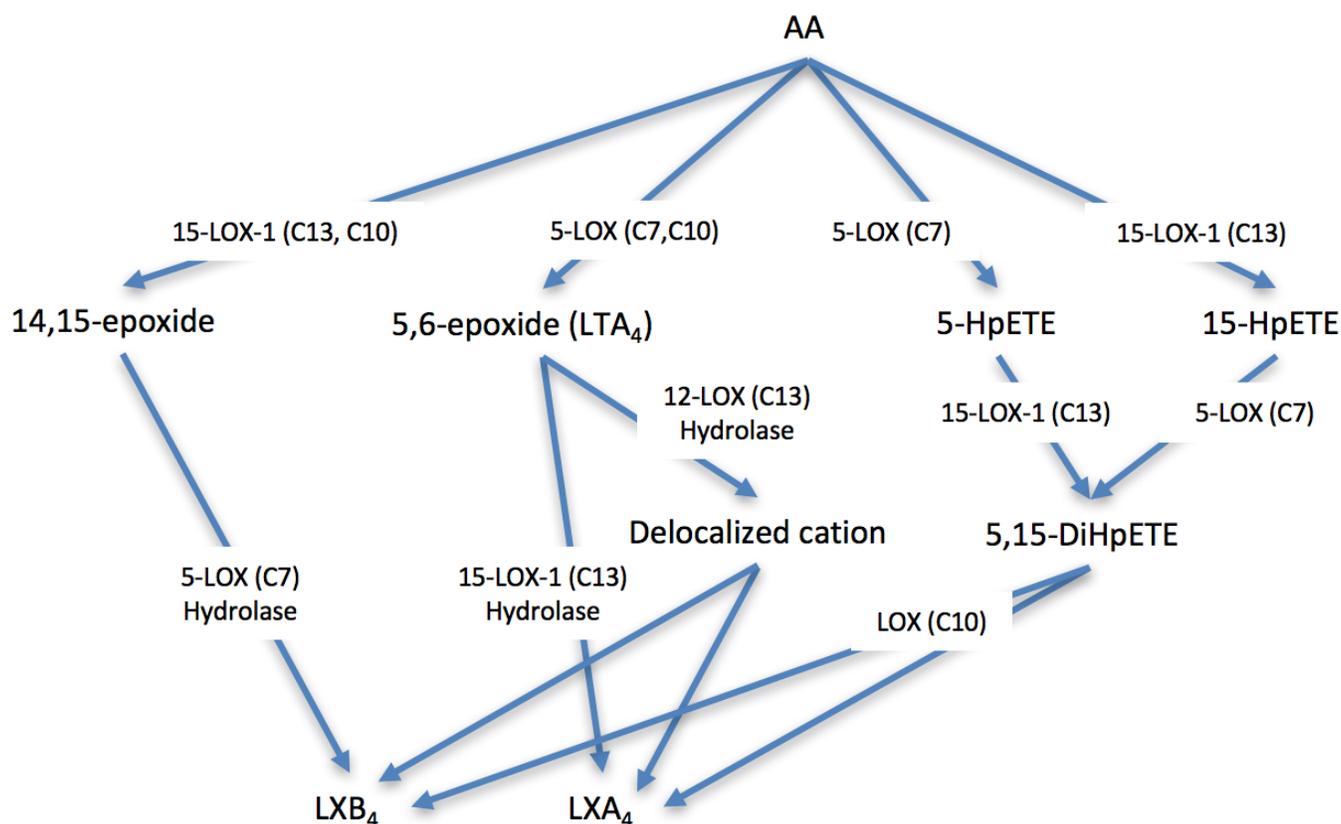
Introduction:

Lipoxins (LXs) were discovered in 1984^{1, 2} and are a class of specialized pro-resolving mediators (SPMs) derived from arachidonic acid (AA), which are involved in inflammation resolution. The first two LXs discovered were lipoxinA₄ (LXA₄) and lipoxinB₄ (LXB₄), with the epi-lipoxins, 15-epi-LXA₄ and 15-epi-LXB₄, being discovered later.³ The initial step of acute inflammation is to recruit macrophages with the generation of leukotrienes (LTs) and hydroxyeicosatetraenoic acids (HETEs). Eventually, SPMs, such as LXs, are generated via transcellular pathways to reverse the inflammatory response and initiate cellular repair.⁴ Researchers have investigated the biosynthetic pathway for the production of LXs and the generally accepted biosynthesis of LXs requires two distinct enzymatic reactions with AA. The first step is the oxygenation of AA to form a hydroperoxide, which is then converted to an epoxide. This can be accomplished by either 5-LOX or 15-LOX, generating either the 5,6-epoxide or the 14,15-epoxide, respectively. The second step is oxygenation at the other end of the molecule from the epoxide, adding a hydroperoxide moiety. There is then a subsequent hydrolysis of the epoxide and reduction of the hydroperoxide, creating either the 5,6,15-trihydroxy- or the 5,14,15-trihydroxy-eicosatetraenoate, depending on the initiating LOX isozyme (Figure 1).^{5, 6} The specific pathway depends on the cell type involved, either with one cell type that has both LOXs, or a transcellular biosynthetic pathway, which involves the transfer of an intermediate between two cell types. Another biosynthetic pathway for LXs involves 5-LOX in neutrophils and 12-LOX in platelets. The hypothesis is that 5-LOX generates LTA₄ in the neutrophil, which is then transferred to the platelet, where 12-LOX subsequently abstracts a hydrogen atom from C13 of LTA₄, with oxygen attacking C15. The epoxide ring is subsequently opened by a hydrolase, generating a delocalized cation, which is attacked by water, generating either LXA₄ with a C6 attack or LXB₄ with a C14 attack (Figure 1).^{7, 8}

Another pathway to making LXs, involves 5,15-diHpETE as an intermediate. A hydrogen atom is abstracted from C10 of 5,15-diHpETE, generating a delocalized radical, which can convert either the C5 hydroperoxide or the C15 hydroperoxide to an epoxide (5,6- or 14,15-epoxide). Subsequent hydrolysis of the

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expoxide and reduction of the hydroperoxide generates either the 5,6,15-trihydroxy- or the 5,14,15-trihydroxy-eicosatetraenoates. Like the cation intermediate discussed above, both LXA₄ and LXB₄ can be generated in this manner since the resulting radical is delocalized over 9 carbons due to the full conjugation of the tetraene system (Figure 1).⁹ The reactivity of this chemical step is enhanced by the fact that 5,15-diHpETE is an “activated” intermediate. The energy required to abstract the C10 hydrogen atom from 5,15-diHpETE is markedly lower than the hydrogen atom from AA or 5-hydroperoxyeicosatetraenoic acid (5-HpETE), due to the greater conjugation of its intermediate, 9-carbons for 5,15-diHpETE versus 5-carbons for AA.⁹ Therefore, the ease of hydrogen atom abstraction from 5,15-diHpETE could enhance the rate of either LXA₄ or LXB₄ biosynthesis.



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Figure 1: Biosynthetic pathways for LX production. The carbon whose hydrogen atom is abstracted is listed next to the LOX isozyme, such as C7, C10 or C13. For the conversion of 5,15-DiHpETE to the two lipoxins, the LOX isozyme is not specified because that is the subject of this publication.

