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A functional role for eicosanoid-lysophospholipids in activating monocyte signaling

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Recently, eicosanoid-lysophospholipids were identified as novel metabolites generated from the direct cyclooxygenase- or lipoxygenase-catalyzed oxidation of 2-arachidonoyl-lysophospholipids produced from either phospholipase A_1-mediated hydrolysis of diacyl arachidonoyl-phospholipids or through the cytochrome c-catalyzed oxidative hydrolysis of the vinyl ether linkage of arachidonoyl-plasmalogens. Although the metabolic pathways generating eicosanoid-lysophospholipids have been increasingly appreciated, the signaling functions of eicosanoid-lysophospholipids remain largely unknown. Herein, we demonstrate that 2-12(S)-HETE-lysophospholipids as well as nonesterified 12(S)-HETE are potent lipid mediators that activate THP-1 human monocytic cells to generate tumor necrosis factor α (TNFα) and interleukin 8 (IL8). Remarkably, low nanomolar concentrations of 12(S)-HETE-lysophospholipids, but not other oxidized signaling lipids examined activated THP-1 cells resulting in the production of large amounts of TNFα. Moreover, TNFα release induced by 12(S)-HETE-lysophospholipids was inhibited by the TNFα converting enzyme inhibitor TAPI-0 indicating normal processing of TNFα in THP-1 cells stimulated with these agonists. Western blotting analyses revealed that 12(S)-HETE-lysophospholipids activated the phosphorylation of NFκB p65, suggesting activation of the canonical NFκB signaling pathway. Importantly, activation of THP-1 cells to release TNFα was stereoselective with 12(S)-HETE favored over 12(R)-HETE. Furthermore, the EC_{50} of 2-12(S)-HETE-lysophosphatidylcholine in activating THP-1 cells was 2.1 nM, whereas the EC_{50} of free 12(S)-HETE was 23 nM. Additionally, lipid extracts of activated platelets were separated by RP-HPLC demonstrating the coelution of 12(S)-HETE with fractions initiating TNFα release. Collectively, these results demonstrate the potent signaling properties of 2-12(S)-HETE-lysophospholipids and 12(S)-HETE by their ability to release TNFα and activate NFκB signaling thereby revealing a previously unknown role of 2-12(S)-HETE-lysophospholipids in mediating inflammatory responses.

Oxidized lipid second messengers are of critical importance for the homeostatic regulation of multiple physiologic functions that are cell type and context dependent (*e.g.* arthritis, cancer, and cardiovascular disease) (1–4). The biosynthesis of inflammatory lipid mediators, such as prostaglandins, and hy-

droxyeicosatetraenoic acids (HETEs) are crucial for the initiation and amplification of inflammatory processes (5, 6). Specific molecular species of proinflammatory lipid mediators have distinct functions during both the acute phase of the inflammatory response and during its chronic evolution. The canonical pathway for the generation of lipid second messengers is through the regiospecific *sn*-2 hydrolysis of arachidonate-containing phospholipids by phospholipases A_2, which can be activated within seconds during elevation of cellular calcium ion concentrations. This results in the release of nonesterified arachidonic acid (AA) and lysophospholipids (7–11). The released AA is rapidly oxidized by a plethora of intracellular oxidases (*e.g.* cyclooxygenases, lipoxygenases, or cytochromes P450) generating a diverse array of potent signaling molecules (12–16). In contrast, the production of protein inflammatory mediators, such as cytokines, are typically transcriptionally regulated which often requires hours to generate a robust cellular response (17).

Proinflammatory cytokines comprise an extensively characterized class of protein-based modulators of inflammation that are critical participants in inflammation and the immune response (18–20). During bacterial infection, the production of proinflammatory cytokines is activated by bacterial products, such as lipopolysaccharides, lipoproteins, and dsRNA (21–23). These responses are mediated by Toll-like receptors, and distinct endotoxins that serve as ligands for different Toll-like receptors (24). Proinflammatory cytokines have multiple functions that work in concert to facilitate an inflammatory response. In most cases, the inflammatory response is appropriate to combat the infection. However, an excessive response results in chronic inappropriate inflammation that can damage cells and compromise the function of specific organs.

Cytokines and oxidized fatty acids also play pivotal roles in the resolution of inflammation. Anti-inflammatory cytokines primarily function by inhibiting the synthesis of IL1, TNFα, and other major proinflammatory cytokines (25). IL10 is the most important anti-inflammatory cytokine in the human immune system. IL10 is a very potent inhibitor of monocyte/macrophage cytokine synthesis including TNFα, IL-1, IL-6, IL-8, IL-12, granulocyte colony-stimulating factor, MIP-1α, and MIP-2α (26–28). In addition to IL10, IL1ra, IL4, IL6, IL11, IL13, and TGFβ also have anti-inflammatory activities (25). The metabolites of docosahexaenoic acid and eicosapentaenoic acid (*e.g.* resolvins and protectins) are the major lipid mediators that function in the resolution of inflammation (29, 30). Resolvin E1 (RvE1) can inhibit the

infiltration of polymorphonuclear leukocytes at nanomolar concentrations (31). RvE1 also can stimulate the macrophage-mediated phagocytosis of apoptotic polymorphonuclear leukocytes (32), inhibit dendritic cell migration and cytokine release (33), and up-regulate CCR5 expression in leukocytes (34).

Previously, we discovered that calcium-independent phospholipase $A_2\gamma$ (iPLA$_2\gamma$, also known as PNPLA8) is predominantly an *sn*-1 lipase for phospholipids containing a polyunsaturated fatty acyl chain at the *sn*-2 position generating 2-arachidonoyl-lysophospholipids (35). Remarkably, we found that 2-eicosanoid-lysophospholipids including 12(*S*)-HETE-, 15(*S*)-HETE-, 11-HETE-, and PGE$_2$-lysophospholipids have emerged as novel lipid natural products that are synthesized through direct oxidation of 2-arachidonoyl-lysophospholipids by 12-lipoxygenase (12-LOX), 15-lipoxygenase (15-LOX), and cyclooxygenase-2 (COX2) (36, 37). The discovery of the ability of cellular oxidases to oxidize lysophospholipids containing polyunsaturated fatty acids suggested a previously unknown pathway for the synthesis of novel lipid metabolites that could potentially serve as novel effectors of specific biologic signaling processes. Additionally, we demonstrated that eicosanoid-lysophospholipids could serve as effective substrates for cPLA$_2\alpha$ thereby contributing to the release of nonesterified eicosanoids (36). Furthermore, we have recently demonstrated that eicosanoid-lysophos-pholipids could be acylated by a microsomal *sn*-1 acyltransferase activity resulting in the production of oxidized phospholipids that have been suggested to serve as damage associated molecular patterns that have potent effects on multiple cellular processes (38). The scope of the significance of these recently identified oxidized lysophospholipid metabolites, their pathways of synthesis, and their potential signaling properties are only now beginning to be explored.

Herein, we report that 12-LOX–generated products of arachidonic acid and arachidonoyl-lysophospholipids (*i.e.* 12(*S*)-HETE-lysophospholipids, 12(*S*)-HETE, and their corresponding hydroperoxides) can induce the release of TNFα and IL8 from THP-1 human monocytic cells. We provide further evidence that 12(*S*)-HETE-lysophospholipid–induced release of mature cleaved TNFα is mediated by TNFα converting enzyme (TACE), which can be inhibited by its inhibitor TAPI-0. Furthermore, we identify the stereoselectivity of 12(*S*)-HETE *versus* 12(*R*)-HETE in the activation of THP-1 cells, suggesting that 12(*S*)-HETE induced signaling processes are due to specific ligand-receptor interactions. In addition, we show that the NFκB pathway is activated by 12(*S*)-HETE-lysophospholipids. Collectively, these results demonstrate the signaling properties and underlying mechanisms of 12(*S*)-HETE-lysophospholipid- and 12(*S*)-HETE-induced TNFα release from THP-1 cells and identify a previously uncharacterized role of oxidized lysophospholipids in activating immune cells promoting inflammatory cascades.

Results

Stimulation of THP-1 cells with 12-LOX–generated metabolites of arachidonoyl-lysophospholipids and arachidonic acid induce the release of TNFα

To determine the effect of various oxidized lipids in activating THP-1 cells, purified individual oxidized lipids of interest including HETEs, epoxyeicosatrienoic acids (EETs), oxidized lysophospholipids, as well as nonoxidized lipids, including arachidonoyl-lysophospholipids, arachidonic acid, or oleic acid (each at 200 nM concentration), were incubated with THP-1 cells grown in suspension. After 1 h of incubation, the cells were pelleted by centrifugation, and the amount of TNFα in the supernatant was quantified by ELISA. Notably, THP-1 cells released considerable amounts of TNFα in response to 2-12(*S*)-HETE-lysophospholipids (*i.e.* 2-12(*S*)-HETE-lysophosphatidylcholine and 2-12(*S*)-HETE-lysophosphatidylethanolamine) as well as nonesterified 12(*S*)-HETE, but not by other oxidized lipids nor non-oxidized fatty acids (Fig. 1). It is intriguing that 11(*S*)-HETE also induced the release of TNFα, although not as robustly as 12(*S*)-HETE, indicating that the position of the hydroxy group on the fatty acyl chain is critical to the signaling process(es) mediating TNFα release.

