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Cyclooxygenase-2 generates anti-inflammatory mediators from omega-3 fatty acids

Alison L. Groeger^{1,*}, Chiara Cipollina^{1,*}, Marsha P. Cole¹, Steven R. Woodcock¹, Gustavo Bonacci¹, Tanja K. Rudolph², Volker Rudolph², Bruce A. Freeman¹, and Francisco J. Schopfer¹

¹ Department of Pharmacology & Chemical Biology, University of Pittsburgh, Pittsburgh, PA 15261, USA

² Cardiovascular Research Center, University Heart Center Hamburg, Hamburg, Germany

Summary

Here we report that Cyclooxygenase-2 (COX-2) mediates the formation of electrophilic fatty acid oxo-derivatives (EFOXs) from the omega-3 fatty acids (ω -3 FA) docosahexaenoic, docosapentaenoic and docosatetraenoic acid. EFOXs produced by activated macrophages were discovered by a mass spectrometry-based “fishing” method, using the nucleophile β -mercaptoethanol (BME) as bait. Intracellular EFOX concentrations ranged from 65 to 350 nM, with acetylsalicylic acid (ASA, aspirin) increasing both rate of production and intracellular concentrations. Due to an electrophilic nature, EFOXs adducted protein cysteine and histidine residues and the cysteine of glutathione (GSH) in activated macrophages. A role for EFOXs as signalling mediators was confirmed by the observations that 17-EFOX-D₆ and 17-EFOX-D₅ are PPAR γ agonists and activate Nrf2-dependent antioxidant responses. They also inhibited cytokine production and inducible nitric oxide synthase (NOS-2) expression in activated macrophages within a biological concentration range. Thus, EFOXs are signalling mediators that can transduce the clinically beneficial effects of ω -3 FA, COX-2 and ASA.

The major ω -3 FAs (5Z,8Z,11Z,14Z,17Z)-eicosapentaenoic acid (EPA) and (4Z,7Z,10Z,13Z,16Z,19Z)-docosahexaenoic acid (DHA) have been associated with beneficial health effects in humans. For example, brain and retina are enriched with DHA in healthy individuals, with DHA being necessary for the normal development and function of these tissues^{1, 2}. Moreover, the consumption of DHA in the diet has been implicated in reducing neurocognitive decline³, improving insulin resistance in diabetics⁴, decreasing incidence of myocardial infarction⁵ and reducing inflammation⁶. Despite these salutary effects, the molecular mechanisms of ω -3 FA actions remain largely uncharacterized.

Multiple anti-inflammatory lipid mediators have recently been reported that promote resolution of inflammation by suppressing NF- κ B activation, modulating cytokine expression, activating G-protein coupled receptors⁷ and promoting cyto-protective responses⁸. Oxygenases, including COX-2 and lipoxygenases (LOXs) are involved in both

Correspondence to: Bruce A. Freeman; Francisco J. Schopfer.

*These authors contributed equally to this work

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Nomenclature: To be consistent with prostaglandin and isoprostane nomenclature EFOX are designated according to the length of the carbon chain (eicosanoids and docosanoids) and “x” number of double bonds (EFOX-D_x and EFOX-E_x). In the case of regioisomers the number preceding the EFOX species indicates the location of the carbonyl group.

the initiation of inflammatory events and in an orchestrated resolution⁹. Other bioactive lipids generated by non-enzymatic redox reactions include reactive electrophilic species (RES), such as nitro-fatty acids (NO₂-FAs) and cyclopentenone isoprostanes and neuroprostanes^{10, 11}.

Biological RES are characterized by an electron-withdrawing functional group that renders the β -carbon electron-poor, promoting Michael addition reactions with cellular nucleophiles (*e.g.* cysteine and histidine residues of protein). By adducting nucleophilic protein residues, RES modulate protein activity, subcellular localization, and electrophile-sensitive gene expression. The reactivity of the electrophile influences downstream signaling actions¹², with irreversible adducts conveying toxic effects¹³. RES can also modulate cellular redox status by changing GSH/GSSG levels and ratios, further impacting cellular metabolism. Recently, there has been a move towards employing RES in the prevention or treatment of various diseases such as neurodegeneration, cancer, and other pathologies presenting a significant inflammatory component. For example, electrophilic neurite outgrowth-promoting prostaglandin compounds display protective effects during cerebral ischemia/reperfusion, an event attributed to their accumulation in neurons and subsequent activation of the Keap1/Nrf2 pathway¹⁴. Other RES (*e.g.* avicins¹⁵ and Bis(2-hydroxybenzylidene)acetone¹⁶, isothiocyanates¹⁷) are potential chemopreventative agents, due to their abilities to induce apoptosis of precancerous cells and tumor cells. Additionally, the electrophile 15-deoxy- Δ (12,14)-prostaglandin J₂ (15d-PGJ₂) is protective in animal models of acute lung injury¹⁸.

