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Methods for imaging and detecting modification of proteins by reactive lipid species

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ABSTRACT

Products of lipid peroxidation are generated in a wide range of pathologies associated with oxidative stress and inflammation. Many oxidized lipids contain reactive functional groups that can modify proteins, change their structure and function, and affect cell signaling. However, intracellular localization and protein adducts of reactive lipids have been difficult to detect, and the methods of detection rely largely on antibodies raised against specific lipid–protein adducts. As an alternative approach to monitoring oxidized lipids in cultured cells, we have tagged the lipid peroxidation substrate arachidonic acid and an electrophilic lipid, 15-deoxy- $\Delta^{12,14}$ -prostaglandin- J_2 (15d-PG J_2), with either biotin or the fluorophore BODIPY. Tagged arachidonic acid can be used in combination with conditions of oxidant stress or inflammation to assess the subcellular localization and protein modification by oxidized lipids generated in situ. Furthermore, we show that reactive lipid oxidation products such as 15d-PG J_2 can also be labeled and used in fluorescence and Western blotting applications. This article describes the synthesis, purification, and selected application of these tagged lipids in vitro.

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Polyunsaturated fatty acids (PUFAs), such as arachidonic acid, are primary components of the diet, biological membranes, and lipoproteins, and they are substrates for lipid peroxidation (LPO) [1–3]. They readily undergo nonenzymatic peroxidation during oxidative stress or are oxidized through reactions involving enzymes such as cyclooxygenase, cytochrome P450, or the lipoxygenases [4–8]. A role for oxidized lipids in pathophysiology is supported by the increased levels of LPO apparent in several diseases, including Alzheimer disease [9,10], renal failure [11,12], and atherosclerosis [13,14].

Many LPO products contain functional groups capable of covalently modifying proteins [15–18]. For example, both nonenzymatic and enzymatic oxidation of PUFAs results in the formation of reactive lipid species that have electrophilic carbons, usually in the β -position of an α,β -unsaturated carbonyl (Fig. 1), that are reactive with protein nucleophiles. Electrophilic products of nonenzymatic lipid oxidation include aldehydes such as 4-hydroxynonenal (HNE), malondialdehyde, acrolein, and the J- and A-series isoprostanes (Fig. 1). Other products include the isoketals, which occur through nonenzymatic LPO by rearrangement of endoperoxide intermediates of the iso-

prostane pathway [19]. Isoketals are highly electrophilic γ -ketoaldehydes that rapidly and covalently modify lysine residues, forming a variety of adducts including lactam, hydroxylactam, imine, and pyrrole species. The enzymatic oxidation of arachidonic acid also results in the formation of reactive LPO products. However, in this case the variety and reactivity of these species is constrained by the active site of enzymes such as lipoxygenase, which limits the number of stereoisomers generated during the enzymatic reaction.

Studying the complex mixture of reactive lipids formed in vivo during oxidative stress or inflammation has been limited by existing techniques, which have focused primarily on following the reactions of a defined lipid in conjunction with a candidate target protein approach. The covalent modification of proteins by oxidized lipids is an important mechanism by which LPO may initiate cell signaling as well as contributing to tissue injury [15,20–22]. Although many studies have shown that the formation of oxidized lipids is increased in disease, it is unclear whether they represent the footprints of oxidative stress or are mediators of pathology. A major obstacle to a complete understanding of the biological roles of oxidized lipids is the unambiguous detection and quantification of biologically relevant protein modifications. Hence, improved technologies that allow a more comprehensive definition of the reactive lipid proteome are needed to determine the mechanism, the extent, and under what circumstances oxidized lipids affect cell signaling and physiology.

Owing to the immunogenic potential of some lipid peroxidation products such as isoketals, levuglandins, and aldehydes, antibodies against specific protein–lipid adducts that allow for their detection by immunological methods have been raised [23–27]. This approach has

Abbreviations: AA, arachidonic acid; BAEC, bovine aortic endothelial cell; BD-AA, BODIPY arachidonic acid; BODIPY FL-EDA, 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene-3-propionylethylenediamine, hydrochloride; Bt-AA, biotinylated arachidonic acid; DMSO, dimethyl sulfoxide; EDC, 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride; ESI-MS, electrospray ionization mass spectrometry; LPO, lipid peroxidation; PVDF, polyvinylidene difluoride; PUFA, polyunsaturated fatty acid; 15d-PG J_2 , 15-deoxy- $\Delta^{12,14}$ -prostaglandin- J_2 .

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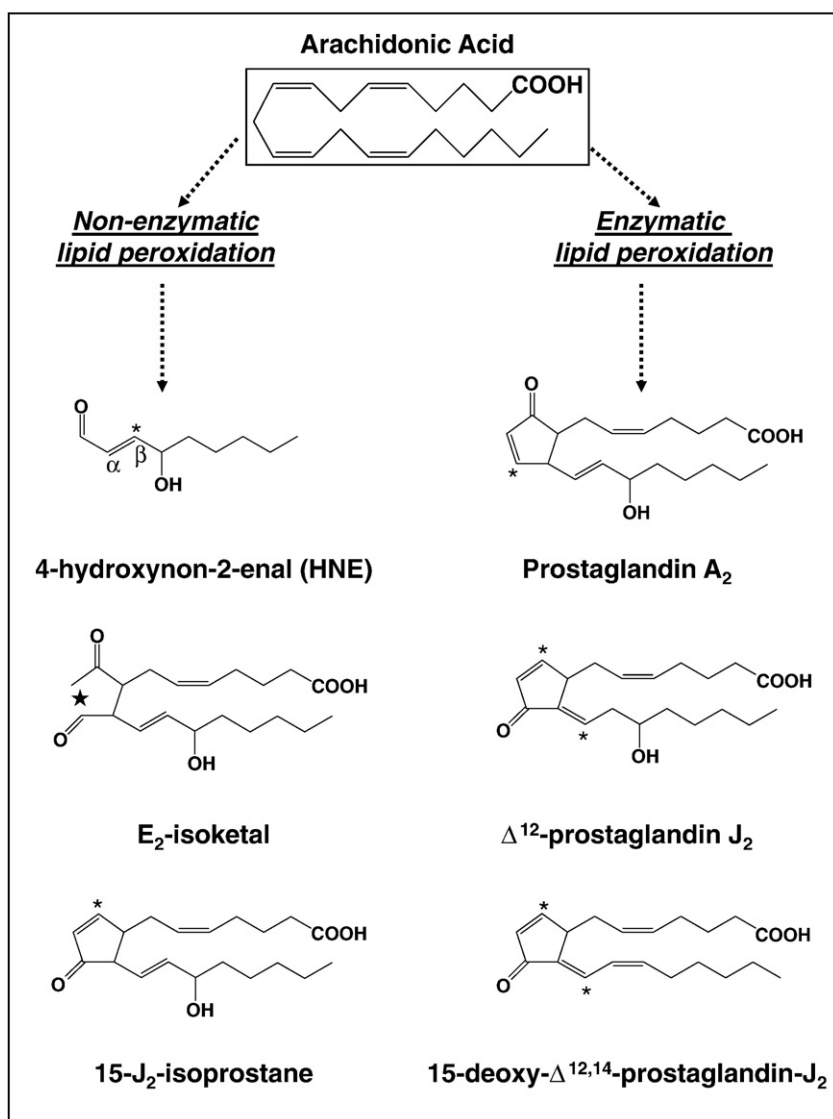


Fig. 1. Examples of reactive lipid species produced by enzymatic or nonenzymatic oxidation of arachidonic acid. Small stars denote the electrophilic carbon (β -carbon) present in lipids containing α,β -unsaturated carbonyls. The large star denotes the carbon on the isoketals that is reactive with lysyl residues of proteins.

been used to identify specific lipid modifications using proteomics and mass spectrometry techniques [28–30]. However, many antibodies to protein–lipid adducts are susceptible to epitope bias; thus protein modifications detected using antibodies may represent a small subset of the reactive lipid proteome. Another disadvantage of this approach is that because complex lipid mixtures are typically generated in cells and tissues during oxidative stress, an antibody to a specific lipid–protein adduct will necessarily give only a partial picture of the reactive lipid proteome.

