Lipidomics Reveals Dramatic Physiological Kinetic Isotope Effects during the Enzymatic Oxygenation of Polyunsaturated Fatty Acids Ex Vivo

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Supporting Information

ABSTRACT: Arachidonic acid (AA, 20:4) is an omega-6 polyunsaturated fatty acid (PUFA) and the main precursor to the class of lipid mediators known as eicosanoids. The enzymes that catalyze the oxygenation of AA begin by abstracting hydrogen from one of three bis-allylic carbons within 1,4-cis,cis-diene units. Substitution of deuterium for hydrogen has been shown to lead to massive kinetic isotope effects (KIE) for soybean lipoxygenase (sLOX) oxygenation of linoleic acid (LA, 18:2). Yet, experimental determination of the KIE during oxygenation of AA and LA by mammalian enzymes including cyclooxygenase (COX) and lipoxygenase (LOX) has revealed far lower values. All prior studies investigating the KIE of PUFA oxygenation have relied on in vitro systems using purified enzymes and were limited by availability of deuterated substrates. Here we demonstrate the use of macrophages as an ex vivo model system to study the physiological KIE (PKIE) during enzymatic AA oxygenation by living cells using a newly synthesized library of deuterated AA isotopologues. By extending lipidomic UPLC-MS/MS approaches to simultaneously quantify native and deuterated lipid products, we were able to demonstrate that the magnitude of the PKIE measured in macrophages for COX and LOX oxygenation of AA is similar to KIEs determined in previous reports using the AA isotopologue deuterated at carbon 13 (C13). However, for the first time we show that increasing the number of deuterated bis-allylic carbons to include both C10 and C13 leads to a massive increase in the PKIE for COX oxygenation of AA. We provide evidence that hydrogen(s) present at C10 of AA play a critical role in the catalysis of prostaglandin and thromboxane synthesis. Furthermore, we discovered that deuteration of C10 promotes the formation of the resolving lipid mediator lipoxin B₄, likely by interfering with AA cyclization and shunting AA to the LOX pathway under physiological conditions.

INTRODUCTION

Polyunsaturated fatty acids (PUFAs) are essential metabolites of cells, playing critical roles in cellular metabolism and membrane structure and serving as precursors for lipid signaling molecules. One of the primary routes of PUFA metabolism is through their oxygenation by enzymes of the cyclooxygenase (COX) and lipoxygenase (LOX) pathways. Both COX and LOX enzymes initiate oxygenation by stereospecifically abstracting a hydrogen atom from a bis-allylic carbon, creating a delocalized radical which then reacts with molecular oxygen. Substrate probes containing deuterium at reactive bis-allylic carbons have been utilized to study the mechanisms by which these enzymes catalyze the addition of oxygen to PUFAs. The LOX enzymes have received much attention due to the extremely high kinetic isotope effects (KIE) observed during the oxygenation of linoleic acid (LA), with values of 50–100 being reported.¹ These investigations have focused mainly on the hydroxylation of LA by soybean LOX (sLOX), but large KIEs for hydroxylation of LA by human 15-lipoxygenase (15-hLOX) have also been reported.² However, in mammalian systems, the major PUFA that undergoes enzymatic oxygenation is arachidonic acid. It has been reported that the KIE for sLOX oxygenation of AA is still quite large at ∼50.³ In contrast to the very large KIEs measured for sLOX and 15-hLOX oxygenation of LA, studies have found that the KIE for oxygenation of AA by 15-hLOX and COX-2 show far lower values, on the order of 10 and 2–5, respectively.⁴,⁵

The enzymatic oxygenation of AA begins with its release from phospholipid membranes via hydrolytic cleavage by cytosolic phospholipase A2 (PLA₂).⁶,⁷ For COX and LOX enzymes, the addition of molecular oxygen to AA requires abstraction of a bis-allylic hydrogen atom, creating a delocalized radical. The position of hydrogen abstraction varies depending on the identity of the oxygenase enzyme. Formation of...
prostaglandin G2 (PGG2) and H2 (PGH2) by COX-2, the precursors of all prostaglandins and thromboxanes, is initiated by abstraction of the 13-pro(S) hydrogen, followed by sequential oxygenation and cyclization of AA. 15-hLOX hydroxylation of AA is also initiated by abstraction of hydrogen from C13, while 5-hLOX and 12-hLOX begin by abstraction of hydrogen from C7 and C10, respectively. A schematic showing the enzymes and lipid products of both the COX and LOX pathways using (D6)-AA as a substrate is shown in Scheme 1A, B.

The investigation of deuterium KIEs during auto- and enzymatic-oxidation of AA have lagged behind those with LA due to a lack of available deuterated substrates. While LA only has one bis-allylic carbon AA has three, making the synthesis of a full library of variably deuterated AA isotopologues more challenging. The synthesis and analytical characterization of a full library of (bis-allyl)-deuterated AA (Table 1, compounds 1−7) was recently published and generously provided to us for this study.8 Using this library of variably deuterated AA, we were able to perform experiments to address the contribution of each individual, or combination of, bis-allylic site(s) involved in AA oxygenation.