EFOX are produced by activated macrophages

To search for electrophilic products formed during inflammation a screening method was applied that is based on BME reaction with electrophiles, followed by resolution with reverse phase-high pressure liquid chromatography and detection by tandem mass spectrometry (RP-HPLC-MS/MS)¹⁹. Alkylation with BME standardizes the MS/MS conditions for adducted RES, conferring similar ionization and fragmentation properties on a range of RES, each with their own particular MS/MS characteristics. This permits the sensitive detection of free RES and RES that a) are reversibly adducted to nucleophiles, b) fragment poorly during MS/MS, c) were previously at or below limits of detection, d) have not been previously predicted. Using this method to screen fatty acid-derived RES, the neutral loss of BME (78 amu) by RP-HPLC-MS/MS revealed six new and abundant RES following activation of RAW264.7 cells (a murine monocyte/macrophage cell line) by phorbol myristic acetate (PMA), LPS, and IFN γ (Fig. 1a). Identical RES were also detected in PMA, Kdo₂-lipid A (Kdo₂) and IFN γ -activated THP-1 cells (a human monocyte cell line) and in primary murine bone marrow-derived macrophages (BMDMs, Fig. 1b and Supplementary Fig. 2–3). Although relative abundance of these RES differed between the two cell lines, MS/MS spectra showed similar characteristic neutral losses and fragment ion intensity ratios (not shown). The BME reaction was further tested for the detection and quantification of reversibly reactive RES by comparing the mass spectrometric characteristics of several RES containing α,β -unsaturated carbonyls using three different approaches: the BME method, selected ion monitoring (SIM) and multiple ion monitoring (MRM) for loss of CO₂ (Supplementary Fig. 4). In all cases, the BME method was unique in specificity for RES and superior in terms of signal intensity, low background levels and linearity of signal response versus concentration. For these reasons and ease of identification, electrophile-BME adducts were studied for identifying unknown RES in biological samples.

Macrophages were activated with various combinations of LPS, IFN γ , PMA, fMLP, and Kdo₂ (Fig. 1c and Supplementary Fig. 5). Kdo₂, a synthetic endotoxin, was used to avoid the contribution of potential LPS contaminants. Since the combination of Kdo₂ and IFN γ

induced responses similar to LPS, it was used for subsequent experiments. EFOX formation under different inflammatory conditions was confirmed by treating the cells with a variety of stimuli. Intracellular concentrations obtained for different species ranged from 65 to 350 nM (Table 1). EFOX formation became detectable 4–6 h post-activation and reached maximum levels approximately 10 h after activation (Fig. 1d and Supplementary Fig. 6).

EFOXs are oxo-derivatives of ω -3 FA

Accurate mass TOF data (mass accuracy ≤ 10 ppm), elution profile and loss of CO_2 upon fragmentation indicated EFOX-D₅ was a mono-oxygenated derivative of a 22-carbon FA with five double bonds. The MS/MS spectrum for BME-adducted EFOX-D₅ (m/z 421 [M-H]⁻) displayed characteristic fragment ions at m/z 403 ([M-H-H₂O]⁻), 377 ([M-H-CO₂]⁻), 343 ([M-H-BME]⁻), 325 ([M-H-BME-H₂O]⁻), and 299 ([M-H-BME-CO₂]⁻) (Fig. 2a). EFOX-D₆ and EFOX-D₄ were identified as mono-oxygenated derivatives of 22-carbon FAs with a total of 6 and 4 double bonds, respectively. To elucidate metabolic precursors *in vivo*, cells were supplemented with different FAs. EFOX-D₅ was significantly increased in activated RAW264.7 cells supplemented with 18:3 ω -3 (α -linolenic acid) and 20:5 ω -3 (EPA), and slightly decreased when ω -6 FA were provided (Fig. 2b), indicating that EFOX-D₅ was derived from ω -3 FA. Addition of 22:6 ω -3 (DHA) did not increase EFOX-D₅ levels, consistent with the precept that mammalian cells can desaturate and elongate shorter chain FAs and generally do not resaturate DHA. The formation of EFOX-D₆ in activated RAW264.7 cells was increased only by supplementation of 22:6 ω -3, while EFOX-D₄ was increased by both ω -3 and ω -6 FA supplementation, indicating precursors could be either ω -3 or ω -6 DTA (Supplementary Fig. 7a–b). Overall, EFOX-D₆, EFOX-D₅ and a percentage of EFOX-D₄ were derivatives of the ω -3 FA DHA, (7Z,10Z,13Z,16Z,19Z)-docosapentaenoic acid (DPA) and docosatetraenoic (DTA) respectively, while EFOX-D₃, EFOX-E₃ and EFOX-E₂ were synthesized from ω -6 and ω -9 FAs (Table 1).

