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Detection and Quantification of Protein Adduction by Electrophilic Fatty Acids: Mitochondrial Generation of Fatty Acid Nitroalkene Derivatives

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Abstract

Nitroalkene fatty acid derivatives manifest a strong electrophilic nature, are clinically detectable and induce multiple transcriptionally-regulated anti-inflammatory responses. At present, the characterization and quantification of endogenous electrophilic lipids is compromised by their Michael addition with protein and small molecule nucleophilic targets. Herein, we report a trans-nitroalkylation reaction of nitro-fatty acids with γ -mercaptoethanol (BME) and apply this reaction to the unbiased identification and quantification of reaction with nucleophilic targets. Trans-nitroalkylation yields are maximal at pH 7 to 8, and occur with physiological concentrations of target nucleophiles. This reaction also amenable to sensitive mass spectrometry-based quantification of electrophilic fatty acid-protein adducts upon electrophoretic resolution of proteins. In-gel trans-nitroalkylation reactions also permit the identification of protein targets without the bias and lack of sensitivity of current proteomic approaches. Using this approach, it was observed that fatty acid nitroalkenes are rapidly metabolized in vivo by a nitroalkene reductase activity and mitochondrial ω -oxidation, yielding a variety of electrophilic and non-electrophilic products that could be structurally characterized upon BME-based transnitroalkylation reaction. This strategy was applied to the detection and quantification of fatty acid nitration in mitochondria in response to oxidative inflammatory conditions induced by myocardial ischemia-reoxygenation.

Keywords

Nitroalkenes; nitrated fatty acid; Michael addition; electrophiles; protein adduction; fatty acids

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Introduction

Electrophilic fatty acid derivatives, generated by both auto-catalytic and enzymatic oxidation of unsaturated fatty acids, are emerging as endogenous signaling mediators that induce a broad array of anti-inflammatory and chemotherapeutic responses [1, 2]. This class of compounds include fatty acid hydroperoxides and their products 4-hydroxynonenal (4-HNE) [3] and 4-oxo-2-nonenal (4-ONE) [4] amongst others. Also, the oxidation of arachidonic, eicosapentaenoic and docosahexaenoic acid yields electrophilic products including isoprostane A₃/J₃ [5], neuroprostane cyclopentenone A₄/J₄-NP [6] and 15-deoxy-prostaglandin J₂ (15-d-PGJ₂) [7, 8]. Enzymatically-derived electrophilic lipids include products of lipoxygenase (LOX), cyclooxygenase (COX) and cytochrome P450 oxidation of arachidonic, eicosapentaenoic and docosahexaenoic acids, including lipoxins, resolvins and protectins [9].

The downstream signaling actions of electrophilic fatty acid derivatives are predominantly ascribed to post-translational Michael addition with critical nucleophilic amino acids located in a sub-proteome of electrophile-sensitive proteins. Specifically, adducts are formed with the nucleophilic amino acids lysine, histidine and cysteine [4, 10, 11]. The redox-dependent formation of electrophiles and subsequent DNA adduction and post-translational protein modification reactions have typically been viewed as pathogenic [12-14]. In the context of inflammation however, the reactions of redox-derived electrophiles are emerging as adaptive events that link redox reactions with the resolution of inflammation. Specifically, the modification of functionally-significant nucleophilic moieties of signaling proteins and transcription factors including NF- κ B, heat shock factors 1-4 and Keap1 induces a net salutary modulation of gene translation and protein expression [15-17]. Consequently, the redox-dependent formation of electrophiles orchestrates cell and tissue responses to metabolic and inflammatory challenges as diverse as heat shock, ischemia-reperfusion, sepsis and trauma [18-21].

Recently, description of the *in vitro* reactions and *in vivo* formation of nitrated fatty acids (NO₂-FA) added new dimension to fatty acid signaling by further linking the inflammatory generation of oxides of nitrogen with the formation of electrophilic byproducts [22-29]. Vinyl nitro derivatives of fatty acids that include 9- or 10-NO₂-octadecenoic acid and 9, 10, 12 or 13-NO₂-octadecadienoic acid are highly reactive electrophiles [28]. This is a consequence of the nitro group being one of the strongest electron-withdrawing biological functional groups [22, 30, 31], rendering the olefinic carbon to the nitro group particularly reactive to nucleophiles. Thus, vinyl nitro-fatty acids react with glutathione (GSH) with a faster reaction constant than most other biological electrophiles (k_{obs} 90-300 M⁻¹ sec⁻¹ compared to ~1 M⁻¹ sec⁻¹ for 4-HNE and 15-d-PGJ₂) [22]. The reaction between NO₂-FA and nucleophilic amino acids (termed nitroalkylation) is also unique in that adduction reactions are reversible. This also is an important criteria if a reactive species is to be considered an effective signaling mediator, in that redox-dependent modulation of enzymatic activity and protein distribution would be transient and reversible [28]. The reversibility of nitroalkylation reactions also supports the transfer of adduction reactions between different nucleophilic targets, thus further influencing the kinetics of cell signaling and enzyme activity responses.