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This last biosynthetic pathway is supported by fact that the LX intermediate, 5,15-diHpETE, is observed in a variety of biological samples. One of the earliest observations of 5,15-diHpETE was in

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2 activated porcine leukocytes, when either AA or 15-HpETE were added as precursors.¹⁰ 5,15-diHpETE was
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4 also observed with isolated porcine 5-LOX, which reacted with 15-HpETE, at a 30% rate of that with AA.¹¹
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6 5,15-diHpETE could then be further modified to LXB₄ with purified rabbit reticulocyte 15-LOX-1 (r15-
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8 LOX-1), presumably due to an epoxide intermediate.¹² r15-LOX-1 could also convert 15-HETE to LXB₄,
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10 which was due to double oxygenation at C5 and C15, as evidenced by labeled O₂ being found on both
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12 carbons.^{12, 13} Porcine leukocyte 12-LOX also converts 5,15-diHpETE to LXB₄, which was deduced by the
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14 observation of four isomers of LXB₄, due to the non-enzymatic hydrolysis of 5-OH,14,15-LTA₄.¹⁴ LXB₄ was
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16 also produced by incubation of 15-HpETE with human leukocytes, with labeled O₂ incorporating into only
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18 C5, and not C14, indicating the presence of a hydrolyzed 14,15-epoxide.¹⁵ 5,15-diHpETE can also be
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20 generated by the addition of AA to human eosinophils, where both 15-LOX-1 and 5-LOX are present.¹⁶
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26 Previously, we have shown that human epithelial 15-LOX-2 does not react with 5,15-diHpETE and
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28 thus it is not implicated in producing LXs through this intermediate.⁹ In the present study, we expand this
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30 investigation into the biosynthetic pathway of LXs generation from 5,15-diHpETE by comparing the relative
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32 activities of human 5-LOX, human platelet 12-LOX and human reticulocyte 15-LOX-1. These results will
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34 help establish a better understanding of the relative roles of these three LOX isozyme in the biosynthesis of
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36 LXs from 5,15-diHpETE.
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38 39 **Methods**

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41 **Chemicals.** The lipid mass spectrometry standards, 5S,15S-dihydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid
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43 (5,15-diHETE), 5S,6R,15S-trihydroxy-7E,9E,11Z,13E-eicosatetraenoic acid (LipoxinA₄ (LXA₄)), and 5S,
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45 14R,15S-trihydroxy-6E,8Z,10E,12E-eicosatetraenoic acid (LipoxinB₄ (LXB₄)), were purchased from
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47 Cayman Chemical. Arachidonic acid (AA) was purchased from Nu Chek Prep, Inc. and used to synthesize
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49 5S-hydroxy-6E,8Z,11Z,14Z-eicosatetraenoic acid (5-HETE), 13S-hydroperoxy-9Z,11E-octadecadienoic acid
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51 (13-HPODE), 5S,15S-hydroxy-6E,8Z,11Z,13E-eicosatetraenoic acid (5,15-diHETE), 5S-hydro-15S-peroxy-
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53 6E,8Z,11Z,13E-eicosatetraenoic acid (5OH,15-HpETE), and 5S,15S-dihydroperoxy-6E,8Z,11Z,13E-
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55 eicosatetraenoic acid (5,15-diHpETE). Synthesis of 5-HETE, 13HPODE, and 5,15-diHpETE were performed
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2 as previously described with 5-LOX and AA, 15-LOX-1 and LA, 15-HPETE and 5-LOX, respectively.^{9, 17, 18}
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4 The synthesis of 5,15-diHETE was performed as done for 5,15-diHpETE, with the exception that
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6 trimethylphosphite was added in 2:1 molar excess as a reductant prior to the HPLC purification. 5OH,15-
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8 HpETE was synthesized from 5-HETE as follows. 60 μ M of 5-HETE was reacted in 1 L of 100mM
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10 NaBorate, pH 9.2 and 4 mg of lipoxygenase from soybean (Sigma). This reaction was monitored at 254 nm
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12 to completion, quenched with 1% (v/v) glacial acetic acid, extracted with 1 L of DCM, and evaporated to
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14 dryness. The 5OH,15-HpETE was then purified isocratically via high performance liquid chromatography
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16 (HPLC) on a Higgins Haisil Semi-preparative (5 μ m, 250mm x 10mm) C18 column with 45:55 of 99.9%
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18 acetonitrile, 0.1% acetic acid and 99.9% water, 0.1% acetic acid. Purity was assessed via liquid
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20 chromatography-mass spectrometry to be greater than 90%.
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25 **Expression and Purification of 15-LOX-1, 12-LOX, and 5-LOX.** Overexpression and purification of
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27 wild-type human reticulocyte 15-lipoxygenase-1 (15-LOX-1), platelet 12-lipoxygenase (12-LOX), and 5-
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29 lipoxygenase (5-LOX) were performed as previously described.^{17, 19, 20} The purity of 15-LOX-1 and 12-LOX
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31 were assessed by SDS gel to be greater than 85%, and metal content was assessed on a Finnigan inductively-
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33 coupled plasma-mass spectrometer (ICP-MS), via comparison with iron standard solution. Cobalt-EDTA
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35 was used as an internal standard. 5-LOX was isolated as an impure ammonium sulfate precipitant from E.
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37 coli expression due to its dramatic loss of activity upon isolation.
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41 **Product Analysis of LOX isozyme reactions with 5,15-diHpETE, 5-OH,15-HpETE, and 5,15-diHETE.**
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43 15-LOX-1 (120 nM), 12-LOX (250 nM), or 5-LOX (25mg of ammonium sulfate precipitated protein) was
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45 reacted in 12 mL of 25 mM HEPES, pH 7.5, at ambient temperature, with 10 μ M of oxylipin (5,15-
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47 diHpETE, 5OH,15-HpETE, or 5,15-diHETE) for one hour and quenched with 0.5% glacial acetic acid and 5
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49 μ M of 7-hydroxy-docosahexaenoic acid as an extraction standard. 3 μ M of 13-HPODE was added to the
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51 reactions with 5,15-diHETE to activate iron as described in.²¹ Each quenched reactions was extracted with
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53 12 mL of DCM, resuspended in 1 mL methanol and split 2:1, with the 30% portion being reduced with
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55 trimethylphosphite, while the other 60% was left unreduced. The samples were then evaporated under a
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2 stream of N₂ to dryness, reconstituted in 50 μL of methanol, and further diluted with 50 μL of 0.1% formic
3 acid in water prior to UPLC-MS/MS analysis. Control reactions without enzymes were also conducted and
4 used for background subtraction, ensuring oxylipin degradation products were removed from analysis.
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6 Chromatographic separation was performed on a Dionex UltiMate 3000 UHPLC with a C₁₈ column
7 (Phenomenex Kinetex, 1.7 μm, 150mm x 2.1mm). The autosampler was held at 4° C and injection volume
8 was 90 uL. Mobile phase A consisted of water with 0.1% (v/v) formic acid and mobile phase B was
9 acetonitrile with 0.1% formic acid. Flow rate was 0.400 mL/min. The initial condition (30% B) was
10 maintained for 2.3 minutes. Mobile phase B was then ramped to 65% over 28.7 minutes, held at 65% for 1
11 minute, ramped to 100% over 0.1 min, held at 100% for 7 minutes, and finally returned to 30% to equilibrate
12 for 7 minutes. The chromatography system was coupled to a Dionex Ultimate-3000 DAD and Velos Pro
13 linear ion trap (Thermo Scientific) for UV and mass spectrum analysis. Analytes were ionized via heated
14 electrospray ionization with -4.0 kV spray voltage, 60, 10, and 0 arbitrary units for sheath, auxiliary and
15 sweep gas, respectively. The RF amplitude of the S-Lens was 49%, and the probe and capillary temperatures
16 were 50° C and 380° C, respectively. All analyses were performed in negative ionization mode at Normal
17 resolution setting. MS² was performed at 35% normalized collision energy in a targeted manner with a mass
18 list containing the following m/z ratios ± 0.1: 319.2, 335.2, 343.2, 351.2, 367.2, 383.2, and 399.2. Products
19 were identified by matching retention times, UV spectra and fragmentation patterns to known standards. Or
20 in the cases where MS standards were not available, structures were deduced from comparison to known and
21 theoretical fragments.
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46 **Steady State Kinetics of 5-LOX, 12-LOX and 15-LOX-1 with 5,15-diHpETE, 12-LOX with 5,15-**
47 **diHETE.** 15-LOX-1 and 5-LOX reactions were performed, at ambient temperature, in a 1 cm² quartz cuvette
48 containing 2 mL of 25 mM HEPES, pH 7.5 with substrate (AA, 15HpETE, or 5,15-diHpETE). 12-LOX
49 reactions were also performed in a 1 cm² quartz cuvette containing 2 mL of 25 mM HEPES with substrate
50 (AA, 5,15-diHpETE, or 5,15-diHETE), and the pH was 8.0. Additionally, 12-LOX reactions with 5,15-
51 diHETE contained 3 μM 13-HPODE and 5-LOX reactions contained 200 μM ATP. AA concentrations were
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2 varied from 0-29 μM , 15-HpETE concentrations were varied from 0-80 μM , 5,15-diHpETE concentrations
3 were varied from 0-45 μM , and 5,15-diHETE concentrations were varied from 0-130 μM . 5-LOX reactions
4 were compared using 10 μM substrate for AA, 15-HpETE, and 5,15-diHpETE with product formation per
5 time analyzed on UV-Vis as well as LC/MS. Concentration of AA was determined by measuring the amount
6 of 15-HpETE produced from complete reaction with soybean lipoxygenase-1 (sLO-1). Concentrations of
7 5,15-diHpETE and 5,15-diHETE were determined by absorbance at 247 nm. Reactions were initiated by the
8 addition 15-LOX-1 (120 nM) or 12-LOX (250 nM) or 5-LOX (approx. 200 nM of crude AS preparation) and
9 were monitored on a Perkin-Elmer Lambda 45 UV/VIS spectrophotometer. Product formation was
10 determined by the change in absorbance at 234 nm for 15-HpETE/5HpETE ($\epsilon_{234\text{nm}} = 25,000 \text{ M}^{-1} \text{ cm}^{-1}$), 5,15-
11 diHpETE ($\epsilon_{254\text{nm}} = 21,90000 \text{ M}^{-1} \text{ cm}^{-1}$),⁹ and 302 nm for lipoxins ($\epsilon_{234\text{nm}} = 50,000 \text{ M}^{-1} \text{ cm}^{-1}$). KaleidaGraph
12 (Synergy) was used to fit initial rates (at less than 20% turnover), as well as the second order derivatives
13 ($k_{\text{cat}}/K_{\text{M}}$) to the Michaelis-Menten equation for the calculation of kinetic parameters.