12-LOX catalyzes the stereospecific oxidation of AA to generate 12(*S*)-HpETE, which is readily converted to 12(*S*)-HETE by cellular peroxidases (39, 40). Recently, we have demonstrated that 2-arachidonoyl-lysophospholipids are excellent substrates for 12-LOX to generate 2-12(*S*)-HpETE-lysophospholipids, which are similarly converted to 2-12(*S*)-HETE-lysophospholipids (37). In addition, because lysophospholipids exist as one of either two regioisomers (*i.e.* 1-acyl-lysophospholipid or 2-acyl-lysophospholipid) that can be interconverted by internal acyl-migration, we tested each regioisomer of 12(*S*)-H(p)ETE-LPC, 12(*S*)-H(p)ETE-LPE, 15(*S*)-H(p)ETE-LPC, and 15(*S*)-H(p)ETE-LPE (*i.e. sn*-1 *versus sn*-2). Accordingly, THP-1 cells were incubated with 200 nM of the purified 12-LOX– or 15-LOX–generated 1-eicosanoid- or 2-eicosanoid-lysophospholipids (both hydroxy and hydroperoxyl derivatives) (Fig. S1) prior to quantifying the amount of TNFα released from the cells by ELISA. The results indicated that both regioisomers (*sn*-1 and *sn*-2) of 12(*S*)-HETE-lysophospholipids and 12(*S*)-HpETE-lysophospholipids are equally potent in mediating the release of TNFα. In stark contrast, despite their close structural similarity to the 12-LOX–derived eicosanoid-lysophospholipids, 15(*S*)-HpETE-lysophospholipids and 15(*S*)-HETE-lysophospholipid regioisomers as well as nonesterified 15(*S*)-HpETE were ineffective in promoting the release of TNFα from THP-1 cells under the conditions examined.

To determine whether the observed TNFα release was due to cell injury or cell death, lactate dehydrogenase (LDH) activity in the media was used as a marker for cell injury/death. Incubation of the cells for 1 h with 12(*S*)-HETE or 2-12(*S*)-HETE-LPC did not induce measurable LDH release from THP-1 cells, indicating TNFα release is not due to cell death or injury (Fig. 1*B*).

To determine whether the increase in TNFα release was regulated by increased mRNA expression, TNFα mRNA was quantified in the THP-1 cells after incubation with 12(*S*)-HETE or 2-12(*S*)-HETE-LPC. As shown in Fig. S2, 40 min or 2 h of incubation with 12(*S*)-HETE or 2-12(*S*)-HETE-LPC did not significantly increase the expression of TNFα mRNA, indicating that TNFα release under these conditions is not transcriptionally regulated.

Screening of proinflammatory cytokines released by THP-1 cells after incubation with 2-12(S)-HETE-lysophosphatidylcholine (2-12(S)-HETE-LPC)

Next, to determine whether 12(*S*)-HETE-lysophospholipids could induce the release of other proinflammatory cytokines, a
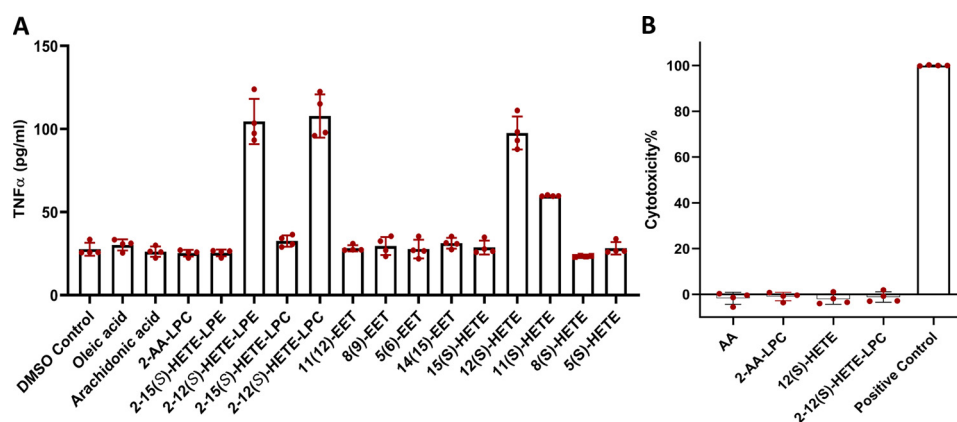
Figure 1. 12(*S*)-HETE-lysophospholipids and 12(*S*)-HETE activate THP-1 cells to release TNFα. After 4 h incubation in serum-free RPMI 1640 media at 37 °C under a 5% CO_2 atmosphere, serum-starved THP-1 cells (1×10^6 cells/ml) were treated with the indicated lipids (200 nM, delivered in dimethyl sulfoxide (DMSO)) or DMSO vehicle alone as control (0.1% v/v) and incubated for 1 h under the same conditions, followed by centrifugation of the cell suspension at $200 \times g$ for 3 min. The TNFα present in the supernatant was quantified by ELISA as described under "Experimental procedures" (*A*). The cytotoxicity of 12(*S*)-HETE and 2-12(*S*)-HETE-LPC were evaluated by measuring the lactate dehydrogenase activity in the supernatant (*B*). 1% Triton X-100 was used as a positive control for the LDH assay. Values are the average of four independent preparations. *Error bars* represent S.D.
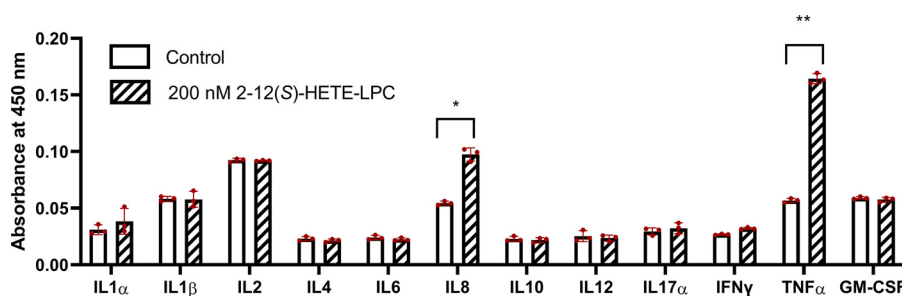

Figure 2. 2-12(*S*)-HETE-lysophosphatidylcholine activates THP-1 cells to release proinflammatory cytokines. After 4 h incubation in serum-free RPMI 1640 media at 37 °C under a 5% CO_2 atmosphere, serum-starved THP-1 cells (1×10^6 cells/ml) were activated by 200 nM 2-12(*S*)-HETE-lysophosphatidylcholine for 1 h under the same conditions in comparison to DMSO vehicle alone (Control). Proinflammatory cytokines present in the supernatant were analyzed by a Multianalyte ELISA Array Kit as described under "Experimental procedures." *, $p < 0.05$; **, $p < 0.01$. Values are the average of four independent preparations. *Error bars* represent S.D.

panel of cytokines was examined including interferon γ (IFNγ), interleukins (ILs), and granulocyte-macrophage colony-stimulating factor (GM-CSF) by an ELISA array. Similar to TNFα, increased amounts of IL8 were also secreted by THP-1 cells after treatment with 200 nM 2-12(*S*)-HETE-lysophosphatidylcholine (Fig. 2). Significant increases in 12(*S*)-HETE-LPC induced release of other proinflammatory cytokines were not observed under the conditions employed.

Dose-response profiles of 12(S)-HETE-lysophospholipids and 12-HETE– mediated activation of THP-1 cells to release TNFα

To determine the relative potencies of the two regioisomers (1-acyl- *versus* 2-acyl-) of 12(*S*)-HETE-LPC and the stereoisomers of 12-HETE that mediate the activation THP-1 cells to release TNFα, various concentrations of 1-12(*S*)-HETE-LPC, 2-12(*S*)-HETE-LPC, 12(*S*)-HETE, and 12(*R*)-HETE were administered to THP-1 cells for 1 h and the amount of TNFα in the media was analyzed by ELISA (Fig. 3). Overall, the oxidized lysophospholipids have 10-fold higher potency (*i.e.* lower EC$_{50}$ values) than the nonesterified oxidized fatty acids examined (Table 1). No significant differences in EC$_{50}$ values for the release of TNFα were observed between 1-12(*S*)-HETE-LPC and 2-12(*S*)-HETE-LPC (Table 1).

Comparisons of the dose-response profiles revealed the ability of 12(*S*)-HETE to induce the release of TNFα from THP-1 cells at lower effective concentrations than 12(*R*)-HETE. Because 12(*S*)-HETE is the naturally occurring stereoisomer generated by 12-LOX (also known as ALOX12), the stereoselectivity of 12(*S*)-HETE in the activation of THP-1 cells suggests that this process is mediated by a specific ligand-receptor interaction rather than by nonspecific effects of fatty acids or oxidized lysophospholipids on membrane dynamics.