Current data indicate that NO₂-FA potently inhibit inflammatory responses. In particular, OA-NO₂ and LNO₂ inhibit cytokine and inducible nitric oxide synthase expression in LPS and interferon- γ -stimulated monocytes by alkylating critical thiols in the DNA binding region of the NF- κ B p65 subunit [32]. In addition, nitro-arachidonic acid and cholesteryl nitrolinoleate down-regulate inducible nitric oxide synthase (NOS2) expression during macrophage activation [33]. Proteomic analysis of glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) as a model protein revealed that the principal nucleophilic targets of NO₂-FA are His and Cys, resulting in reversible inhibition of GAPDH activity and promoting translocation to membranes [28]. More recently, cholesteryl-nitrooleate formation by LPS and cytokine-activated activated macrophages is reported to suppress NOS2 expression and cytokine secretion and induce heme oxygenase-1 expression [29].

The characterization and quantification of lipid mediators is typically performed using either GC- or LC-based mass spectrometry with high accuracy. Although less sensitive and specific, the immuno-detection of electrophilic protein adducts is also performed by EIA and ELISA analysis [34] using antibodies developed against new epitopes formed after adduction reactions [35-37]. The poor sensitivity and specificity of these affinity-based methods, confounded by the multiplicity of electrophilic lipid derivatives and the array of regioisomers formed by redox reactions, render antibody-based approaches impractical. Moreover, in the case of 4-HNE, the preponderance of Michael addition products over Schiff's base adducts supports that the determination of 4-HNE adducts in biological samples via hydrazine or hydroxylamine displacement of HNE from adducted proteins underestimates adducted levels of electrophilic lipids.

Insight into the formation, metabolism, distribution and signaling actions of biological electrophiles, including NO₂-FA, would benefit from an analytical method that permits qualitative and quantitative analysis of the array of products being formed, as well as the relative distribution of biomolecule-adducted vs. free derivatives. In this regard, traditional mass spectrometry-based proteomic approaches are time-consuming, lack quantitative rigor and only provide information about selected and identifiable targets. Herein a strategy is reported that reveals the ratios of nitroalkylated (to both protein and low molecular weight targets) vs. free NO₂-FA derivatives and that identifies specific protein adducts. This trans-nitroalkylation reaction is a strategy applicable to other reversibly-adducted electrophiles and is applied herein to the identification and quantification of NO₂-FA-albumin adducts formed in mice. In addition, we identified the formation of electrophilic oxo-fatty acid derivatives and NO₂-FA in rat heart mitochondria subjected to ischemia-reoxygenation. Overall, the reactions of electrophilic fatty acids reported herein are biologically-relevant and present a new pathway by which nitroalkenes can be placed in reserve, protected from decay and transported to remote anatomic locations to exert pluripotent signaling actions.

Experimental Procedures

Materials and chemicals

Rabbit muscle GAPDH and diethylenetriamine-pentaacetic acid (DTPA) were purchased from Sigma (St Louis, MO). Potassium phosphate and citric acid was from Mallinckrodt (Hazelwood, MO). Nitro-linoleic acid (9-, 10-, 12, and 13--9,12-*cis*-octadecadienoic acids; LNO₂), nitro-oleic acid (9- and 10-nitro-9-*cis*-octadecaenoic acids; OA-NO₂) and their corresponding internal standards ([¹³C₁₈]LNO₂ and [¹³C₁₈]OA-NO₂) were prepared as previously described [23, 24]. Biotin-OA-NO₂ was prepared as described [32]. GAPDH concentration was determined at 280 nm ($\epsilon = 1.46 \times 10^5 \text{ M}^{-1}\cdot\text{cm}^{-1}$) [38].

GS-OA-NO₂ and GS-LNO₂ synthesis

Nitroalkene adducts of glutathione (GSH) were synthesized as standards for LC separation and quantitative MS analysis, and included GS-OA-NO₂, GS-[¹³C₁₈]OA-NO₂, GS-LNO₂, GS-[¹³C₁₈]LNO₂. For syntheses, GSH (300 mM) was solvated in 500 mM potassium phosphate buffer (final pH 7.4) and treated with 1.5 mM of either LNO₂[¹³C₁₈]LNO₂, OA-NO₂, or [¹³C₁₈]OA-NO₂ at 37°C for 30 min. The reaction was stopped by acidifying to pH 2 with formic acid. GS-nitroalkene adducts were purified from non-reacted GSH by reverse

phase chromatography. Products were analyzed by electrospray ionization triple quadrupole mass spectrometry (ESI MS/MS) with and without HPLC separation. The concentration of purified standards was determined by elemental nitrogen analysis following pyrolysis, using an Antek chemiluminescent nitrogen detector (Houston, TX).

Structural Characterization and quantification of BME-adducted NO₂-FA by ESI MS/MS

Qualitative analysis of OA-NO₂ by ESI MS/MS was performed using a hybrid triple quadrupole-linear ion trap mass spectrometer (4000 Q trap, Applied Biosystems/MDS Sciex). To characterize synthetic BME-OA-NO₂ adducts, a reverse-phase HPLC-based methodology was developed using a 20 × 2 mm C18 Mercury MS column (3 μm). NO₂-FA were separated and eluted from the column 2 using a gradient solvent system consisting of A (H₂O containing 0.1% NH₄OH) and B (CNCH₃ containing 0.1% NH₄OH) at 750 μl/min under the following conditions: hold at 0% B for 2 min, then 0-90 % B (3 min; hold for 2 min); and 90-0% B (0.1 min; hold for 2 min). Enhanced product ion analysis (EPI) was performed in the negative ion mode to generate characteristic and identifying fragmentation patterns of adducted eluting species with precursor masses of *m/z* 404.4, BME-OA-NO₂; 402.4, BME-LNO₂; 422.4, BME-[¹³C₁₈]OA-NO₂; 420.4, BME-[¹³C₁₈]LNO₂, 633.5, GS-OA-NO₂; 631.5, GS-LNO₂.