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30 **5,15-diHpETE and LXB₄ titration into human platelets.** The University of Michigan Institutional Review
31 Board approved all research involving human volunteers. Washed human platelets (250 μL at 3.0×10^8
32 platelets/mL) were dispensed into glass cuvettes and incubated with 0-10 μM of either LXB₄ or 5,15-
33 diHpETE for 10 minutes at 37°C. Oxylipin-treated platelets were then stimulated with 0.25 $\mu\text{g/mL}$ of
34 collagen (Chrono-log), stirring at 1100 rpm and 37°C, in a Chrono-log Model 700D lumi-aggregometer and
35 platelet aggregation was recorded for six minutes.

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44 In order to determine the biosynthetic products of 5,15-diHpETE ex vivo, platelets were isolated
45 from human whole blood via serial centrifugation and adjusted to 3.0×10^8 platelets/mL in Tyrode's buffer
46 (10 mM HEPES, 12 mM NaHCO_3 , 127 mM NaCl , 5 mM KCl , 0.5 mM NaH_2PO_4 , 1 mM MgCl_2 , and 5 mM
47 glucose), as previously published.²² One ml of platelets was incubated with varying concentrations of 5,15-
48 diHpETE (0-20 μM) or vehicle (DMSO) for 10 minutes at 37° C, and then the platelets were pelleted by
49 centrifugation at 1000 x g for 1 minute. The supernatant was transferred to a fresh tube and snap frozen.
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60 Oxylipins were extracted and analyzed via UPLC-MS/MS as described previously,⁹ with the following

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2 exceptions. The m/z transitions for 5,15-diHETE, LTB_4 - d_4 , RvD_2 - d_5 , and Lipoxin B_4 were $335.2 \rightarrow 115$,
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4 $339.2 \rightarrow 197$, $380.5 \rightarrow 141$, and $351.2 \rightarrow 221$, respectively.
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7 **Results and Discussion**

