REVERSIBLE POST-TRANSLATIONAL MODIFICATION OF PROTEINS BY NITRATED FATTY ACIDS IN VIVO

Carlos Batthyany1-3, Francisco J. Schopfer1♦, Paul R.S. Baker1, Rosario Durán3, Laura M.S. Baker1, Yingying Huang4, Carlos Cerveñansky3, Bruce P. Branchaud5 and Bruce A. Freeman1*

From the Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, 15213

Running Title: In Vivo Nitroalkylation of Proteins

Address correspondence to: Bruce A Freeman, Ph.D., Department of Pharmacology, E1340 BST, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA; Tel: 412-6489319; Fax: 412-6482229; Email: freerad@pitt.edu

1Department of Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA. 2Departamento de Bioquímica, Facultad de Medicina, Universidad de la República, Uruguay. 3Unidad de Bioquímica Analítica, Instituto de Investigaciones Biológicas, Ministerio de Educación y Cultura, Uruguay. 4Thermo Electron, San Jose, CA95134, USA. 5Department of Chemistry, University of Oregon, Eugene, OR 97403, USA.

♦ These authors contributed equally to this manuscript

Nitric oxide (⋅NO)-derived reactive species nitrate unsaturated fatty acids, yielding nitroalkene derivatives including the clinically abundant nitrated oleic and linoleic acid. The olefinic nitro group renders these derivatives electrophilic at the carbon β to the nitro group, thus competent for Michael addition reactions with cysteine and histidine. Using chromatographic and mass spectrometric approaches, we characterized this reactivity using in vitro reaction systems and demonstrate that nitroalkene-protein and glutathione (GSH) adducts are present in vivo under basal conditions in healthy human red cells. LNO2 (m/z 324.2) and OA-NO2 (m/z 324.2) reacted with GSH (m/z 306.1), yielding adducts with m/z of 631.3 and 633.3, respectively. At physiological concentrations, nitroalkenes inhibited glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which contains a critical catalytic Cys (Cys-149). GAPDH inhibition displayed an IC50 of 3 μM for both nitroalkenes, an IC50 equivalent to the potent thiol oxidant peroxynitrite (ONOO−) and an IC50 30-fold less than H2O2, indicating that nitroalkenes are potent thiol-reactive species. LC-MS analysis revealed covalent adducts between fatty acid nitroalkene derivatives and GAPDH, including at the catalytic Cys-149. LC-MS-based proteomic analysis of human red cells confirmed that nitroalkenes readily undergo covalent, thiol-reversible post-translational modification of nucleophilic amino acids in GSH and GAPDH in vivo. The adduction of GAPDH and GSH by nitroalkenes significantly increased the hydrophobicity of these macromolecules, both inducing translocation to membranes and explaining why these abundant derivatives had not been previously detected via traditional HPLC analysis. The occurrence of these electrophilic nitroalkylation reactions in vivo indicates that this reversible post-translational protein modification represents a new pathway for redox regulation of enzyme function, cell signaling and protein trafficking.

Nitric oxide (⋅NO) exerts a broad influence on cell and inflammatory signaling via both cGMP-dependent and -independent oxidative, nitrosative and nitrative reactions (1,2). The nitration of polyunsaturated fatty acids present in both membranes and lipoproteins is now emerging as a novel mechanism for transducing NO-dependent redox signaling (3,4). Recent evidence indicates that all major unsaturated fatty acids present in human blood contain some proportion of alkanyl nitro derivatives, [R1HC=C(NO2)R2], also termed nitroalkenes. Due to the prevalence of fatty acid nitroalkenes in
healthy humans, these species are now appreciated as the single largest pool of bioactive oxides of nitrogen in the vasculature (5). The two most clinically abundant nitroalkene fatty acid derivatives, nitro-oleic acid (9- and 10-nitro-9-cis-octadecenoic acids; OA-NO2) and nitro-linoleic acid (10-nitro-9,12-octadecadienoic and 12-nitro-9,12-octadecadienoic acids; LNO2) are present in net concentrations of >1 μM in membrane and lipoprotein lipid extracts prepared from healthy human blood. These nitroalkene concentrations far exceed the <10 nM concentrations reported for other NO derivatives, including S-nitrosothiols, nitrosyl heme and 3-nitrotyrosine, and the <300 nM concentrations reported for nitrite (NO2-; (6)).