Simply stated, the KIE is the change in the rate of a chemical reaction when one atom of the reactants has been substituted with one of its isotopes. KIEs regarding enzymatic reactions are typically shown as a ratio of the reaction rates conducted with native (light, L) versus isotope labeled substrate (heavy, H) \( \frac{\text{L}_k}{\text{H}_k} \). All studies to date investigating the deuterium KIE on the enzymatic oxygenation of AA have been done in cell-free systems containing purified enzymes and substrates. This approach has yielded accurate kinetic rate constants required for the determination of KIEs. However, many of these studies have relied on indirect measures of enzymatic activity such as oxygen consumption. This can be a reasonable proxy for enzymatic activity and product formation during simple reactions consisting of a single addition of molecular oxygen such as those of LOX enzymes. However, this approach has the potential to be misleading when more complex reactions are taking place, like those for cyclooxygenase enzymes, which feature two oxygen additions and cyclopentane ring formation. Therefore, we sought to develop a method to measure the physiological KIE on the oxygenation of AA within a cell-based system by using mass spectrometry to precisely identify final products.9

Macrophages are a critical component of the mammalian immune system and some version of a macrophage is present in nearly every organ system in humans. Macrophages also possess the enzymatic systems for agonist induced eicosanoid biosynthesis, making them an ideal model to study AA metabolism.10 In addition, our group has developed an ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) platform to simultaneously quantify up to 180 eicosanoids in biological samples.11 Using this platform, we quantified both native and deuterated eicosanoids produced by macrophages that were labeled with AA isotopologues (Table 1). We were able to demonstrate herein the ex vivo deuterium KIEs upon the enzymatic oxygenation of AA by the COX and LOX systems in living cells. The values we have obtained for the physiological deuterium isotope effects for individual carbons were comparable to what has been observed by others in vitro. However, we discovered unexpectedly large isotope effects toward oxygenation of AA by COX when both C10 and C13 were deuterated. These data suggest that hydrogen present at C10 may be important in the oxygenation and cyclization of AA by COX enzymes and that deuteration of C10 promotes the formation of Lipoxin B4.

Deuteration of essential PUFAs at bis-allylic positions was proposed as a way of controlling metabolic pathways.12

**Table 1. Arachidonic Acid Isotopologues Used in This Study**

| compound | MRM [M−H]+ |
|----------|-------------|
| 1. arachidonic acid | 303/259 |
| 2. 7,7-(D2) | 305/261 |
| 3. 10,10-(D2) | 305/261 |
| 4. 13,13-(D2) | 305/261 |
| 5. 7,7,13,13-(D4) | 307/263 |
| 6. 7,7,13,13-(D4) | 307/263 |
| 7. 10,10,13,13-(D4) | 307/263 |
| 8. 7,7,10,10,13,13-(D6) | 309/265 |
| 9. 5,6,8,9,11,12,14,15-(D8) | 311/267 |

*Position and number of deuterons listed with the masses of the parent and fragment ion used for MRM detection.*

The molecules depicted are those produced from 7,7,10,10,13,13-(D6)-arachidonic acid.

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**Scheme 1. Enzymatic Eicosanoid Production via the (A) Cyclooxygenase and (B) Lipoxygenase Pathways**

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method was used to downregulate nonenzymatic lipid peroxidation (LPO) in various disease models. The findings reported herein lend further support to the D-PUFA approach by demonstrating favorable enzymatic shifting from pro- to anti-inflammatory lipid mediators, providing additional justification to clinical trials of D-PUFAs in several human diseases.

RESULTS

D<sub>2</sub>-Arachidonic Acid Displays Normal Esterification and Release Dynamics. Previous work has suggested that simply adding mass to AA (through addition of deuterium at exchangeable sites) does not alter normal uptake and release dynamics in macrophages. However, unlike the preceding report, the AA compounds used in this study are deuterated at bis-allylic carbons. No studies to date have addressed the fate of AA species containing deuterium at bis-allylic carbons in living cells. Therefore, we first needed to establish that bis-allylic deuteration of AA does not alter its uptake, esterification and release from phospholipids compared to native arachidonic acid. RAW264.7 macrophages were labeled with 25 μM native or 25 μM of one of the 7 AA isotopologues for 24 h, and then levels of AA and D<sub>2</sub>-AA esterified in complex lipids were measured by UPLC-MS/MS. See Table 1 for a list of the MRM pairs used to quantify AA and D<sub>2</sub>-AA species. Macrophages incubated with D<sub>2</sub>-AA isotopologues displayed an accumulation of esterified D<sub>2</sub>-AA, comprising approximately ~60–80% of all esterified AA under these labeling conditions (Figure 1A, D).

In order for AA to be converted into eicosanoids by COX and LOX enzymes, it must first be released via enzymatic hydrolysis by PLA<sub>2</sub>. It is well documented that toll-like receptor 4 (TLR4) agonists result in the activation of cPLA<sub>2</sub>, release of AA and production of eicosanoids. RAW264.7 macrophages were labeled with 25 μM native or D<sub>2</sub>-AA for 24 h followed by stimulation with the TLR4-specific agonist Kdo2-Lipid A (KLA). Cells were collected prior to, or 24 h after stimulation with KLA and esterified AA species were quantified by UPLC-MS/MS. Stimulation of macrophages with KLA lead to the release of esterified AA and D<sub>2</sub>-AA at similar levels (Figure 1B, E). The amount of released AA, or Δesterified AA, following KLA treatment is shown in Figures 2C and F.

