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Linoleic acid esters of hydroxy linoleic acids are anti-inflammatory lipids found in plants and mammals

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Fatty acid esters of hydroxy fatty acids (FAHFAs) are a recently discovered class of biologically active lipids. Here we identify the linoleic acid ester of 13-hydroxy linoleic acid (13-LAHLA) as an anti-inflammatory lipid. An oat oil fraction and FAHFA-enriched extract from this fraction showed anti-inflammatory activity in a lipopolysaccharide-induced cytokine secretion assay. Structural studies identified three LAHLA isomers (15-, 13-, and 9-LAHLA) as being the most abundant FAHFAs in the oat oil fraction. Of these LAHLAs, 13-LAHLA is the most abundant LAHLA isomer in human serum after ingestion of liposomes made of fractionated oat oil, and it is also the most abundant endogenous LAHLA in mouse and human adipose tissue. As a result, we chemically synthesized 13-LAHLA for biological assays. 13-LAHLA suppresses lipopolysaccharide-stimulated secretion of cytokines and expression of pro-inflammatory genes. These studies identify LAHLAs as an evolutionarily conserved lipid with anti-inflammatory activity in mammalian cells.

Fatty acid esters of hydroxy fatty acids (FAHFAs)⁴ are a recently discovered class of lipids with anti-diabetic and

anti-inflammatory activity (1). Because there are numerous FAHFAs, they are classified into families based on the composition of fatty acid and hydroxy fatty acid. For example, palmitic acid esters of hydroxy stearic acids (PAHSAs) and oleic acid esters of hydroxy stearic acids (OAHSAs) are two FAHFA families. Furthermore, within a FAHFA family, there are multiple regioisomers that differ in the position of the ester linkage (e.g. 5-PAHSA and 9-PAHSA) (1).

Biological testing of 5- and 9-PAHSA revealed potent anti-diabetic and anti-inflammatory activity (1–3). Mechanistic studies revealed that FAHFAs regulate several cellular and physiological pathways, with at least some of the biology being attributable to agonism of GPR120 and GPR40, two G protein-coupled receptors (1, 3). Other ligands for these G protein-coupled receptors include saturated and polyunsaturated fatty acids (4, 5). GPR120 is the endogenous receptor for omega-3 fatty acids, and it mediates the anti-inflammatory effects of these lipids (5).

The anti-inflammatory activity of FAHFAs has been reported *in vitro* and *in vivo* (1, 2). Initially, cellular experiments with bone marrow-derived dendritic cells showed that treatment of cells with 9-PAHSA reduced the amplitude of cytokine secretion and expression of cellular inflammation markers. In addition, administration of 9-PAHSA to mice on a high-fat diet reduced inflammation in adipose tissue of treated mice (1).

9-PAHSA also showed robust anti-inflammatory activity in a mouse colitis model. Administration of 9-PAHSA to mice undergoing chemically induced colitis improved clinical and molecular inflammation (2). Moreover, an analysis of the impact of 9-PAHSA on the immune system revealed effects on the innate and adaptive immune system (2). Most recently, Kuda *et al.* (6) demonstrated that docosahexaenoic acid of 13-hydroxy linoleic acid (13-DHAHLA), a novel FAHFA, inhibits LPS-induced cytokine secretion in the mouse macrophage cell line RAW 264.7. Furthermore, 13-DHAHLA is more potent than 9-PAHSA, highlighting the need to find more biologically active FAHFAs to determine structure–activity relationships.

Including all the regioisomers, there are at least 80 known FAHFAs, but only three of these have been tested for their

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This article contains Figs. S1–S7 and supporting text.

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⁴ The abbreviations used are: FAHFA, fatty acid ester of hydroxy fatty acid; PAHSA, palmitic acid ester of hydroxy stearic acid; OAHSA, oleic acid ester of hydroxy stearic acid; 13-DHAHLA, docosahexaenoic acid ester of 13-hydroxy linoleic acid; DGDG, digalactosyldiacylglycerol; LAHLA, linoleic acid ester of hydroxy linoleic acid; LPS, lipopolysaccharide; SPE, solid-phase extraction; HLA, hydroxy linoleic acid; SQWAT, subcutaneous white adipose tissue; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 13-HODE, 13-hydroxyoctadecadienoic acid; iNOS, inducible

nitric oxide synthase; wt.%, weight percentage; RT-qPCR, quantitative RT-PCR.

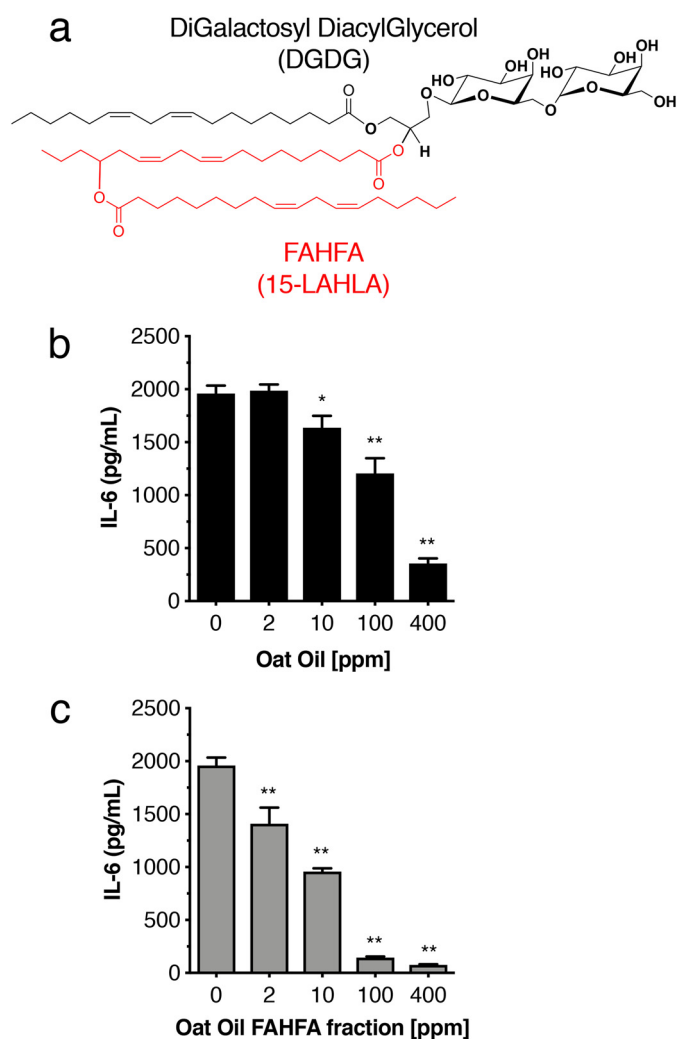


Figure 1. Anti-inflammatory activity of oat oil. *a*, the reported structure of a DGDG from oat seed. *b* and *c*, activity of oat oil fraction called T1 (*b*) and solid-phase-extracted sample of the T1 fraction (*c*) on IL-6 secretion from LPS-stimulated RAW 264.7 cells. Data represent mean \pm S.E. for three biological replicates. **, $p \leq 0.0001$; *, $p \leq 0.01$; versus LPS by one-way ANOVA.