Quantitative analysis of BME-adducted molecules was performed in the multiple reaction monitoring (mrm) scan mode. BME adducts were detected by monitoring for molecules that undergo a M⁻/[M - BME]⁻ transition. Thus, the transitions used were as follows: BME-OA-NO₂, *m/z* 404.4/326.3; BME-LNO₂, *m/z* 402.4/324.3; BME-[¹³C₁₈]OA-NO₂, *m/z* 422.4/344.3; BME-[¹³C₁₈]LNO₂, *m/z* 420.4/342.3. The declustering potentials were -90 V and -50 V for GS- and BME- adducts, respectively. The collision energies were set at -35 and -17 for GS and BME adducts, respectively. The different GSH-adducted species were detected using an mrm scan mode by reporting molecules that undergo a M⁻/[M - nitroalkene]⁻ transition. Zero grade air was used as source gas, and nitrogen was used in the collision chamber. Data were acquired and analyzed using Analyst 1.4.2 software (Applied Biosystems, Framingham, MA). Quantification was achieved by comparing peak area ratios between analytes and their corresponding internal standards, and then calculating analyte concentration using an internal standard curve.

β-Mercaptoethanol reaction with nitroalkenes

The reaction between LNO₂ (100 nM) and OA-NO₂ (100 nM) was performed in citrate (for pH 2-5) and potassium phosphate buffer (for pH 6-13) (50 mM, 100 μM DTPA) containing 1 mM β-mercaptoethanol (BME) for 10 min at room temperature. The reaction was stopped by adding formic acid (final concentration 1 %, to give a pH lower than 3 in all cases). Then, internal standards were added, including β-mercaptoethanol-LNO₂ (BME-[¹³C₁₈]LNO₂, 5.4 nM) and β-mercaptoethanol-OA-NO₂ (BME-[¹³C₁₈]OA-NO₂, 2.7 nM). The samples were quantified by HPLC ESI MS/MS, as described.

Trans-nitroalkylation reaction

GS-OA-NO₂ and GS-LNO₂ were incubated at different pH using the same conditions described for the nitroalkylation reaction, but with the addition of 0.5 M β-mercaptoethanol for 60 min at 40°C. Reactions are stopped by adding formic acid (final volume fraction of 1%, giving pH < 3 in all cases) followed by BME-[¹³C₁₈]LNO₂ (15 nM) and BME-[¹³C₁₈]OA-NO₂ (15 nM) addition as internal standards. The samples were quantified by HPLC ESI MS/MS as detailed below.

pH dependence of alkylation reactions

LNO₂, OA-NO₂, GS-LNO₂ and GS-OA-NO₂ were subjected to alkylation and/or trans-alkylation reactions at different pH values. The buffers used were 50 mM sodium citrate, 100 μM DTPA for pH 2-5 and 50 mM potassium phosphate, 100 μM DTPA for higher pH values. Reactions were stopped by addition of 10% (v/v) formic acid and [¹³C]-labeled BME-nitroalkene adducts were added as internal standards (final concentrations, 1.36 ng/ml BME-[¹³C₁₈]OA-NO₂ and BME-[¹³C₁₈]LNO₂) in each sample. Samples were kept at 4°C until quantified by HPLC ESI MS/MS. Alkylation and trans-nitroalkylation reactions were performed with BME concentrations of 1 and 500 mM, respectively.

Analysis of adduct stability in gel electrophoresis-based studies

GAPDH (5 mg/ml) was incubated for 60 min at 37°C in 50 mM phosphate buffer in the presence of 100 μM DTPA and biotin-OA-NO₂. After incubations, sample buffer in the presence or absence of 5 mM TCEP was added and samples subjected to different temperatures and time periods to study adduct stability under these conditions. NO₂-FA-adducted GAPDH was then resolved by gel electrophoresis, transferred onto a PVDF membrane, and developed using HRP-labeled streptavidin.

Analysis of nitroalkylated proteins in vitro

GAPDH (5 mg/ml) was incubated for 60 min at 37°C in phosphate buffer (50 mM, pH 7.4) in the presence of 100 μM DTPA and varying concentrations of OA-NO₂. After incubation, sample buffer and 5 mM TCEP were added and heated for 7 min at 75°C. The samples were then loaded onto gels, electrophoretic separations performed and the GAPDH band identified (by Coomassie staining) and excised. The gel band was then washed three times with H₂O to remove the Coomassie (10 min each wash) and 500 μl K₄PO₄ (50 mM containing 1.5 ng [¹³C₁₈]OA-NO₂ and 500 mM BME) was added and incubated for 1 h at 37 °C.