The Luche reaction, where NaBH_4 selectively reduces conjugated carbonyls to allylic alcohols without loss of regioselectivity²⁰ (Fig. 2c), confirmed that the EFOX electrophilic functional group was an α,β -unsaturated carbonyl. Lipid extracts from activated RAW264.7 cells were first fractionated by HPLC and then fractions containing EFOX-D₅ were purified and reduced with NaBH_4 . This significantly decreased the signal corresponding to EFOX-D₅ and induced the presence of a previously absent peak at the transition 345/327 (reduced EFOX-D₅, hydroxy-DPA; Fig. 2d). The enhanced MS/MS fragmentation of hydroxy groups also revealed the location of the carbonyl group in precursor EFOXs. In addition to the commonly observed ion fragments m/z 327 ([M-H]-H₂O) and 283 ([M-H]-H₂O-CO₂), the following diagnostic ions for 13-hydroxy-DPA (13-OH-DPA) were observed in the EFOX-D₅ enriched fraction reduced with NaBH_4 : m/z 223, 205 (223-H₂O) and 195 (Fig. 2e). These findings revealed that EFOX-D₅ corresponded to 13-oxoDPA and EFOX-D₄ was an oxo-derivative of DTA (Table 1).

COX-2 mediates EFOX production

EFOX levels were quantified in activated RAW264.7 cells treated with a variety of inhibitors (Fig. 3a and Supplementary Fig. 8). Both genistein, a general tyrosine kinase inhibitor, and methyl arachidonyl fluorophosphonate (MAFP) inhibited EFOX production by over 50%. Inhibition by MAFP, a selective irreversible inhibitor of both calcium-dependent (cPLA₂) and calcium-independent cytosolic phospholipase A₂ (iPLA₂), indicated that a significant proportion of FA substrates for EFOX generation came from the hydrolysis of complex lipids. MK886, an inhibitor of 5-LOX activating protein (FLAP)-dependent activation of 5-LOX and had no significant effect on EFOX formation. Eicosatetraynoic acid (ETYA), a nonspecific inhibitor of COX and LOX enzymes, strongly inhibited EFOX formation. The general phosphatase inhibitor, okadaic acid (OKA), only slightly increased

EFOX formation. To further characterize EFOX formation by COX, EFOX levels were quantified in activated RAW264.7 cells treated with COX inhibitors at concentrations ≥ 5 -fold the IC_{50}^{21} (Fig. 3b and Supplementary Fig. 9). Indomethacin and diclofenac completely inhibited EFOX formation, while ibuprofen inhibition was 80%. The selective COX-2 inhibitor NS-398, fully inhibited EFOX formation, and in contrast ASA significantly increased production of all EFOX derivatives, with the exception of EFOX-D₃ and EFOX-D₂. This effect of ASA is consistent with ASA acetylation of COX-2 Ser530 favoring the mono-oxygenation of long chain FAs²².

The role of COX-2 in EFOX formation was affirmed by observing that purified ovine COX-2 generated the EFOX-D₅ precursor, hydroxy-DPA (OH-DPA), from DPA (Fig. 3c–e) and the EFOX-D₆ precursor, hydroxy-DHA (OH-DHA), from DHA (Supplementary Fig. 10a–b). ASA increased the rate and extent of formation of OH-DPA (Fig. 3c) and shifted the distribution of hydroxy regioisomers from C-13 to C-17 (Fig. 3d–e, and Supplementary Fig. 10a–b). The fragmentation pattern of COX-derived 13-OH-DPA showed the characteristic m/z 195 and 223 ions, corresponding to the hydroxyl-induced fragmentation observed in $NaBH_4$ -reduced RAW264.7 cell-derived products (Fig. 3d and 2e). In contrast, when COX-2 was treated with ASA, characteristic fragment ions corresponding to a 17-OH group were detected (Fig. 3e). In activated RAW264.7 cells ASA addition similarly promoted the production of 17-EFOX-D₅ (Fig. 3f) and 17-EFOX-D₆ (Supplementary Fig. 10c).