Temporal dependence of 12(S)-HETE-lysophospholipid induced release of TNFα

To determine the temporal dependence of TNFα secretion induced by 12-LOX–generated eicosanoid-lysophospholipids, either 2-12(*S*)-HETE-lysophospholipids or 2-12(*S*)-HpETE-lysophospholipids were incubated with THP-1 cells for up to 2 h followed by measurement of TNFα present in the media by ELISA. As shown in Fig. 4*A*, the majority of TNFα was released after 1 h incubation with 10 nM 2-12(*S*)-HETE-LPC, 2-12(*S*)-HpETE-LPC, 2-12(*S*)-HETE-LPE, or 2-12(*S*)-HpETE-LPE with negligible additional TNFα release after a 2 h incubation. The temporal course of TNFα release activated by these 12-LOX–generated eicosanoid-lysophospholipid products were
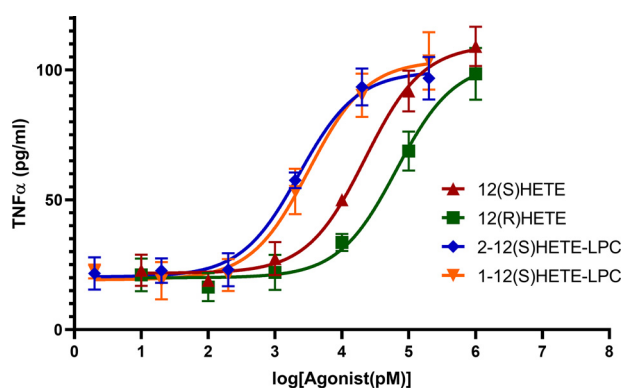
Figure 3. Dose-response profile for 12(*S*)-HETE-lysophospholipids and nonesterified 12-HETE–mediated activation of THP-1 cells to release TNFα. After 4 h of incubation in serum-free RPMI 1640 media at 37 °C under a 5% CO_2 atmosphere, serum-starved THP-1 cells (1×10^6 cells/ml) were treated with 200 nm, 20 nm, 2 nm, 200 pm, 20 pm, 2 pm 1-12(*S*)HETE-LPC or 2-12(*S*)HETE-LPC, or 1 μm, 100 nm, 10 nm, 1 nm, 100 pm, 10 pm 12(*S*)HETE or 12(*R*)HETE. After 1 h of incubation under the same conditions, the samples were centrifuged at 200 × *g* for 3 min, and the amount of TNFα present in the supernatant was determined by ELISA as described under "Experimental procedures." Values are the average of three independent preparations. *Error bars* represent S.D.

indistinguishable. In comparison, TNFα secretion activated by 10 ng/ml of lipopolysaccharide (LPS) continued to increase after 1 h, which contrasts with that observed with the tested oxidized lysophospholipids (Fig. 4*B*). We note that the amount of TNFα released in the presence of 2-12(*S*)-HETE-lysophospholipids after 1 h incubation was ~10% of the total amount of TNFα released after stimulation by 10 ng/ml of LPS.

In addition, we tested TNFα secretion from THP-1 cells after 24 h incubation with either 12(*S*)-HETE-LPC or 12(*S*)-HETE. As shown in Fig. 4*C*, after 24 h incubation, the level of TNFα in the cell media of all tested samples returned to baseline. Results in Fig. 4, *A* and *C*, demonstrated that TNFα levels reached a maximum after 1-2 h incubation with 12(*S*)-HETE or 12(*S*)-HETE-LPC, and then decayed to background levels thereafter. We also tested IL1β secretion from THP-1 cells after 24 h incubation with 12(*S*)-HETE-LPC or 12(*S*)-HETE. As shown in Fig. 4*D*, there was no detectable IL1β secretion from THP-1 cells even after 24 h incubation with 12(S)-HETE or 12(*S*)-HETE-LPC. Collectively, these results demonstrate that 12(*S*)-HETE-lysophospholipids are potent (low nM) stimulators of THP-1 cells, which can rapidly induce (<1 h) the release of TNFα.

2-12(S)-HETE- and 2-12(S)-HpETE-lysophospholipid–induced TNFα secretion is mediated by TACE

The secretion of TNFα from immune cells in response to inflammatory stimuli is mediated by the cleavage of the membrane form of TNFα (25 kDa) by TACE, releasing the soluble form of TNFα (18 kDa). To determine whether 12(*S*)-HETE-lysophospholipid-induced release of TNFα is mediated by TACE, THP-1 cells were preincubated with 1 or 5 μm TAPI-0, a specific TACE inhibitor, prior to treatment with 2-12(*S*)-HETE-LPC, 2-12(*S*)-HpETE-LPC, 2-12(*S*)-HETE-LPE, or 2-12 (*S*)-HpETE-LPE. Pretreatment of THP-1 cells with 1 or 5 μm TAPI-0 substantially decreased TNFα release induced by 12-LOX–generated eicosanoid-lysophospholipids indicating the role of TACE in the processing and secretion of TNFα from

THP-1 cells treated with 12(*S*)-HETE- or 12(*S*)-HpETE-lysophospholipids (Fig. 5).

12(S)-HETE- and 12(S)-HpETE-lysophospholipids induce production of the membrane-bound form of TNFα in THP-1 cells

To substantiate the expression level of the membrane-bound form of TNFα after stimulation with 12-LOX–generated eicosanoid-lysophospholipids, THP-1 cells were treated with various non-oxidized lipids or 12-LOX–generated metabolites (Fig. 6). After 1 h of incubation at 37 °C, THP-1 cells were centrifuged, and the cell pellets were resuspended in lysis buffer for Western blotting analysis of the soluble and membrane forms of TNFα. Incubation of THP-1 cells with 10 nM 12(*S*)-HETE-lysophospholipids, 12(*S*)-HpETE-lysophospholipids, or nonesterified 12(*S*)-HETE resulted in a significant increase in the level of the membrane form TNFα (25 kDa) as well as its active proteolytic product, TNFα (18 kDa). In sharp contrast, non-oxidized 2-arachidonoyl-lysophospholipids or free arachidonic acid did not affect the protein expression level of the membrane form of TNFα (Fig. 6).

12(S)-HETE- and 12(S)-HpETE-lysophospholipids activate the nuclear factor κB (NFκB) pathway in THP-1 cells

The transcription factor NFκB serves as a central mediator in many inflammatory responses (41). The activation of the NFκB pathway induces the expression of multiple proinflammatory genes including cytokines and chemokines, as well as regulation of the inflammasome (42). In addition, NFκB plays a critical role in determining cell survival and the activation or differentiation of innate immune cells (43, 44). To determine whether the NFκB pathway is activated by 2-12(*S*)-HETE-lysophospholipids or 12(*S*)-HETE, the relative contents of phospho-NFκB p65 and phospho-IκB in control and stimulated THP-1 cells were determined by Western blotting analysis. In the presence of 10 nM 2-12(*S*)-HETE-lysophospholipids or 2-12 (*S*)-HpETE-lysophospholipids, the phosphorylation of NFκB p65 is substantially increased, indicating activation and nuclear translocation of NFκB (Fig. 7*A*). Compared with the tested oxidized lysophospholipids, 10 nM nonesterified 12(*S*)-HETE and 12(*S*)-HpETE are less potent in activating the phosphorylation of NFκB p65 or IκB, whereas non-oxidized arachidonoyl-lysophospholipids or arachidonic acid did not have any measurable effect on NFκB p65 or iκB phosphorylation.

To substantiate the difference between 10 nM 2-12(*S*)-HETE-LPC and 10 nM 12(*S*)-HETE in activating THP-1 cells, 10 nM 2-12(*S*)-HETE-LPC, 10 nM 12(*S*)-HETE, or DMSO control (0.1%, v/v) were incubated with THP-1 cells in triplicate. The cells were pelleted, lysed, and the phosphorylation of NFκB p65 was analyzed by Western blotting analysis. The results show that 10 nM 2-12(*S*)-HETE-LPC induces greater overall phosphorylation of NFκB p65 (normalized to β-tubulin) compared with 10 nM 12(*S*)-HETE, indicating that 12(*S*)-HETE-LPC is more potent in activating THP-1 cells compared with nonesterified 12(*S*)-HETE (Fig. 7, *C* and *D*). Collectively, these results demonstrate that 12(*S*)-HETE- and 12(*S*)-HpETE-lysophospholipids activate the canonical NFκB

Table 1

Half-maximal effective concentrations (EC$_{50}$) of 12(*S*)-HETE-lysophosphatidylcholine regioisomers and 12-HETE in activating THP-1 cells to release TNFα

The data from Fig. 3 were analyzed by nonlinear regression and the EC$_{50}$ values were determined as described under "Experimental procedures."