Quantification of nitro-alkylated proteins in OA-NO₂-treated mice

All animal studies were approved by the University of Pittsburgh Institutional Animal Care and Use Committee (Approval 0605735-A3). Intraperitoneal injection of OA-NO₂ (4.8 mg) or LNO₂ (4.8 mg) in saline containing 20% ethanol was performed and samples obtained 3 h after injection. Alternatively, osmotic mini-pumps (ALZET Model 1007D, Durect Corporation, CA, USA) containing either vehicle or OA-NO₂ (20 mg/kg/d) were subcutaneously implanted in C57BL/6 male mice, 8-10 wks of age (Jackson Laboratories, Bar Harbor, ME), using isoflurane as anesthesia. Stability of OA-NO₂ in aqueous milieu is limited, thus polyethylene glycol 400 (PEG 400) with 15% ethanol was determined to be optimal for NO₂-FA stabilization and giving macromolecule release rates comparable to those observed for water-solvated macromolecules [39]. The mini-pumps delivered solutes for 7 days, had a volume of 0.1 ml and an estimated flow rate of 0.3 μl/h using PEG/EtOH as a vehicle. After 5 days, mice were anesthetized with Nembutal® sodium solution (65 mg/kg) (Ovation Pharmaceuticals, Deerfield, IL). Blood was removed via right ventricular cardiac puncture and collected in heparinized tubes. Plasma samples were obtained by centrifugation diluted 5 fold with phosphate buffer pH 7.4, BME reactions performed in the presence of internal standard and samples were assessed for albumin nitroalkylation as described for GAPDH.

Formation of electrophilic fatty acids by ischemia-reoxygenation

Rat hearts were perfused in an isolated Langendorff preparation and subjected to either 45 min normoxic perfusion or 20 min equilibration followed by 3 × 5 min ischemia interspersed with 5 min reperfusion as previously [40]. Mitochondria were immediately

isolated at the end of each perfusion protocol, protein concentration measured and lipids extracted from 5 mg mitochondrial protein [40]. Extracts were treated with 500 mM BME for 30 min at 37°C and analyzed by HPLC-ESI-MS/MS. HPLC separations were performed on a C₁₈ reverse phase column (150 × 2 mm, 3 μm particle size) with a mobile phase consisting of A (0.1% acetic acid in H₂O) and B (0.1 % acetic acid in ACN). Nitroalkenes were resolved from the column and compared with standards using the gradient program: 45% B for 1 min., 45-80% B over 44 min., 80-100% B over 1 min., 100% B for 7 min., 100-45% B over 6 s., 45% B for 10 min. to reequilibrate the column.

Results

The reaction between BME and LNO₂ or OA-NO₂ was monitored spectrophotometrically at their absorbance maximum of 270 nm in KPO₄ at pH 7.4 (Fig. 1). Adducts between BME and LNO₂ or OA-NO₂ gave maximal absorbance at 236 and 240 nm, respectively (Fig. 1, A and B). This reaction reached equilibrium by 3 min under these conditions (Fig. 1, C and D). Reaction products were characterized by HPLC ESI MS/MS. The BME-OA-NO₂ and BME-LNO₂ adducts were resolved from each other by C-18 reverse phase chromatography and quantified (Fig. 2 upper panels). Analysis of fragment ions showed a predominant product ion identified as the neutral loss of BME (−78 amu; [M-BME][−]). Other fragments include the loss of water (−18 amu; [M-H₂O][−]), loss of BME and water (−96; [M-BME + H₂O][−]) and the loss of BME and HNO₂ (−125; [M-BME + HNO₂][−]) (Fig. 2, lower panels). The fragmentation pattern of BME adducts was also analyzed in the negative ion mode for the reaction between GSH and OA-NO₂ or LNO₂. In this case, fragmentation followed a different pattern than with BME, with the main product ion arising from the neutral loss of the NO₂-FA (Fig. 3).

The reaction rate of the Michael addition reaction between the electrophile (NO₂-FA) and the nucleophile will be dependent on the concentration of thiolate present, the nitroalkene and the reaction media. Thus, reaction conditions including the concentration and pK_a of the nucleophile and pH will influence the reaction rate. Under the conditions used in Fig. 4, with BME as the nucleophile, maximal adduct formation was achieved at pH > 6.

Michael addition reactions are reversible, with the ratio of products to substrates under different reaction conditions (i.e., buffering agent, solvent and pH) determined by the equilibrium constant. In this regard, perturbation of the system with a high concentration of an acceptor nucleophile (e.g., BME) will displace the equilibrium of the NO₂-FA towards the formation of a thioether adduct with the introduced nucleophile. First, for characterizing this exchange reaction, the pH dependence was determined. For purified GS-OA-NO₂ and GS-LNO₂, BME displaced glutathione to yield BME-NO₂-FA adducts at pH > 5, with maximal formation at pH 7.5 (Fig. 5A). At pH > 7.5, decreased rates and yields of the trans-nitroalkylation reaction occurred. From this insight, the pH for the remaining characterization steps was set at 7.4 because of combined biological relevance and optimal trans-nitroalkylation yields.

The trans-nitroalkylation of GSH to BME showed a pronounced decrease in parent GSH-OA-NO₂ and GSH-LNO₂ adducts upon BME addition (Fig. 5B), with concomitant formation of BME-NO₂-FA derivatives. Maximal BME adduct formation occurred at 1 hr, with most GS-OA-NO₂ and GS-LNO₂ concomitantly consumed.

Most reducing agents used in protein chemistry and molecular biology applications rely on nucleophilic thiols (e.g., DTT, BME) that can induce the reduction of post-translational electrophile-protein adducts. Also, gel electrophoresis is typically performed using reducing conditions designed to enhance protein resolution. To devise alternative reducing conditions

that would not affect extents of protein nitroalkylation, GS-NO₂-FA adduct stability was evaluated in phosphine-based (TCEP) reducing conditions. When compared with thiol-based reductants (e.g., DTT), phosphines more effectively reduced low molecular weight thiols and sterically-hindered protein thiols [41]. GS-OA-NO₂ and GS-LNO₂ incubated with TCEP in electrophoresis sample buffer at different temperatures and pH were more stable at neutral pH and at temperatures lower than 60°C, since more than 50% of adduct formation was lost within 7 min at 100 °C (Fig. 6 A). To evaluate the impact of TCEP on nitroalkylated proteins, GAPDH was treated with biotin-labeled OA-NO₂ and incubated for various times at different temperatures in the absence or presence of TCEP (Fig. 6 B-C). TCEP did not induce a decrease in GAPDH nitroalkylation when heated at 75 °C for 7 min, revealing that this reducing condition could be utilized for electrophoretic analysis of protein nitroalkylation.