Since purified COX-2 produced hydroxy rather than carbonyl derivatives of FA, a hydroxy-dehydrogenase reaction was viewed as necessary. When non-activated RAW264.7 cell lysates were incubated with OH-DPA in presence of NAD^+ , there was a time-dependent production of EFOX-D₅. This indicates that the dehydrogenase activity responsible for the conversion of OH-DPA to its oxo-derivative was constitutively expressed (Fig. 3g–i). Only activated cells converted DPA into oxo-DPA, due to the requirement of COX-2 for the first oxidation step.

EFOXs adduct to proteins and GSH

Typically, electrophiles form adducts with nucleophilic amino acid residues in the cell, including the cysteine of GSH^{23–26}. Total EFOX content was quantified and compared with the pool of free EFOXs (including small molecule EFOX adducts, such as GSH). The difference between these two EFOX pools gave the percentage of EFOXs adducted to proteins (~50%) (Fig. 4a). The difference in the kinetics of BME reaction with free and adducted EFOXs further confirmed the distribution of intracellular EFOXs. Reaction rates of BME with free electrophiles were relatively fast, with a pseudo first order reaction rate constant between 3×10^{-3} and $5 \times 10^{-3} \text{ sec}^{-1}$ for different α,β -unsaturated carbonyl derivatives (15d-PGJ₂, 17-EFOX-D₅, 17-EFOX-D₆ and 15-oxoeicosatetraenoic acid, 15-oxoETE)(Supplementary Fig 11a). In contrast, BME exchange reactions with adducted electrophiles were slower, a consequence of the k_{off} reaction rate of Cys-EFOX and His-EFOX adducts. The time-dependence of these reactions further confirmed that biomolecule-adducted populations constituted ~50% of the total EFOX pool present in cell lysates (Supplementary Fig. 11b). To more specifically test the capability of EFOXs to react with nucleophilic residues of proteins, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was utilized. GAPDH is a well-characterized target for electrophiles that becomes inactivated by *s*-nitrosation, oxidation or nucleophilic addition. Proteomic analysis revealed adducts between 17-EFOX-D₅ and the Cys244, Cys149, His163 and His328 residues of GAPDH *in vitro* (Supplementary Fig. 12).

Since RES react with GSH, EFOXs were tested as substrates for glutathione S-transferase (GST) and the cellular formation of GS-EFOX products were evaluated. Incubation of EFOXs without or with GST resulted in EFOX-GSH adduction rates dependent on the

amount of GST added, confirming that EFOXs are GST substrates (Supplementary Fig. 13). GS-EFOX adducts were measured in activated RAW264.7 cell lysates and media, and compared with synthetic standards of GS-17-EFOX-D₅ and GS-17-EFOX-D₆ (Fig. 4b–c and Supplementary Fig. 14). The fragmentation patterns and retention times observed for cellular GSH adducts of EFOX-D₅ and EFOX-D₆ corresponded with their synthetic homologues, confirming cellular GS-EFOX adduction. The addition of ASA enhanced the formation of GS-EFOX adducts to extents consistent with the concomitant increase in EFOX synthesis. GS-EFOX adducts were also found extracellularly, in the absence of cell lysis, suggesting multi-drug resistance protein transport.

Anti-inflammatory signaling actions of EFOXs

Many RES can promote expression of the Nrf2-dependent phase II genes²⁴ by thiol-dependent modification of the Nrf2 inhibitor Keap1. 17-EFOX-D₅ and 17-EFOX-D₆ induced dose-dependent cellular Nrf2 nuclear accumulation and expression of the cytoprotective enzymes heme oxygenase 1 (HO-1) and NAD(P)H:quinone oxidoreductase 1 (NQO1) (Fig. 5a–b). Besides activating the Nrf2-dependent anti-oxidant response, 17-EFOX-D₅ and 17-EFOX-D₆ modulated the inflammatory response generated by Kdo₂ and IFN γ treatment. 17-EFOX-D₅ and 17-EFOX-D₆ dose-dependently repressed IL-6, MCP-1 and IL-10 expression (Fig. 5c), inducible nitric oxide synthase (NOS-2) expression (Fig. 5d) and accumulation of the nitric oxide metabolites, nitrate (NO₃⁻) and nitrite (NO₂⁻), in the media of both RAW264.7 cells and BMDMs (Supplementary Fig. 15). COX-2 expression levels were not affected by EFOXs.

Oxo-FAs, including 15d-PGJ₂²⁷, 5-oxoEPA, and the synthetic 4-oxoDHA²⁸ covalently bind and activate the peroxisome proliferator-activated receptor γ (PPAR γ). PPAR γ beta-lactamase reporter assays revealed that 17-EFOX-D₅ and 17-EFOX-D₆ activated PPAR γ within a physiological concentration range (Fig. 5e), with a slightly higher EC₅₀ (~40 nM) than 15d-PGJ₂ (~25 nM) and orders of magnitude lower than the EFOX precursor, 17-OH-DPA, DHA and DPA.