![Figure 1](image-url)

**Figure 1.** Arachidonic acid isotopologues are esterified and released from macrophage complex lipids. RAW264.7 macrophages were left untreated (control), or incubated for 24 h with native arachidonic acid (AA, 25 μM) or (D<sub>2</sub>)-arachidonic acid isotopologues (25 μM). Esterified native (□) and deuterated (■) AA was quantified by UPLC-MS/MS before (A, D) or after (B, E) 24 h KLA (100 ng/mL) treatment. The amount of released arachidonic acid (Δesterified AA) over this time frame is shown in panels C and F. Graphs represent the mean ± SEM of 2–3 independent experiments.

![Figure 2](image-url)

**Figure 2.** Time course of prostaglandin production by KLA stimulated macrophages. RAW264.7 macrophages were labeled for 24 h with vehicle (blue), AA (red, 25 μM) or (D<sub>2</sub>)-AA (green, 25 μM) before stimulation with KLA (100 ng/mL). Supernatants were collected at 6, 12, or 24 h for analysis. Native (□) and deuterated (■) eicosanoids were then quantified by UPLC-MS/MS. Graphs show the mean ± SEM of a single experiment containing technical triplicates that is representative of three independent experiments.

Furthermore, by labeling macrophages with 5,6,8,9,11,12,14,15-(D<sub>8</sub>)-AA, we demonstrated that deuteration itself does not alter KLA-elicited eicosanoid production (data not shown). These data suggest that D<sub>2</sub>-AA isotopologues are taken up by macrophages, esterified into phospholipids and released by cPLA<sub>2</sub> to similar levels as native AA. Therefore, this is a tractable model for the investigation of the deuterium KIE during the enzymatic oxygenation of arachidonic acid by mammalian cells.

Tandem Mass Spectral Analysis of Eicosanoids Derived from Isotopologues of Arachidonic Acid. To quantify the amount of eicosanoids derived from each of the AA isotopologues listed in Table 1, we started with the optimized MRM pairs for these molecules and adjusted the mass of the parent and fragment ions according to the expected position of deuterium. Fragmentation patterns for native eicosanoids obtained from the LIPID MAPS database (lipidmaps.org) were used as a guide to determine the mass of the fragment ions. An example of this calculation for PGD<sub>2</sub> is shown in Figure S1 of the Supporting Information (SI).

A complete list of MRMs and acquisition parameters for each metabolite measured in this study can be found in Table S1. One challenge encountered during the quantification of
eicosanoids derived from (D₆)-AA isotopologues, whose product is one mass unit more than the native molecule, is the coincident ¹³C isotope effect. Molecules with mass M detected by the mass spectrometer will display a peak at M+1 with an intensity equal to 0.0109^n, where n is the total number of carbons.¹⁷ Thus, a fraction of the signal identified as an eicosanoid containing one deuterium actually comes from the native eicosanoid pool containing one ¹³C. The eicosanoids affected are noted in Table S1 with an asterisk. All values shown in the manuscript have been corrected for the influence of ¹³C when required.

Substantial Deuterium Isotope Effect for the Enzymatic Oxygenation of (D₆)-AA by Macrophages. Previous in vitro studies investigating the KIE of oxygenation of AA by enzymes have used purified COX or LOX of mammalian origin as well as the plant lipoxygenase, sLOX.¹⁻⁵,¹⁸,¹⁹ The results of these studies varied, but all reported fairly low KIEs of AA oxidation by COX and LOX enzymes. The highest reported KIE (⁷⁵kcat) for AA oxygenation by COX-2 (using 13,13-(D₆)-AA as substrate) was 3.1 ± 0 when the concentration of O₂ was limited to physiological levels.⁴ To test the hypothesis that enzymatic oxidation of AA by macrophages would be reduced by bis-allylic deuteration of AA, we first started with 7,7,10,10,13,13-(D₆)-AA (D₆-AA; compound 7). We reasoned that we should see a reduction in the agonist-induced production of all eicosanoids derived from this isotopologue. RAW264.7 macrophages were labeled with 25 μM native AA or D₆-AA for 24 h before stimulation with KLA or ATP. Supernatants were collected at the indicated time points and the major eicosanoids of the COX and LOX pathway were quantified by UPLC-MS/MS (Figures 2 and 3). Vanishingly low amounts of all COX and LOX products derived from D₆-AA were detected following KLA or ATP stimulation compared to native eicosanoids (Figures 2 and 3). One possible explanation for these results is that 24 h is not enough time for the supplemented D₆-AA to reach the intracellular sites of eicosanoid biosynthesis. To test this hypothesis, supplementation with D₆-AA was allowed to continue for 48 h, allowing more time for intracellular distribution. No change in the production of KLA-elicited eicosanoids derived from D₆-AA was observed compared to 24-h supplementation (data not shown). Following these time course experiments, we selected a single time point for KLA (24 h) or ATP (30 min) stimulation to investigate isotope effects with D₂ and D₄ isotopologues.