Determination of electrophile-protein adducts following resolution by gel electrophoresis informs about key targets and, when used in concert with BME exchange reaction and internal standards, permits quantification of protein adducts under different conditions. GAPDH was nitroalkylated in vitro using different concentrations of OA-NO₂ and then was resolved by gel electrophoresis using previously-defined conditions (Fig. 6 BC). Protein-containing samples were resolved by electrophoresis, identified by Coomassie staining, cut from gels and macerated in the presence of BME. Upon removal of acrylamide debris by sedimentation, BME-containing extracts were transferred into vials and directly injected into the mass spectrometer. Detectable BME-OA-NO₂ adducts were linear over 3-4 orders of magnitude, with respect to OA-NO₂ concentrations added to GAPDH (Fig. 7). Using this strategy, the presence of nitroalkylated proteins, with a focus on albumin, was determined in control and OA-NO₂-treated mice. Plasma was obtained, subjected to gel electrophoresis, the Coomassie-stained albumin band excised, BME exchange reactions performed in-gel and the BME-albumin adduct analyzed by mass spectrometry. A significant increase in albumin-derived BME-OA-NO₂ adducts was detected in OA-NO₂ treated animals (Fig. 8).

The metabolism of OA-NO₂ to potentially electrophilic products was evaluated following bolus IP injection of OA-NO₂ in mice. Two hours after administration, multiple chemical modifications of OA-NO₂ occurred, including reduction of the nitroalkene bond to yield nitro-octadecanoic acid, further desaturation to nitro-octadecenoic acid and the generation of -oxidation products, as previously (Fig. 9, [42]). The electrophilic reactivity of the parent molecule and metabolites was evaluated herein following intraperitoneal injection by scanning for BME-reactive product formation in concert with the disappearance of the ion corresponding to the BME-reactive species of interest. For OA-NO₂, it was confirmed that the initial reduction to nitro-octadecanoic acid occurs at the double bond adjacent to the nitro group, thus eliminating BME reactivity. The subsequent -oxidation of nitro-octadecanoic acid gave nitro-fatty acid metabolites containing 16, 14 and 12 carbons that were not BME-reactive (nitro-hexanoic acid, nitro-tetradecanoic acid and nitro dodecanoic acid, respectively). In contrast, -oxidation of the parent OA-NO₂ yielded BME-reactive adducts 16 and 14 carbons long (nitro-hexenoic acid and nitro-tetradecenoic acid, respectively).

Upon IP injection of nitro-octadecenoic acid (LNO₂), the structural nature of metabolites and their Michael addition products with thiols becomes more complex, owing to a second double bond and a nitro group that is now positioned at olefinic carbons 9, 10, 12 or 13. The parent LNO₂ and a reduced metabolite losing 1 double bond (m/z 322.3) were BME-reactive (Fig. 9). There were also species formed that were isobaric with the loss of either 2 or 1 double bond that were not BME-reactive, diagnostic of nitro-octadecanoic acid (m/z 326.3) and a mono-unsaturated nitro derivative having an allylic nitro configuration (m/z 328.3). This conclusion is further supported by the different HPLC retention time of the latter LNO₂

metabolite compared with its isobaric synthetic vinyl nitro OA-NO₂ species. The α -oxidation products of the BME-reactive OA-NO₂ also displayed electrophilic reactivity. In contrast, the α -oxidation products of nitro-octadecanoic acid, derived from both OA-NO₂ and LNO₂ (m/z 328.3) and the mono-unsaturated nitro derivative having an allylic nitro configuration, were all not BME-reactive. Thus, BME reactivity permits identification of a broad range of electrophilic fatty acid metabolites and allows further distinction of isobaric species with or without electrophilic reactivity.

The structural characterization of BME-adducted parent molecules and metabolites was performed (Fig. 10). In the case of both LNO₂ and OA-NO₂, the only detectable BME-adducted derivatives were species that disappeared upon BME treatment when monitoring for free nitro-fatty acids. Strong signals for BME-adducted α -oxidation products of LNO₂ were evident (m/z 402-18 carbons, 374-16 carbons, 346-14 carbons, 318-12 carbons) and OA-NO₂ (m/z 404-18 carbons, 376-16 carbons, 346-14 carbons, 320-12 carbons). No transitions were detectable for BME-OA-NO₂ or the BME adducts of its α -oxidation products in LNO₂-injected mice, confirming an absence of electrophilic reactivity of species formed upon reduction of the nitroalkene to nitroalkane.

Further analysis of BME-exchangeable species adducted to electrophoretically-resolved plasma albumin from LNO₂-treated mice also revealed electrophilic LNO₂ α -oxidation products upon MS/MS analysis (Fig. 10B). The characteristic product ions, similar to those obtained from direct analysis of LNO₂ α -oxidation products, confirmed the structures (Fig. 10 B). Due to their retention of electrophilic reactivity, α -oxidation products of nitroalkene derivatives of unsaturated fatty acids may also display cell signaling activity.