A BME “trapping” strategy revealed six COX-2-derived EFOXs, (as well as oxoETE, not shown) produced by activated macrophages. These ω -3 FA-derived species have not been described before as mediators of inflammation or as metabolic products of mammalian cells, although EFOX-E₃ may correspond to the oxoETrE produced by plants²⁹. Interestingly, concomitant monitoring of 15d-PGJ₂ generation showed this species was below the limit of detection, implying that EFOXs may in some cases be responsible for effects often attributed to endogenous 15d-PGJ₂.

EFOXs were discovered by a new chemical profiling strategy as negatively-charged hydrophobic molecules with reversible electrophilic activity. This BME-based method increased MS/MS sensitivity for diverse RES and standardized their behavior during MS/MS analysis. For example, oxo-FA derivatives do not fragment as well as their corresponding hydroxy-derivatives, preventing structural identification. In particular, EFOX-D₄, EFOX-D₅ and EFOX-D₆ correspond to oxoDHA, oxoDPA and oxoDTA (with different isomers being favored by addition of ASA). EFOX-D₃, EFOX-E₃ and EFOX-E₂ are derived from ω -6 and ω -9 FAs, with detailed structural characterization of these latter species not yet performed.

While COX-2 accounts for EFOX formation, additional mechanisms cannot be excluded. Concerning initial oxygenation, LOX and cytochrome p450 (CYP) monooxygenase activities can also catalyze FA hydroxylation^{9, 30}. While the formation of monohydroxy-derivatives of FAs has already been described^{22, 31}, further oxidation to the corresponding oxo-species has only been observed for hydroxy-ETA³². Moreover, despite an appreciation

of oxoETE and oxo-octadecadienoic acid generation, similar 22-carbon species have not been described.

The enzymatic oxidation of hydroxyls on bioactive lipids has been generally viewed as a step in metabolic inactivation, but present data indicate that this reaction instead confers new biologic activity. This report also reveals an alternative reaction for the hydroxy-derivatives of ω -3 FAs that are proposed to be further oxidized by LOXs to resolvins and lipoxins. Alternatively, we observe that monohydroxy-FA derivatives can be readily converted to EFOXs by cellular dehydrogenases or by purified dehydrogenases *in vitro*. For example, 3 α -hydroxysteroid dehydrogenases (3 α -HSDs) converted 13- and 17-OH-DPA and 13- and 17-OH-DHA into corresponding EFOXs in the presence of NAD(P)⁺ *in vitro* (Supplementary Fig. 10). Members of the aldo/keto reductase superfamily, 3 α -HSDs, regulate steroid hormone levels in mammals³³. Other candidate dehydrogenases leading to EFOX generation could be the 5- and 15-hydroxyeicosanoid dehydrogenases (5- and 15-HEDH), which convert LOX products to 5- and 15-oxoETE³². The formation of 5-oxoETE is favored by a high NADP⁺:NADPH ratio, a condition symptomatic of cells under oxidative stress. Notably, while HEDH activity is present in myeloid cells, it is further induced following differentiation to macrophages using PMA³². Thus, 15-HEDH can confer electrophilic reactivity to EFOX precursors, since it catalyzes the oxidation of hydroxyl groups to carbonyl products for a wide range of substrates, including AA-derived prostaglandins, 15-hydroxyeicosatetraenoic acid, EPA-derived Resolvin E1 and DHA-derived Resolvin D1 at position 17³⁴⁻³⁶.

Approximately 50% of the EFOXs extracted from activated RAW264.7 cells were adducted to protein, but this value did not include EFOXs that were bound to small molecules such as GSH. These covalent adducts of EFOXs to protein and GSH revealed a capacity to modulate protein function and mediate electrophilic signaling by covalently binding to transcriptional regulators. The modulation of several signaling mechanisms by EFOXs confirmed a role as endogenously produced anti-inflammatory signaling mediators. 17- EFOX-D₅ and 17- EFOX-D₆ induced Nrf2-dependent translocation to the nucleus and the expression of two key Nrf2 target genes, HO-1 and NQO-1. Biologic concentrations of the 17-EFOX standards also activated PPAR γ , implying potential anti-inflammatory actions through PPAR γ -regulated genes³⁷. Anti-inflammatory signaling properties of EFOXs were also observed when 17- EFOX-D₅ and 17- EFOX-D₆ dose-dependently inhibited IFN γ and Kdo₂-induced cytokine production and iNOS expression. While recognized electrophile-sensitive signaling pathways were tested, it is probable that EFOXs display additional unique signaling profiles and receptors.