biological activity (1, 6). To expedite the discovery of additional bioactive FAHFAs, we wondered whether there exist other natural sources of biologically active lipids that would lead to bioactive FAHFAs. Oat oil emerged as a potential source of bioactive FAHFAs because colloidal oatmeal has anti-inflammatory activity (7), and physiological studies with liposomes made of fractionated oat oil revealed beneficial metabolic effects (8). Furthermore, a digalactosyldiacylglycerol (DGDG) lipid found in oat oil contains a FAHFA (also referred to as an estolide), the linoleic acid ester of 15-hydroxy linoleic acid (15-LAHLA) (Fig. 1*a*) (9–11).

We reasoned that the existence of a FAHFA-containing DGDG meant that oat oil is a potential source of additional biologically active FAHFA families. We hypothesized that FAHFAs might contribute to the anti-inflammatory activity of oat extracts. We identified free linoleic acid esters of LAHLAs in an oat oil fraction rich in neutral lipids with anti-inflammatory activity (Fig. 1*b*) and synthetic 13-LAHLA as another anti-inflammatory FAHFA.

Results

Testing the anti-inflammatory activity of oat oil fractions

Previous reports have identified anti-inflammatory activity of colloidal oatmeal (7) and beneficial metabolic effects on consumption of liposomes made of fractionated oat oil rich in polar lipids (8, 12). Crude oat oil, obtained by ethanol extraction of oats, can be fractionated using different concentrations of ethanol, water, and sugar (12). The resulting fractions are rich in neutral lipids (called the T1 fraction) or rich in polar lipids (called the T2 fraction). We measured the anti-inflammatory effects of oat oil fraction T1 by measuring IL-6 release from RAW 264.7 cells upon treatment with lipopolysaccharide (LPS). This assay was used previously to measure the anti-inflammatory activities of 9-PAHSA and 13-DHAHLA (6). The T1 fraction exhibited dose-dependent anti-inflammatory activity, with significant suppression of LPS-stimulated IL-6 secretion starting at 10 ppm ($\sim 15\%$ inhibition) (Fig. 1*b*).

Solid-phase extraction (SPE) of the T1 fraction using a protocol for FAHFA enrichment (13) resulted in a fraction (SPE-T1 fraction) with more potent anti-inflammatory activity than T1. For example, the SPE-T1 fraction resulted in $\sim 50\%$ IL-6 inhibition at 10 ppm and more than 90% IL-6 inhibition at 100 ppm (Fig. 1*c*). To show that these effects were due to activity and not cytotoxicity, we performed an MTT viability assay, and there was no observed cellular death at the tested concentrations of the T1 oat oil fraction and SPE-T1 fraction (Fig. S1).

The SPE-T1 fraction contains LAHLAs

We measured FAHFA levels in the SPE-T1 fraction by LC-MS. These measurements were designed to detect different FAHFA families, including PAHSAs, OAHSAs, and LAHLAs (Fig. 2*a*). LAHLAs are the most abundant FAHFAs in the SPE-T1 fraction (~ 300 pmol/mg), whereas PAHSAs and OAHSAs are detected at much lower concentrations (~ 1 pmol/mg) (Fig. 2*a*). We detected three different LAHLA peaks in the LC-MS chromatogram (Fig. 2*b* and Fig. S2), indicative of three different LAHLA isomers. No other FAHFAs were as abundant as the LAHLAs, and therefore we focused on the structural and functional characterization of the LAHLAs.

LAHLA regioisomers

To be able to study these LAHLAs in greater detail, we needed to know which regioisomers were present and then synthesize a pure version of these lipids for biological assays. To identify the structures of these LAHLA regioisomers, we applied a recently reported strategy for structural elucidation of 13-DHAHLA (6). This method utilizes multiple fragmentation steps (MS3) to identify the position of the hydroxy group (Fig. 3*a*). Because LAHLAs and DHAHLAs both contain hydroxy linoleic acid (HLA), the method should be directly transferable to identify LAHLA regioisomers.

We did not have access to an instrument capable of MS3, so we performed pseudo-MS3 instead (13). The source voltage of the mass spectrometer was increased to induce in-source fragmentation of LAHLAs to linoleic acid and HLA, and the HLA was further fragmented in the mass spectrometer to reveal the position of the hydroxy group (Fig. 3*a*). Analysis of the oat oil

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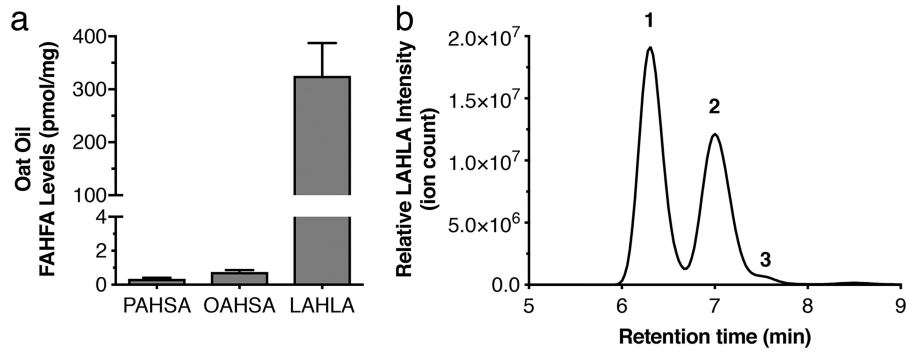


Figure 2. LAHLAs are primary oat oil FAHFAs. *a*, LAHLAs are more abundant than PAHSAs or OAHSAs in oat oil. *b*, LC-MS chromatogram showing three LAHLA regioisomers.