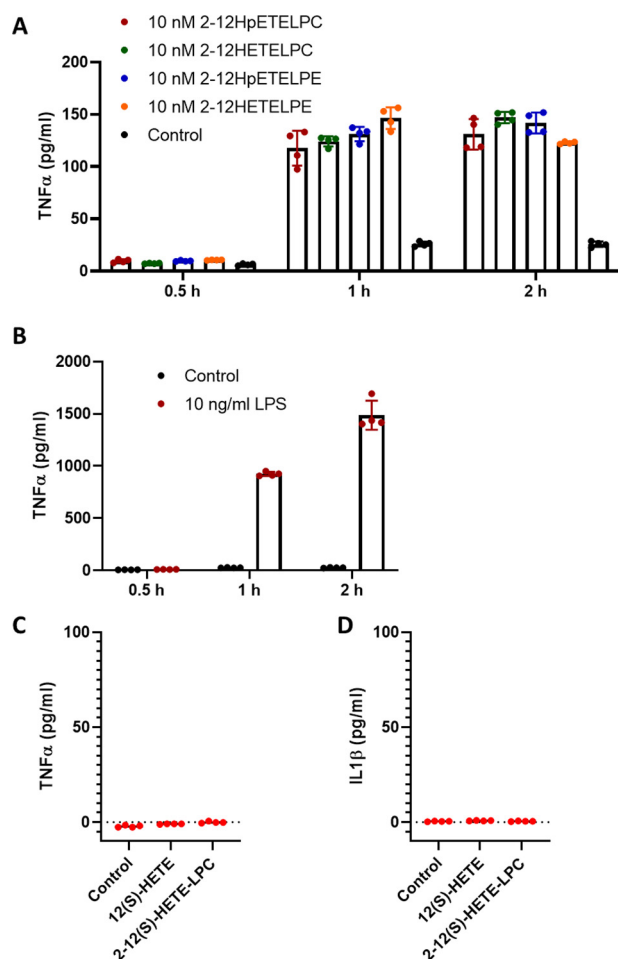| Agonist	12(*S*)-HETE	12(*R*)-HETE	2-12(*S*)-HETE-LPC	1-12(*S*)-HETE-LPC
EC$_{50}$ (best fit)	22 nM	65 nM	2.3 nM	3.2 nM
EC$_{50}$ (95% confidence interval)	14-34 nM	36-112 nM	1.5-3.5 nM	1.9-5.6 nM

Figure 4. Time-dependent release of TNFα from THP-1 cells activated by 12(*S*)-H(p)ETE-lysophospholipids or 12(*S*)-HETE. THP-1 cells (1 × 10^6 cells/ml) were serum-starved in serum-free RPMI 1640 media for 4 h at 37 °C under a 5% CO$_2$ atmosphere prior to incubation with the indicated oxidized-lysophospholipids (10 nM each) (*A*) or 10 ng/ml of O55:B5 *Escherichia coli* LPS (*B*) for 0.5, 1, or 2 h. DMSO vehicle alone (0.1% v/v) served as control for each experiment. After incubation, the samples were centrifuged at 200 × *g* for 3 min, and the amount of TNFα in the supernatant was determined by ELISA as described under "Experimental procedures." Cells were also incubated with the indicated oxidized lipids for 24 h prior to measurement of TNFα (*C*) and IL1β (*D*) by ELISA. Values are the average of four independent preparations. *Error bars* represent S.D.

Figure 5. 12(*S*)-HETE-lysophospholipid–induced release of TNFα is mediated by TACE. THP-1 cells (1 × 10^6 cells/ml) were serum-starved in serum-free RPMI 1640 media for 4 h at 37 °C under a 5% CO$_2$ atmosphere prior to incubation with the indicated oxidized lysophospholipids (10 nM each) or DMSO vehicle (0.1%, v/v) alone as control in the absence or presence of different concentrations of the TACE inhibitor TAPI-0. After 1 h of incubation under the same conditions, the samples were centrifuged at 200 × *g* for 3 min and the TNFα present in the supernatant was quantified by ELISA as described under "Experimental procedures." Values are the average of four independent preparations. *Error bars* represent S.D.

source of eicosanoids and other potent oxidized lipid mediators of inflammation (45, 46), we treated isolated mouse platelets with thrombin, extracted lipids from the thrombin-activated platelets, and resolved the extracted lipids on a C18 HPLC column (Fig. 8*A*). Separate eluent fractions were then collected, dried under nitrogen stream, resuspended in serum-free media, and incubated with THP-1 cells. Of the tested fractions, only fraction 4 from the HPLC-separated lipid extract of thrombin-activated platelets was able to appreciably stimulate THP-1 cells to release TNFα (Fig. 8*C*). Next, fraction 4 from the first purification step was further fractionated (resolved) by collecting smaller volume fractions of the column eluent (Fig. 8*B*). These eluent fractions were then dried, resuspended in serum-free media, and incubated with THP-1 cells. As shown in Fig. 8*D*, only fractions 4-5 and 4-6 were capable of activating THP-1 cells to release TNFα.

To identify the molecule(s) in fraction 4-5 responsible for the activation of THP-1 cells, the lipids in fraction 4-5 were analyzed by LC–MS. It was demonstrated that 12(*S*)-HETE is the major species in this fraction (Fig. 8*E*). The identity of 12(*S*)-HETE was substantiated by *N*-(4-aminomethylphenyl)-pyridinium (AMPP) derivatization and comparison of the obtained tandem mass spectra with a commercial 12(*S*)-HETE standard (Fig. 8*F*). Next, the 12(*S*)-HETE content in each fraction was analyzed by LC-MS/MS, which showed that only fraction 4 (from the initial purification), fraction 4-5, and fraction-4-6 (further resolved fractions from fraction 4) contained 12(*S*)-

signaling pathway for the transcriptional regulation of inflammatory responses in immune cells.

Activation of THP-1 cells by 12(S)-HETE produced by thrombin-activated platelets

In addition to testing various known oxidized signaling lipids (including HETEs, EETs, HETE-lysophospholipids, and HpETE-lysophospholipids), we sought to identify other potentially unknown oxidized lipid mediators capable of activating THP-1 cells. Because activated platelets are a well-established
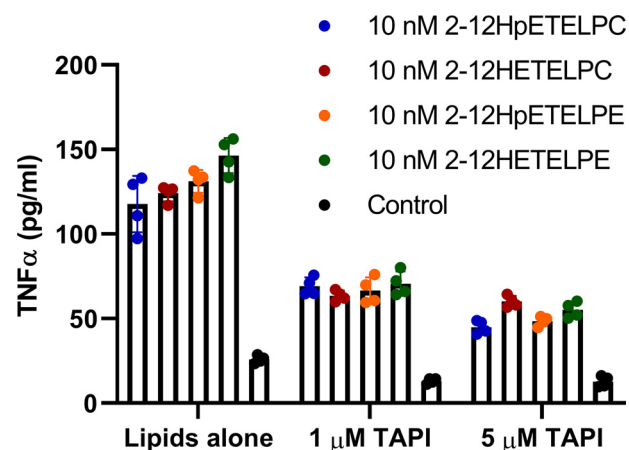
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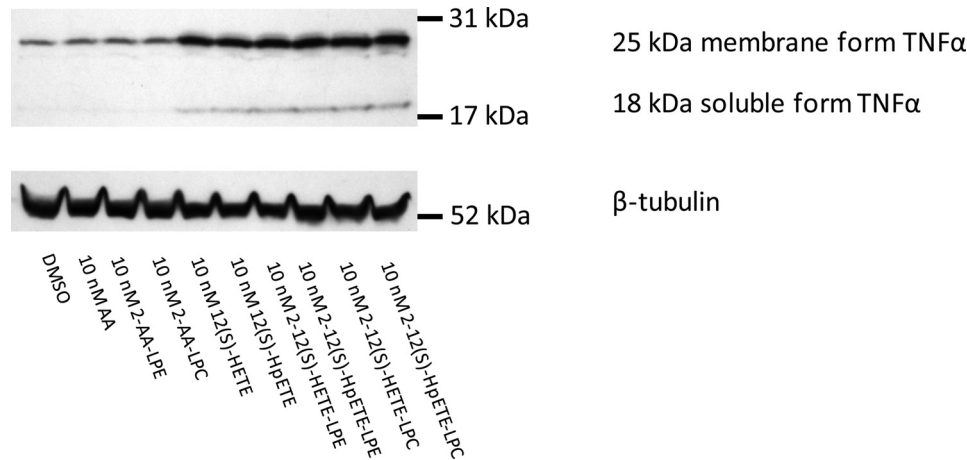*J. Biol. Chem.* (2020) 295(34) 12167–12180 **12171**