Detection of the carbonyl moiety present on proteins conjugated to α,β -unsaturated aldehydes and ketones has been a useful marker of lipid peroxidation and oxidative stress [31,32]. Aldehydes and ketones can react with protein nucleophiles such as histidine, cysteine, and lysine by a Michael addition reaction to form stable adducts [18]. This type of adduct can be detected by derivatizing the carbonyl group using hydrazine or hydrazide chemistry to form a stable hydrazone product [31,33,34]. In particular, the use of avidin or streptavidin detection techniques in conjunction with biotin hydrazide increases the sensitivity for detecting low-abundance proteins. One important caveat, however, is that protein carbonyls can be introduced by reaction with oxidants other than reactive lipids, decreasing the specificity of this approach for the unique detection of the oxidized lipid proteomes.

Other methods of detection, such as the radioactive labeling of precursor lipids, have been used to measure protein–lipid adducts [35–37]. Nevertheless, the intracellular targets for reactive lipid oxidation products remain largely undefined owing to a lack of suitable detection reagents and protocols. Herein, we describe a nonradioactive method for the labeling, purification, and utilization of substrates for LPO (i.e., arachidonic acid) and of a specific oxidized lipid, 15-deoxy-prostaglandin J₂ (15d-PGJ₂). In previous studies, we and others have shown that conjugation of 15d-PGJ₂ to biotin or fluorescent tags can be used both to monitor the subcellular localization of the lipid and to identify specific protein targets [16,17,38–42]. Using this approach, signaling proteins such as Keap-1 were shown to form covalent adducts with 15d-PGJ₂, which mediated the induction of antioxidant defenses including glutathione and heme oxygenase-1 [16,17,38]. The insertion of the tags on the lipid via the carboxyl group has little or no impact on the potency of the lipid electrophiles to induce either antioxidant defenses at low concentrations or apoptosis at higher levels. The BODIPY analog of 15d-PGJ₂ has been particularly interesting, revealing mitochondrial targeting of the lipid, and biotin-tagged 15d-PGJ₂ has been used to identify the proteome modified by reactive prostaglandins in mitochondria and cells [41,42]. This paper describes the methods for

detecting protein–lipid adducts using fluorescence and Western blotting approaches.

Principles

To follow the formation of lipid adducts with proteins, we describe a protocol to conjugate biotin or BODIPY to the carboxylic acid group of the unsaturated lipid arachidonic acid or the lipid electrophile. This method was adapted from two previously published methods that describe the biotinylation of prostaglandin A₂ [43] and 15d-PGJ₂ [44]. Tagging arachidonic acid offers the important advantage of detecting proteins that are reactive with multiple LPO species. As shown in Fig. 2, tagged arachidonic acid can be oxidized to lipid peroxidation products in the presence of catalytic metals or oxidants or in response to an inflammatory stimulus. Many of these tagged products contain functional groups such as reactive aldehydes, ketones, and electrophilic carbons capable of modifying protein nucleophiles. Hence, this technique enables the detection of protein-reactive lipid products containing the BODIPY or biotin tag. Incorporation of the fluorescent BODIPY tag also allows the detection of protein modification by oxidized lipids using in-gel fluorescence, thereby eliminating the need for protein transfer and allowing a more high-throughput proteomics approach.

Materials

- (1) Arachidonic acid (Calbiochem, Product No. 181198)
- (2) EZ-Link 5-(biotinamido)pentylamine (Pierce Biotechnology, Product No. 21345)
- (3) 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC; Pierce Biotechnology, Product No. 22980)
- (4) 4,4-Difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionylethylenediamine, hydrochloride (BODIPY FL-EDA; Invitrogen, Product No. D2390)

- (5) 15d-PGJ₂ (Cayman Chemical, Product No. 18570)
- (6) Ethanol, high-performance liquid chromatography (HPLC) grade
- (7) Acetonitrile, HPLC grade
- (8) Methanol, HPLC grade
- (9) Chloroform, HPLC grade, preserved with 0.75% ethanol
- (10) Acetic acid, glacial, certified ACS-plus grade
- (11) Chambered coverglass, four-well (Nunc, Product No. 155383)
- (12) Hoechst 33258 (Invitrogen, Product No. H-3569)
- (13) Protein assay reagent (Bio-Rad, Product No. 500-0006)
- (14) 30% Acrylamide/bis solution (Bio-Rad, Product No. 161-0158)
- (15) Tris base (Fisher, Product No. BP152-1)
- (16) Triton X-100 (Sigma-Aldrich, Product No. T-9284)
- (17) 1-Butanol (Fisher, Product No. A399-500)
- (18) TEMED (Acros Organics, Product No. 420580050)
- (19) Sodium dodecyl sulfate (SDS; Fisher Scientific, Product No. BP166-500)
- (20) Nitrocellulose (Bio-Rad, Product No. 162-0112)
- (21) Supersignal West Dura (Pierce Biotechnology, Product No. 34076)
- (22) Amber glass vials, 1.5 ml (SUN SRI, Product No. 200 252)
- (23) Caps with rubber septa (SUN SRI, Product No. 500 062)
- (24) Borosilicate glass tubes, 16 × 100 mm (Fisher, Product No. 14-961-29)
- (25) Hemin chloride (MP Biomedical, Product No. 15489-47-1)
- (26) DMSO (Sigma-Aldrich, Product No. D2438)
- (27) Hybond-LFP, low-fluorescence PVDF (GE Healthcare, Product No. RPN303LFP)
- (28) PVDF (Millipore, Product No. IPVH00010)

Instruments

- (1) Mass balance (sensitive to 0.1 mg)
- (2) Pipettes
- (3) Rocker (tilting shaker)

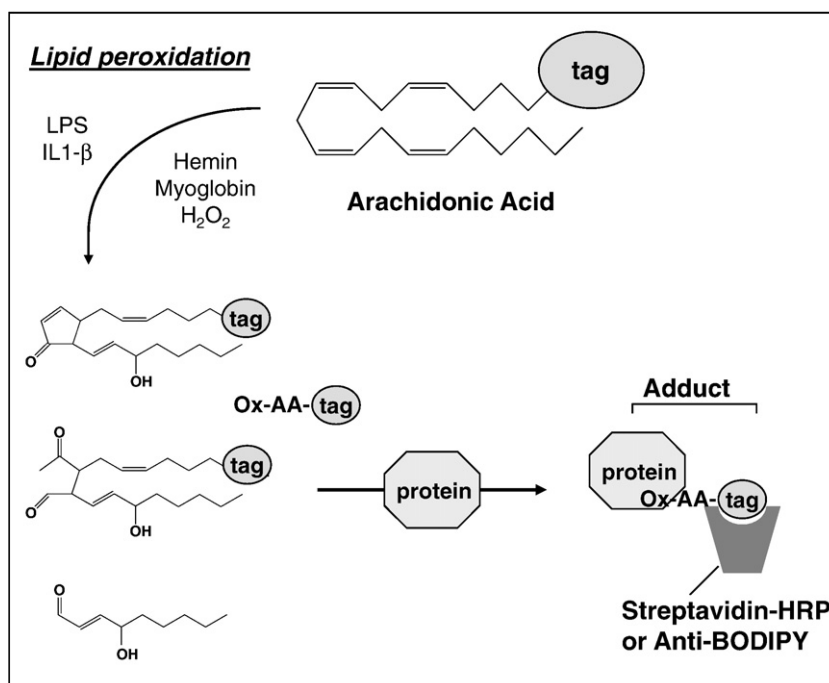


Fig. 2. Proteins modified by reactive lipids can be detected or enriched using tagged arachidonic acid. Tagged arachidonic acid (Bt-AA or BD-AA) can undergo lipid peroxidation to form various products when subjected to oxidative stimuli, such as hemin, lipopolysaccharide, or hydrogen peroxide. Lipid species formed during this process that react with proteins and remain tagged are detected by probing for the tag. Reactive lipids such as HNE that lose the tag during lipid peroxidation, for example, owing to β -scission, cannot be detected by this technique. If using biotinylated arachidonic acid, modified proteins can be detected by probing for biotin using streptavidin. Lipid-modified proteins can also be detected by fluorescence if using BODIPY; alternatively, an anti-BODIPY antibody may be used for detection of the BODIPY tag by Western blotting.