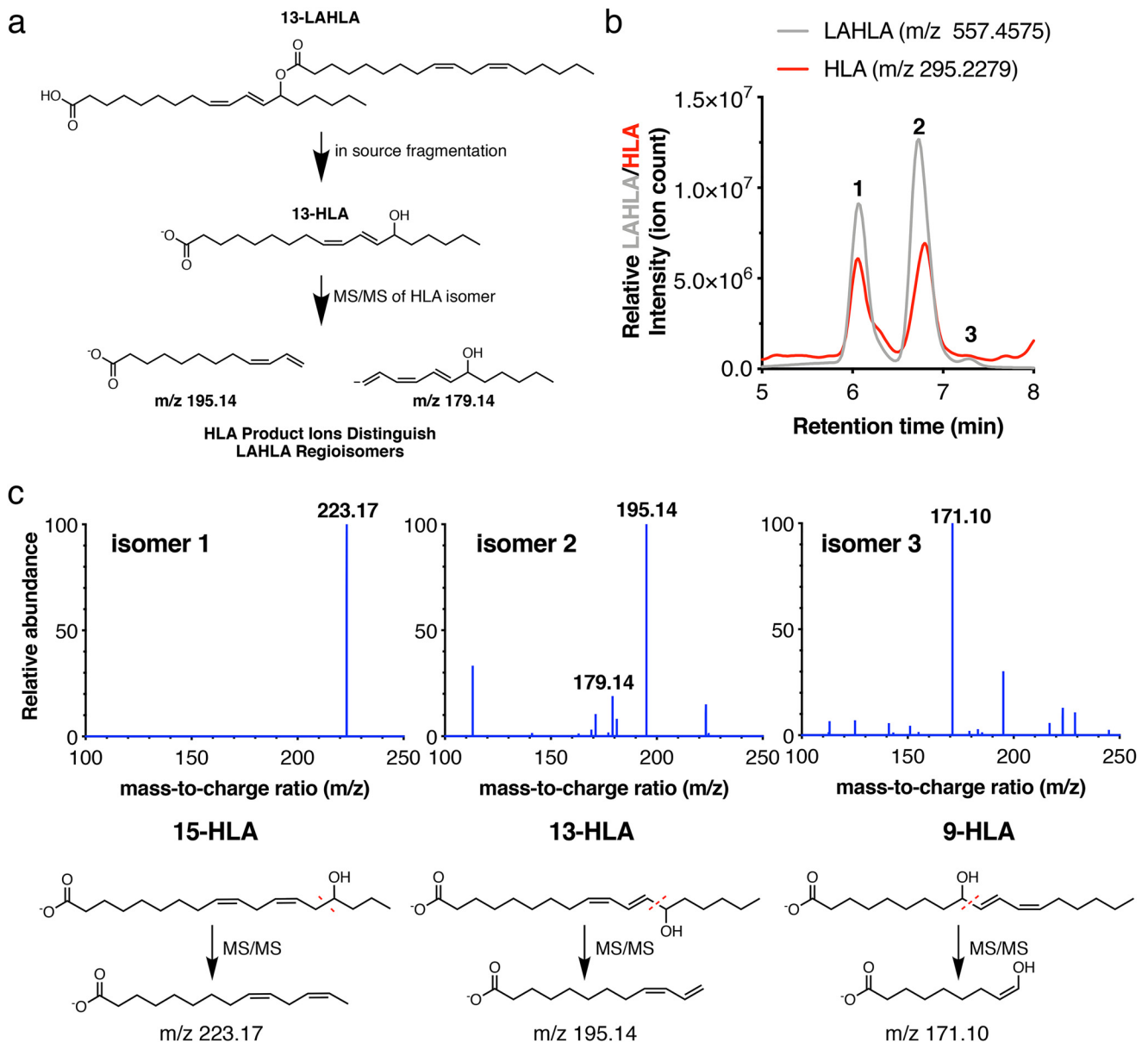


Figure 3. Identification of LAHLA regioisomers in oat oil. *a*, schematic of a pseudo-MS3 approach to identify the LAHLA regioisomers. *b*, LC-MS chromatograms of FAHFA-enriched oat oil with a source energy of 80 eV, showing the presence of LAHLAs (*m/z* 557.4575, gray) and HLA fragments from the LAHLAs (*m/z* 295.2279, red). *c*, MS/MS of HLAs from regioisomers 1–3 identify the LAHLAs as 15-LAHLA (1), 13-LAHLA (2), and 9-LAHLA (3).

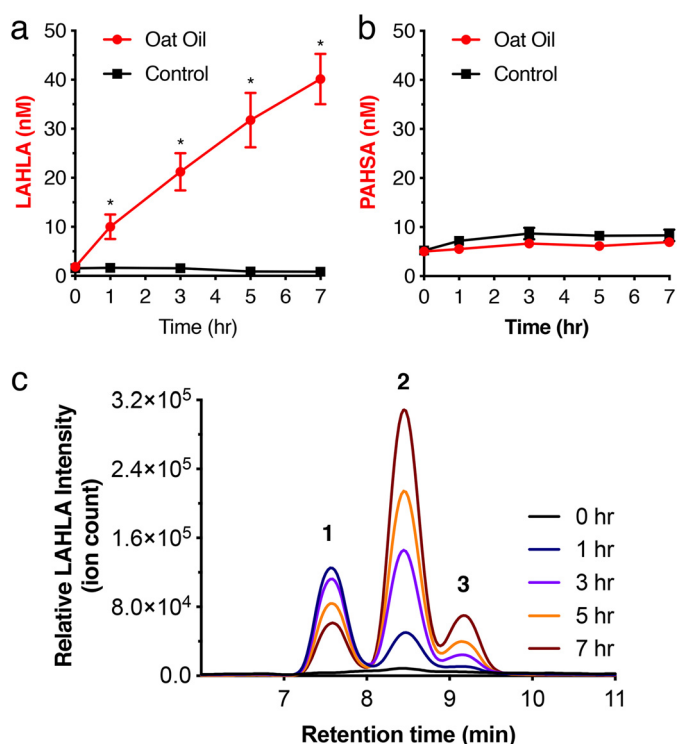


Figure 4. LAHLAs in plasma after ingestion of liposomal oat oil. *a* and *b*, levels of plasma LAHLAs (*a*) and *b*, PAHSAs in subjects fed liposomal oat oil versus control-fed subjects over 7 h. *c*, LC-MS chromatograms of LAHLA regioisomers in human plasma over a 7-h time period after liposomal oat oil ingestion. Time 0 h represents plasma FAHFs after an overnight fast. Data represent mean \pm S.E. ($n = 10-12$). *, $p \leq 0.05$ by two-sided Student's *t* test.

samples using this approach led to HLA fragments that co-elute with the different LAHLA isomer peaks (Fig. 3, *b* and *c*). Using this approach, we identified the LAHLA isomers to contain 15-, 13-, and 9-HLA (Fig. 3*c*). The 13- and 9-LAHLA product ions are identical, with reported fragments for 13- and 9-DHAHLAs (6), providing strong evidence for these regioisomer assignments.