Physiological KIEs. The physiological kinetic isotope effect (PKIE) discussed in this manuscript is simply the ratio of native eicosanoid versus deuterated eicosanoid measured following stimulation. To obtain an accurate representation of the PKIE, the substrate availability following 25 μM AA supplementation was determined by measuring the amount of arachidonic acid esterified in complex lipids. In the absence of any isotope effect, we would expect the amount of deuterated vs native eicosanoids detected to reflect the abundance of released (D₆)-AA and AA. Taking the results for D₆-AA as an example, the data presented in Figure 1 demonstrate that this ratio (D₆-AA/AA) is approximately 3 to 1. This expected ratio of heavy to light substrate does not match the experimental results of the products presented in Figure 2, where very little (D₆)-AA-derived eicosanoids are detected. To determine the PKIE of (D₆)-AA oxidation, as well as oxidation of all other AA isotopologues discussed in this study, eq 1 was used:

\[
\frac{\text{PKIE}}{\text{PKIE}} = \frac{\text{eAA}}{\text{eAA}}
\]

Where L is the native, or light metabolite; eAA is esterified AA; and PKIE is the physiological isotope effect. This takes into account the abundance of the native and deuterated metabolite (⁶EICO/⁶EICO) as well as substrate availability (⁶eAA/⁶eAA). This is in contrast to KIEs calculated during in vitro studies, in which the KIE is the ratio of reaction velocities (kcat (s⁻¹))/kcat (s⁻¹) obtained from careful kinetic studies by varying substrate concentration. We feel this is a fair representation of the PKIE since we have demonstrated that there is no significant preference for AA vs (D₆)-AA by cPLA². These data suggest that there is a substantial PKIE during oxidation of (D₆)-AA by both COX and LOX enzymes in macrophages.

Determination of PKIEs upon Oxygenation of D₆-AA Isotopologues. The results obtained from experiments using the D₆-AA isotopologue were somewhat surprising given previous studies reported such low deuterium isotope effects for oxygenation of 13,13-(D₆)-AA. We then completed a more targeted interrogation of enzyme specific deuterium isotope effects using the D₆-AA isotopologues: 7,7-(D₆)-AA (1), 10,10-(D₆)-AA (2), and 13,13-(D₆)-AA (3). We hypothesized that the PKIE measured during oxidation of these isotopologues by COX and LOX enzymes would be greatest when the known position of hydrogen abstraction was deuterated (Abstract Graphic). RAW264.7 macrophages were labeled with AA or one of the (D₆)-AA isotopologues for 24 h before stimulation with KLA (100 ng/mL) or ATP (2 mM). Culture media was collected at 24 h (KLA) or 30 min (ATP) and eicosanoids were quantified by UPLC-MS/MS. We observed low levels of COX products derived from 13,13-(D₆)-AA compared to AA while

![Figure 3](image-url)
equal production of 7,7-(D₂)-AA-derived COX products were detected under similar conditions (Figure 4A). This was due to deuterium incorporation into the AA substrate, resulting in similar enzyme-substrate interactions.

Table 2A. Physiological Isotope Effects: COX Products

| physiological isotope effect (mean ± SEM) | TXB₂ | PGF₂α | PGE₁ | PGD₂ | 11-HETE | 15-HETE |
|------------------------------------------|------|-------|------|------|---------|---------|
| 7,7-(D₂)                                 | 4 ± 1| 5 ± 2 | 5 ± 2| 5 ± 2| 3 ± 1   | 3 ± 1   |
| 10,10-(D₂)                               | 5 ± 2| 270 ± 140| 8 ± 2| 11 ± 3| 3 ± 1   | 230 ± 40|
| 13,13-(D₂)                               | 22 ± 7| 32 ± 7| 17 ± 7| 20 ± 6| 280 ± 100| 14 ± 3 |
| 7,7,10,10-(D₄)                           | 20 ± 6| ∞     | 12 ± 9| 35 ± 20| 7 ± 3   | 210 ± 170|
| 7,7,13,13-(D₄)                           | 52 ± 44| ∞     | 9 ± 5| 25 ± 8| 115 ± 57| 18 ± 4 |
| 10,10,13,13-(D₄)                         | 150 ± 65| ∞     | 34 ± 7| 57 ± 18| 120 ± 74| ∞       |
| (D₆)-AA                                  | 120 ± 76| 330 ± 53| 40 ± 12| 76 ± 18| 250–∞   | 400–∞   |