- (4) Vortex mixer
- (5) HPLC system: BioCad perfusion chromatography system (PerSeptive Biosystems, Inc.) or other HPLC system equipped with ultraviolet/visible spectrophotometer and fraction collector
- (6) Preparative column: Gemini C18 reversed-phase column with 10- μ m particle size and dimensions of 250 \times 21.2 mm (Phenomenex, Product No. 00G-4436-P0)
- (7) Semipreparative column: Luna C18 reversed-phase column with 5- μ m particle size and dimensions of 250 \times 10 mm (Phenomenex, Product No. 00G-4041-N0)
- (8) Microscope: Leica DMIRBE microscope with TCS SP laser scanning confocal system or other confocal microscopy system equipped with lasers suitable for fluorescein and DAPI excitation and detection (Leica; Wetzlar, Germany)
- (9) Gel imaging system with CCD camera (Alpha Innotech)
- (10) Fluorescence imager: Typhoon or other fluorescence imaging system suitable for gel scanning and equipped with a filter and laser suitable for BODIPY excitation and detection (GE Healthcare)

Protocol

Biotinylation of arachidonic acid

The structures for unlabeled and biotinylated arachidonic acid are shown in Fig. 3A. The biotin moiety is covalently attached to the fatty acid at the carboxyl group using the activating compound EDC, causing it to be reactive with the amino group of 5-(biotinamido)pentylamine. This condensation reaction results in the formation of a stable amide bond between the fatty acid and the biotin tag. To minimize oxidation, exposure to room air and light must be avoided and all solvents should be purged with an inert gas such as argon or nitrogen before use. In addition, new glassware that has been prerinse with ethanol and dried should be used to prevent contamination and loss of lipid. The reaction should be performed in an amber or foil-covered glass vial to further limit light-dependent oxidation of the lipid.

- (1) Prepare 10 mg/ml EDC in 100% acetonitrile. Vortex until in solution.

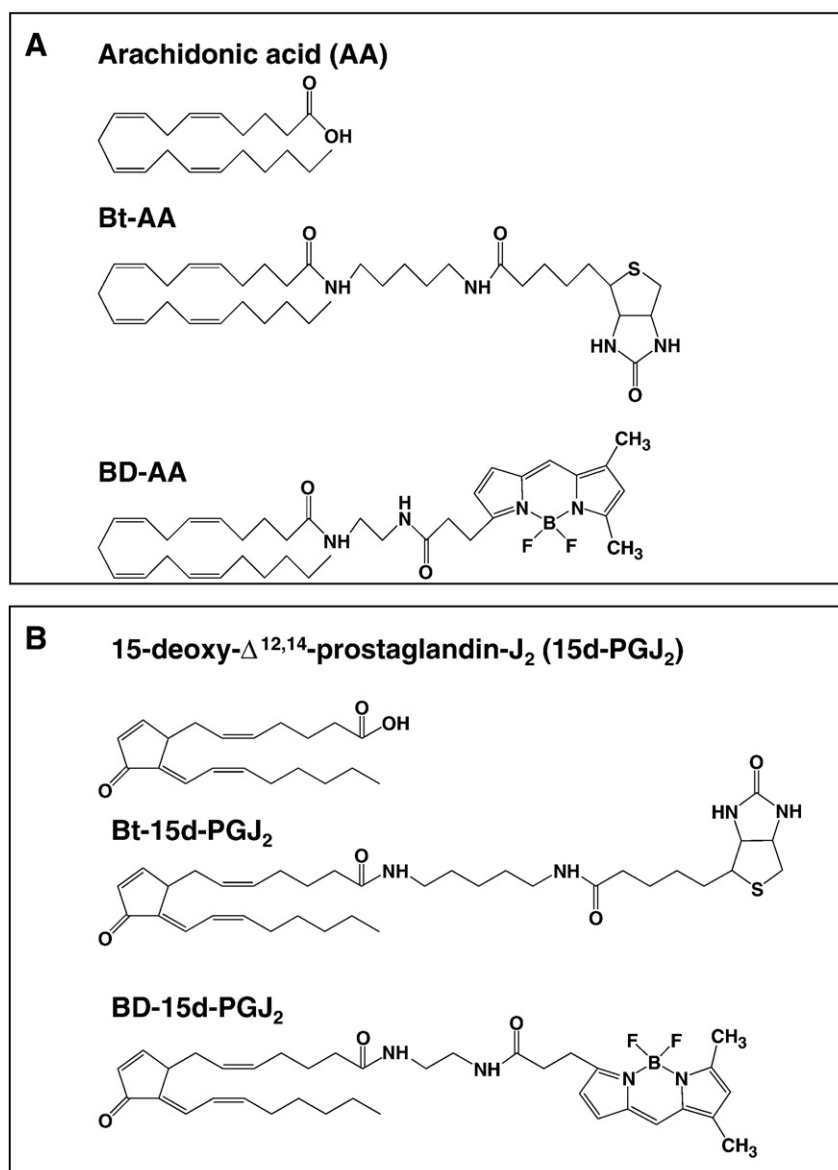


Fig. 3. Structures of lipids and tagged versions. (A) The structures of arachidonic acid and labeled derivatives. (B) The structures of 15-deoxy-prostaglandin J_2 and its biotinylated and BODIPY-tagged derivatives.

- (2) Make a solution of 10 mg/ml 5-(biotinamido)pentylamine in 44% ethanol/44% acetonitrile/12% water (v/v).
- (3) Add components to the glass vial in the following order:
 - (A) 6 μ l arachidonic acid (neat oil), approximately 5 mg;
 - (B) 500 μ l EDC solution (10 mg/ml in acetonitrile);
 - (C) 400 μ l 5-(biotinamido)pentylamine solution (10 mg/ml in ethanol, acetonitrile, and water).
 - (D) Add 94 μ l acetonitrile to bring the volume up to final 1-ml reaction volume.
- (4) Blow argon over the headspace in the vial and cap tightly.
- (5) Incubate covered in foil for 16–18 h at room temperature with gentle rocking.
- (6) Store at -80°C , if necessary. Otherwise purify immediately by HPLC.

Synthesis of BODIPY–arachidonic acid

This synthesis of BODIPY-labeled arachidonic acid is also performed using a carbodiimide-mediated conjugation reaction. The structure of BODIPY–arachidonic acid is shown in Fig. 3A. The reaction should be performed in a new, prerinsed amber or foil-covered glass vial to prevent light-dependent oxidation of the unsaturated lipid.

- (1) Prepare EDC (10 mg/ml) in acetonitrile. Vortex until in solution.
- (2) Resuspend BODIPY FL-EDA (5 mg vial) in 150 μ l of ethanol.
- (3) Add components to a clean glass vial in the following order:
 - (A) 6 μ l arachidonic acid (neat oil), approximately 5 mg;
 - (B) 500 μ l EDC;
 - (C) 150 μ l BODIPY FL-EDA dye.
- (4) Rinse the original BODIPY FL-EDA vial with 344 μ l acetonitrile. Pipette the suspension into the light-protected glass vial to bring the total volume to 1 ml.
- (5) Blow argon over the headspace in the vial and cap tightly.
- (6) Incubate the vial for 16–18 h at room temperature with gentle rocking.
- (7) Purify immediately by HPLC or store at -80°C until purification.