LAHLA levels after ingestion of liposomal oat oil

Liposomes made from fractionated oat oil rich in polar lipids (fraction T2) were used as a dietary supplement to look for beneficial postprandial metabolic effects (8). Liposomal oat oil experiments correlated with lower glucose levels and elevated levels of beneficial hormones, including peptide YY, glucagon-like peptide 1, glucagon-like peptide 2, and cholecystokinin. We measured FAHFA levels in human serum samples acquired in a previous study (8) to determine whether any LAHLAs from ingested oat oil could be detected in human serum.

LAHLA levels in subjects ingesting liposomal oat oil were much higher than in control subjects, indicating that oat oil was the source of plasma LAHLA levels (Fig. 4*a*). Furthermore, we saw no change in PAHSAs or OAHSAs, consistent with oat oil having high LAHLA levels (Fig. 4*b* and Fig. S3). In subjects that received liposomal oat oil, we detected 15-, 13-, and 9-LAHLA (Fig. 4*c*). The levels of the LAHLA isomers are dynamic, with 13- and 9-LAHLA increasing with time and 15-LAHLA decreasing (Fig. S4). Overall, 13-LAHLA is the most abundant of all LAHLAs (Fig. 4*c*). The serum concentrations of LAHLAs reached ~ 40 nM, which is 45 times higher than in control

subjects. These data identify 13-LAHLA as the primary serum LAHLA after ingestion of liposomal oat oil. Although 13-LAHLA increases in serum in correlation with the physiological effects of oat oil in these individuals (8), we doubt that serum 13-LAHLA at 40 nM can account for the physiological response to oat oil because we show that 13-LAHLA has an IC_{50} of ~ 20 μ M in a cellular anti-inflammatory assay (see below). Thus, additional studies in animal models will be required to determine what, if any, effects dietary LAHLAs have in biology.

LAHLAs are endogenous mammalian lipids

We have shown previously that PAHSAs are endogenous FAHFs, with the highest levels found in adipose tissue (1). Multiple PAHSA regioisomers exist, with 9-PAHSA being the most abundant. Although PAHSAs are present in some foods, they can be synthesized endogenously. We explored whether LAHLAs were also present in adipose tissues. We examined subcutaneous white adipose tissue (SQWAT) of WT mice fed *ad libitum* for the presence of LAHLAs and observed that multiple LAHLA regioisomers were present, with 13-LAHLA the most abundant (Fig. 5, *a* and *b*). We also observed a similar finding in human white adipose tissue, with 13-LAHLA being the most abundant (Fig. 5, *c* and *d*). The discovery of 13-LAHLA as a naturally occurring lipid in rodents, humans, and oat oil pointed to 13-LAHLA as an ideal candidate for further investigation of LAHLA biology.

Synthesis of 13-LAHLA

To determine whether 13-LAHLA has direct effects on inflammation, we first needed to synthesize 13-LAHLA (Fig. 6*a*). Synthetic FAHFs have exclusively contained saturated HFA fragments because of the early identification and relative ease of synthesis of this class of FAHFs (1, 4, 15, 16). For laboratory synthesis of 13-LAHLA, the challenge was preparing large quantities of 13-hydroxy linoleic acid (13-HLA is synonymous with 13-HODE and coriolic acid) with the correct olefinic configuration. Although there have been several multistep syntheses of 13-HLA (17–21), we utilized a concise approach implementing selective selenium dioxide oxidation of the methyl ester of linoleic acid (22) (Fig. 6*a*). Although the oxidative transformation proceeded in low yield, the approach dramatically simplified synthesis. Importantly, standard phase column chromatography could be applied to separate the multiple products, including olefin isomers, with the desired 13-HLA methyl ester produced as the major product. This reaction enabled access to ample quantities of 13-HLA methyl ester from readily available methyl linoleate. From this intermediate esterification of the secondary, allylic alcohol of 13-HLA methyl ester with the acid chloride of linoleic acid yielded the methyl ester of 13-LAHLA. Selective saponification of the methyl ester in preference to the fatty acid ester because of steric effects generated synthetic 13-LAHLA for the first time. Comprehensive NMR analysis confirmed that the correct olefinic isomer was present and that the placement of the ester linkage was at the 13th carbon.

The characteristic precursor-to-product ion transitions and the retention times were the same for the synthetic and natural

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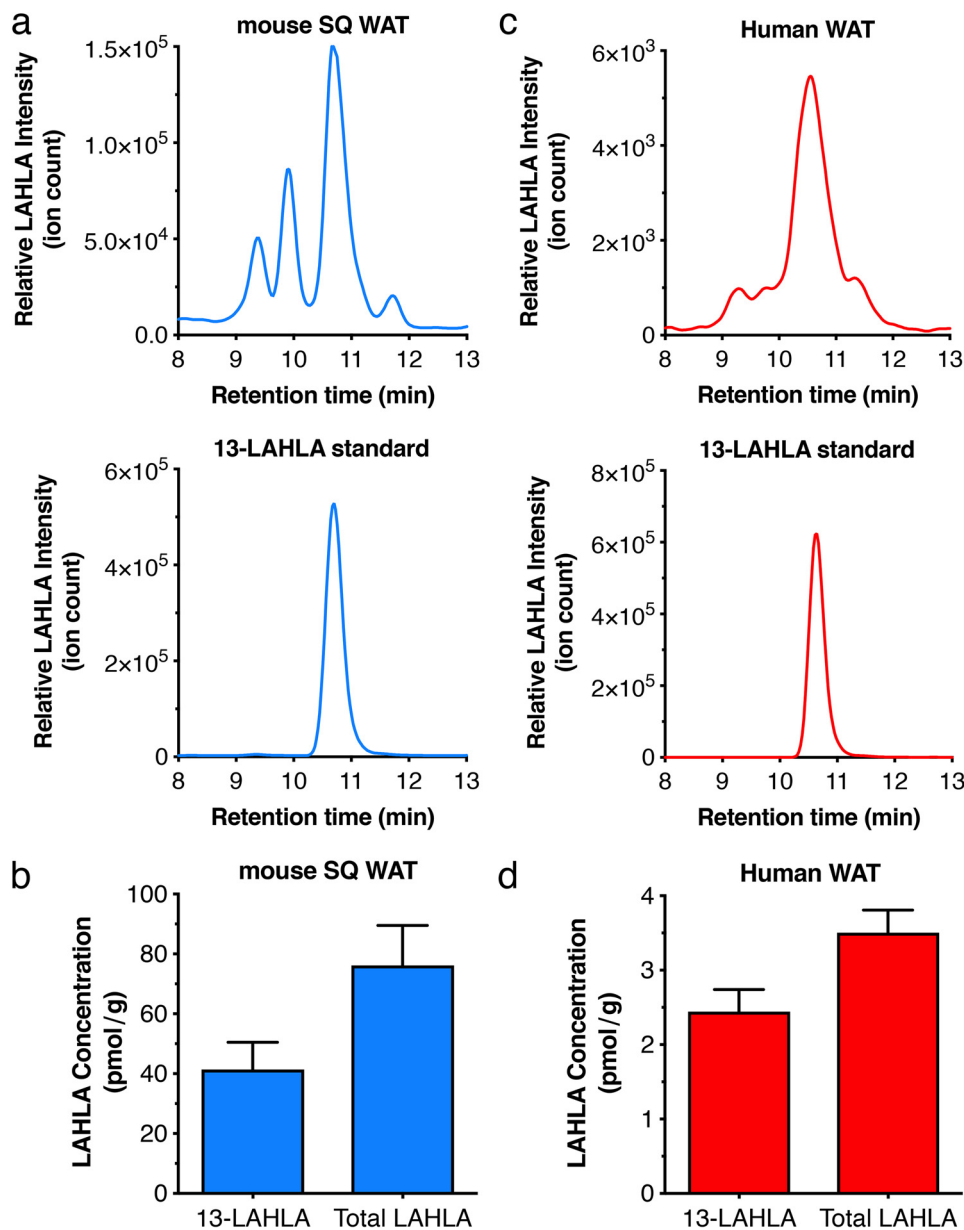