expected as hydrogen atoms present at C7 are not expected to play a role in the catalysis of AA oxygenation by COX enzymes, while the 13-pro(S) hydrogen plays a critical role. However, we did observe a PKIE toward the formation of COX products from 10,10-(D₂)-AA. This PKIE was slightly lower in magnitude compared to that measured for C13 deuteration, and was higher than 7,7-(D₂) when considering cyclized COX products (Figure 4A, Table 2A). A notable exception was PGE₂, where the strongest effect was observed with 10,10-(D₂)-AA (Figure 4A). The COX-2 side product 11-HETE showed expected results with little effect of C7 or C10 AA deuteration and a very strong effect with C13 deuteration (Figure 4A). We also observed a PKIE for COX-2 oxygenation of 13,13-(D₂)-AA to form its major side product, 15-HETE (Figure 4A). This was expected, as this hydroxylation event is understood to proceed through initial hydrogen abstraction from C13. Interestingly, an even larger PKIE was observed for 15-HETE produced from 10,10-(D₂)-AA (Figure 4A, Table 2A). 5-LOX begins by abstracting a hydrogen atom from C7 to form 5-HETE. Accordingly, no (D₁)-5-HETE derived from 7,7-(D₂)-AA was detected; while (D₂)-5-HETE was readily made from 10,10-(D₂) or 13,13-(D₂)-AA precursors by ATP-stimulated macrophages (Figure 4B). Like 5-HETE, the formation of leukotrienes also begins with a LO abstraction of a hydrogen atom from C7, thus very little (D₁)-LTB₄/LTE₄ derived from 7,7-(D₂)-AA was detected (Figure 4B). The dehydration of 5-HpETE that forms LTA₄, a short-lived intermediate in the synthesis of LTB₄ and LTE₄ (Scheme 1), leads to the loss of a hydrogen atom from C10. Accordingly, we measured a PKIE for the production of LTB₄ and LTE₄ from 10,10-(D₂)-AA. Finally, our data supports the understanding that there are no precluded hydrogen abstraction events at C13 during the formation of LTB₄ and LTE₄ as (D₂)-LTB₄/LTE₄ are both readily formed from 13,13-(D₂)-AA (Figure 4B).

**Determination of KIEs upon Oxygenation of D₂-AA Isotopologues.** Macrophages were labeled with deuterated AA isotopologues 7,7,10,10-(D₄)-AA (5), 7,7,13,13-(D₄)-AA (6), and 10,10,13,13-(D₄)-AA (7). The PKIE of enzymatic AA oxygenation observed when C10 or C13 deuteration is combined with C7 (5,6) is similar to deuteration of C10 or C13 alone (3,4). However, when C10 and C13 are both deuterated (7), the effect is amplified (Figure 5). These data are consistent with the known role of C13 in the formation of COX products and further support a role for C10 in prostaglandin and thromboxane formation. For products of the LOX pathway, virtually no native product was detected following labeling with (D₄)-AA isotopologues and ATP treatment, thus these graphs are not shown in Figure 5 and PKIE values for these substrates have not been included in Table 2B. Taken together, these data demonstrate that there is a PKIE for the enzymatic oxidation of D₄-AA species corresponding to the known site of hydrogen abstraction for COX and LOX enzymes. Further, the apparent PKIE toward oxygenation of 10,10,13,13-(D₂)-AA by COX or LOX is considerably greater than any KIE value reported for purified enzyme/substrate experiments carried out in vitro with 13,13-(D₂)-AA as a substrate, and suggest an important contribution of C10 to the catalysis of cyclized COX products.

**Deuteration at Carbon 10 Reduces Prostaglandin Formation, Promoting Lipoxin Formation.** As discussed above, we observed a PKIE for the formation of prostaglandins...
Figure 5. KLA and ATP-elicited production of eicosanoids derived from (D13)-arachidonic acids. RAW264.7 macrophages were labeled for 24 h with vehicle (control), AA (25 μM), (D13)-AA or one of the (D13)-AA isotopologues (25 μM) before mock treatment (−) or stimulation with KLA (100 ng/mL) for 24 h. Production of native (□) and deuterated (■) eicosanoids were then quantified by UPLC-MS/MS. Bar graphs display the mean ± SEM of a single experiment containing technical triplicates that is representative of two independent experiments.

Table 2B. Physiological Isotope Effects: LOX Products

| physiological isotope effect (mean ± SEM) | 5-HETE | 15-HETE | LTB4 | LTE4 |
|-----------------------------|--------|--------|------|------|
| 7,7-(D2)                  | 120 ± 2| 4 ± 0.5| 33 ± 2| 47 ± 17 |
| 10,10-(D2)                | 2 ± 1 | 220 ± 68| 22 ± 1| 43 ± 18 |
| 13,13-(D2)                | 3 ± 1 | 50 ± 32| 3 ± 0 | 2 ± 1  |
| (D13)-AA                  | ∞      | ∞      | ∞    | ∞     |

Figure 6. Enhanced production of (D13)-Lipoxin B4. RAW264.7 macrophages were labeled for 24 h with vehicle (control), AA (25 μM), or D13-AA isotopologues (25 μM) before mock treatment or stimulation with KLA for 24 h (100 ng/mL). Native (□) and deuterated (■) LXB4 were then quantified by UPLC-MS/MS. Bar graph displays the mean ± SEM of a three independent experiments, each containing technical triplicates.

when C10 is deuterated (Figure 4A) and an enhanced PKIE when combining deuteration at C10 and C13 (Figure 5). This was noteworthy because the currently accepted radical model of arachidonate cyclization and oxygenation by COX enzymes does not predict a role for C10.20,22 However, Fried and co-workers observed that COX was unable to produce any cyclic products when using 10,10-difluoroarachidonic acid as a substrate, but was able to form the acyclic alcohols, 10,10-difluoro-11-HETE and 10-fluoro-8,15-DiHETE.24 Furthermore, it has been proposed that the mechanism of cyclization could proceed through a carboxation intermediate centered at C10.25 Indeed, we also detected the formation of the multihydroxylated product, (D13)-LXB4, by macrophages labeled with 10,10-(D13)-AA following KLA stimulation that was not seen in unlabeled controls (Figure 6). These data are consistent with prior reports25,26 and support the hypothesis that C10 is important for cyclization of AA by COX.