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9 **Lipoxin synthesis from 5,15-diHpETE, 5-OH,15-HpETE, and 5,15-diHETE.** To study the conversion of
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11 5,15-diHpETE to LX through product identification, 5,15-diHpETE was reacted with human 5-LOX, 12-
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13 LOX, and 15-LOX-1. For the reaction of 5-LOX with 5,15-diHpETE, no activity was detected at 302 nm
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15 (the absorbance of conjugated lipoxins) and LXA_4 and LXB_4 were both undetected via LC-MS. This data
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17 indicates that 5,15-diHpETE is a poor substrate for 5-LOX *in vitro*. In order to determine the relative
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19 turnover of 5,15-diHpETE with 5-LOX, the rate of AA, 15-HpETE and 5,15-diHpETE were compared under
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21 the same reaction conditions. 5-LOX converted AA to 5-HpETE at 5 ng/sec and 15-HpETE to 5,15-
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23 diHpETE at 0.5 ng/sec, but there was no conversion of 5,15-diHpETE to LXA_4 observed (the detection limit
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25 of the experiment is 0.0003 ng/sec). This data indicates that the oxidation reaction of 5-LOX with 5,15-
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27 diHpETE is more than 1700-fold slower than that with 15-HpETE. This was unexpected because 5-LOX
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29 abstracts the C10 hydrogen atom from 5-HpETE to generate leukotriene A_4 , (LTA_4) which is the same
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31 hydrogen atom presented by 5,15-diHpETE. In addition, the hydrogen atom on C10 for 5,15-diHpETE is
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33 more reactive than that of 15-HpETE, due to conjugation,⁹ making this lack of reactivity even more
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35 noteworthy. However, 5-LOX is able to convert 15-HpETE into LXA_4 at a rate of 0.5 ng/sec. This difference
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37 in reactivity of 5,15-diHpETE versus 15-HpETE is most likely due to a difference in the rate of substrate
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39 capture, as defined by Northrop et al.^{23, 24} Previously, we have shown that the k_{cat} for generating LTA_4 is the
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41 same when starting with arachidonic acid (AA) or 5-HpETE. However, the k_{cat}/K_M is 10-fold slower for 5-
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43 HpETE than AA, due to a slow binding step.¹⁷ We propose that 5-LOX's lack of reactivity with 5,15-
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45 diHpETE is due to an even slower binding step for 5,15-diHpETE relative to 5-HpETE, limiting reactivity
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47 completely. We therefore propose that 5-LOX produces LXA_4 from 15-HpETE by generating 5,15-diHpETE
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49 in situ and subsequently generates LXA_4 without releasing the 5,15-diHpETE intermediate, similar to the
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51 mechanism for LTA_4 synthesis.
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In contrast, both 12-LOX and 15-LOX-1 reacted with 5,15-diHpETE, manifesting observable rates at 302 nm, which is consistent with LXs being formed. LC-MS analysis of the reduced products confirmed that both 12-LOX and 15-LOX-1 made LXB₄ (RT = 8.8 min), but not LXA₄ (Figure 2, dashed traces). We also observe LXB₄ isoforms, with the all-trans-LXB₄ (RT = 8.1 min), 14S-LXB₄-epimer (RT = 8.9 min), and the

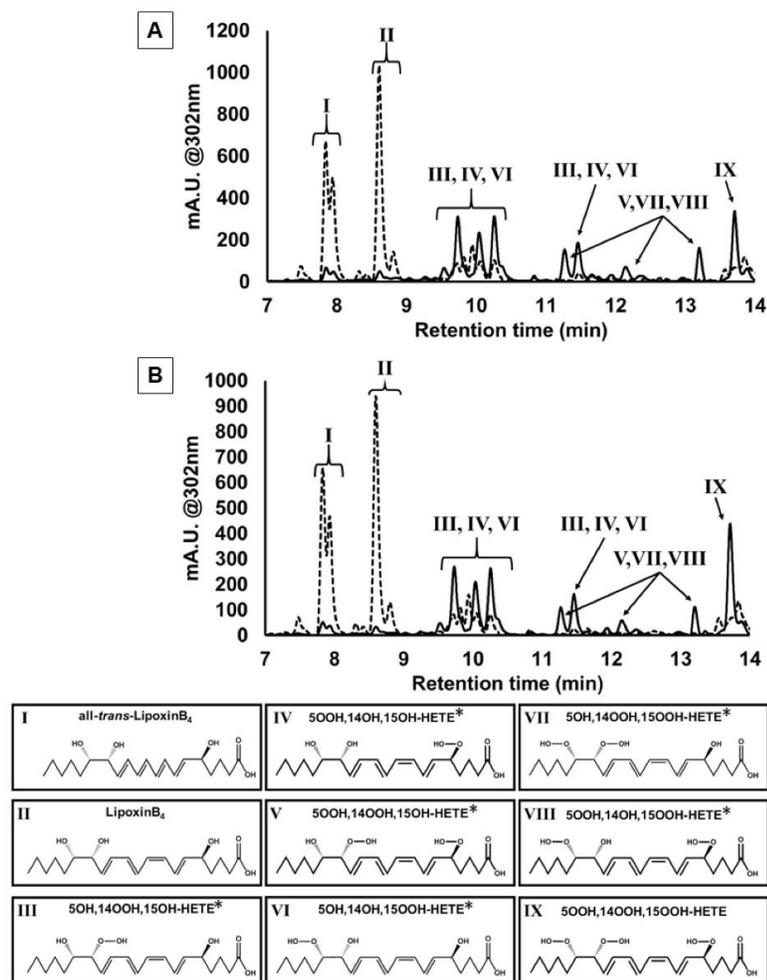


Figure 2: UPLC-UV-Vis chromatogram of h12-LOX (A) and h15-LOX-1 (B) products from 5,15-diHpETE. The wavelength of 302 nm is the absorbance maximum for lipoxins (Supplemental S1). At 302 nm, 11 peaks are observed in the unreduced (solid line) reaction that coalesce to 2 clusters of peaks upon reduction (dashed line). Based on the UV-Vis spectrum, retention times, parent masses, and MS/MS spectra (Supplemental S2), the identities of these peaks have been determined to be all-trans-LxB₄ and its 14S-epimer at 7.9 min, LxB₄ and its 14S-epimer at 8.6 min and 5,14,15-trihydroperoxy-LxB₄ at 13.6 min. The peaks at 9.5, 9.8, 10.0, 10.3 and 11.5 min are the monohydroperoxide products and the peaks at 11.3, 12.2, and 13.2 min are the dihydroperoxide products. *Note: although only one double bond stereochemistry is depicted for these compounds, the differing retention times suggest rearrangements. MS/MS spectra contain fragments that indicate the hydroperoxide moiety's position may differ between these mono- and dihydroperoxy-HETEs.

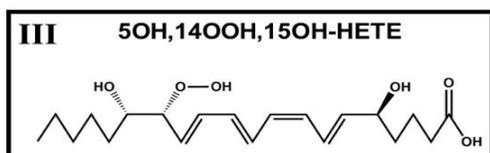
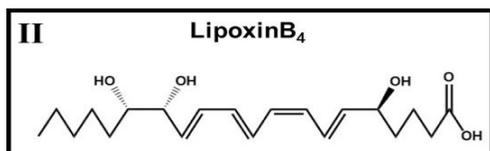
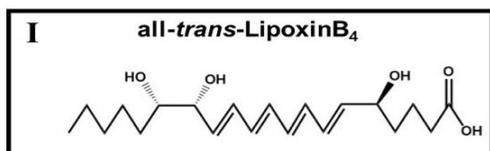
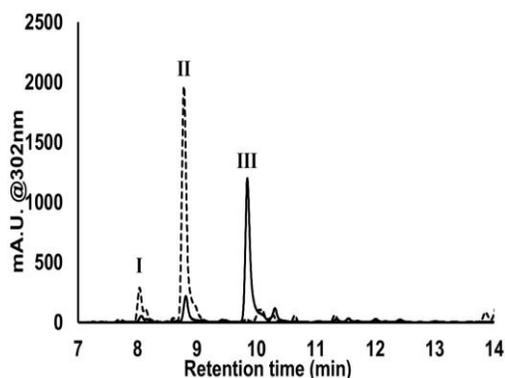
14S-all-trans-LXB₄-epimer (RT = 8.2 min). These products are synthesized by the opening of the epoxide via dehydration, as previously observed with purified rabbit 15-LOX-1 and 15-HpETE as the substrate.¹²