Synthesis of a biotinylated electrophilic lipid

The electrophilic prostaglandin 15-deoxy-prostaglandin- J_2 can also be conjugated to biotin; the structure of biotinylated 15-deoxy-prostaglandin- J_2 is shown in Fig. 3B. The protocol for this conjugation reaction is as follows:

Evaporate solvent from a 1-mg vial of 15d-PG J_2 under nitrogen gas. Resuspend the neat oil immediately in 600 μ l acetonitrile. *Note: It is recommended that a pure preparation of 15d-PG J_2 that does not contain mixed isomers is used (product information is given under Materials).*

- (1) Prepare 4 mg/ml 5-(biotinamido)pentylamine in 77% aqueous acetonitrile.
- (2) Make a 5 mg/ml stock solution of EDC in acetonitrile.
- (3) In an amber glass vial combine the following:
 - (A) 600 μ l 15d-PG J_2 (1 mg),
 - (B) 200 μ l of EDC in acetonitrile (1 mg),
 - (C) 200 μ l 5-(biotinamido)pentylamine (0.8 mg).
- (4) Fill the headspace with argon, and close the vial with a Teflon-lined cap.

- (5) Incubate the vial for 18 h at room temperature in the dark with constant, gentle agitation.
- (6) Purify the reaction mixture by HPLC or store at -80°C until purification.

Synthesis of a BODIPY-tagged electrophilic lipid

The protocol used for this conjugation reaction is as follows:

- (1) Evaporate solvent from five 1-mg vials of 15d-PG J_2 under nitrogen gas. Resuspend each vial immediately in 340 μ l acetonitrile.
- (2) Add 1 ml acetonitrile to a 5-mg vial of BODIPY FL-EDA, mix, and add 300 μ l of ethanol to facilitate solubility.
- (3) Make a 5 mg/ml stock solution of EDC in acetonitrile.
- (4) In five clean amber glass vials combine the following (the total volume in each vial should be 1 ml to allow for adequate mixing):
 - (A) 340 μ l 15d-PG J_2 (1 mg),
 - (B) 400 μ l of EDC in acetonitrile (2 mg),
 - (C) 260 μ l BODIPY FL-EDA dye (1 mg).
- (5) Fill headspaces with argon, and close vials with Teflon-lined caps.
- (6) Incubate the reaction mixture for 18 h at room temperature in the dark with constant, gentle agitation.
- (7) Purify the reaction mixture by HPLC or store at -80°C until purification.

Purification of tagged lipids by HPLC

After synthesis, the reaction preparations have a mixture of the original unreacted lipid, the tag, the expected product, and the priming compound, EDC. HPLC with UV–Vis detectors is needed to purify the tagged lipid. After chromatographic separation, product purity is verified using electrospray ionization mass spectrometry (ESI-MS). The mobile phases used for HPLC separations are prepared as follows:

- (1) Solvent A (10% acetonitrile/0.24% acetic acid/90% H_2O (v/v))
- (2) Solvent B (100% acetonitrile)
- (3) Solvent C (100% ultrapure water)

A summary of all HPLC parameters used for each lipid is given in Table 2. For all purifications, the column is equilibrated in the initial mobile phase, which varies depending on which tagged lipid is being separated (Table 1). The mass of each lipid, typical retention times, and optimum ionization mode for mass spectrometry are given in Table 1.

HPLC separation of arachidonic acid conjugates

For purification of tagged arachidonic acid, reversed-phase HPLC should be performed on a C18 semipreparative column as indicated under Instruments. Typically, an aliquot of the reaction mix containing approximately 1 mg of starting material is eluted at a flow rate of 4.5 ml/min using a linear gradient of 50–95% B after equilibration in 50% solvent A/50% solvent B (Table 2, Gradient I). Fractions containing the peak of interest are collected and verified using ESI-MS.

Table 1
Parameters for tagged lipids

Product	Gradient	Retention time	λ_{max} (nm)	λ_{obs} (nm)	ϵ at λ_{obs} ($\text{M}^{-1} \text{cm}^{-1}$)	Mass (Da)	Ionization mode
Arachidonic acid (AA)	I		205	215	9,400	305	
Bt-AA		13 min	205	215	9,400	615	Positive
BD-AA		29 min	504	504	76,000	621	Negative
15d-PG J_2	II		306	306	12,000	316	
Bt-15d-PG J_2		19 min	306	306	12,000	627	Positive
BD-15d-PG J_2		26 min	504	306	14,900	633	Negative

Table 2
HPLC gradient profiles for purification of tagged lipids

Gradient I			Gradient II		
Time (min)	A (%)	B (%)	Time (min)	A (%)	B (%)
0	50	50	0	100	0
15	50	50	13.2	100	0
25	5	95	27	5	95
35	5	95	40	5	95

Figure 4A represents an example separation of the Bt-AA reaction mixture by HPLC, with the product typically eluting at a retention time of 13 min (peak b), arachidonic acid eluting late (27 min, peak c), and oxidized products of arachidonic acid eluting early (peak a). To verify separation of Bt-AA, a wavelength scan from 200 to 300 nm (Fig. 4B) should be performed using a spectrophotometer. Fig. 4C is a characteristic spectrum obtained from ESI-MS in the positive ionization mode with the major peak at 616 ($M+H$)⁺, the m/z value for Bt-AA.

BD-AA can also be separated to high purity by HPLC (Fig. 5A) using the same HPLC gradient profile as for Bt-AA. As with Bt-AA, oxidized products containing the tag elute before the product (peak a). BD-AA elutes at approximately 29 min, and purity can be verified by UV-Vis spectrophotometry (Fig. 5B) at 200–600 nm. The maximum absorbance is observed at 504 nm (for BODIPY), with the second largest

peak at 205 nm (for arachidonic acid). Other components in the spectra are contributions of the BODIPY tag. Product purity must be verified using ESI-MS in the negative ionization mode as shown in Fig. 5C. The m/z value for BD-AA is 619 ($M+H$)⁺.

HPLC separation of tagged electrophilic lipids

For purification of biotinylated and BODIPY-tagged 15d-PGJ₂, HPLC separation should be carried out on a C18 preparative column as indicated under Instruments. Approximately 2 mg of starting material can be separated using Gradient II, at a flow rate of 20 ml/min. The absorbance of the eluant should be measured at 306 nm (λ_{\max} for 15d-PGJ₂). Fractions containing the peak of interest are collected and verified using ESI-MS.

BD-15d-PGJ₂ is purified using the HPLC Gradient II profile, which begins at a concentration of 100% A/0% B and after 13 min increases over a linear gradient to 5% A/95% B. BD-15d-PGJ₂ elutes during the linear gradient as peak c with a retention time of 26 min (Fig. 6A). A characteristic UV-Vis wavelength scan from 200 to 600 nm is shown in Fig. 6B, with major peaks observed at 504 nm (BODIPY) and 306 nm (15d-PGJ₂). The purity of BD-15d-PGJ₂ should be verified using ESI-MS in the negative ionization mode (Fig. 6C). BD-15d-PGJ₂ is apparent at 632 ($M-H$)⁻. A formic acid adduct formed during the mass spectrometry can also be seen in the spectrum (m/z 678).