Figure 5. LAHLAs are present in mouse and human adipose tissue. *a*, LC-MS chromatograms of mouse SQWAT LAHLAs (*top panel*) and the 13-LAHLA standard (*bottom panel*). *b*, 13-LAHLA and total LAHLA levels in mouse SQWAT. *c*, LC-MS chromatogram of human WAT LAHLAs (*top panel*) and the 13-LAHLA standard (*bottom panel*). *d*, 13-LAHLA and total LAHLA in human WAT. Data represents mean \pm S.E. ($n = 3$).

13-LAHLA, indicating a structural match (Fig. S5). Using the synthetic 13-LAHLA, we validated the structure of the LAHLA isomers in oat oil and human plasma as 13-LAHLA (Fig. 6b).

Oat oil contains both stereoisomers of 13-LAHLA, as determined by hydrolysis

We set out to determine the stereochemistry of 13-LAHLA, as we determined previously that absolute configuration of FAHFs is important in their regulation (15). With commercially available enantiopure 13-HLA/HODE standards, we reasoned that we could purify and hydrolyze LAHLAs to determine their HLA backbone stereochemistry using LC-MS. We developed a chiral separation method that resolved the *S*-9-, *R*-9-, *S*-13-, and *R*-13-HLAs (Fig. S6). We then purified FAHFs from oat oil, subjected this sample to alkali hydrolysis,

and analyzed it for the presence of HLAs (Fig. S6). In this hydrolyzed sample, we observed equal amounts of *S*- and *R*-13-HLA. 9-HLA was below our limit of detection under these conditions. Based on the data, we hypothesize that the formation of 13-LAHLAs is nonenzymatic because of both the *S*- and *R*-13-LAHLA stereoisomers in oat oil.

13-LAHLA is an anti-inflammatory lipid

9-PAHSA and 13-DHAHLA have been shown to have anti-inflammatory effects. 9-PAHSA blocks LPS-stimulated dendritic cell (1) and macrophage (6) activation and reduces inflammation in a mouse colitis model (2). We analyzed the effects of 9-PAHSA and 13-LAHLA on suppression of LPS-stimulated cytokine expression in RAW 264.7 macrophages. Both 9-PAHSA and 13-LAHLA at 10 μ M significantly sup-

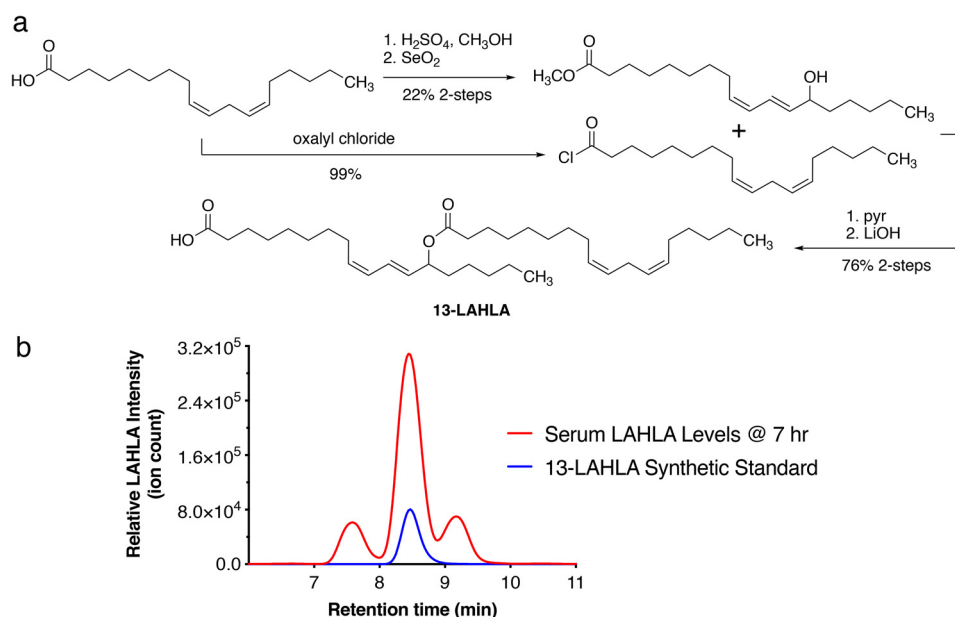


Figure 6. Synthesis of 13-LAHLA and confirmation that this is the major isomer in human serum after liposomal oat oil ingestion. *a*, synthesis of 13-LAHLA was initiated by conversion of linoleic acid to methyl linoleate under Fisher esterification conditions. Allylic oxidation of methyl linoleate with stoichiometric selenium dioxide afforded methyl 13-hydroxy linoleate. Linoleoyl chloride, prepared from linoleic acid and oxalyl chloride, was combined with methyl 13-hydroxy linoleate to generate the methyl ester of 13-LAHLA. Selective hydrolysis of the methyl ester yielded 13-LAHLA in 17% overall yield starting from linoleic acid. *b*, chromatogram of LAHLA isomers in human plasma 7 h after liposomal oat oil ingestion (*red*) compared with the 13-LAHLA synthetic standard (*blue*) provided confirmation of this regioisomer.