The present discovery gains physiological relevance, considering that EFOXs derived from ω -3 FA are produced by aspirin-enhanced COX-2 oxidation reactions. The beneficial roles of COX-2 and ω -3 FA in the resolution of inflammation and the demonstrably significant role of ω -3 FA in cardiovascular protection³⁸ suggest that COX-2-derived EFOXs can contribute to a component of these actions.

Methods Summary

Cell Culture and treatment

RAW264.7 and THP-1 cell lines were obtained from ATCC (USA) and maintained according to ATCC guidelines. For treatment with EFOXs coupled with pro-inflammatory stimulation, 17- EFOX-D₅ and 17- EFOX-D₆ was followed by addition of Kdo₂ and IFN γ at 6h.

Trans-alkylation reaction of electrophiles with BME

Upon thawing, lysates were exposed to BME (500 mM + internal standard, 5-OxoETE-d7 (1.25 ng/ml)) and incubated at 37 °C for 1 h in 50 mM phosphate buffer (pH= 7.4) as previously described¹⁹. Proteins were precipitated with cold acetonitrile and the supernatant was analyzed by HPLC-ESI-MS/MS.

HPLC-ESI-MS/MS

For experimental details see Methods. Samples were separated by reverse-phase HPLC. The analysis and quantification of BME adducts were performed using a hybrid triple quadrupole-linear ion trap mass spectrometer (4000 Q trap, Applied Biosystems/MDS Sciex) in the neutral loss (NL) scan mode, MRM scan mode, and the enhanced product ion analysis (EPI) mode. EFOXs were quantified using external synthetic standards, when available, and by comparing peak area ratios between analytes and a 5-OxoETE-d7 internal standard. EFOX values represented as percentages were normalized to internal standard and cell lysates protein concentration.

COX-2 reactions

COX-2 reactions were performed as described previously^{21, 22}. The reactions were initiated by addition of FA (10 μM) and terminated by addition of ice-cold acetonitrile (9 x volume) and protein removed by centrifugation. Product formation was monitored by RP-HPLC-MS/MS in MRM mode following the loss of CO₂ (*m/z* 345/301 and *m/z* 343/299 for OH-DPA and OH-DHA, respectively).

Nitrate/Nitrite measurement

Total nitrite and nitrate concentration was measured in cell culture media by Griess reaction using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical).

Measurement of glutathione adducts

For experimental details see Methods. GS-adducts were analyzed by nano-LC-MS/MS using nanoACQUITY UltraPerformance LC coupled with Thermo-Fisher LTQ.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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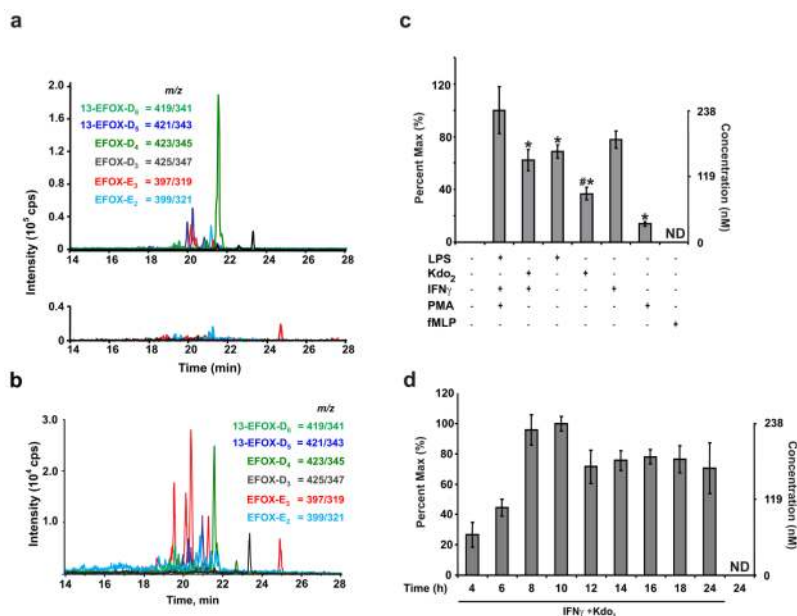