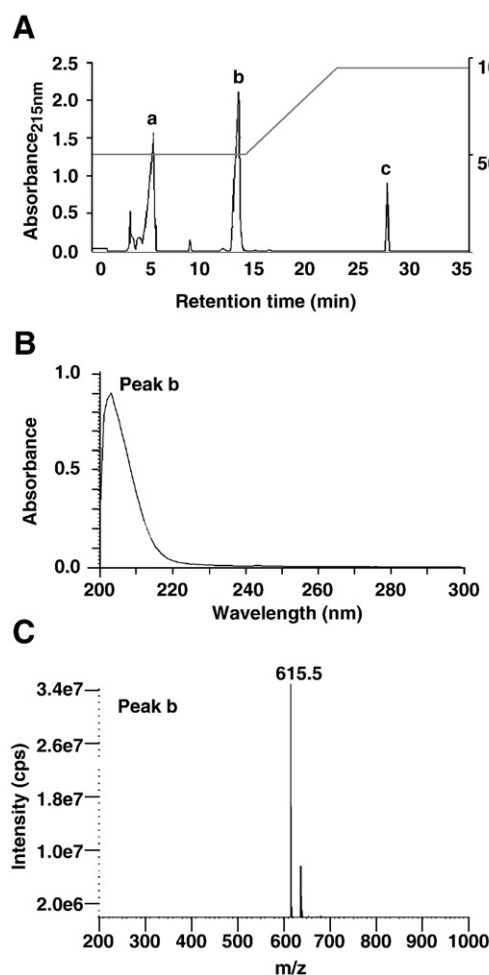


Fig. 4. HPLC purification, UV spectra, and mass-spectrometric verification of biotinylated arachidonic acid. (A) HPLC purification chromatogram showing the separation of arachidonic acid from biotinylated arachidonic acid after synthesis. (B) Representative UV spectrum of Bt-AA in ethanol, showing the characteristic absorbance λ_{\max} at 205 nm. (C) ESI-MS in the positive ion mode with the characteristic m/z peak for Bt-AA.

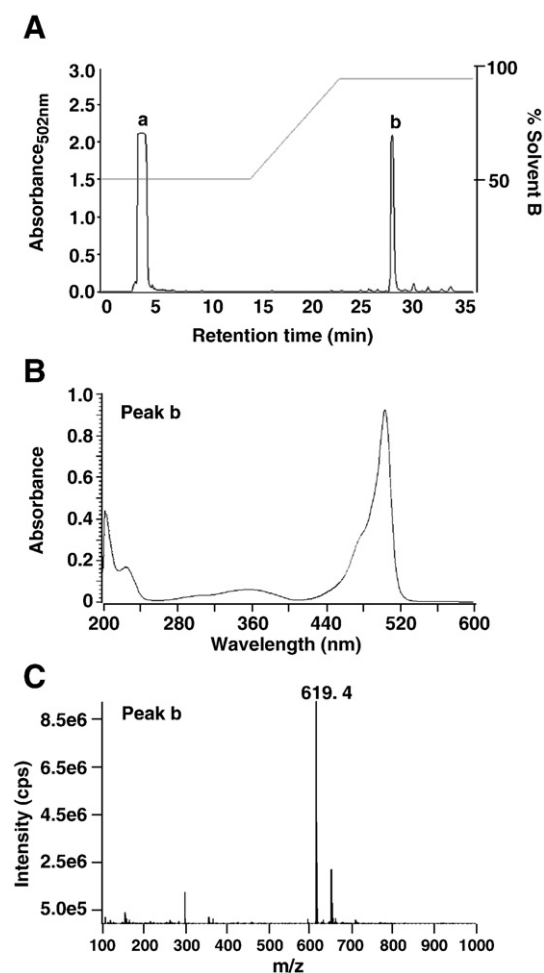


Fig. 5. HPLC purification, UV spectra, and mass-spectrometric verification of BODIPY-arachidonic acid. (A) HPLC purification chromatogram showing the separation of BODIPY from BD-AA after synthesis. (B) Representative UV spectrum of BD-AA in ethanol, showing the characteristic absorbance λ_{\max} at 205 nm for arachidonic acid and λ_{\max} at 504 nm for BODIPY. (C) ESI-MS in the negative ion mode showing the characteristic m/z peak for BD-AA.

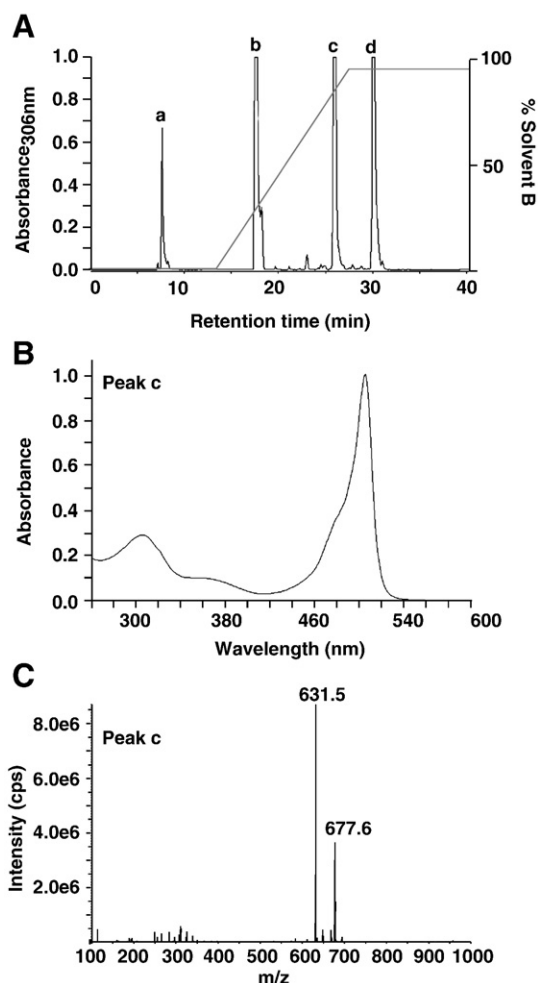


Fig. 6. HPLC purification, UV spectra, and mass-spectrometric verification of BODIPY-15d-PGJ₂. (A) HPLC purification chromatogram showing the separation of BODIPY and 15d-PGJ₂ from BD-15d-PGJ₂ after synthesis. (B) Representative UV spectrum of BD-15d-PGJ₂ in ethanol, showing the characteristic absorbance λ_{max} at 306 nm for 15d-PGJ₂ and λ_{max} at 504 nm for the BODIPY tag. (C) ESI-MS in the negative ion mode showing the characteristic m/z peak for BD-15d-PGJ₂.

Extraction of tagged lipids after HPLC purification

After HPLC purification, the tagged lipids are in a solution containing acetic acid, acetonitrile, and water. To eliminate the water and exchange solvents, it is necessary to extract the product using the following protocol:

- (1) Combine fractions of interest in a glass separatory funnel. Record the volume.
- (2) Mix with a 2:1 volume of 1:1 methanol:chloroform.
- (3) Add 1 drop of 1 M acetic acid per 5-ml fraction. Shake until a uniform layer has formed and vent.
- (4) Add 1 volume of 1 M sodium chloride and 1 volume of chloroform.
- (5) Shake well and vent.
- (6) Allow phases to equilibrate for 10 min.
- (7) Discard the aqueous (top) layer, wash the bottom lipid-containing layer with water, and repeat the extraction. Collect the bottom layer containing the lipid product in glass tubes.
- (8) Evaporate the solvent (from bottom layer) in a chemical hood under a steady stream of nitrogen gas.
- (9) Reconstitute the purified product in ethanol, and measure the concentration with a spectrophotometer using the absorbance and extinction coefficient given in Table 1.

- (10) Store tagged lipids at -80°C under inert gas. Verify the quality by spectrophotometry and mass spectrometry before use. In our experience, if care is taken to exclude exposure to both light and air the compounds are stable for several months.

Mass spectrometry verification

Once synthesized, the tagged lipids are purified and fully characterized by mass spectrometry as shown for Bt-AA in Fig. 4C. Typically, derivatives of arachidonic acid are diluted to $1\ \mu\text{M}$ in ethanol and injected in an infusion mobile phase of 50% acetonitrile containing 10 mM ammonium acetate for ESI-MS. Tagged 15d-PGJ₂ can be diluted to $1\ \mu\text{M}$ in ethanol and injected in an infusion mobile phase of 50% acetonitrile containing 0.1% formic acid. The mass for each lipid and optimal ionization mode for each are reported in Table 1.