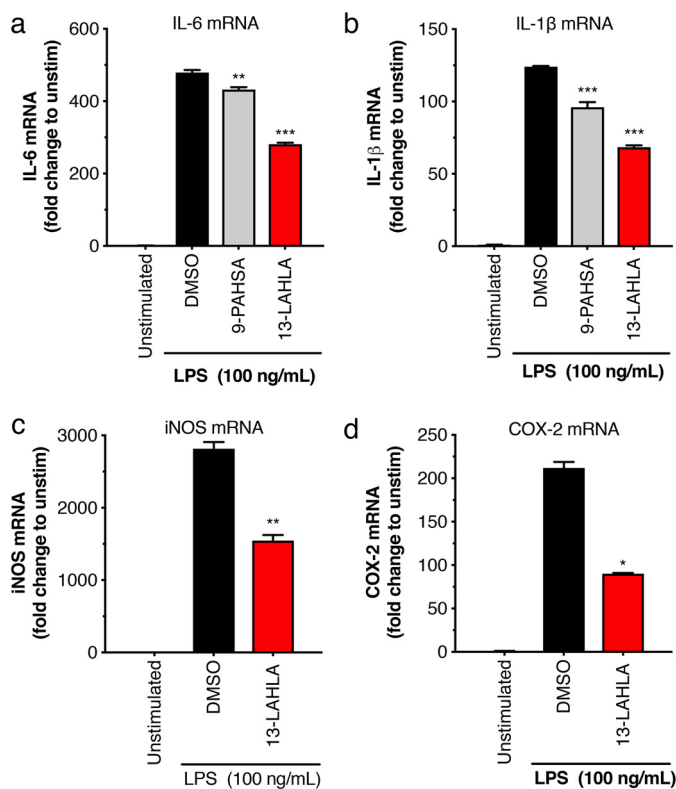


Figure 7. Anti-inflammatory effects of 13-LAHLA. *a* and *b*, 9-PAHSA (10 μ M) and 13-LAHLA (10 μ M) reduce IL-6 and IL-1 β mRNA levels in RAW 264.7 macrophages treated with LPS (100 ng/ml). *c* and *d*, effects of 13-LAHLA (10 μ M) on iNOS and COX-2 mRNA levels in RAW 264.7 macrophages treated with LPS (100 ng/ml). Data represents mean \pm S.E. ($n = 3-4$). *, $p \leq 0.05$; **, $p \leq 0.001$; ***, $p \leq 0.0001$ by two-sided Student's *t* test for FAHFA-treated versus LPS-treated alone.

pressed the mRNA levels of IL-6 and IL-1 β , with 13-LAHLA having a stronger effect (Fig. 7, *a* and *b*). Administration of 13-LAHLA to RAW 264.7 cells in the absence of LPS had no

impact on control IL-6 levels (data not shown). In addition, 13-LAHLA (10 μ M) also suppressed the mRNA levels of iNOS and COX-2 in RAW 264.7 cells when stimulated with LPS (Fig. 7, *c* and *d*). The reduction in expression of pro-inflammatory cytokines as well as the downstream regulators of inflammation iNOS and COX-2 indicate that 13-LAHLA has broad anti-inflammatory effects.

Three hydrolases (CEL, AIG1, and ADTRP) have been identified *in vitro* as FAHFA-specific lipases (23, 24), and these enzymes led us to perform additional experiments to ensure that 13-LAHLA and not LAHLA hydrolysis products are responsible for the activity we observe. First we measured the activity of 13-LAHLA and 13-HLA in this macrophage stimulation assay and found that 13-LAHLA is significantly more active than 13-HLA (Fig. 8). Neither 13-LAHLA nor 13-HLA were toxic toward cells. Comparison of linoleic acid and 13-HLA showed that they are equipotent in this assay (Fig. S7). These experiments demonstrate that 13-LAHLA is more active than either breakdown product, supporting the hypothesis that LAHLA is the biologically active species.

Discussion

Lipids are fundamental regulators in biology, with roles in membrane structure, sources of energy, and signaling molecules. Lipids in the latter category are often classified into families, such as prostaglandins (25) or oxysterols (26), that share a structural feature but have many distinct members. Individual members in a family can have distinct activities or different potencies for the same activity. For example, with FAHFAs, 9-PAHSA is anti-inflammatory but 5-PAHSA is not (1), and similar differences in structure-activity relationships are observed with other lipid classes (25). Analytical experiments suggest that there are many FAHFA families (1, 6, 27, 28), but

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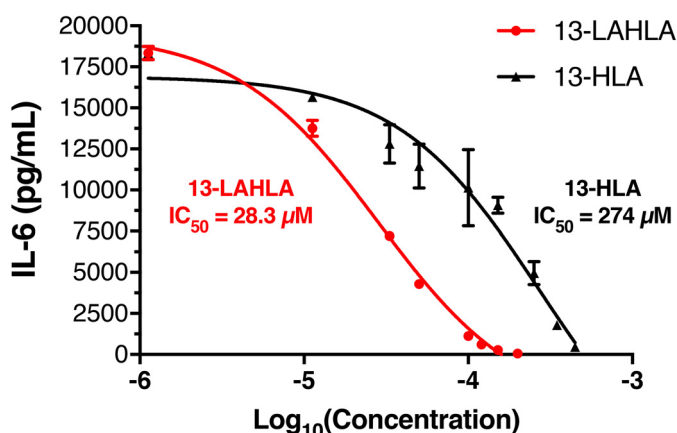


Figure 8. 13-LAHLA structure and activity. Comparison of 13-LAHLA and 13-HLA reveals greater activity for 13-LAHLA. Cells were viable over the entire dose range (data not shown).

only a few of these have been functionally characterized (1, 6). FAHFAs have been detected in foods, including vegetables (1), and recent work has detected these lipids in rice and in the plant model organism *Arabidopsis thaliana* (29). We hypothesized that there might be additional, uncharacterized FAHFAs in bioactive lipid extracts from plants.

The historical record reports the use of oats (*Avena sativa*) to treat skin conditions thousands of years ago. Today, oats, in particular colloidal oats, are present in many commercial personal care products (e.g. soaps, shampoos, and shaving creams) (30, 31). Identification of active molecules in oats identified avenanthramide alkaloids (32, 33), which have antioxidant and anti-inflammatory activity *in vitro* (34) and *in vivo* (35). Our interest in oat oil was kindled by a physiological study in humans that found beneficial metabolic and hormonal effects of a liposomal oat oil dispersion (8).

We hypothesized that oat oil might contain biologically active FAHFAs because some of the observed effects of liposomal oat oil in humans mirrored that of FAHFAs in mice (1, 3), and oats contained a complex lipid, a DGDG, that contains an esterified 15-LAHLA (Fig. 1a) (9). In this work, we identified free FAHFAs in oat oil, with LAHLAs being the most pronounced. Our hypothesis was bolstered by the observation that 13-LAHLA is anti-inflammatory.