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2 However, some of these products could also be formed by the competing reaction, oxygenation. In order to
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4 differentiate these two reaction mechanisms, the reaction products were analyzed without reductant added
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6 during product extraction (Figure 2, solid traces). Both dehydration (i.e. epoxide formation) and oxygenation
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8 require abstraction at C10, with migration of the radical to C14. Dehydration occurs upon reaction of the
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10 radical with the hydroperoxide in an anti-periplanar orientation, while oxygenation occurs with antarafacial
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12 attack of the radical by molecular oxygen. Both reactions require the active site ferrous-water species to
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14 reduce the radical intermediate and form the final product. Comparing reduced and un-reduced products can
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16 contribute to differentiating the two sets of products, hydroperoxides and alcohols, produced by oxygenation
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18 and dehydration, respectively. Analysis of the data clearly demonstrates that hydroperoxides are present, due
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20 to the fact that many of the peaks migrated to shorter retention times upon reduction. Specifically, the tri-
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22 hydroperoxy species (5,14,15-triHpETE, RT = 13.6 min, Figure 2) is observed, which could only be formed
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24 via oxygenation at C14. It should be noted that while we observe the parent mass of 5,14,15-triHpETE (399
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26 m/z, hydrogen loss in the negative mode), we also observe the neutral loss of water from 5,14,15-triHpETE
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28 (Supplemental S2), resulting in the most intense ion for 5,14,15-triHpETE at 381 m/z. Additionally, because
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30 there are three hydroperoxide moieties, multiple peaks that correspond to the loss of up to three waters upon
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32 ionization (Supplemental S1 & S2) are observed. The additional hydroperoxide peaks correspond to both
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34 oxygenation products, which have lost an oxygen, most likely due to reduction during the long reaction time
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36 in buffer. These products include: 5-OOH,14-OOH,15-OOH-triHpETE (RT = 13.7 min), 5-OOH,14-OH,15-
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38 OOH-triHETE, 5-OH,14-OOH,15-OOH-triHETE, 5-OH,14-OH,15-OOH-triHETE, 5-OOH,14-OOH,15-
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40 OH-triHETE, and 5-OH,14-OOH,15-OH-triHETE. These products exhibit multiple peaks, which most likely
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42 correspond not only to the hydroperoxide position in these molecules, but also to the all-trans, S-epimers,
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44 and other isomers resulting from the non-enzymatic epoxide opening at C14 and C15. The non-enzymatic
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46 epoxide opening is also supported by comparing the ratio of the sum of all-trans-LXB₄, 14S-LXB₄ and 14S-
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48 all-trans-LXB₄ to LXB₄ in the reduced samples of 5,15-diHpETE (LXB₄ isomers and epimers/LXB₄ = 0.91 ±
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50 0.2) and 5,15-diHETE (LXB₄ isomers and epimers = 0.19 ± 0.02) as substrates with 12-LOX. The
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2 dehydration and oxygenation peaks have been quantified, demonstrating no preference for dehydration over
3 oxygenation, with 12-LOX having a dehydration/oxygenation ratio of 1.3 ± 0.2 and 15-LOX-1 having a ratio
4 of 1.2 ± 0.2 (Figure 2, supplemental S2). Since only LxB₄ is produced by 12-LOX and 15-LOX-1 from
5 5,15-diHpETE, with it being generated either through oxygenation or dehydration (i.e. via the epoxide), the
6 data indicates similar LX reactivities for both LOX isozymes. It should be noted that due to the reactive
7 nature of these di- and tri-hydroperoxides, the data was scanned for Hock rearrangement decomposition,
8 however, no evidence was observed for these products.²⁵

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10 To eliminate the possibility of epoxide generation, 12-LOX and 15-LOX-1 were reacted with 5,15-
11 diHETE, the reduced form of 5,15-diHpETE, which is incapable of forming epoxides. 12-LOX reacted with
12 5,15-diHETE producing LxB₄ and its isoforms, but 13-HPODE was required. 13-HPODE is a known
13 oxidant of lipoxygenase that reduces lag times and expands the substrate range of lipoxygenases by
14 activating the LOX isozyme when the endogenous hydroperoxide product cannot oxidize the intermediate
15 ferrous ion.^{18, 26} For this particular reaction, the conversion of 5,15-diHETE to LxB₄ implies that 12-LOX
16 oxygenates at C14 because the 14,15-epoxide cannot be formed from the alcohol. The requirement of 13-
17 HpODE is because the ferrous-water intermediate cannot efficiently reduce the hydroperoxide radical at
18 C14, thus releasing the radical intermediate and generating the inactive ferrous-water species. The ferrous-
19 water intermediate is subsequently oxidized by 13-HpODE, generating the active form of 12-LOX. This
20 conclusion is supported by the comparison of the reduced and un-reduced products (Figure 3). The un-
21 reduced reaction manifests a large peak (RT = 9.8 min), which elutes after LxB₄ (RT = 8.8 min). This peak
22 has an MS/MS fragmentation pattern of a C14 hydroperoxy moiety (supplemental S3), which becomes LxB₄
23 upon reduction, as seen by its change in retention time and its MS/MS fragmentation (supplemental S3) and
24 confirms an oxygenation reaction on C14. It should be noted that we observe a large reduction in the number
25 of isoforms of LxB₄, as compared to the reaction with 5,15-diHpETE as the substrate. This is appropriate
26 given that this is a region- and stereo-specific oxygenation reaction and no epoxide was formed that could
27 undergo non-enzymatic hydrolysis to generate the LxB₄ isoforms.

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3 Interestingly, 15-LOX-1 cannot perform an oxygenation reaction with 5,15-diHETE, even with 13-
4 HPODE present, indicating that 15-LOX-1 cannot oxygenate at C14 with 5,15-diHETE as the substrate. This
5 is unexpected since 15-LOX-1 can oxygenate 5,15-diHpETE. Therefore, the hydroxides at C5 and C15
6 appear to change substrate binding so that either the hydrogen atom abstraction at C10 is prohibited or the
7 oxidation of the active site ferrous-water intermediate to the active ferric-hydroxide moiety by the
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Figure 3: UPLC-UV-Vis chromatogram of h12-LOX products from 5,15-diHETE. The wavelength of 302 nm is the absorbance maximum for lipoxins (Supplemental S1). At this wavelength, three peaks, at times 8.1, 8.8, and 9.8 min, are observed in the unreduced reaction (solid line) that coalesce to two peaks, 8.1 and 8.8 min, upon reduction (dashed line). Based on the UV-Vis spectrum, retention times, parent mass, and MS/MS spectra (Supplemental S2), the identities of these peaks have been determined to be all-*trans*-LxB₄ at 8.1, LxB₄ at 8.8 and 14-hydroperoxy-LxB₄ at 9.8 min.

hydroperoxyl radical is prohibited. This hypothesis is supported by the fact that hydroperoxyl moieties in these eicosanoids behave more hydrophobically than hydroxyl moieties, as evident by their longer retention on a C18 column (Supplemental S1). In addition, 15-LOX-1 will not oxygenate 15-HETE either, but it will oxygenate 15-HpETE, indicating a difference in reactivity with the reduced oxylipin.