General tips on using tagged lipids

Because tagged lipids are typically used for *in vivo* or *in vitro* detection of lipid products, lipid derivatives should be resuspended and stored at -80°C in a vehicle suitable for cell culture, such as ethanol or DMSO. As with any new experimental treatment, it must be verified that the vehicle used does not change the biological outcome observed in your particular model.

Owing to evaporation of the storage solvent, the concentration of tagged lipids may change over time and should be measured before each experiment using a spectrophotometer. For this reason, it is also not recommended to make small aliquots of the stock, because they evaporate more quickly. The absorbance and extinction coefficient for calculating the concentration of each lipid in ethanol is given in Table 1. In addition, a wavelength scan of arachidonic acid derivatives can be used to detect oxidation of the lipid, which will be apparent

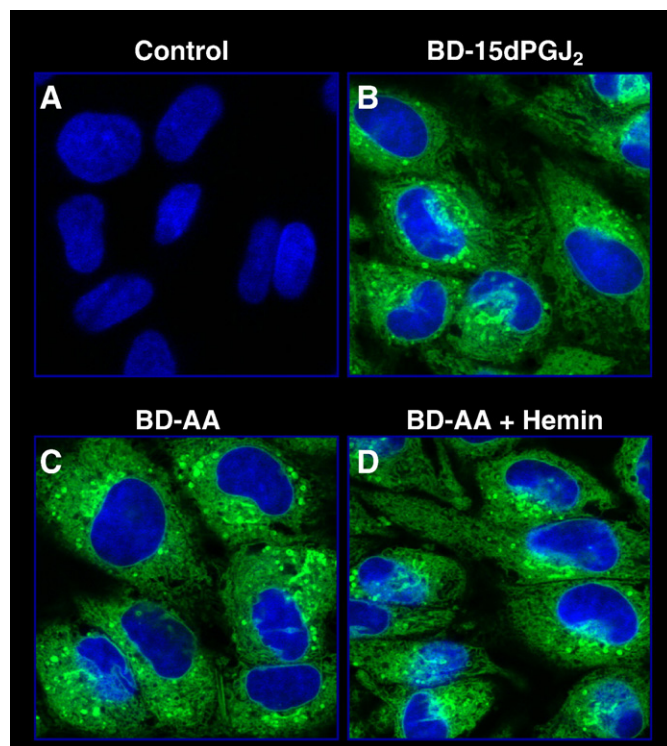


Fig. 7. Confocal microscopy of BAEC demonstrating live-cell visualization of fluorescently tagged lipids. (A) Control cells stained with Hoechst 33258 nuclear stain; (B) BAEC treated with $5\ \mu\text{M}$ BD-15d-PGJ₂ for 2 h before staining with Hoechst 33258; (C) BAEC treated with $10\ \mu\text{M}$ BD-AA for 2 h before staining with Hoechst 33258; (D) BAEC treated with $10\ \mu\text{M}$ BD-AA and $10\ \mu\text{M}$ hemin for 2 h before staining with Hoechst 33258. Images were acquired and merged as described under Fluorescence microscopy with tagged lipids.

through the presence of a peak at 233 nm due to conjugated dienes [45,46].

Fluorescence microscopy with tagged lipids

The BODIPY FL-EDA moiety allows for visualization of lipid products in situ. This can be combined with tracker dyes to determine the subcellular localization of the lipids during experimentation. To detect BODIPY-tagged lipids using microscopy, the following protocol is used:

- (1) Typically, cells are grown in chambered coverglasses to allow live-cell visualization of fluorescence. In the example shown in Fig. 7, bovine aortic endothelial cells (BAEC) were used as previously described [47].
- (2) Deprive the cells of serum for 16 h before the experiment by changing the medium to Dulbecco's modified Eagle's medium (DMEM) containing 0.5% FBS.
- (3) Treat the cells with 10 μ M BD-AA or 5 μ M BD-15d-PGJ₂.
- (4) If using AA, treat the BAEC with a pro-oxidant to induce lipid peroxidation (in this case hemin).
- (5) Incubate for 2 h at 37°C, 5% CO₂.
- (6) Stain nuclei with 1 μ g/ml Hoechst 33258 for 20 min at room temperature.
- (7) Wash cells twice with warm 0.5% FBS-DMEM (500 μ l per well).
- (8) Detect BODIPY FL fluorescence using an argon laser with a 488-nm laser line. Emission should be detected between 500 and 570 nm.
- (9) Detect Hoechst nuclear stain using an ultraviolet laser. Emission should be detected between 350 and 450 nm.
- (10) Single-channel images can be merged using Adobe Photoshop or Image J.

SDS-PAGE and Western blotting

The detection of covalent protein adducts by oxidized lipids using tagged lipids is facilitated by the biotin or BODIPY moiety present on the lipid. Both types of tagged lipids offer unique advantages and should be considered depending on the research goal, available laboratory equipment, and experimental conditions. Biotinylated lipids allow for sensitive detection as well as accurate quantification of protein modification using Western blot analysis [39]. BODIPY-labeled lipids can also be detected using Western blot analysis; however, an advantage of using fluorescent tags is that the gel can be imaged immediately after electrophoresis using "in-gel" imaging, eliminating the need for transfer to a membrane. For both biotin- and BODIPY-tagged lipids, high-resolution digital imaging techniques are advantageous. In our experience, the use of film is rarely optimal, primarily owing to the fact that, even at low exposures, the film easily saturates. The result is that quantification obtained from a film image is often nonlinear and may underestimate experimental changes. The dynamic range of digital cameras and fluorescence imagers (e.g., Typhoon imagers) is far superior, and they are therefore recommended for Western blotting applications using biotin tags and/or in-gel fluorescence imaging. Protocols specific for each tagged technology (biotin or BODIPY) are detailed below.

Detecting protein modification using biotinylated lipids

One advantage of using biotinylated lipids is that the amount of lipid-protein adducts may also be calculated using a standard, such as biotinylated cytochrome c (Bt-cyt c). It is recommended to include such a standard on the same gel to quantitate the biotin content in experimental samples [39]. Because each mole of lipid is tagged with one mole of biotin, the quantification of biotin on a streptavidin blot serves as a quantitative marker for the lipid adduct, therefore allowing the calculation of the mol lipid/mol protein in proteomics format. The

following protocol is used to detect proteins modified by biotinylated lipids:

- (1) After experimentation using Bt-AA or Bt-15d-PGJ₂, the protein samples are separated by 1D or 2D SDS-PAGE.
- (2) To blot proteins, transfer to a nitrocellulose or PVDF membrane at 100 V for 2 h with cooling or at 25 V overnight at 4°C. *Note: If chemifluorescence imaging will be used, a low-fluorescence PVDF membrane such as Hybond-LFP is recommended. See Materials.*
- (3) Block the membranes with 5% milk in Tris-buffered saline (25 mM Tris, 137 mM sodium chloride, 2.6 mM potassium chloride, 0.05% Tween 20) for 1 h.
- (4) Wash membranes thoroughly to remove all milk. *Note: Some milk blotting formulations contain biotin. Because this could interfere with streptavidin binding to biotinylated proteins, milk should not be included after the blocking step.*
- (5) Incubate blots with streptavidin-HRP (1:10,000 dilution in TBS-T) for 1 h at room temperature to ensure even distribution of streptavidin-HRP.
- (6) Wash the Western blots with TBS-T 3 \times 10 min at room temperature.
- (7) Add the chemiluminescent or chemifluorescent substrate evenly to the blot, ensuring coverage of the entire surface.
- (8) If imaging using chemiluminescence, acquire a series of images using a CCD camera imager (Alpha Innotech). For chemifluorescence imaging, use a fluorescence scanner such as the Typhoon imager (GE Healthcare).
- (9) An image that is not saturated and is within the linear range for band intensity should be chosen for absolute quantitation with the standard.
- (10) Refer to [39] for the complete method for using Bt-cyt c as a standard. Briefly, a range of Bt-cyt c concentrations should be used to construct a standard curve. The concentrations chosen will depend on the exact experimental protocol and amount of biotin labeling; however, in our experience 0.01–10 pmol is the ideal range. Quantitate the band intensity per lane, and plot this versus the known amount of biotin for each lane of Bt-cyt c to obtain a standard curve. A representative standard curve and quantitated blot are shown in Fig. 8A.