**DISCUSSION**

Evidence for the Involvement of C10 during Cyclooxygenation of AA. With the exception of PGF2α, our experiments revealed equivalent PKIEs for the formation of cyclized eicosanoids of the COX pathway from 10,10-(D13)-AA and 13,13-(D13)-AA, between 5 and 20. The results obtained from experiments using 13,13-(D13)-AA were expected, as it is well understood that COX abstracts the 13-pro(S) hydrogen during catalysis. What was not expected was a PKIE with 10,10-(D13)-AA, especially since there is no role for C10 in the widely accepted radical-based mechanism for AA oxygenation and cyclization by COX enzymes.20 Further evidence suggesting that C10 contributes to the formation of COX products was the large PKIE observed with 10,10,13,13-(D13)-AA as a substrate, yielding virtually no detectable prostaglandins or thromboxanes by KLA treated macrophages. The classical mechanism of AA cyclooxygenation by COX enzymes put forth by Hamberg and Samuelsson20 suggested that a tyrosyl radical stereospecifically abstracts the 13-pro(S) hydrogen from AA, generating a radical centered on C13. This mechanism is supported by a number of studies, which show AA within the COX active site and demonstrate the existence of tyrosyl and arachidonic acid radicals during catalysis.24–30 Subsequent cyclization and oxygenation steps proposed by this mechanism do not suggest any involvement of C10. In an attempt to resolve inconsistencies in the radical based mechanism, Dean and Dean25 proposed that cyclization of AA proceeds through a carboxation intermediate centered at C10. The theoretical framework and full explanation of carboxation formation and the mechanism can be found in ref 23. Briefly, the carboxation hypothesis predicts that AA oxygenation by COX-2 begins with the generation of a radical at C13 via abstraction of the 13-pro(S) hydrogen, followed by a sigmatropic hydrogen transfer from C10 to C13; thus quenching the C13 radical. Subsequent removal of an electron from the C10 radical by heme allows for the generation of the reactive carboxation centered at C10. C10 carboxation formation is immediately followed by conrotatory ring closure between C8 and C12,31 and a shift of the carboxation center from C10 to C11. The return of an electron to the carboxation at C11 yields a C11 radical, which then reacts with molecular oxygen, immediately forming the dioxygen bridge between C9 and C11, leaving a radical center.
at C10. A final hydrogen shift from C13 to C10 regenerates the C13 radical required for peroxidation of C15. Considering 10,10-(D2)-AA as the substrate, these transfer reactions become less favorable. It could be hypothesized that the reason we measured a small isotope effect with 10,10-(D2)-AA is that the hydrogen transfer to C13 is made more difficult due to increased C–D bond strength vs C–H. However, the isotope effect is magnified when both C10 and C13 are deuterated (10,10,13,13-(D4)-AA) because both the initial abstraction of the 13-pro(S) deuterium and the deuterium transfers between C10 and C13 all become less likely to occur. It should be noted however that unless C10 and C13 deuteration changes the rate-limiting step of the reaction from the initial hydrogen abstraction to the C10–C13 hydrogen shift, we would not expect any additional changes in reaction rate/product distribution. Further experiments would be required to determine if combining C10 and C13 deuteration alters the rate-limiting step of this reaction. Furthermore, the carbocation mechanism proposed by Dean and Dean is not fully supported by experimental evidence arguing against the existence of a pentadienyl radical spanning C8–C12. Detailed electron paramagnetic resonance (EPR) experiments using deuterated AA isotopologues and COX2 under anaerobic conditions established that the pentadienyl radical structure spans C11–C15. This result fully supports the radical mechanism posited by Hamberg and Samuelsson.

One possible explanation for the PKIE observed for production of cyclic eicosanoids from 10,10-(D2)-AA is that deuteration of C10 increases its $K_w$. Hydrogen bonding can help stabilize AA within the active site, and if deuteration reduces these stabilizing interactions, then it may promote dissociation out of the active site. This could explain the relatively small, but consistent PKIE observed for 7,7-(D2)-AA, a site that is too far away from the site of hydrogen abstraction and cyclization to have an effect on catalysis. However, the main stabilizing hydrogen bond interaction occurs at the carboxy end of AA with a tyrosine residue near the entrance to the active site. Additionally, hydrophilic interactions between C10 and Ser530 have been suggested to occur, however mutation of this site to alanine in COX1 only reduces enzymatic activity by $\sim$20% and does not change the $K_w$. These reports suggest that interactions between C10 and Ser530 are not necessary for catalysis or AA binding. While it is conceivable that deuteration decreases AA binding/stability in the active site, we find it unlikely that this could explain the PKIE observed for cyclooxygenation of 10,10-(D2)-AA and 10,10,13,13-(D4)-AA.