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3 It should be reiterated that 15-LOX-1 and 12-LOX do not make LXA₄ from 5,15-diHpETE, however,
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5 they do make LXB₄. This is an interesting observation considering that the radical intermediate of 5,15-
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7 diHpETE can potentially form either the 5,6- or 14,15-epoxide. However, the active sites of both 12-LOX
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9 and 15-LOX-1 preferentially generate only the 14,15-epoxide. This restricted reactivity could be related to
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11 the fact that the oxygen tunnel for both 15-LOX-1 and 12-LOX is located near C14, as observed by the C14
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13 oxygenation products.^{27, 28} Therefore, the opening to the oxygen tunnel in the active site could provide a dual
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15 function. Primarily to oxygenate 5,15-diHpETE but also to provide the space for the 14,15-epoxide to form,
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17 thus giving the oxygen tunnel an added functionality.
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Steady-state kinetics of 15-LOX-1 and 12-LOX with 5,15-diHpETE and 12-LOX with 5,15-diHETE. Considering that hydrogen atom abstraction is typically rate-limiting in lipoxygenase reactions,²⁹⁻³¹ the steady-state kinetics of 15-LOX-1 and 12-LOX with 5,15-diHpETE revealed an interesting difference between their relative rates. When reacted with AA, 12-LOX abstracts the hydrogen atom at C10, generating more than 95% 12-HpETE.¹⁹ On the other hand, 15-LOX-1 abstracts the hydrogen atom at C10 only $\approx 10\%$ of the time, with C13 being the major target.³²⁻³⁴ We confirmed this result by observing approximately 10% of 12-HpETE and 90% of 15-HpETE being generated from AA reacted with 15-LOX-1. Therefore, 12-LOX has a 78-fold larger k_{cat}/K_M than 15-LOX-1 for abstracting the hydrogen atom from C10 of AA and generating 12-HpETE, $18 \mu\text{M}^{-1}\text{s}^{-1}$ and $0.23 \mu\text{M}^{-1}\text{s}^{-1}$, respectively (Table 1). It should be noted that these 15-LOX-1 kinetic values for C10 abstraction are estimated from the total rate observed for 15-LOX-1 and its product percentages. This indicates that 12-LOX is more efficient in both product release (k_{cat}) and substrate capture (k_{cat}/K_M) than 15-LOX-1 in abstracting a hydrogen atom from C10 on AA (13-fold for k_{cat} and 78-

Table 1: Steady State Parameters for 15-LOX-1 and 12-LOX reaction with 5,15-diHpETE and 5,15-diHETE

Enzyme	Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\mu\text{M}^{-1}\text{s}^{-1}$)
12-LOX	AA (C10 abstraction)	18 ± 0.8	1.0 ± 0.1	18 ± 2
15-LOX-1	AA (C10 abstraction)	$1.4 \pm 0.1^*$	6 ± 1	$0.23 \pm 0.03^*$
12-LOX	5,15-diHpETE	0.17 ± 0.007	14 ± 1	0.011 ± 0.007
15-LOX-1	5,15-diHpETE	4.6 ± 0.1	23 ± 2	0.21 ± 0.01
12-LOX	5,15-diHETE**	0.18 ± 0.02	113 ± 19	0.0015 ± 0.0001
15-LOX-1	5,15-diHETE**	nr***	nr	nr

* k_{cat} and k_{cat}/K_m values were confirmed daily relative to the k_{cat} and k_{cat}/K_m values of AA. The C10 abstraction kinetic values of 15-LOX-1 are 10% of the AA kinetic values, which include both the 15-HpETE and 12-HpETE kinetics (k_{cat} and k_{cat}/K_m for AA are $14 \pm 1 \text{ s}^{-1}$ and $2.3 \pm 0.3 \mu\text{M}^{-1} \text{ s}^{-1}$, respectively)

** $3 \mu\text{M}$ of 13-HPODE is added to activate 12-LOX.

*** nr = no reaction

fold for k_{cat}/K_M). However, despite the preference of 12-LOX for abstracting at C10 on AA, 15-LOX-1 has both a better product release rate (k_{cat}) and substrate capture rate (k_{cat}/K_M) than 12-LOX, for abstracting the

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2 C10 hydrogen atom from 5,15-diHpETE (Table 1). For k_{cat} , the 12-LOX rate for C10 abstraction from 5,15-
3 diHpETE is 106-fold less than that of AA ($k_{\text{cat}} = 0.17 \text{ s}^{-1}$), while the 15-LOX-1 rate is 3.3-fold greater than
4 that of C10 abstraction from AA ($k_{\text{cat}} = 4.6 \text{ s}^{-1}$). For $k_{\text{cat}}/K_{\text{M}}$, the 12-LOX rate for C10 abstraction from 5,15-
5 diHpETE is 1600-fold less than that of AA ($k_{\text{cat}}/K_{\text{M}} = 0.011 \mu\text{M}^{-1}\text{s}^{-1}$), while the $k_{\text{cat}}/K_{\text{M}}$ for 15-LOX-1 is the
6 same as that of C10 abstraction from AA ($k_{\text{cat}}/K_{\text{M}} = 0.21 \mu\text{M}^{-1}\text{s}^{-1}$). Effectively, there is no loss of activity for
7 15-LOX-1 abstracting the hydrogen atom from C10 of 5,15-diHpETE relative to AA, but 12-LOX has a
8 large loss of activity with 5,15-diHpETE relative to AA, as seen by a significant decrease in k_{cat} and $k_{\text{cat}}/K_{\text{M}}$
9 (Table 1). This data indicates that the hydroperoxy moieties on C5 and C15 of 5,15-diHpETE, disrupt its
10 positioning in the 12-LOX active site for C10 abstraction significantly, while not affecting 15-LOX-1
11 appreciably. It should be noted that for both 12-LOX and 15-LOX-1, only 10% of the total 5,15-diHpETE in
12 the reaction is converted to LXB₄ and addition of 12-LOX, 15-LOX-1 or 5,15-diHpETE at 10% conversion
13 did not improve the turnover percentage. This implies that there is some form of suicide product inhibition,
14 which is a common observation in LOX reactions, due to the reactive nature of the radical intermediates, and
15 has previously been observed with 12-LOX and LTA₄.^{35, 36}
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As noted above, LX formation from the reduced intermediate, 5,15-diHETE, is only observed with 12-LOX in the presence of 13-HPODE, and no rate was observed for 15-LOX-1. However, the rate of 12-LOX with 5,15-diHETE is extremely slow, manifesting a k_{cat}/K_M that is 10-fold less than that seen for 5,15-diHpETE ($k_{\text{cat}}/K_M = 0.0015 \mu\text{M}^{-1}\text{s}^{-1}$). Since the k_{cat} of 12-LOX with 5,15-diHETE is essentially the same as