Figure 6. 12(*S*)-HETE-lysophospholipid–induced production of the membrane–associated form of TNFα and the soluble form of TNFα protein. THP-1 cells (1 × 10⁶ cells/ml) were serum-starved in serum-free RPMI 1640 media for 4 h at 37 °C under a 5% CO_2 atmosphere prior to treatment with the indicated lipids or oxidized lysophospholipids (10 nᴍ each) or DMSO vehicle alone (0.1%, v/v) as control. After 1 h of incubation under the same conditions, the samples were centrifuged at 200 × *g* for 3 min. The resultant cell pellets were lysed in cell lysis buffer. Proteins in the cell lysates were separated by SDS-PAGE and the amount of TNFα (25-kDa membrane associated and 18-kDa soluble forms) present in each sample were determined by Western blotting analysis as described under "Experimental procedures." The same blot was probed with an antibody against β-tubulin, which served as a loading control.
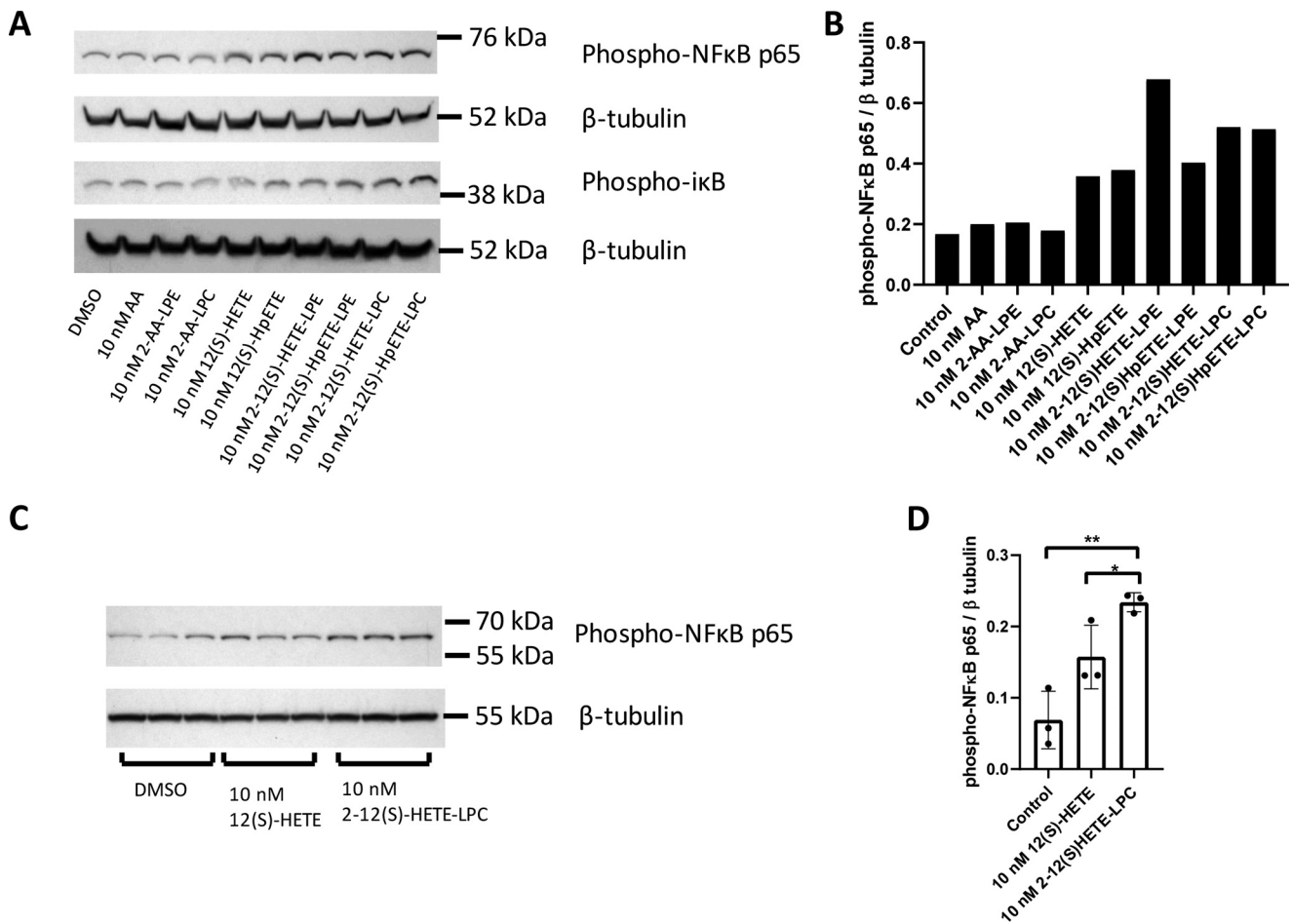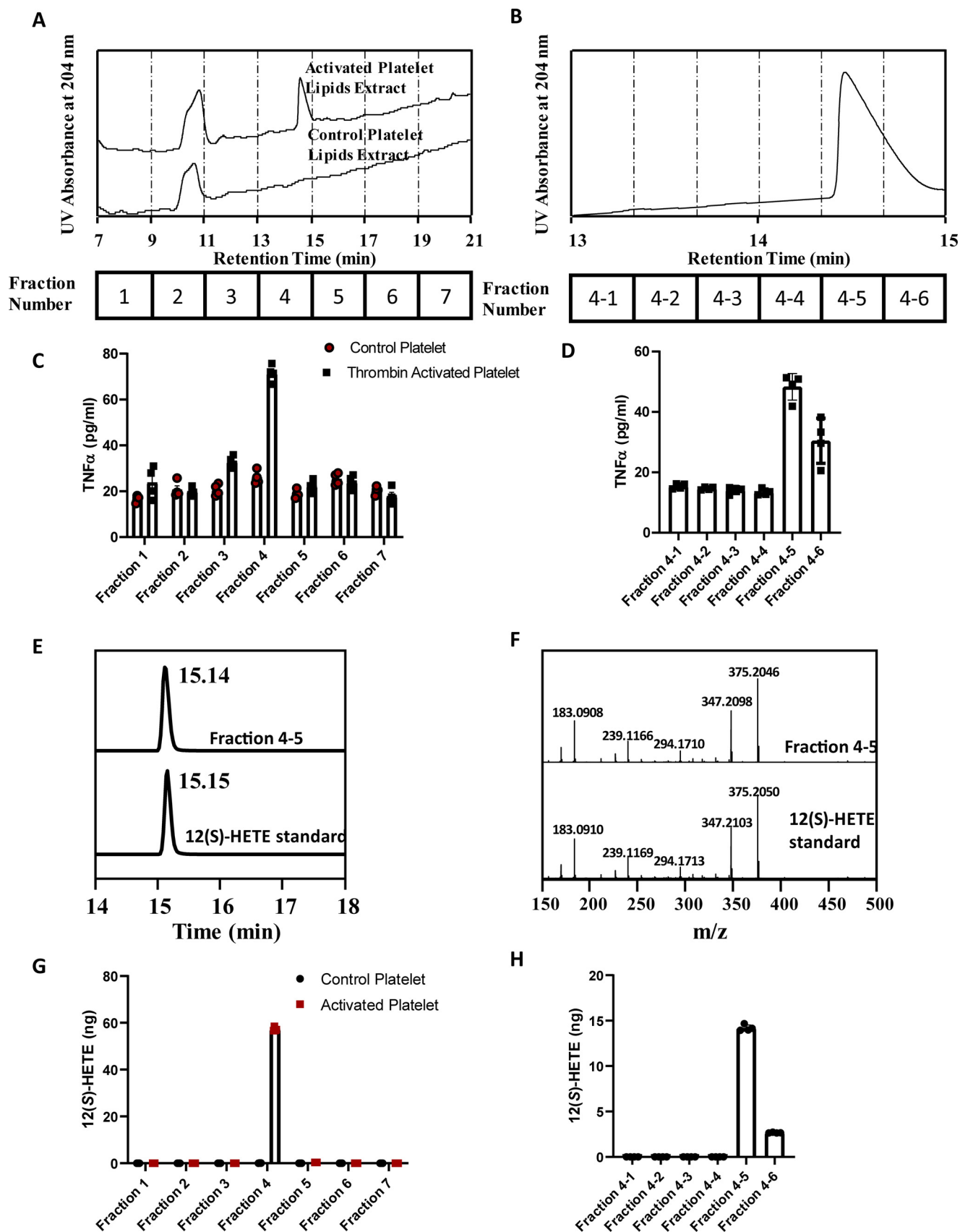

Figure 7. 12(*S*)-HETE-lysophospholipids activate the NFκB pathway in THP-1 cells. THP-1 cells (1 × 10⁶ cells/ml) were serum-starved in serum-free RPMI 1640 media for 4 h at 37 °C under a 5% CO_2 atmosphere prior to treatment with the indicated lipids or oxidized lysophospholipids (10 nᴍ each) or DMSO vehicle alone (0.1% v/v). After 1 h of incubation under the same conditions, the cell suspensions were centrifuged at 200 × *g* for 3 min. The cell pellets were then lysed in cell lysis buffer prior to separation of cellular proteins by SDS-PAGE and transfer to a PVDF membrane. *A*, phospho-NFκB p65, phospho-IκB, and β-tubulin were probed by Western blotting as described under "Experimental procedures." *B*, densitometric quantitation of NFκB p65 phosphorylation based on the blot shown in *panel A*. *C*, comparison of NFκB p65 phosphorylation in cells treated with DMSO, nonesterified 12(*S*)-HETE, or 2-12(*S*)-HETE-LPC performed in triplicate. *D*, densitometric quantitation of NFκB p65 phosphorylation based on the blot shown in *panel C*. *, $p < 0.05$; **, $p < 0.01$. The corresponding blots were probed with an antibody against β-tubulin, which served as a loading control. In *panel C*, values are the average of three independent preparations. *Error bars* represent S.D.

HETE (Fig. 8, *G* and *H*), which is consistent with the ability of these C18 HPLC fractions to activate THP-1 cells to release TNFα.

The distribution of 12(*S*)-HETE or 12(*S*)-HETE-lysophospholipids is likely cell-type and context dependent. 12(*S*)-HETE is the major eicosanoid product in activated platelets because platelets have high levels of cPLA$_2$α, 12-lipoxygenase, and relatively low levels of iPLA$_2$γ (39). Furthermore, the lysophospholipase activity of cPLA$_2$α is higher than its phospholipase activity. Thus, even if lysolipids were produced, it is unlikely they would be observed because cPLA$_2$α is an excellent *sn*-2 acyl lysophospholipase (47) and the lysophospholipid product would likely be hydrolyzed before it can accumulate (47, 48). Collectively, these results demonstrate that 12(*S*)-HETE is the major proinflammatory lipid mediator produced by thrombin-activated platelets that can stimulate THP-1 cells to release TNFα.