We also detected LAHLAs as endogenous lipids and increased LAHLA concentrations upon ingestion of liposomal oat oil (fraction T2) (Fig. 4, a and c). Because the *in vitro* biological activity has an IC_{50} of $\sim 28 \mu M$ in a cell-based anti-inflammatory assay (Fig. 8), we doubt that a serum concentration of 13-LAHLA at 40 nM is regulating any biology. Future studies that utilize pure LAHLAs pharmacologically or perturb LAHLA effects metabolically will be necessary to understand what role, if any, LAHLAs have in mammalian biology. This is the second observation of LAHLAs *in vivo*; a previous study that developed a higher-resolution method to measure FAHFA levels also reported these lipids (28).

Recent work that characterized the function of 13-DHAHLA provided a guide (6) for *in vitro* characterization of 13-LAHLA. We suspected that the 13-LAHLA and 13-DHAHLA might have similar activities because of their similar structures of a 13-HLA core esterified with a polyun-

saturated fatty acid. Testing of 13-LAHLA for its ability to inhibit LPS-stimulated macrophage activity revealed that this lipid is active with an IC_{50} of $\sim 30 \mu M$. Although 9-PAHSA is active in this assay, we found that it is much less so than 13-LAHLA (Fig. 7).

We extended these measurements to include iNOS and COX-2 as additional inflammatory markers (Fig. 7, c and d). NO signaling is a key trigger in the immune system to help get rid of infection, but in some cases, overactive NO signaling is thought to damage healthy cells (36, 37). There are three NO-producing enzymes: endothelial, neuronal, and inducible NOS (38). iNOS is the primary regulator of NO signaling in macrophages, and we measured changes in the expression of iNOS in the presence and absence of LPS. These experiments revealed modest inhibition of iNOS expression (50%) at 10 μM 13-LAHLA. We also measured COX-2 levels to infer changes in the prostaglandin pathway (39). We find that administration of 13-LAHLA (10 μM) inhibits COX-2 expression by over 50%. Together, these data indicate that 13-LAHLA affects many branches of the cellular inflammatory response, including cytokine production and secretion, nitric oxide signaling, and prostaglandin production.

One challenge when testing endogenous lipids such as LAHLAs is that these metabolites are susceptible to endogenous metabolism, and caution must be taken to conclude that a specific metabolite is biologically active. For instance, 13-DHAHLA has been shown to be produced upon administration of docosahexaenoic acid to cells and mice (6), suggesting that at least some of the biological activity of docosahexaenoic acid might come from 13-DHAHLA (6). With 13-LAHLA, it is possible that the breakdown products 13-HLA or linoleic acid are active in these assays.

We measured the IC_{50} values of 13-LAHLA and 13-HLA on IL-6 secretion and found that 13-HLA is less active than 13-LAHLA ($\sim 30 \mu M$ for 13-LAHLA versus 274 μM for 13-HLA) (Fig. 8). In a separate experiment, we found that 13-HLA and linoleic acid have similar IC_{50} values (Fig. S8), indicating that both of these lipids are at least an order of magnitude less active than the intact 13-LAHLA. The data argue that, even when 13-LAHLA is hydrolyzed, the products are less active and therefore less likely to contribute to the observed activity in our assays.

In summary, our findings demonstrate that FAHFAs are an evolutionary conserved class of lipids, from plants to humans, with anti-inflammatory activity. Moreover, the characterization of 13-LAHLA here and the reported activity of 13-DHAHLA begin to reveal a trend that polyunsaturated FAHFAs are more active than the saturated FAHFAs tested so far (i.e. 9-PAHSA).

In previous work on the stereochemistry of hydroxy linoleic acids, specifically 15-HLA, they were identified as possessing R stereochemistry (9). The existence of a stereocenter on this lipid indicates that the lipid is the product of an enzymatic and therefore stereospecific pathway instead of a nonenzymatic pathway. Future efforts will look to synthesize LAHLAs enantiomers to determine whether these lipids from oat oil and tissues are enantiopure and whether the different stereoisomers vary in their biological activity. The identification of anti-in-

flammatory activity of a natural lipid that is nontoxic when consumed and the ability to modify this lipid while retaining activity indicate that we should continue to explore the biology and functions of FAHFAs, especially 13-LAHLA, as we examine the functional roles in the body and explore the therapeutic potential of this pathway.

Experimental procedures

Chemicals

9-PAHSA and the enantiopure 9- and 13-HODEs (synonymous with HLA) were purchased from Cayman Chemical.

Oat oils

Ethanol-extracted crude oat oil was produced on a large scale at Swedish Oat Fiber (Bua, Sweden). This crude oat oil was fractionated using different mixtures of water, ethanol, and sugar, resulting in the neutral lipid-rich T1 fraction, the polar lipid-rich T2 fraction, and an oat syrup containing sugar and ethanol-soluble oat protein (12). The main lipid classes in the T1 oil were triglycerides (90 wt.%); slightly polar lipid-like free fatty acids, sterols, diglycerides, and monoglycerides (6 wt.%); galactolipids (2 wt.%); and phospholipids (2 wt.%). The T2 fraction contained triglycerides (40 wt.%); slightly polar lipid-like free fatty acids, sterols, diglycerides, and monoglycerides (3 wt.%); galactolipids (29 wt.%); and phospholipids (29 wt.%). The SPE-T1 fraction was enriched using a solid-phase extraction method described previously (40).

Mouse and human tissue for FAHFA measurements

WT C57BL/6J mice were purchased from The Jackson Laboratory. All animals were housed in groups on a 14-h light, 10-h dark schedule at the Salk Institute for Biological Sciences. All animal care and experimental procedures were in accordance with the standing committee on the Use of Animals in Research and Teaching at the Salk Institute for Biological Sciences, the Institutional Animal Care and Use Committee, and the National Institutes of Health Guidelines for the Humane Treatment of Laboratory Animals. SQWAT was collected immediately after euthanasia and snap-frozen using liquid nitrogen.

Human adipose tissue from a 46-year-old African American female was purchased (BioreclamationIVT, Hicksville, NY). Human plasma samples were obtained and prepared as described previously (8). All participants gave their written informed consent prior to the study. The study was approved by the Regional Human Ethics Committee of Lund-Malmö, Sweden (registration numbers 2010/18 and 2011/55). Initial blood samples were taken after the subjects fasted overnight. Subsequent blood samples were taken 1, 3, 5, and 7 h after ingestion of a controlled breakfast supplemented with liposomal oat oil or a control diet. The breakfasts contained 35 g of lipids; the breakfast with oat oil contained 2 mmol DGDG with FAHFA. An institutional review board letter of exemption for the deidentified human plasma samples used in this study is on file at the Salk Institute for Biological Sciences.