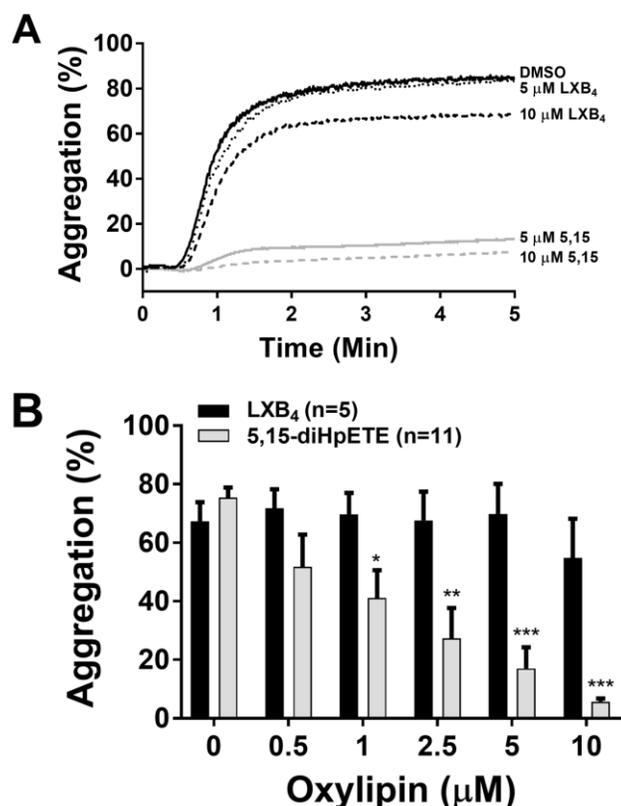


Figure 4: 5,15-diHpETE, but not LXB₄, inhibits collagen-mediated platelet aggregation. Isolated human platelets were treated with increasing concentrations of either LXB₄ or 5,15-diHpETE for ten minutes and then stimulated with collagen (0.25 $\mu\text{g}/\text{mL}$). **A**) Representative tracings of 5,15-diHpETE or LXB₄ treated platelets stimulated with collagen. **B**) Data represents mean \pm S.E.M. Statistical analysis was performed comparing oxylin concentration to vehicle control using one-way ANOVA with Dunnett's multiple comparison test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

its k_{cat} for 5,15-diHpETE, this lower rate of substrate capture (k_{cat}/K_M) is manifested in a 10-fold increase in K_M , ($113 \pm 19 \mu\text{M}$). As discussed above, 12-LOX only oxygenates 5,15-diHETE but it oxygenates and dehydrates 5,15-diHpETE. Since the k_{cat} for both 5,15-diHETE and 5,15-diHpETE are similar, it indicates that the rate of product release for both oxygenation and dehydration are also similar. This implies that the reaction steps after the irreversible hydrogen abstraction step, such as the reduction of the radical intermediate for oxygenation or dehydration by the ferrous-water moiety, are either not rate-limiting or comparable in rate for the two reactions. It is also observed that the rate of substrate capture (k_{cat}/K_M)²³ for

12-LOX is decreased by a factor of 10 for 5,15-HETE relative to 5,15-HpETE, indicating that there is a rate difference in a reaction step prior to the irreversible hydrogen atom abstraction step, such as substrate binding. As mentioned above, this could be due to its smaller size or the more polar hydroxyl moiety relative to the hydroperoxide, as seen by its RP-HPLC retention time.

5,15-diHpETE and LXB₄ titration into human platelets. To determine whether or not 5,15-diHpETE inhibits platelet activation, platelets from 11 separate donors were treated with varying concentrations of 5,15-diHpETE (0.5-20 μ M) or vehicle control (DMSO) prior to stimulation with collagen, a GPVI and $\alpha_2\beta_1$ agonist. Compared to vehicle control, 5,15-diHpETE inhibited platelet aggregation in response to collagen (0.25 μ g/mL) stimulation with an IC₅₀ of 1.3 μ M (Figure 4). Furthermore, over 90% reduction in collagen-

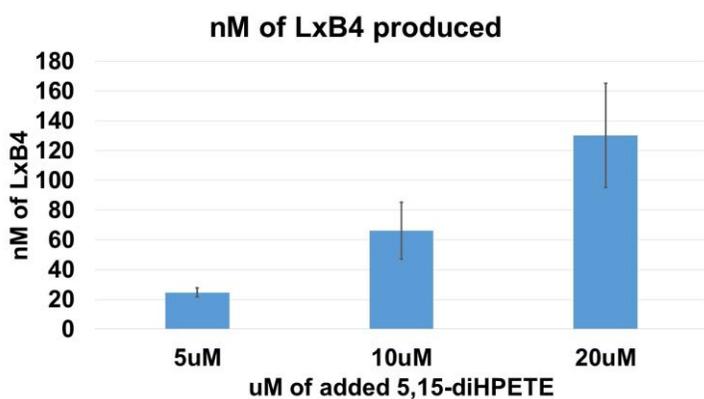


Figure 5: Platelets produce LxB₄ from 5,15-diHPETE. The amount of LxB₄ produced by platelets treated with 5, 10 or 20 μ M of 5,15-diHPETE are plotted in this histogram. The standard deviation of each value is represented by error bars.

mediated platelet aggregation was observed in platelets treated with a concentration greater than 10 μ M of 5,15-diHpETE. The antiplatelet effects of LXB₄ were also investigated; however, in contrast to 5,15-diHpETE, LXB₄ did not significantly inhibit collagen-mediated platelet activation at the concentrations tested.

With respect to the biosynthetic pathway, human platelets were treated with 5,15-diHpETE and shown to produce LXB₄ (Figure 5), but they did not produce LXA₄. This is consistent with the in vitro results, where purified 12-LOX only makes LXB₄. However, the amount of LXB₄ that we observe from 5,15-diHpETE treated platelets is very small compared to the unreacted 5,15-diHpETE (less than 1.2 %), indicating that this is not an efficient pathway for the generation of LXB₄. In addition, since LXB₄ does not