Physiological Shunting of Metabolites. One possible interpretation of the large PKIEs measured for KLA-elicited production of eicosanoids from 13,13-(D2)-AA and 10,10,13,13-(D4)-AA (Table 2A) is that these values appear large due to metabolite shunting rather than enzymatic blockade. Under physiological conditions these AA species can serve as substrate for multiple enzymatic pathways, thus there is competition for the same pool of AA. It stands to reason that 13,13-(D2)-AA could be a more favorable substrate for enzymes that initiate oxygenation via abstraction of hydrogen from C7 or C10. Thus, if substrate shunting is responsible for the PKIE observed, we should see a substantial increase in metabolites derived from C7 and/or C10 abstraction during 13,13-(D2)-AA and 10,10,13,13-(D4)-AA labeling. We do observe an increase in S-HETE derived from 13,13-(D2)-AA and 10,10,13,13-(D4)-AA following KLA stimulation (data not shown). However, the amount of deuterated S-HETE detected is not sufficient to explain the large PKIEs measured for cyclized COX products derived from 13,13-(D2)-AA and 10,10,13,13-(D4)-AA. We did not detect an increase in 12-HETE under any conditions tested. Our data does suggest that a consequence of raising the energy barrier for cyclization of 10,10-(D2)-AA by COX-2 is a shunting of this substrate toward lipoxygenase reactions, specifically 5-LOX and 15-LOX. Shunting of 10,10-(D2)-AA away from cyclooxygenation favors the activity of S-LOX and the 15-LOX activity of COX-2 (Scheme 2). This is supported by our results presented in Figure 6 that show elevated production of 10,10-(D2)-LXB4 (5,14,15-trihydroxy-eicosatetraenoic acid). This is similar to the inhibition of COX activity by aspirin, which acetylates the cyclooxygenase active site, thereby promoting the production of pro-resolving lipid mediators such as the lipoxins via its 15-lipoxygenase activity.

Alternative Path for PGF$_{2\alpha}$ Synthesis? As mentioned above, one of the inconsistencies in our data set is the extremely high PKIE calculated for PGF$_{2\alpha}$ (270) derived from 10,10-(D2)-AA, while the PKIEs for PGE$_2$ and PGD$_2$ are both small ($\sim$5, Table 2A). This is not expected as all three of these prostaglandins are derived from the intermediate PGH$_2$ via reduction of the endoperoxide by prostaglandin synthase enzymes. In addition to PGF synthase, PGF$_{2\alpha}$ can be derived via ketoreduction of PGE$_2$ or PGD$_2$. Some ketoreductase enzymes preferentially catalyze the reduction of the enol vs the ketone, and tautomerization between these states would be inhibited by adjacent deuterium atoms. If the majority of PGF$_{2\alpha}$ detected in our system is derived via ketoreduction of PGE$_2$/PGD$_2$, then it is possible that the large PKIE is due to inhibited keto–enol tautomerization caused by deuterium present at C10. Further experiments are underway to determine the major route(s) of PGF$_{2\alpha}$ synthesis by KLA stimulated macrophages.

Additionally, PGF$_{2\alpha}$ and structurally similar isoprostanes can be derived from AA via nonenzymatic peroxidation reactions.
However, KLA-elicited production of 8-iso PGF$_2$α-III was ~400 fold lower than enzymatically derived prostaglandins under the same experimental conditions (data not shown). This suggests that nonenzymatic lipid peroxidation events only contribute a very minor fraction of the total eicosanoid output by KLA-stimulated macrophages. Thus, it is unlikely that production of isoprostanes has a significant effect on the PKIIs measured in this system.

### CONCLUSIONS

We present a novel, lipidomics-based UPLC-MS/MS method to quantify the ex vivo deuterium isotope effect for the enzymatic oxidation of AA by macrophages. This was accomplished by using a newly synthesized library of AA isotopologues, variably deuterated at bis-allylic carbons. Applying a sensitive, UPLC-MS/MS MRM detection method allowed us to identify and accurately measure low abundant deuterium-containing metabolites of arachidonic acid. The data collected revealed relevant results in a cell-based system in agreement with the current understanding of the COX and LOX mechanisms of action. We also provide novel evidence for the contribution of C10 to the formation of cyclized COX products.

### EXPERIMENTAL SECTION

#### Cell Culture and Stimulation.

The RAW264.7 murine macrophage cell line (ATCC #TIB-71) was maintained at 37 °C, 5% CO$_2$ in DMEM containing 10% FBS (Gemini Bio) Penicillin/streptomycin, Sodium Pyruvate and t-glutamine. For experiments, cells were plated 12-well tissue culture clusters in 1 mL phenol red-free DMEM at a concentration of 5 × 10$^5$ macrophages per well and were allowed to adhere for 24 h. Cells to be labeled with D$_2$-arachidonic acids were incubated during this initial 24-h period. Media was then removed and the cells washed 2 times with 1 mL phenol red-free DMEM. One mL of phenol red-free DMEM was added to each well and incubated at 37 °C for 30 min. Cells were then left untreated or treated with either 100 ng/mL KLA (Avanti) or 2 mM ATP (SIGMA). After treatment, supernatant was collected and stored at −80 °C until analysis. Cell material was collected in 1 mL PBS for protein and fatty acid analysis.