Discussion

In this study, we report that 12(*S*)-H(p)ETE-lysophospholipids as well as nonesterified 12(*S*)-H(p)ETE activate THP-1 cells to release TNFα in a dose- and time-dependent manner. Importantly, this process is stereoselective and TNFα release is blocked by pharmacologic inhibition of TACE. Furthermore, 12(*S*)-H(p)ETE-lysophospholipids and nonesterified 12(*S*)-HETE activate the phosphorylation of NFκB p65, indicating the transcriptional activation of inflammatory programs in THP-1 cells. Collectively, these findings demonstrate that 12(*S*)-HETE, 12(*S*)-HETE-lysophospholipids, and their corresponding hydroperoxides are potent lipid agonists that promote the activation of THP-1 cells to release TNFα and underscore the importance of specific oxidized lipids as mediators of inflammation.

Calcium-independent phospholipase A$_2$γ (iPLA$_2$γ, also known as PNPLA8) is a critical membrane-associated calcium-regulated phospholipase known to participate in mitochondrial signaling and bioenergetics (4, 49). This enzyme has the unusual property of catalyzing both *sn*-1 and *sn*-2 hydrolysis of phospholipids containing a saturated or monounsaturated fatty acyl chain at the *sn*-2 position. In sharp contrast, when polyunsaturated aliphatic constituents (*e.g.* arachidonic acid) are present at the *sn*-2 position, iPLA$_2$γ catalyzes the highly regioselective cleavage of phospholipids at the *sn*-1 position resulting in the generation of 2-arachidonoyl-lysophospholipids (35). In addition, we have demonstrated that cytochrome *c* can initiate the oxidative cleavage of the vinyl ether bond in plasmalogens under oxidative stress, generating 2-arachidonoyl-lysophospholipids and α-hydroxy aldehydes (50). Importantly, the unanticipated regiospecificity of iPLA$_2$γ and plasmalogenase

activity of cytochrome *c* represent previously unknown convergent metabolic pathways resulting in the generation of 2-AA-LPC and 2-arachidonoyl-lysophosphatidylethanolamine (2-AA-LPE), which serve as a central branch point metabolites for the production of eicosanoid-lysophospholipids by cellular oxidases.

In previous work, we have demonstrated that 2-arachidonoyl-lysophospholipids can be directly oxidized by cyclooxygenase 2, 12-lipoxygenase, or 15-lipoxygenase, generating PGE$_2$-lysophospholipids, 12(*S*)-HpETE-lysophospholipids, or 15(*S*)-HpETE-lysophospholipids, respectively (36, 37). In this study, we have demonstrated that 2-12(*S*)-HETE-lysophospholipids can serve as signaling molecules that induce monocyte cells to release TNFα and activate the NFκB pathway, indicating potential signaling functions of eicosanoid-lysophospholipids in inflammation and the immune response. The dose-response profiles indicate increased affinity of eicosanoid-lysophospholipids to initiate these signaling pathways compared with their nonesterified counterparts, suggesting the biologic importance of an iPLA$_2$γ/cyt *c* → 2-AA-LPC → 2-12(*S*)-HETE-LPC signaling axis as shown in Fig. 9.

It is well-established that cytokines can promote the synthesis of inflammatory lipid mediators through the transcriptional activation of phospholipases and cyclooxygenase (51). For example, treatment with IL1 in combination with TNFα results in the elevated expression of cPLA$_2$α and cyclooxygenase-2, increasing the production of prostaglandins and HETEs thereby establishing an autoamplification network that is cell-specific and context-dependent (51–57). Molina-Holgado *et al.* (51) suggested that IL1β-induced PGE$_2$ synthesis is mediated by PKC and mitogen-activated protein kinases in murine astrocytes. Fournier *et al.* (52) demonstrated the critical function of cAMP in TNFα-activated PGE$_2$ production in murine macrophages. Additionally, Nakao *et al.* (54) showed that TNFα-induced PGE$_2$ release in human fibroblasts is controlled by NFκB.

Lipid second messengers such as prostaglandins and thromboxanes typically function by binding to a family of G protein–coupled receptors, resulting in increased cAMP, calcium flux, and/or inositol triphosphate concentrations (6). It has also been demonstrated that lipid second messengers can modulate the expression of proinflammatory cytokines. For example, it was previously shown that exogenous PGE$_2$ activated the production of IL6 and TNFα in murine peritoneal macrophages (58, 59).

Previously published work using genetically engineered mice has suggested the relationship between 12/15-LOX and inflammation. For example, Middleton and co-workers (60) found that 12/15-LOX–deficient macrophages produced reduced

Figure 8. Activation of TNFα production by lipids extracted from activated platelets. Mouse platelets were isolated and activated by thrombin. After platelet activation, lipids were extracted using chloroform/methanol/water (1:1:1, v/v/v) and purified using C18 HPLC. Eluent fractions of were collected as shown and used to stimulate THP-1 cells as described under "Experimental procedures." *A*, UV absorbance chromatogram of lipid extracts resolved by C18 HPLC from control and thrombin-activated platelets. Indicated eluent fractions were collected to test their ability to activate THP-1 cell to release TNFα. *B*, UV absorbance chromatogram of lipids in fraction 4 with collected subfractions (4-1 through 4-6). *C* and *D*, fractions from the first (*A*) and second (*B*) purification were dried by a flow of nitrogen, resuspended in RPMI 1640 media, and incubated with THP-1 cells (1 × 10⁶ cells/ml) for 1 h. The TNFα released was quantified by ELISA. *E* and *F*, the identity of 12(*S*)-HETE in fraction 4-5 was confirmed by comparison of extracted ion retention time (*E*) and MS/MS spectra (*F*) with authentic 12(*S*)-HETE standard as indicated. *G* and *H*, 12(*S*)-HETE in fractions from the first (*A*) and second (*B*) purification were quantified by LC-MS/MS. For *C*, *D*, *G*, and *H*, values are the average of four independent preparations. *Error bars* represent S.D.
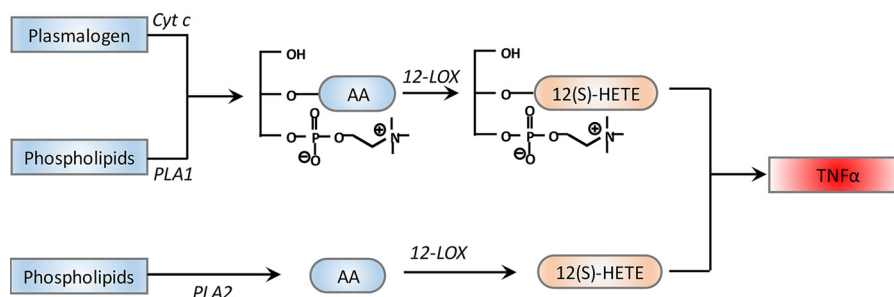
Figure 9. Synthesis of 2-12(*S*)-HETE-lysophospolipids and 12(*S*)-HETE. 2-Arachidonoyl-lysophospholipids are generated by PLA₁-mediated hydrolysis of phospholipids or by cytochrome *c*- catalyzed oxidative cleavage of plasmalogens. 2-Arachidonoyl-lysophospholipids can be oxidized by 12-LOX, generating 2-12(*S*)-HETE-lysophospholipids. Nonesterified 12(*S*)-HETE is synthesized by sequential PLA₂-catalyzed hydrolysis of arachidonoyl-phospholipids and 12-LOX catalyzed oxidation of the released arachidonic acid (AA).

levels of cytokines in response of lipopolysaccharide. Additionally, Dioszeghy *et al.* (61) reported that peritoneal lavage of 12/15-LOX$^{-/-}$ mice showed decreased levels of several cytokines including TNFα, indicating the involvement of 12/15-LOX in peritoneal macrophage cytokine production. Wen *et al.* (62) showed that 12(*S*)-HETE and 12(*S*)-HpETE can induce a mild increase of proinflammatory cytokines' mRNA in macrophages. To the best of our knowledge, there are only a few studies that have investigated the regulation of proinflammatory cytokines by 12(*S*)-HETE and those studies have quantified cytokine mRNA but not increases in cytokine protein amounts. The present results demonstrate the regiospecific and stereospecific potency of 2-12(*S*)-HETE-lysophospholipids and 12(*S*)-HETE in activating THP-1 cells to synthesize/release TNFα and IL8 and promote downstream signaling pathways of inflammation.

At least three different mechanisms for secretion of cytokines have been described in innate immune cells. First, as expected, cytokine protein synthesis can be controlled by transcriptional increases in cytokine mRNA (63, 64). The transcriptional alterations resulting in cytokine synthesis through this mechanism usually requires hours for synthesis. Second, many innate immune cells contain preformed cytokines, which are stored in secretory vesicles or granules (65–67). These preformed cytokines may be released within minutes upon stimulation along the secretory pathway. The third mechanism involves translational regulation in which the mRNA encoding the target protein is present but not translated due to binding of an inhibitory polynucleotide to the translation initiation site that can be removed. After cellular activation, the inhibitor detaches from its cognate mRNA. Cells including macrophages and dendritic cells do not have typical secretory granules that can store preformed cytokines (68). Thus, they employ this process now termed constitutive exocytosis where the cells contain a pool of constitutively transcribed cytokine mRNA that is not translated. Upon cellular stimulation, cytokine mRNAs are "activated"/disinhibited and rapidly translated to produce cytokines that can be detected in the Golgi apparatus within 20 min and released within 1 h (69). Based on our results that increased TNFα can occur in 1 h, the mRNA of TNFα is not increased, and the membrane form TNFα was newly synthesized after activation, we believe that TNFα secretion is mediated by disinhibition of previously synthesized mRNA where it can be translationally activated. Through this process, TNFα can be quickly synthesized from existing mRNA and trafficked through the Golgi apparatus for secretion.