Detecting protein modification by BODIPY-tagged lipids with Western blotting

The following protocol is used to detect proteins modified by BODIPY-tagged lipids by Western blotting. Alternatively, if access to a fluorescence imager is available, in-gel fluorescence imaging may be used. Refer to Visualizing BODIPY-tagged adducts using in-gel fluorescence, below.

- (1) Protein samples are separated by 1D or 2D SDS-PAGE.
- (2) To blot proteins, transfer to nitrocellulose or PVDF membrane at 100 V for 2 h with cooling or at 25 V overnight at 4°C.
- (3) Block the membranes with 5% milk in TBS-T for 1 h at room temperature.
- (4) Incubate with anti-BODIPY antibody (1:1000 dilution in 5% milk) for 3 h at room temperature to ensure even distribution of the BODIPY antibody.
- (5) Wash the Western blots with TBS-T 3 \times 10 min at room temperature.
- (6) Incubate with anti-rabbit antibody (1:3000 dilution in 5% milk) for 1 h at room temperature.
- (7) Wash the blots with TBS-T 3 \times 10 min to decrease nonspecific binding.
- (8) Add the chemiluminescent or chemifluorescent substrate evenly to the blot, ensuring coverage of the entire surface.
- (9) For chemiluminescence imaging, acquire a series of images using a CCD camera such as the Alpha Imager (Alpha Innotech).

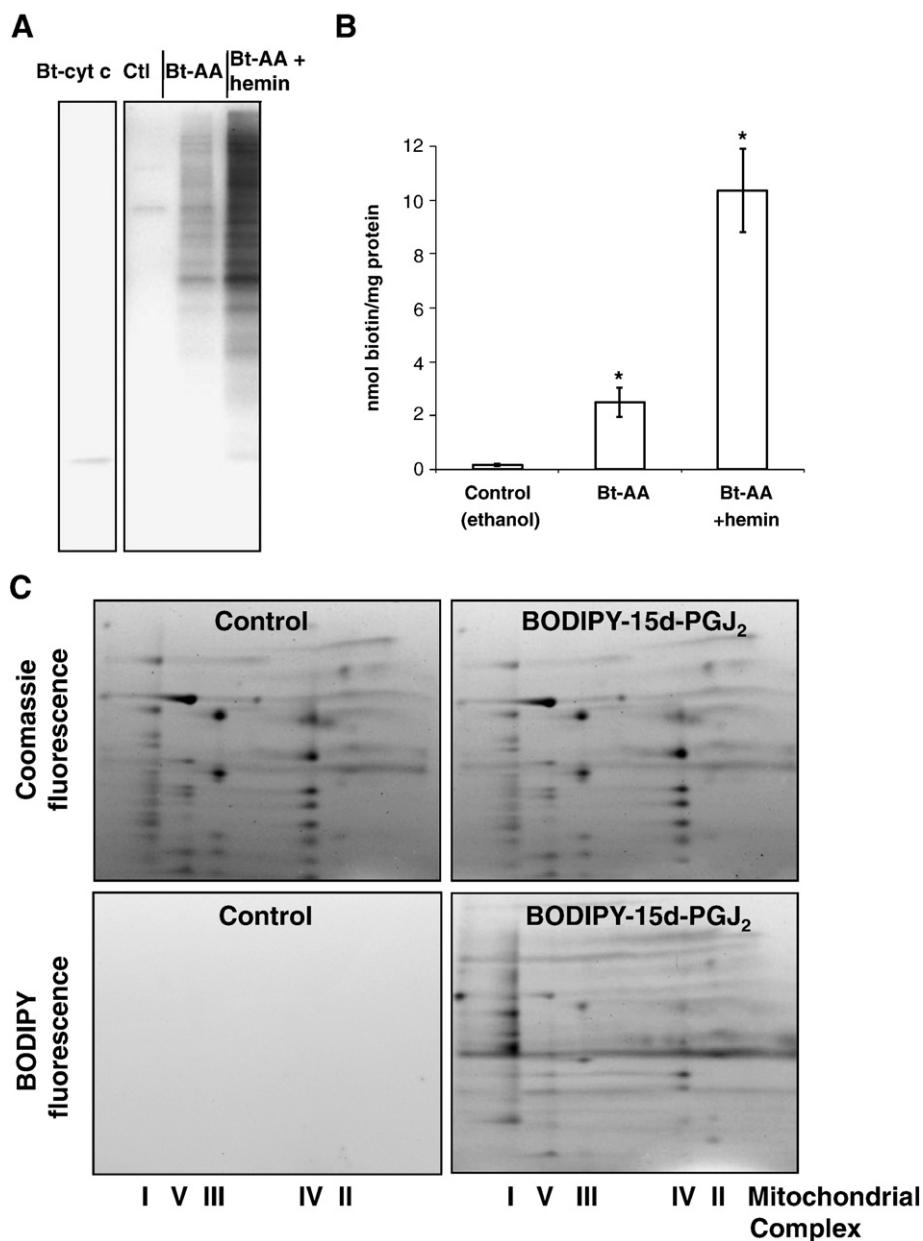


Fig. 8. Detection of proteins modified by tagged lipids by Western blotting and in-gel fluorescence techniques. (A) RAW 264.7 macrophages were treated with Bt-AA in the presence or absence of hemin chloride (10 μ M) for 4 h. Protein adducts were detected using streptavidin–HRP. The standard (0.2 pmol), biotinylated cytochrome c (Bt–cyt c), was included on the same Western blot for quantification. A representative immunoblot is shown. (B) Quantification of biotin-labeled adducts using the internal standard, Bt–cyt c. $n = 3$, $*p < 0.05$ compared with control by student's t-test. (C) Two-dimensional blue native polyacrylamide gels of mouse heart mitochondrial proteins. Mitochondria isolated from mouse heart were left untreated (Control) or treated with 20 μ M BODIPY–15d-PGJ₂ for 30 min. Fluorescent images of BODIPY-labeled proteins were obtained on a Typhoon Trio Variable Mode imager using a 526SP emission filter and 488-nm laser at 650 V. For visualization of total protein using Coomassie fluorescence, the same gels were imaged using the 670BP30 emission filter and the 633-nm laser operating at 700 V.

(10) For chemifluorescence imaging, dry membranes after incubating with ECL Plus reagent. In our experience, less background is observed when the membrane is dried completely before imaging. After exposure to ECL Plus reagent, transfer the membrane to a stacked layer of lab tissue and gently blot the membrane dry. Afterward, place the membrane on a piece of filter paper and allow to dry for at least 15 min before imaging. Failure to allow the blot to dry completely will result in uneven background and image artifacts. Lay the blot face down on the Typhoon plate and overlay with a piece of nonfluorescent plastic such as 3 M Dual Purpose transparency film. Scan image using the fluorescence acquisition mode, the 520 BP 40 emission filter, and the blue (488-nm) laser. (Note: If using a Typhoon Trio Plus imager, use the ECL Plus excitation setting.) Set the PMT voltage

to 450–500 V initially. Do not press the sample; the plastic transparency film will adequately press the membrane against the plate. Set the pixel size to 500–1000 μ m initially. Adjust the PMT voltage to obtain an image that is intense but not saturated. Take the final image using a pixel size of 100 μ m or less.