Lipid extraction and SPE

Lipid extraction of samples and solid-phase extraction were performed as described previously (40). Briefly, WAT (150 mg) was Dounce-homogenized on ice in a mixture of PBS, methanol, and chloroform (1.5 ml/1.5 ml/3 ml). 5 pmol of [¹³C₄]9-PAHSA was added to the chloroform prior to lipid extraction as an internal standard. The mixture was vortexed and then centrifuged at 2200 × *g* for 5 min. The organic layer (bottom) was then transferred to a new vial, dried down, and stored at −80 °C for future use. The human plasma was prepared similarly, except no Dounce homogenization was performed. SPE was performed at room temperature using a Strata SI-1 silica cartridge (500 mg silica, 3 ml, Phenomenex). The column was washed using 6 ml of ethyl acetate followed by column equilibration with 6 ml of hexane. The lipid extract from the prior step was then added to the equilibrated column, and neutral lipids were removed using 6 ml of 95:5 hexane:ethyl acetate followed by elution of FAHFAs with 4 ml of ethyl acetate. This eluate was dried down and then subjected to LC-MS analysis.

Cell culture methods

RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C and 5% CO₂.

IL-6 ELISA

RAW 264.7 cells were seeded using a 48-well plate (2.5 × 10⁴ cells/well). The next day, when cells were ~50% confluent, cells were co-treated with LPS (100 ng/ml) and compound (oat oil extract, FAHFA, 13-HLA, and linoleic acid) in a total volume of 200 μl of medium at 37 °C. After 20 h, the medium was collected, and secreted IL-6 was quantified using the mouse IL-6 ELISA MAXTM Deluxe Kit following the manufacturer's protocol (BioLegend).

Cell proliferation using an MTT assay

After the medium was removed for IL-6 measurements, the remaining cells were assessed for their viability using an MTT assay according to the manufacturer's protocol (Calbiochem). Briefly, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was dissolved in sterile PBS (5 mg/ml) and filtered through a 0.2-μm sterile Millex filter to prepare a 500 μg/ml solution in RPMI 1640 medium. The prepared solution was added to adherent cells and incubated at 37 °C for 4 h. Sterile DMSO was then supplied upon removal of the MTT solution. Relative cell viabilities were quantified using a plate reader at an absorbance of 570 nm.

qRT-PCR

RAW 264.7 cells (6-well plate, 60%–70% confluent) were co-treated with FAHFA and LPS (100 ng/ml) or the equivalent amount of DMSO in cell medium for 20 h. Total RNA was isolated using a Purelink RNA Mini Kit (Thermo Fisher) according to the manufacturer's instructions. RNA from each replicate (2 μg) was reverse-transcribed to double-stranded cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was performed using a LightCycler 480 II

LAHLAs are anti-inflammatory lipids

(Roche) with SYBR Green qPCR Master Mix (Bimake). The qRT-PCR reaction conditions were as follows: 95 °C for 10 min and 35 times (95 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s). The relative quantification of gene expression was calculated using the $2^{-\Delta\Delta C_t}$ approximation method. The primers used in this study were GAPDH, iNOS1 (14), COX2 (14), IL-6 (6), and IL-1 β (6) (the IL-1 β and IL-6 primer sequences were obtained through personal communication): GAPDH forward, 5'-AGG TCG GTG TGA ACG GAT TTG-3'; GAPDH reverse, 5'-TGT AGA CCA TGT AGT TGA GGT CA-3'; COX2 forward, 5'-GGA GAG ACT ATC AAG ATA GT-3'; COX2 reverse, 5'-GGA GAG ACT ATC AAG ATA GT-3'; iNOS forward, 5'-AAT GGC AAC ATC AGG TCG GCC ATC ACT-3'; iNOS reverse, 5'-GCT GTG TGT CAC AGA AGT CTC GAA CTC-3'; IL-6 forward, 5'-AAC CAC GGC CTT CCC TAC TT-3'; IL-6 reverse, 5'-GCC ATT GCA CAA CTC TTT TCT C-3'; IL-1 β forward, 5'-ACC TGG GCT GTC CTG ATG AGA G-3'; IL-1 β reverse, 5'-CCA CGG GAA AGA CAC AGG TAG C-3'.

Targeted LC-MS analysis of FAHFAs

FAHFAs were measured on a TSQ Quantiva LC-MS instrument using multiple reaction monitoring in negative ionization mode as described previously (14). Resolution of FAHFAs was achieved using an Acquity UPLC BEH C18 column (1.7 μ m, 2.1 \times 100 mm, Waters) with a flow rate of 0.2 ml/min and a 93:7 (MeOH:H₂O) mobile phase with 5 mM ammonium acetate and 0.03% ammonium hydroxide. LAHLAs were analyzed by monitoring the precursor-to-product ion transitions m/z 557.5 \rightarrow 279.2 and m/z 557.5 \rightarrow 295.2, which correspond to the parent LAHLA to linoleic acid and LAHLA to hydroxy linoleic acid, respectively.

Pseudo-MS3 for analysis of LAHLA regioisomers

Oat oil (5 μ l) was dissolved in 2:1 CHCl₃:MeOH (400 μ l), and then 5 μ l was subjected to LC-MS analysis. LC separation was achieved using a Gemini 5U C-18 column (Phenomenex). Resolution of LAHLAs was achieved using an Acquity UPLC BEH C18 column (1.7 μ m, 2.1 \times 100 mm, Waters) with a flow rate of 0.2 ml/min with a 93:7 (MeOH:H₂O) mobile phase with 5 mM ammonium acetate and 0.03% ammonium hydroxide. MS analysis was performed using a Thermo Scientific Q Exactive Plus fitted with a heated electrospray ionization source. To facilitate in-source fragmentation, the in-source collision-induced dissociation was set to 60.0 eV. To attain MS² data for HLA, the m/z of the negative HLA ion, m/z 295.227, was added to the inclusion list to of masses to be fragmented.

Targeted LC-MS analysis of HLA enantiomers

HLA enantiomers were measured on a TSQ Quantiva LC-MS instrument in negative ionization mode. Resolution of HLAs was achieved using a Lux Cellulose-3 chiral column (3 μ m, 250 \times 4.6 mm, Phenomenex) with an isocratic flow rate of 0.2 ml/min of 75:25 MeOH:H₂O and 0.1% formic acid solution at 35 °C. HLAs were analyzed by pseudo-multiple reaction monitoring (collision energy of 9 V, radio frequency lens set at 92), monitoring the precursor-to-product ion transition, m/z 295.3 \rightarrow 295.3.

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