Although the ability of 2-12(*S*)-HETE-lysophospholipids and 12(*S*)-HETE to initiate cellular signaling is described here, the receptor(s) that mediate the downstream processes resulting in increased TNFα synthesis and secretion remains to be discovered. It has been suggested that G protein–coupled receptor 31 (GPR31) is a potential target of 12(*S*)-HETE. In previous work, Honn *et al.* (70) identified GPR31 as the receptor for 12(*S*)-HETE and demonstrated that the membrane fractions from cells expressing GPR31 have high affinity binding sites for 12(*S*)-HETE. Zhang *et al.* (74) suggest that GPR31 mediated the hepatic ischemia-reperfusion injury caused by 12-lipoxygenase and 12(*S*)-HETE. Accordingly, the observed effects likely emanate through the GPR31 receptor, but other contributing mechanisms remain possible. Collectively, these results demonstrate the cellular signaling potential of 12(*S*)-HETE-lysophospholipids and 12(*S*)-HETE as assessed by the induced release of TNFα from human monocytic cells and reveal a previously uncharacterized role of 12(*S*)-HETE-lysophospholipids and 12(*S*)-HETE in promoting inflammatory responses.

Experimental procedures

Materials

Plasmenyl-SAPE and plasmenyl-SAPC were obtained from Avanti Polar Lipids (Alabaster, AL). Kinetex 5 μM EVO C18 column (250 × 4.6 mm) and Kinetex 2.7 μM EVO C18 column (150 × 2.1 mm) were purchased from Phenomenex (Torrance, CA). Oxidized fatty acids (HETEs and EETs), LDH cytotoxicity assay kit, and AMP+ MaxSpec Kit were purchased from Cayman Chemical (Ann Arbor, MI). Human TNFα uncoated ELISA kit, Pierce BCA protein assay kit, Halt protease/phosphatase inhibitor mixture, NuPAGE LDS sample buffer, Novex WedgeWell Tris glycine precast gel, horseradish peroxidase-linked anti-mouse IgG antibody, TRIzol reagent, Powerup SYBR green master mix, SuperScript III First-Strand Synthesis SuperMix, and 0.45 μM PVDF membrane were purchased from ThermoFisher Scientific. Phospho-NF-κB p65 (Ser-536) (93H1) rabbit mAb, phospho-IkBα rabbit mAb, TNFα rabbit mAb, and horseradish peroxidase-linked anti-rabbit IgG antibody were purchased from Cell Signaling Technologies. β-Tubulin mouse antibody was purchased from Santa Cruz. Human proinflammatory cytokines MultiAnalyte ELISA Array

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Kit was purchased from Qiagen. LC–MS grade acetonitrile and water were obtained from Fisher Scientific (Pittsburgh, PA). LC–MS grade methanol and isopropyl alcohol were purchased from Burdick & Jackson (Muskegon, MI). HPLC grade methanol and acetonitrile were purchased from Fisher Scientific (Pittsburgh, PA). RPMI 1640 media and fetal bovine serum were purchased from ThermoFisher Scientific. The THP-1 cell line was purchased from American Type Culture Collection. All other chemicals were purchased from Sigma Aldrich.

General animal studies

Animal protocols were conducted in strict accordance with the National Institutes of Health guidelines for humane treatment of animals and were reviewed and approved by the Animal Studies Committee of Washington University.

Activation of THP-1 cells by oxidized lipid agonists

THP-1 cells were grown in suspension in RPMI 1640 media supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 50 μM 2-mercaptoethanol. The cells were cultured in Corning T75 nontreated flasks (431464U) in an upright position under a 5% CO_2 atmosphere at 37 °C. The cells were routinely split at a 1:2 ratio when the cell density reached 6×10^5 cells/ml. When subculturing the cells, the cell suspension in growth media was centrifuged at $250 \times g$ for 7 min. The supernatant was then discarded, and the cell pellet was resuspended in fresh growth media.

For experiments examining activation by various lipid agonists, THP-1 cells in suspension were centrifuged at $250 \times g$ for 7 min prior to resuspending the cell pellet in serum-free RPMI 1640 media without supplements and incubated for 4 h at 37 °C under a 5% CO_2 atmosphere. After serum starvation, the cell suspension was centrifuged again at $250 \times g$ for 7 min and resuspended in fresh serum free RPMI 1640 media without supplements. The cell suspension was then dispensed into test tubes containing DMSO stock solutions of each lipid agonist or DMSO vehicle alone (0.1%, v/v). After adding the cell suspension, the test tubes were gently shaken while being incubated for the indicated times at 37 °C under a 5% CO_2 atmosphere. At the end of the incubation period, the test tubes were gently shaken again to thoroughly mix the media prior to centrifugation at $250 \times g$ for 3 min. An aliquot of the resultant supernatant (100 μl) from each separate sample was transferred to a 96-well–plate coated with a capture antibody for ELISA analysis.

ELISA analysis of cytokines

ELISA to quantify specific cytokines were performed as described by the manufacturer. Briefly, the capture antibody was diluted in coating buffer, and 100 μl of capture antibody solution was added to each well of a high binding 96-well–plate. The plate was incubated at 4 °C overnight and then washed three times with washing buffer (PBS, pH 7.4, 0.05% Tween-20). Blocking solution (200 μl) was added to each well and incubated at room temperature for 1 h. After aspiration of the blocking solution, the wells were then washed once prior to addition of 100 μl of either test sample or cytokine standard. The plate was then sealed with a plate cover and incubated at

4 °C overnight. After removal of the well contents by aspiration, the wells were washed three times. Next, the diluted biotin-conjugated detection antibody (100 μl) was added and the plate was incubated at room temperature for 1 h. The contents of the wells were aspirated, and plate wells were then washed three times. Avidin-conjugated horseradish peroxidase (100 μl) was next added and the plate was then incubated at room temperature for 40 min. Contents of the wells were then aspirated, and the plate was washed seven times. Following the addition of tetramethylbenzidine substrate (100 μl) and incubation at room temperature for 15 min, 1 M phosphoric acid (50 μl) was added to terminate the reaction prior to measurement of the absorbance at 450 nm.

Western blotting analysis

THP-1 cells (2×10^6 cells) were incubated with individual lipid agonists as described under "Activation of THP-1 cells by lipid agonists." After incubation for the indicated times, the cell suspension was centrifuged at $250 \times g$ for 3 min. The supernatant was carefully aspirated to avoid disturbing the cell pellet. The cell pellet was then suspended in 200 μl of cell lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.4) containing $1\times$ Halt™ protease and phosphatase inhibitor mixture (Thermo Scientific). The cell lysate was sonicated with a probe sonicator at 5×1 s (30% power) and centrifuged at $3000 \times g$ for 3 min. The supernatant was collected, and the protein content was assayed by a BCA method as described in manufacturer's manual (Pierce™ BCA protein assay kit, Thermo Scientific). 30 μg of protein of each sample was mixed with 12 μl of $4\times$ NuPAGE™ LDS sample buffer (Thermo Scientific) and 5 μl of 1 M DTT. The sample mixture was boiled for 3 min prior to loading onto an SDS-PAGE gel. After running the gel, the separated proteins were transferred to a 0.45 μM PVDF membrane by electroelution. The membrane was then briefly washed with TBS containing 0.1% Tween 20 (TBST buffer) and incubated with blocking buffer (5% nonfat dry milk in TBST buffer) for 1 h. After blocking, the membrane was washed $3\times$ 10 min in TBST buffer and incubated with a primary antibody (1:1000 dilution of the primary antibody in TBST buffer containing 2% BSA) for 1 h. After incubating with the primary antibody, the membrane was washed $3\times$ 10 min with TBST buffer and incubated with a horseradish peroxidase-conjugated secondary antibody (1:2000 dilution of antibody in TBST buffer containing 5% nonfat dry milk) for 40 min. The membrane was washed $4\times$ 10 min with TBST buffer, incubated with ECL substrate and exposed to X-ray film. For some experiments, immunoblot band intensities were quantified by densitometry using a Kodak Image Station with 2D software.

LDH cytotoxicity assay

The LDH assay was performed according to manufacturer's instructions. Briefly, 100 μl of cell culture media from each sample was added to a 96-well–plate. For the positive control sample, THP-1 cells were incubated in RPMI 1640 media containing 1% Triton X-100 for 1 h. 100 μl of LDH reaction reagent (NAD^+, lactic acid, iodonitrotetrazolium, and diaphorase) was added into each well and the 96-well–plate was incubated at

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37 °C for 30 min. The absorbance at 490 nm (A490) was read with a plate reader. The cytotoxicity was calculated as (sample A490 – control A490)/(positive control A490 – control A490).