To explore the potential of the BME exchange reaction to reveal novel electrophilic lipids, a rodent model of metabolic and inflammatory stress was used. Lipid extracts were prepared from mitochondria isolated from rat cardiac tissue exposed to cycles of ischemia and reperfusion, an event also termed ischemic pre-conditioning (IPC) due its induction of subsequent cardioprotection against future ischemic events [43]. During ischemia, free radical production is elevated, and primary and secondary oxygen and NO-derived species are generated that can mediate the formation of electrophilic lipids in mitochondria. This IPC-induced formation of electrophilic nitroalkene derivatives of nitro-linoleic acid yields mitochondrial levels of 600-800 nM and is partially dependent on NOS activity, indicating a radical-induced nitration pathway [40]. Lipid extracts from control and IPC mitochondria were incubated with BME to form adducts and analyzed by HPLC ESI-MS/MS (Fig. 11 A). Ischemic preconditioning induced the formation of multiple electrophilic lipids as compared to control heart mitochondria when analyzed by mass spectrometry as neutral losses of BME (-78 amu). The major peaks present in the IPC chromatogram have mrm transitions consistent with oxo-fatty acid formation, including oxo-oleic acid, oxo-palmitoleic acid and shorter chain fatty acids containing a ketone functional group. Due to a lack of standards, the structures of these molecules have not been confirmed; however, using the BME technique, potential electrophilic signaling molecules have been revealed that may play a role during IPC cytoprotection (Fig 11 A and Supplementary Table 1) In addition, formation of LNO₂ was detected in mitochondrial lipid extracts following IPC that display chromatographic and mass spectrometric characteristics identical to synthetic BME-LNO₂ standard (Fig. 11B). Moreover, the formation of electrophilic LNO₂ was further confirmed by the analysis and co-chromatography with [¹³C₁₈] LNO₂ internal standard and the nitroalkene structural configuration confirmed by disappearance of the LNO₂ signal after reaction with BME (not shown). Using stable isotope dilution, the mitochondrial LNO₂ content was determined to increase from basal levels of 3.6 $\pm$ 1.1 pg LNO₂/mg protein in control heart mitochondria to 186.3 $\pm$ 41.3 pg LNO₂/mg protein in mitochondria isolated from hearts subjected to IPC.

Discussion

The salutary signaling actions of redox-derived lipid electrophiles indicate that these species can mediate adaptive responses that link gene expression and cell function to the metabolic and inflammatory status of the organism [7, 18-21, 44, 45]. At higher concentrations of endogenous or xenobiotic-derived electrophiles, mutagenic and cytotoxic actions might also be expected [7].

The reactivity of electrophiles towards biological targets differs considerably and can be enhanced by the catalysis of enzymes such as GSH transferases, with GS-electrophile adducts then eliminated via multidrug-resistant protein export mechanisms. In the case of NO₂-FA, which show a high reactivity towards nucleophilic amino acids, reactions involving GSH transferases are less critical [22]. In contrast, for less reactive electrophiles such as eicosanoids, isoprostanes, estrogens, catecholamines, 4-HNE, 4-ONE and other oxo-fatty acid derivatives reactions catalyzed by glutathione transferases will predominate. In this regard, the low plasma or tissue concentrations of many electrophilic compounds has raised questions about their significance as signaling mediators [46], but this concern is offset by the observation that time-dependent accumulation of protein-lipid adducts by 15-PGJ₂ can compensate for reactivity and concentration-related concerns [17]. A method providing quantitative and structural insight into the formation of Michael addition products between target proteins and endogenous nitroalkenes or other electrophilic species is thus of value. The strategy reported here can also be applied to the identification of other novel protein or GSH adducts with electrophilic species, as well as extents and sites of protein adduction by cysteine, GSH and other low molecular weight derivatives.

The reaction of BME with nitroalkenes results in the rapid formation of BME-adducts through a direct nitroalkylation reaction. We expanded the scope of this reaction by applying BME-exchange reactions to NO₂-FA adducts with GSH, GAPDH and albumin. In all cases, full recovery of BME adducts was achieved. The exchange of electrophilic adducts of electrophoretically-resolved proteins to BME was also quantitative. This approach can thus resolve multiple limitations inherent in the identification of molecular targets of endogenous electrophiles.

The detection of small molecules by tandem mass spectrometry is dependent on molecules of interest ionizing and fragmenting efficiently. In this regard, BME adducts of electrophilic fatty acids and low molecular weight thiols ionize efficiently, with in-source and MS/MS fragmentation primarily inducing the loss of the BME adduct. Thus, a BME-based neutral loss-dependent detection strategy provides a powerful tool for “fishing” for reversible electrophilic modifications of relevance to post-translational protein modification and cell signaling. Electrophile-BME adducts were labile during electrospray ionization, thus sensitive to high declustering potentials. To accommodate for, lower declustering potentials were set for the analysis of fatty acids bearing a thioether bond, compared with the parent NO₂-FA. Moreover, the lability of the thioether bond primarily generates the loss of the BME group after collision-induced dissociation. In this regard, the BME adduction of NO₂-FA thus renders these species ~5 times more sensitive for detection, due to the high fragmentation efficiency of the thioether bond. Better structural information is obtained when using a hybrid triple quadrupole spectrometer with a trap function (MS³). Since BME is the major neutral loss, the molecule can thus be recovered intact for MS³ analysis. In-source decay allowed for structural determination in Q3 after fragmentation, where the declustering potential provided an efficient source of molecules displaying a neutral loss of BME that could then be selected, further fragmented and analyzed. In this regard, other species with thioether bonds (e.g. GS-adducts) do not share the same characteristics, mainly because of charge distribution and size.