Visualizing BODIPY-tagged adducts using in-gel fluorescence

Because bromophenol blue is a fluorescence quenching agent [48,49], a dye-free SDS sample loading buffer is recommended if using fluorescence imaging. Our laboratory uses a 5 \times sample buffer containing 250 mM Tris–HCl (pH 6.8), 25% glycerol, and 10% sodium dodecyl sulfate. If another loading buffer is used, it should be adapted so that components that interfere with BODIPY fluorescence are removed or substituted with a similar but noninterfering reagent. The

Typhoon imaging system or another analogous system may be used for imaging BODIPY-labeled proteins. In addition, we recommend staining the gels with Coomassie blue or another stain such as deep purple (GE Healthcare) after acquiring the BODIPY image to verify even protein loading. This allows for simultaneous or postvisualization of the total protein amount as well as the modifications.

- (1) Owing to potential photobleaching, the BD-labeled protein samples should be separated by 1D or 2D SDS–PAGE under low-light conditions. It is important to run the dye front completely off of the gel before imaging because the unreacted BODIPY-labeled lipid agent can interfere with imaging and result in poor image quality in the lower half of the gel.
- (2) After separation, the gel can be left in the glass plates and imaged or removed from the plates and stained with Coomassie, deep purple, or another stain with excitation/emission properties distinct from those of BODIPY.
 - (A) For gel imaging directly in the glass plates using a Typhoon or similar imager, wash the outside of the glass plates well with 70% ethanol, followed by distilled water. Allow plates to dry completely.
 - (B) Place the dry glass plates containing the gel on 1.5-mm spacers, and adjust the focal plane accordingly (e.g., to 2–4 mm to account for the width of the glass plate).
 - (C) If using a Typhoon imager, use the 520 BP40 or 526SP emission filter and the 488-nm blue laser. Use similar settings if using another imager, or capture image using UV light and a camera.
- (3) Alternatively, the gel can be removed from the plates and stained immediately with a total protein stain. The gel can then be imaged using the laser and emission filter described above for BODIPY. Subsequently, a fluorescent Coomassie image can be acquired on the Typhoon, using the 670BP30 emission filter and 633-nm laser. *Note: In our experience, we have found that imaging the BODIPY label while the gel is in the glass plates used for electrophoresis provides the best results.*

Calculations and expected results

Confocal microscopy with fluorescently labeled lipids

As shown in Fig. 7, BD–15d-PGJ₂ and BD–AA are capable of entering BAEC after 2 h. In this experiment, BD–15d-PGJ₂ fluorescence showed a reticular pattern, consistent with previous studies demonstrating its ability to concentrate in mitochondria [41,42]. After entering cells, the fluorescent tag of BD–AA retains its excitation/emission properties and does not seem to be oxidized owing to hemin treatment.

Protein modification by tagged lipids

Both the BODIPY and the biotin moieties allow the detection of protein modification by endogenously produced lipid peroxidation products in lysates prepared from *in vitro* and *in vivo* samples. Hemin has been shown to increase lipid peroxidation, resulting in the formation of protein-reactive lipid species; however, the formation of protein–lipid adducts has not been previously reported. As shown in Fig. 8A, Bt–AA can be used to detect protein modifications by lipid peroxidation products in cells. In this experiment, RAW 264.7 macrophages were treated with the ethanol vehicle (control), Bt–AA, or hemin and Bt–AA. In control lysates, a small number of proteins can be detected on the streptavidin–HRP blot corresponding to endogenous biotin-containing carboxylases. Bt–AA only slightly increases the reactivity with streptavidin–HRP, suggesting that arachidonic acid itself is not reactive with proteins, as expected. However, addition of hemin, which catalyzes lipid peroxidation, results in an increase in streptavidin–HRP signal. BD–AA can be used in a similar manner and also facilitates the detection of protein-

reactive lipid species. In addition, other controls, such as the addition of the chain-breaking antioxidants butylated hydroxytoluene and α -tocopherol, can be used to decrease LPO and protein adduct formation (data not shown).

The electrophilic lipid 15d-PGJ₂ has been shown to modify proteins in several experimental models [17,20,50–54] and is capable of modifying the mitochondrial proteome [40]. The mitochondrial proteome modified by BD–15d-PGJ₂ can be investigated using proteomic techniques. Fig. 8C shows the results from such an experiment. Briefly, isolated cardiac mitochondria were treated with BD–15d-PGJ₂, and the proteins were separated by 2D blue native PAGE. This method uses the protein-binding and charge-conferring characteristics of Coomassie dye to facilitate protein separation in the first dimension [55–57]. After separation in the second dimension, the proteins remain bound to much of the Coomassie dye. Hence, both the BODIPY and the Coomassie fluorescence can be detected using the guidelines under Visualizing BODIPY-tagged adducts using in-gel fluorescence. As shown in Fig. 8C, equal protein loading was verified on 2D blue native PAGE gels of isolated heart mitochondria using the intrinsic Coomassie fluorescence. BODIPY fluorescence could be detected with a Typhoon imager in samples treated with BD–15d-PGJ₂. No interfering fluorescence of proteins was detected at this wavelength in control mitochondria. The blue native PAGE gels shown in Fig. 8C demonstrate the utility of using tagged electrophilic lipids to detect reactive subproteomes. Similar experiments with these tagged lipids can be performed using other 1D and 2D gel-based separation techniques.

Quantification of protein modification by tagged lipids

Protein modification by biotin-tagged lipids can be quantified using standards such as Bt–cyt c. The development of this method is described in [39]. Briefly, addition of Bt–cyt c allows for the absolute determination of the moles of biotin per milligram of protein in a biological sample. Owing to the addition of the biotin tag, this increase in biotin is assumed to be due to protein modifications by the biotin-containing oxidized lipid. Therefore, each biotin molecule is equivalent to one lipid covalently adducted to a protein. Because covalent modification of proteins is an important regulatory mechanism in cell signaling, reliable measurement of these modifications is advantageous for these studies [20,40,52,53]. As shown in Fig. 8A, proteins modified by oxidized products of Bt–AA were detected on streptavidin–HRP Western blot. Quantification of adducts (nmol biotin/mg protein) was facilitated using the internal standard, Bt–cyt c, and is shown in Fig. 8B.

Caveats

Do tagged lipids have biological effects different from those of untagged derivatives?

Tagged derivatives should be compared with the untagged version of the same lipid for all new experimental models to ensure that the biological effects are not changed by the tag. For example, induction of antioxidant defenses and the cytoprotection elicited by the electrophilic lipid 15d-PGJ₂ are not significantly different for the tagged derivatives of this electrophile [17]. Importantly, the covalent addition of BODIPY and biotin occurs at the carboxylic acid distant from the electrophilic carbons on the molecule and will not affect the electrophilicity of the lipid. However, modification of the carboxyl group may change other properties of the lipid [58]. For example, the biotinylated derivative of 15d-PGJ₂ is not an effective ligand for PPAR γ .

Although the use of tagged unsaturated fatty acids allows the identification of lipid–protein adducts derived from lipid peroxidation, there are a number of important limitations. For example, modifications of the fatty acid other than oxidation that lead to covalent modification of proteins could also result in increased protein

adduct formation. As a control for this, the attenuation of modification by chain-breaking antioxidants, which should inhibit lipid peroxidation but not other modifications of the fatty acid such as acetylation, can be used. In interpreting the accumulation of lipid–protein adducts a number of mechanisms should be considered. For example, it is known that electrophilic lipids can inhibit the proteasome, and thus the turnover of adducted proteins may be inhibited [30,51].

Identification of lipid–protein adducts

Use of the tagged lipids described herein enables the discovery and identification of protein targets of reactive LPO products. A limitation of this approach is that the products of LPO from which the tag is lost or oxidized will not be detected. For example, 4-hydroxy-2-nonenal generated from the oxidation of a PUFA will not contain a tag, which is attached to the carboxyl group. In addition, it should be noted that using the tagged substrate arachidonic acid and the oxidation systems described (i.e., hemin) will not enable the investigator to know the specific oxidized lipid species that modified a particular protein without coupling this technology to mass spectrometry analysis. However, use of the tagged substrate does allow a more comprehensive and thorough approach to identifying the proteins modified by LPO in response to oxidative stress or inflammation.

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