Quantitative RT-PCR

qRT-PCR was performed as described previously ([71]). Briefly, total RNA was extracted using TRIzol reagent. cDNA was synthesized using Super-Script III First-Strand Synthesis SuperMix. The resultant cDNA was amplified by RT-PCR (Applied Biosystems) using Powerup SYBR Green master mix and primers (TNFα and GAPDH) purchased from IDT. The following equations were used for normalization: $\Delta C_T = C_T^{TNF\alpha} - C_T^{GAPDH}$, $\Delta\Delta C_T = \Delta C_T^{Activation} - \Delta C_T^{Control}$, and fold-change = $2^{\Delta\Delta C_T}$.

Expression and purification of recombinant human 12-LOX

100 ml of Sf9 cells (cultured in suspension at 1.2×10^6 cells/ml) were infected with baculovirus (multiplicity of infection = 4) encoding recombinant human N-terminal-(His)$_6$-12-LOX and incubated for 48 h at 27°C. Harvested cells were centrifuged at $250 \times g$ for 10 min and washed once with ice-cold PBS. The cell pellet was then resuspended in 2 ml of ice-cold $0.1\times$ PBS and incubated on ice for 5 min. Next, 2 ml of equilibration buffer ($2\times$ PBS containing 10 mM imidazole) containing 40 μl of 0.1 M phenylmethylsulfonyl fluoride and 2 μl of 10 mg/ml of leupeptin were added, vortexed, and the mixture was initially sonicated with 20×1 s pulses at 50% power (Sonics VibraCell sonicator). Following the addition of 10 μl of DNase I and 40 μl of 20% Tween-20, the cell lysate was again sonicated with $10\times$ 1-s pulses at 50% power and incubated on ice for 5 min. The lysate was centrifuged at $15,000 \times g$ for 15 min and the resultant supernatant (3.6 ml) was collected and mixed with an equal volume of $3\times$ PBS prior to loading onto a 1-ml HisPur nickel-nitrilotriacetic acid column previously equilibrated with 20 ml of equilibration buffer. The column was then washed with 20 ml of wash buffer (PBS with 25 mM imidazole) and the recombinant human 12-lipoxygenase was eluted with elution buffer ($2\times$ PBS with 200 mM imidazole). The eluent containing 12-lipoxygenase was mixed with an equal volume of 40% glycerol containing 10 μM $FeCl_2$ prior to being flash frozen in liquid nitrogen and stored at −80°C.

Synthesis and purification of 12(S)-HETE-lysophospholipids

2-Arachidonoyl lysophosphatidylcholine (2-AA-LPC) and 2-arachidonoyl-lysophosphatidylethanolamine (2-AA-LPE) were synthesized by incubating 1-(1Z-octadecenyl)-2-arachidonoyl-sn-glycero-3-phosphocholine or 1-(1Z-octadecenyl)-2-arachidonoyl-sn-glycero-3-phosphoethanolamine, respectively, in methanol/water (9:1, v/v) containing H_2SO_4 (0.4 N) at 70 °C for 5 min. 2-AA-lysophospholipids extracted from the acidic hydrolysis reaction were purified by RP-HPLC. A linear gradient was used as follows using a flow rate of 1 ml/min: 0 min, 70% A (acetonitrile, methanol, 10 mM ammonium acetate buffer, 2:1:1), 0% B (methanol), 30% C (water); 2 min, 70% A, 0% B, 30% C; 15 min, 100% A; 25 min, 100% A; 25.1 min, 100% B; 35 min, 100% B; 35.1 min, 70% A, 0% B, 30% C; 45 min, 70% A, 0% B, 30% C. Purified 2-AA-lysophospholipids were resuspended

in reaction buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA, pH 7.0) by vortexing and sonication (15 pulses × 1 s) at a final concentration of 100 μM. Purified recombinant human 12-lipoxygenase was added to 1 ml of substrate at a final concentration of 50 μg of 12-LOX/ml and incubated at 37 °C for 30 min. The reaction was quenched by adding 2 ml of chloroform/methanol (1:1) containing 0.1% acetic acid. The extraction mixture was vigorously vortexed and then centrifuged at $1000 \times g$ for 10 min. The chloroform phase was collected, and the aqueous phase was re-extracted with 1 ml of chloroform. The chloroform phases were combined and dried under a nitrogen stream. The 12-HpETE-lysophospholipids generated by recombinant 12-LOX were then purified using reversed-phase HPLC. To prepare 12-HETE-lysophospholipids, 100 μl of 1 mg of triphenylphosphine/ml of methanol (to reduce remaining hydroperoxyl groups to their hydroxyl derivatives) was added to the dried 12-HpETE-lysophospholipid products prior to rapid injection onto a RP-HPLC column (Phenomenex Kinetex EVO C18 column (5 μM, 250 mm × 4.6 mm)) for purification. A linear gradient was used as follows using a flow rate of 1 ml/min: 0 min, 70% A (acetonitrile, methanol, 10 mM ammonium acetate buffer, 2:1:1), 0% B (methanol), 30% C (water); 2 min, 70% A, 0% B, 30% C; 15 min, 100% A; 25 min, 100% A; 25.1 min, 100% B; 35 min, 100% B; 35.1 min, 70% A, 0% B, 30% C; 45 min, 70% A, 0% B, 30% C. Fractions containing 12(S)-HETE-lysophospholipids were collected and dried under nitrogen flow. Chloroform/methanol/water (1:1:1, final v/v/v) was added to the dried products and vortexed. The chloroform phase was collected and dried under nitrogen flow. The resulting products were purged with nitrogen and stored at −80 °C.

Platelet isolation and activation

Murine blood was obtained by intracardiac puncture of euthanized mice. Approximately 0.8 ml of blood was drawn into a syringe containing 0.15 ml of 3.8% sodium citrate to prevent platelet activation. The blood was then centrifuged at $150 \times g$ for 10 min and the platelet-rich plasma was subsequently centrifuged at $200 \times g$ for 5 min to remove residual red blood cells. The purified platelet-rich plasma was centrifuged at $1500 \times g$ for 10 min and the supernatant was discarded. The platelet pellet was then resuspended in Tyrode's solution at room temperature and used within 20 min of preparation. The platelet protein concentration was measured using a Bradford protein assay (Bio-Rad). Platelets were activated by 20 μg/ml of mouse thrombin and incubated at 37 °C for 15 min. The reactions were terminated by the addition of chloroform/methanol (1:1, v/v) and vortexed. The chloroform layer was collected and dried under a nitrogen stream. The dried residue was re-dissolved in water/methanol (1:4) and resolved on a Kinetex EVO C18 column (4.6 × 250 mm). A linear gradient was used as follows with a flow rate of 1 ml/min: 0 min, 60% A (acetonitrile/methanol/water 2:1:1), 0% B (methanol), 40% C (2 mM potassium phosphate buffer pH 7.2); 5 min, 60% A, 0% B, 40% C; 25 min, 100% A; 26 min, 100% B; 40 min, 100% B. From 7 to 21 min, eluents were collected every 2 min. Each eluent fraction was dried under a stream of nitrogen and re-dissolved in 200 μl

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of chloroform/methanol (1:1). 10 μl of each fraction was aliquoted into glass test tubes in 4 replicates. The solvent was dried by nitrogen stream and 1 ml of THP-1 cells suspension (1×10^6 cells/ml) in RPMI 1640 media was added. The test tubes were gently shaken and incubated at 37 °C under a 5% CO_2 atmosphere for 1 h. After 1 h incubation, the samples were centrifuged at $200 \times g$ for 3 min and TNFα in the supernatant was measured by ELISA as described above.

AMPP derivatization of oxidized fatty acids and LC–MS/MS analysis

AMPP derivatization was performed using AMP+ MaxSpec Kit as described by Gelb and co-workers (72). In brief, 20 μl of cold acetonitrile/*N,N*-dimethylformamide (4:1, v/v) was added to the dried residue. The sample tube was vortexed, then 20 μl of cold 640 mM *N*-(3-dimethylaminopropyl)-N'-ethylcarbodiimide in water, 10 μl of 20 mM 1-hydroxybenzotriazole in acetonitrile/dimethylformamide (99:1, v/v), and 30 μl of AMPP in acetonitrile were added and vortexed. After 30 min incubation at 60 °C, the sample was cooled to room temperature and subjected to LC–MS/MS analysis as previously described (73). Briefly, LC–MS/MS analysis was performed using an LTQ Orbitrap mass spectrometer connected to a Waters Acquity UPLC system. Lipids were separated using a C18 reversed phase column (Kinetex EVO C18, 2.7 μM, 150×2.1 mm) at 22 °C with a flow rate of 200 μl/min. A linear gradient of solvent A (10 mM ammonium acetate and 0.1% acetic acid (v/v) in water) and solvent B (acetonitrile) were used as follows: 0 min, 25% B; 5 min, 25% B; 20 min, 95% B; 25 min, 95% B; 25.1 min, 25% B; 35 min, 25% B. The autosampler tray temperature was set at 4 °C. The spray voltage in electrospray ionization source was 4.1 kV. The sheath gas flow rate was 40 (arbitrary unit). The capillary temperature was 270 °C. In MS/MS analysis, the CID collision energy is 30, and the isolation width is 3 Th.

Statistical analyses

Results are expressed as mean ± S.D.

Data availability

All data are contained within the manuscript and supporting information.

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A functional role for eicosanoid-lysophospholipids in activating monocyte signaling

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