Figure 1. EFOXs are produced during macrophage activation

a, MRM scans following the neutral loss of 78 reveal EFOXs adducted with BME in cell extracts from activated (upper) and non-activated (N/A, lower) RAW 264.7 cells. **b**, EFOX levels in PMA-differentiated THP-1 cells 8 h post activation. **c**, EFOX-D₅ levels in RAW264.7 cells activated with the indicated compounds at the following concentrations: LPS (0.5 μ g/ml), Kdo₂ (0.5 μ g/ml), IFN γ (200 U/ml), PMA (3.24 μ M), fMLP (1 μ M). Data are expressed as mean \pm S.D. (n=4), where * = significantly different ($p < 0.01$) from “PMA + IFN γ + LPS,” and # = significant difference ($p < 0.01$) between “LPS” and “Kdo₂ + IFN γ ” (one-way ANOVA, post-hoc Tukey’s test). **d**, EFOX-D₅ levels in RAW264.7 cells were quantified at the indicated time points post activation. ND indicates not detectable.

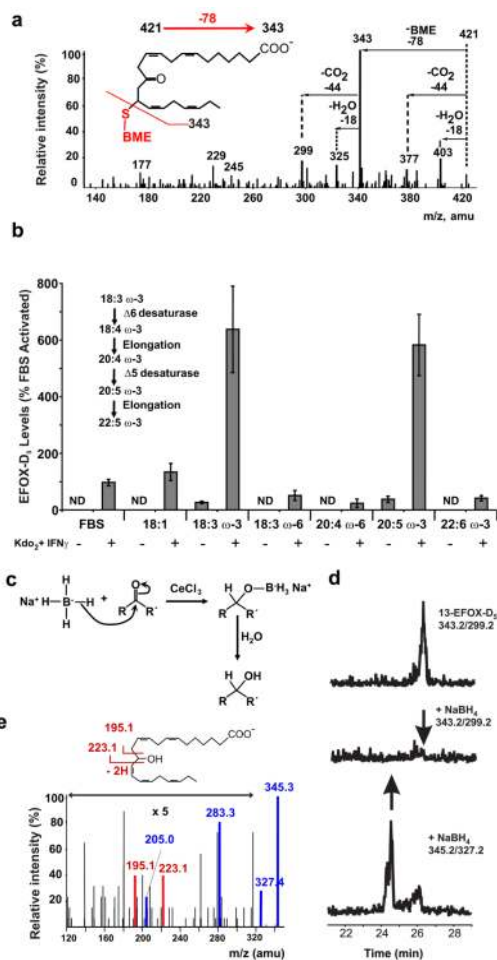


Figure 2. EFOX-D₅ is an α,β -unsaturated oxo-derivative of DPA

a. Characteristic BME-EFOX fragmentation pattern derived from the EPI of BME-EFOX-D₅. **b.** EFOX-D₅ levels in activated RAW264.7 cells grown for 3 days in medium supplemented with the indicated FA. ND indicates not detectable. **c.** Diagram of NaBH₄ reaction. **d.** MRM scans monitoring for the *m/z* transitions 343.2/299.2 (13-EFOX-D₅/loss of CO₂; upper and middle panel) and 345.2/327.2 (hydroxy-DPA/loss of H₂O; lower panel) in RAW264.7 cell lysates purified for EFOX-D₅ \pm NaBH₄. **e.** MS/MS fragmentation of EFOX-D₅ purified from activated RAW 264.7 cells and reduced with NaBH₄.

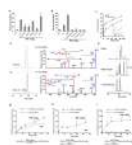


Figure 3. EFOX-D₅ formation is dependent on COX-2 activity

a, b, EFOX-D₅ levels in RAW264.7 activated and treated with the indicated inhibitors at the following concentrations: genistein (25 μM), MAFP (25 μM), MK886 (500 nM), ETYA (25 μM), OKA (50 nM), ASA (200 μM), indomethacin (25 μM), ibuprofen (100 μM), diclofenac (1 μM) and NS-398 (4 μM). ND indicates not detectable. Data are expressed as mean ± S.D. (n=4), where * = significantly different ($p < 0.01$) from “Kdo₂ + IFN γ ” (one-way ANOVA, post-hoc Tukey’s test). **c**, Temporal formation of hydroxy-precursors (MRM 345/327) of EFOX-D₅ synthesized *in vitro* by purified ovine COX-2 + DPA ± ASA. **d, e**, Chromatographic profiles (left panels) and spectra (right panels) of the two isomers formed by COX-2 ± ASA. **f**, BME-adducted 17-EFOX-D₅ standard (MRM 421.2/343.2) and EFOX-D₅ BME-adducts from activated RAW264.7 cells ± ASA. **g-i**, Production of EFOX-D₅ and OH-DPA by RAW264.7 cell lysates supplemented with OH-DPA, DPA, or vehicle alone. Full and empty symbols indicate activated and non-activated cell lysates respectively.