The influence of pH on BME adduction of NO₂-FA revealed that higher pH values, where greater proportions of the thiolate anion were present, favored the reaction. This was not the case for trans-nitroalkylation of NO₂-FA from sites of protein adduction to BME. In this instance, maximal BME-adduction of various products was obtained at pH 7 to 7.8. Higher pH values resulted in decreased formation of BME adducts.

The fate of NO₂-FA in vivo involves four principal metabolic routes that are expected to vary in extent depending on tissue compartmentalization and physiological state. This includes a) Michael addition reaction with nucleophilic targets, b) the formation of vicinal nitrohydroxy-containing derivatives upon electrophilic reaction with water, c) nitroalkene reduction to a nitroalkane and d) mitochondrial ω -oxidation. For polyunsaturated fatty acids (e.g., 18:2), saturation of the nitroalkene can yield an NO₂-FA derivative isobaric with the nitro adduct of 18:1 that lacks electrophilic reactivity. Similar reactions can occur with longer chain and more highly unsaturated fatty acids. Thus, in the analysis of biological samples where different regioisomers and isobaric NO₂-FA species co-exist, useful structural information will stem from the determination of BME reactivity as an indicator of electrophilicity. These considerations are especially relevant in mitochondria, where nitrated fatty acids are produced, can react with nucleophiles and consumed by ω -oxidation [40, 42]. Nitration of fatty acids in mitochondria is NO dependent [40], thus via free radical-mediated reactions, yielding an array of isomers and electrophilic species. As shown herein, multiple isobaric fatty acid derivatives can be generated and by capitalizing on BME reactivity one can determine which were electrophilic and resolve these adducts from non-reactive isobaric species or derivatives already deactivated by isomerization or metabolism.

The analysis of biological samples subjected to inflammatory-derived reactive species typically reveals a wide spectrum of often labile products. Herein, we report a strategy for the structural determination and quantification of free and adducted electrophilic fatty acids formed by NO-dependent oxidative inflammatory conditions. This approach, which can also be applied to other classes of electrophilic species, permits both the screening and targeted analysis of electrophiles and their targets that contribute to the propagation of redox-dependent signaling events.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

OA-NO₂	nitro-oleic acid, 9- or 10-NO ₂ -octadecenoic acid
LNO₂	nitro-linoleic acid, 9, 10, 12 or 13-NO ₂ -octadecadienoic acid
BME-OA-NO₂	-mercaptoethanol adduct of OA-NO ₂
BME-LNO₂	-mercaptoethanol adduct of LNO ₂
GSH	reduced glutathione
GS-OA-NO₂	glutathione adducted to OA-NO ₂

GS-LNO₂	glutathione adducted to LNO ₂
4-HNE	4-hydroxynonenal
4-ONE	4-oxononenal
15-PGJ₂	15-deoxy-12,14-prostaglandin J ₂
HPLC ESI MS/MS	high performance liquid chromatography electrospray ionization triple quadrupole mass spectrometry
TCEP	tris (2-carboxyethyl) phosphine
mrm	multiple reaction monitoring
TIC	total ion count
m/z	mass to charge ratio

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Fig. 1. Spectrophotometric characterization of the reaction between nitroalkenes and BME (A-B) Spectra of the reaction between LNO₂ (30 μ M)(A) and OA-NO₂ (45 μ M)(B) with BME (1 mM) were obtained in the 220-340 nm range every 20 sec. (C-D) The formation of the BME adduct was followed at 240 nm (LNO₂, C) and at 236 nm (OA-NO₂, D). The absorbance values at 290 nm correspond to LNO₂ (C) and OA-NO₂ (D) consumption.

Fig. 2. Mass spectrometric analysis of BME-adducted fatty acid nitroalkene derivatives
 The purified product of the reaction was analyzed by LC-MS/MS. (A) Chromatograms showing the elution profile of the different products. (B) Enhanced product ion formation of the BME adducts shown in A after MS/MS. Inserts: MS/MS analysis showing the formation of m/z 46 (NO_2^- anion) upon fragmentation of the BME adduct.

Fig. 3. Mass spectrometric characterization of the GS-adducted nitroalkenes

The purified product of the reaction was analyzed by LC-MS/MS. (A) Chromatograms showing the elution profile of GS-LNO₂ and GS-OA-NO₂. (B) Enhanced product ion formation in the negative ion mode of the GS-adducts shown in A after MS/MS, resulting in the main fragment of m/z 306.1 (GS⁻), corresponding to the neutral loss of LNO₂ and OA-NO₂ respectively.

Fig. 4. pH dependence of BME reaction with nitroalkenes

LNO₂ and OA-NO₂ (150 nM) were reacted for 10 min with 1 mM BME at different pH. The samples were subjected to reverse phase chromatography using a C18 column and analyzed by LC-ESI-MS/MS. The experiment was performed on four independent occasions, with n=3. A representative experiment is shown, with the data representing the mean value $\pm$ standard deviation.