#### Arachidonic Acid Library.

Library of arachidonic acids variably deuterated at bis-allylic carbons was generously provided by M.S. Shchepinov and colleagues; the synthesis and analytical verification is described elsewhere. Isotopologues of arachidonic acid (AA) used in this study: 7,7-(D$_2$)-AA, 10,10-(D$_2$)-AA, 13,13-(D$_2$)-AA, 7,7,10,10-(D$_4$)-AA, 7,7,13,13-(D$_4$)-AA, 10,10,13,13-(D$_4$)-AA, 7,7,10,13,13-(D$_6$)-AA. These AA compounds were synthesized as ethyl esters for improved stability during storage. AA-ethyl esters were prepared for analysis as previously described. Isotopologues of arachidonic acid (AA) used in this study: 7,7-(D$_2$)-AA, 10,10-(D$_2$)-AA, 13,13-(D$_2$)-AA, 7,7,10,10-(D$_4$)-AA, 7,7,13,13-(D$_4$)-AA, 10,10,13,13-(D$_4$)-AA, and 7,7,10,13,13-(D$_6$)-AA. These AA compounds were synthesized as ethyl esters for improved stability during storage. AA-ethyl esters were hydrolyzed to free acid by base hydrolysis for experiments. Briefly, a solution of AA-ethyl ester in methanol (3 mg/mL) was hydrolyzed with 0.5 M KOH in a round-bottom flask, capped under argon gas for 2 h at 70 °C. The reaction solution was then diluted by 1/3 with HPLC-grade water, and then acidified with 160 mM H$_2$SO$_4$. The solution was then extracted with hexane 3 times. The organic phase was transferred to a borosilicate tube and evaporated under a flow of nitrogen. The residual was dissolved in 1 mL of 200 proof ethanol and stored at −80 °C under argon. The concentration of free D$_2$-AA following hydrolysis was determined via UPLC-MS/MS using native AA (Cayman) for the reference standard curve.

#### Eicosanoid Extraction from Cell Supernatant.

Supernatants were thawed on ice and then spiked with 3.57 ng of internal standards in ethanol (Cayman). Samples were purified via solid-phase extraction and prepared for analysis as previously described.

#### Extraction and Analysis of Esterified Arachidonic Acid from Cell Membranes.

Cell material in PBS was thawed on ice and then sonicated with a probe sonicator for 30 s. 250 μL (~250 000 cells), 500 μL methanol and 25 μL of 1 M HCl were combined in a borosilicate tube and extracted 2 times with 1 mL of isooctane to remove free fatty acids. Lipids were then extracted from the remaining aqueous (methanol/PBS) phase via a modified Bligh and Dyer procedure. 250 μL dichloromethane (DCM) was added to the methanol/PBS aqueous phase and vortexed 5–4 times and placed on ice for 30 min. 250 μL DCM and 250 μL 0.1 M HCl was then added and tubes vortexed. Phases were separated by centrifugation at 5000 rpm for 1 min. The organic layer was collected and transferred to a new borosilicate tube. Solution was extracted with an additional 1 mL of DCM. Combined organic fractions were dried on a speed-vec for 30 min. Residue was then saponified by resuspension in 250 μL methanol and 250 μL 1 M KOH and incubation at 37 °C for 1 h. After saponification, the solution was acidic by the addition of 200 μL 1× glycine-HCl and 225 μL 1 M HCl. The solution was then spiked with 5,6,8,9,11,12,14,15-(D$_4$)-AA internal standard (Cayman), diluted with 4 mL H$_2$O, and purified as described for eicosanoids above. AA was quantified by UPLC-MS/MS.

#### Liquid Chromatography Tandem Mass Spectrometry.

Arachidonic acid and eicosanoid analysis was performed as described in detail previously. Briefly, 10 μL of each sample was separated by reversed phase liquid chromatography using a gradient of mobile phase A [water:acetonitrile-acetic acid (60:40:0.02; v/v/v)] and mobile phase B [acetonitrile/isopropanol (50:50; v/v)] on a 2.1 × 100 mm Acuity UPLC BEH Shield RP18 1.7 μm column. Online LC-electrospray ionization MS/MS was performed on a QTRAP 6500 hybrid quadrupole/linear ion-trap mass spectrometer (AB Sciex) via multiple reaction monitoring (MRM) in negative ion mode. Eicosanoids were quantified by comparing the selected MRM signal and retention time to a pure standard.

### ASSOCIATED CONTENT

#### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.7b09493.

An example of mass spectrum fragment analysis and calculation of MRM pairs for deuterated eicosanoid species used to generate the data represented in Figures 2–7 (Figure S1). A table of all MRMs used in this study can be found in Table S1 (PDF).

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#### Notes

The authors declare the following competing financial interest(s): EAD discloses that he served as a consultant and advisor to Retrotope and holds equity in the company. M.S. is an employee of Retrotope, Inc.

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