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2 have a strong antiplatelet effect, it is reasonable to conclude that 5,15-diHpETE, and not its downstream
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4 product, LXA₄, is the active oxylipin species that affects platelet biology in our experiments.
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3 In summary, the reaction of 5,15-diHpETE with various human lipoxygenase isozymes was
4 investigated to determine the relative rates of producing LXs. Previously, it was established that 15-LOX-2
5 did not react with 5,15-diHpETE, consistent with its enhanced substrate selectivity relative to the other LOX
6 isozymes.⁹ 15-LOX-2 abstracts a hydrogen atom from C13 of AA, but does not abstract from C10, the only
7 available methylene carbon of 5,15-diHpETE. In the current work, it was determined that 5-LOX does not
8 react with 5,15-diHpETE either. However, 5-LOX can produce LXA₄ if 15-HpETE is the substrate (Figure
9 6), indicating that substrate binding is impaired when the hydroperoxide is on both C5 and C15, consistent
10 with our previous work with 5-LOX where oxygenation of the oxylipin affects substrate binding
11 significantly.¹⁷ In contrast, both 12-LOX and 15-LOX-1 react with 5,15-diHpETE (Figure 6), albeit at slow
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2 rates relative to AA. Surprisingly, 15-LOX-1 has a 27-fold greater k_{cat} and a 20-fold greater k_{cat}/K_M than 12-
3 LOX. This result is unusual because C10 is the preferred location for hydrogen abstraction for 12-LOX, so it
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5 appears that the active site of 12-LOX is less tolerant of oxygenated fatty acids than 15-LOX-1, which
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7 lowers its catalytic rates, as already seen for 5-LOX.¹⁷ The low *in vitro* catalytic rate for 12-LOX with 5,15-
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9 diHpETE is corroborated by the fact that when 5,15-diHpETE is added to platelets, LXB₄ production is
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11 extremely low (1.2 ± 0.2 % total conversion), consistent with the poor *in vitro* LXB₄ production rate. This
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13 result is in contrast to the conventional wisdom that assumes 12-LOX is the biosynthetic enzyme for LXs
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15 production from 5,15-diHpETE, due to its preference for abstracting a hydrogen atom from C10.⁸
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21 Nevertheless, LXs are highly active molecules so low production levels in platelets could still have large
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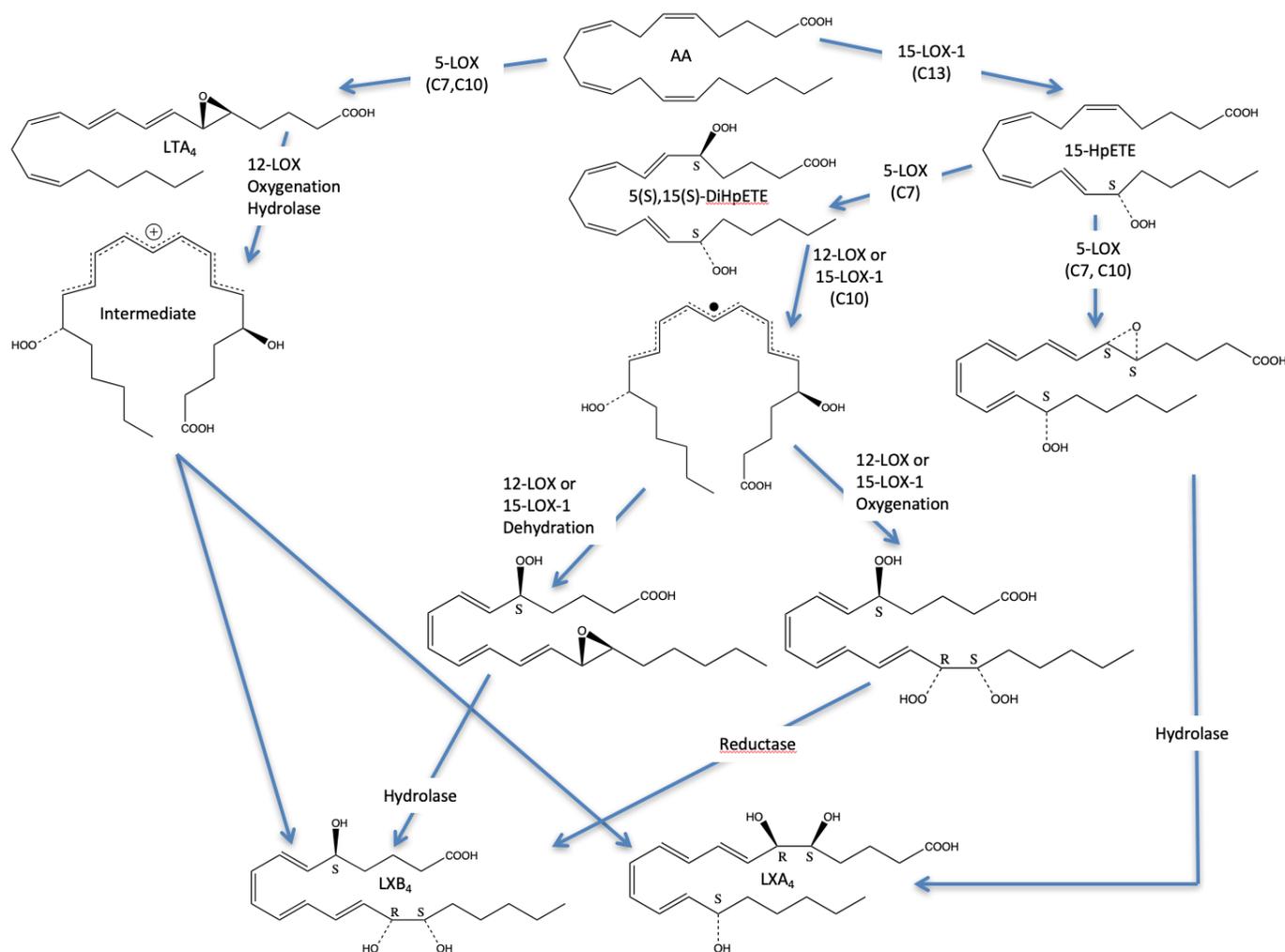


Figure 6: Reaction scheme for LXA₄ and LXB₄ production. The production of LXA₄ and LXB₄ is dependent on both the order of LOX reactivity and the nature of the substrate. The carbon atom from which the hydrogen atom is abstracted is indicated below the LOX isozyme label, such as C7, C10 or C13. The central pathway which involves 5(S),15(S)-diHpETE is the subject of this publication.

effects on the cellular response, resulting in 12-LOX still being a potentially biosynthetically relevant LOX isozyme for making LXs. For comparison, Serhan and coworkers determined that human 12-LOX reacted with LTA₄, producing both LXA₄ and LXB₄, with a V_{\max} of 24.5 nmol/min/mg (or a k_{cat} of 0.031 sec⁻¹) and a V_{\max}/K_m of 3.1 nmol/min/mg/ μM (or a k_{cat}/K_M of 0.004 sec⁻¹ μM^{-1}).³⁵ These values are markedly slower than the current observed rates of 15-LOX-1 with 5,15-diHpETE (150-fold slower for k_{cat} and 50-fold slower for k_{cat}/K_M), suggesting that 15-LOX-1 may be a more predominant source of LXs than 12-LOX, depending on the cells involved. Another interesting difference between 5,15-diHpETE and LTA₄ as LOX substrates is that with LTA₄, both LXA₄ and LXB₄ are made, but with 5,15-diHpETE as the substrate, only LXB₄ is made.

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2 Clearly, the mechanisms of LX production are distinct, which could help in the future identification of the
3 source of LX biosynthesis. For example, if 5,15-diHpETE is the substrate, LXB₄ is the primary product of
4 12-LOX and 15-LOX-1 catalysis, whereas LXA₄ is the primary product with 5-LOX. However, if LTA₄ is
5 the substrate, then 12-LOX generates both LXA₄ and LXB₄. Therefore, it appears that cells have multiple
6 pathways for making LXs, depending on the available LOX isozyme and the available substrate, 5,15-
7 diHpETE or LTA₄. We are currently investigating the molecular mechanism of these reactions with active
8 site mutants in order to gain better insights into the differences between these distinct LX biosynthetic
9 pathways.
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23 **Supplemental information.** The supplemental figures that are referenced in this article (S1, S2, S3 and S4)
24 along with their corresponding legends are available online. These materials are supplied free of charge at
25 <http://pubs.acs.org>.
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