Fig. 5. pH- (A) and time- (B) dependent trans-nitroalkylation of GS-LNO₂ and GS-OA-NO₂ to BME

GS-LNO₂ (150 nM) and GS-OA-NO₂ (150 nM) were treated for 60 min with 500 mM BME at different pH (A) or for different times at pH 7.4 (B). The reactions were stopped by acidification with formic acid (pH < 3), then BME internal standards were added, specifically BME-[¹³C₁₈]OA-NO₂ and BME-[¹³C₁₈]LNO₂. The samples were then chromatographed using a reverse phase C18 column and quantified by LC-ESI-MS/MS by monitoring the following transitions: BME-OA-NO₂, *m/z* 404.4/326.3; BME-LNO₂, *m/z* 402.4/324.3; BME-[¹³C₁₈]OA-NO₂, *m/z* 422.4/344.3; BME-[¹³C₁₈]LNO₂, *m/z* 420.4/342.3, 633.5/306.3, GS-OA-NO₂, 631.5/306.3, GS-LNO₂. Reactions and measurements were performed on three independent occasions, with n=4. A representative experiment is shown with data expressed as mean ± standard deviation.

Fig. 6. Stability of nitroalkylated thiols in phosphine-based reducing agents
 (A) Adduct stability was tested using nitroalkylated glutathione that was exposed to different temperatures. Nitroalkylated glutathione was measured using LC-MS/MS. (B-C) GAPDH was nitroalkylated using biotinylated OA-NO₂, sample buffer containing TCEP was added (A) or not (B) and then heated as indicated for different times.

Fig. 7. In-gel trans-nitroalkylation reaction with BME

GAPDH was incubated in the presence of different concentrations of OA-NO₂. The samples were resolved by electrophoresis using TCEP as the reducing agent. After Coomassie staining, the GAPDH band was excised and subjected to maceration and BME transnitroalkylation. The BME adducts were directly measured from the reaction mixture by LC-MS/MS.

Fig. 8. Detection of nitroalkylated albumin in mice treated with OA-NO₂ via implanted osmotic mini-pumps

The proteins from 5 μ l of plasma were separated by gel electrophoresis under TCEP reducing (5 mM) conditions. The gel was stained using Coomassie, the band corresponding to albumin was excised and transnitroalkylated using BME in the presence of [¹³C₁₈]OA-NO₂ (that is immediately converted to BME-[¹³C₁₈]OA-NO₂). The BME-adducted nitroalkenes were quantified by LC-MS/MS. Data show the individual levels, expressed as mean $\pm$ standard deviation from n=7 control and treated animals.

Fig. 9. Determination of electrophilic nitrated fatty acids and products upon LNO_2 and OA-NO_2 metabolism in mice

Lipids from IP injected mice (2 h; LNO_2 , OA-NO_2 or vehicle) were extracted from serum using cold acetonitrile. Samples were directly analyzed by HPLC-MS-MS and also reacted for 1 hr with BME and then analyzed. Neutral loss of HNO_2 was followed after collision induced dissociation of the different nitro-containing fatty acid metabolites. Light grey boxes show the corresponding peaks for electrophilic NO_2 -18:3; NO_2 -18:2; NO_2 -18:1; NO_2 -16:1; NO_2 -16:2; NO_2 -14:2; and NO_2 -14:1 species. The ovals denote the corresponding peaks for non-electrophilic NO_2 -FA upon reduction by an apparent nitroalkene reductase activity and are the following: NO_2 -18:1; NO_2 -18:0; NO_2 -16:1; NO_2 -16:0; NO_2 -14:1; NO_2 -14: and NO_2 -12:0.

Fig. 10. Formation of BME adducts with free and albumin-adducted electrophilic ω -oxidation products of LNO₂ and OA-NO₂

The ACN extracted serum samples from treated mice were reacted with BME and then analyzed by HPLC MS-MS. The electrophilic nitro-fatty acid identified in Fig. 9 was found adducted to BME when the transitions for loss of BME were followed. No BME adduction was observed for the non-electrophilic adducts. The small peaks observed in the chromatogram obtained following the neutral loss of BME from 404, 376, 348 and 320 correspond to the isotopic distribution of the electrophilic nitro-fatty acid counterparts (402, 374, 346 and 318). Albumin from LNO₂-injected mice was resolved by gel electrophoresis, detected by Coomassie staining, removed from gels and macerated in the presence of BME. Adducts of albumin were identified for ω -oxidation products of LNO₂ (NO₂-18:2, NO₂-16:2, NO₂-14:2 and NO₂-12:2 were detected).

Fig. 11. Formation of electrophilic fatty acid derived compounds in ischemic preconditioned (IPC) Rat Heart Mitochondria

(A) Lipid extracts isolated from control rat heart mitochondria or from mitochondria isolated from rat hearts exposed to IPC. Extracts were treated with 500 mM BME at 37°C for 30 min. Experimental transitions from BME neutral loss experiments and theoretical mrm transitions designed to detect keto-derivatives of multiple different fatty acids were monitored in extracts from control and IPC heart mitochondria (supplemental table 1). Multiple oxo-fatty acids are generated during IPC including oxo-oleic acid, oxo-palmitoleic acid and other short chain fatty acid derivatives among others. (B) Formation of electrophilic nitrated linoleic acid is upregulated during IPC as determined by the formation of BME adducts of the four positional isomers of LNO₂. Samples were analyzed by HPLC ESI-MS/MS in the mrm mode using 402/324 as the mrm transition specific to BME-LNO₂. Synthetic BME-LNO₂ was used as a confirmatory standard. Insets expand the HPLC elution profile to highlight co-elution of LNO₂ positional isomers in IPC-treated mitochondria vs. synthetic standard. Data are representative of n=3 samples.