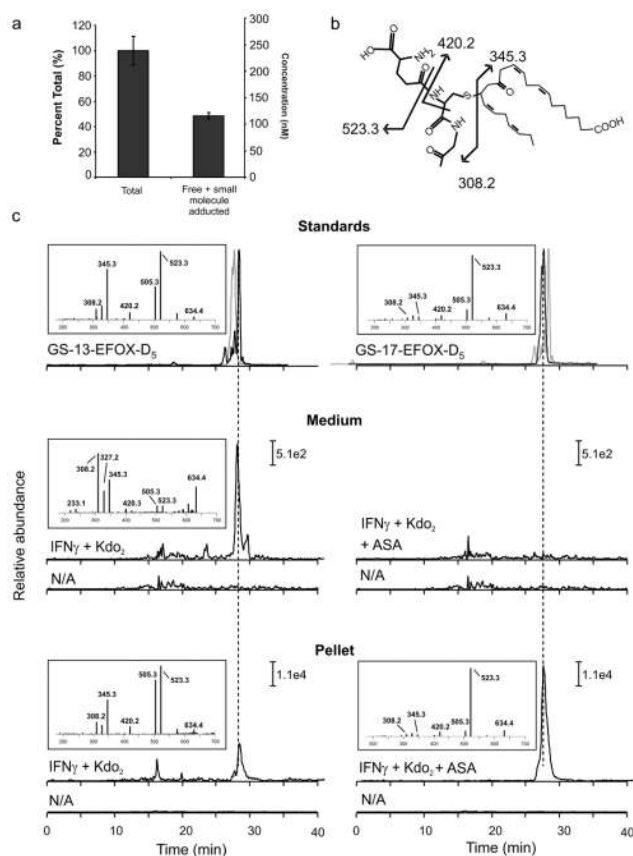


Figure 4. EFOXs form adducts with proteins and GSH following activation of RAW264.7
a. Cell lysates from activated RAW264.7 cells were split into two groups: treatment with BME followed by protein precipitation with acetonitrile (“Total”) and protein precipitation followed by BME treatment (“Free + small molecule adducted”). **b.** Chemical structure and fragmentation pattern of GS-13-EFOX-D₅. **c.** Chromatographic profiles and positive ion mass spectra of GSH adducts of 13-EFOX-D₅ and 17-EFOX-D₅ derived from standards (upper panels), cell medium (middle panel) and cell pellet (lower panel), N/A profiles correspond to non-activated cell samples. Grey chromatograms represent GS-17-EFOX-D₅ (upper panel left) and GS-13-EFOX-D₅ (upper panel right) standards. Fragments 345.3 and 523.3 were monitored in cell media and cell pellet samples respectively.

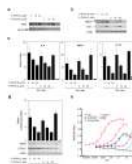


Figure 5. 17-EFOX-D₆ and 17-EFOX-D₅ modulate anti-oxidant and inflammatory responses
a, b, RAW264.7 cells treated with 17-EFOX-D₆ and 17-EFOX-D₅ were harvested at 1 h for nuclear Nrf2 (**a**) and 18 h for of HO-1 and NQO-1 (**b**). **c, d**, RAW264.7 cells pretreated for 6 h with 17-EFOX-D₆ and 17-EFOX-D₅ were harvested 12 h post activation and IL-6, MCP-1, IL-10 levels, and nitrite levels were measured in the cell media and normalized to protein. **d**, iNOS and COX-2 levels were detected in cell lysates. **e**, PPAR γ beta-lactamase reporter assays for Rosiglitazone, 17-EFOX-D₆, 17-EFOX-D₅, 15d-PGJ₂, 17-hydroxyDHA, DPA, and DHA.

Table 1

Summary of EFOX concentrations and characteristics.

Name	EFOX-D ₆	EFOX-D ₅	EFOX-D ₄	EFAD-D ₃	EFAD-E ₃
Cellular concentration nM	65 ± 5	238 ± 16	348 ± 26	106 ± 6	326 ± 15
Mass (<i>m/z</i>)	341.2	343.2	345.2	347.2	319.2
FA precursors	22:6	18:3n-6, 20:5	20:4, 18:3n-6, 20:5, 18:3n-3	18:3n-6, 20:4	18:3n-6
Series	ω-3	ω-3	ω-3 and ω-6	ω-6	ω-6
Identity	13-oxoDHA 17-oxoDHA	13-oxoDPA 17-oxoDPA	oxoDTA	oxoDTrA	oxoETrA