Nitroalkenes display pluripotent cell signaling capabilities, acting via both cGMP-dependent and -independent mechanisms (7-9). First, nitroalkenes such as LNO2 decay via the hydrophobically-regulated Nef reaction to release \( \cdot \)NO, thus acting as endogenous \( \cdot \)NO donors that mediate cGMP-dependent cell signaling responses such as relaxation of vascular smooth muscle cells (2). Second, fatty acid nitroalkene derivatives are potent endogenous peroxisome proliferator-activated receptor (PPAR) ligands that act within physiological concentration ranges to modulate key PPAR-regulated signaling events (9). Third, nitroalkenes inhibit human peripheral blood neutrophil activation (\( O_2^- \) production and formylmet-leu-phe stimulated \( Ca^{2+} \) influx, degranulation and CD11b expression) via non-cAMP, non-cGMP-dependent mechanisms (7). Fourth, thrombin-induced platelet aggregation is inhibited by nitroalkene-induced attenuation of cAMP-dependent \( Ca^{2+} \) mobilization and activation of the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) at Ser-157 (8). Current evidence supports a dual regulation of platelet adenyl cyclase and phosphodiesterase E activities by nitroalkenes. Finally, fatty acid nitroalkene derivatives, as opposed to native unsaturated fatty acids, potently regulate the expression of key inflammatory, cell proliferation and cell differentiation-related proteins (5,9).

The broad impact of nitroalkenes on differentiated characteristics of cells and tissues motivated analysis of additional chemical reactivities of nitroalkenes that would account for their pluripotent signaling capabilities. NMR, MS/MS and spectroscopic analysis of nitrated fatty acids indicates the presence of predominantly nitroalkene rather than nitroalkane derivatives in clinical specimens, with this structural configuration conferring unique biochemical and pharmacological qualities. The alkenyl nitro configuration of endogenous nitrated fatty acids indicates potential electrophilic reactivity of the \( \beta \)-carbon adjacent to the nitro-bonded carbon. This would promote nitroalkene reactivity with nucleophiles (i.e. Cys and His residues) via Michael addition reactions, yielding new carbon-carbon or carbon-heteroatom bond frameworks (2,10). This electrophilic property of nitroalkenes was first suggested by the biological detection of \( \alpha,\beta \)-nitro-hydroxy fatty acid derivatives (5). Also, synthetic nitroalkenes generated hydroxy derivatives under aqueous conditions, presumably induced by reaction with the low levels of hydroxide ion present at physiological pH (2).

Other electrophilic lipids, in particular 15-deoxy-\( \Delta^{12,14} \)-PGJ2 (15d-PGJ2) and 4-hydroxy-2-nonenal (HNE), are reactive and form adducts with proteins that contain nucleophilic centers (11). The electrophilic HNE reacts with sulphydryl groups (12), the imidazole of histidine (13) and the \( \varepsilon \)-amino of lysine (14). Covalent modification by electrophilic lipids has been shown to alter the structure and activities of cathepsin B (15), Keap1 (16) and insulin (13).

Herein, the reactions of nitroalkenes with cysteine and glutathione (GSH) are characterized, and GAPDH is evaluated as an exemplary target protein for nitroalkene reaction. GAPDH is of additional relevance, as a) catalytic thiols are essential for GAPDH activity (17), b) GAPDH can be inhibited by electrophilic lipids (18,19) and c) the \( \cdot \)NO-dependent post-translational modification and translocation of GAPDH to specific cellular microenvironments impacts on the roles of GAPDH in intermediary metabolism and apoptotic signaling (20,21).

We reveal herein, via model reaction systems and the proteomic analysis of human red cells, that nitroalkenes readily undergo covalent, thiol-reversible post-translational modification of nucleophilic amino acids in GSH and proteins. The occurrence of these electrophilic nitroalkylation reactions \textit{in vivo} indicates that fatty acid nitroalkene derivatives serve to regulate protein function and trafficking.
EXPERIMENTAL PROCEDURES

Materials and chemicals. Rabbit muscle GAPDH, D-L-glyceraldehyde 3-phosphate (GAP), glutathione (reduced and oxidized forms), ascorbic acid, diethylenetriamine-pentaacetic acid (DTPA), dithiothreitol (DTT), hydrogen peroxide (H₂O₂) and sodium arsenite were from Sigma (St Louis, MO). Nicotinamide adenine dinucleotide (oxidized) was from Boehringer Mannheim GmbH (Mannheim, Germany). Sodium pyrophosphate was from Mallinckrodt. Nitrolinoleic acid (10-nitro,12-octadecadienoic acid and 12-nitro,9,12-octadecadienoic acid; LNO₂), nitro-oleic acid (9- and 10-nitro-9-cis-octadecenoic acids; OA-NO₂) and their corresponding internal standards [13C₁₈]LNO₂ and [13C₁₈]OA-NO₂ were prepared as previously (5,22) (1). Hitrap desalting columns were from Amersham Biosciences Corp. (Piscataway, NJ) was synthesized and. Pipette tips for sample preparation (ZipTip C18, P10) were from Millipore Corporation (Bedford, MA). Sequencing grade modified trypsin was from Promega (Madison, WI). Peroxynitrite (ONOO⁻) was handled as previously (23,24). Hitrap desalting columns were from Amersham Biosciences Corp. (Piscataway, NJ) was synthesized and. Pipette tips for sample preparation (ZipTip C18, P10) were from Millipore Corporation (Bedford, MA). Sequencing grade modified trypsin was from Promega (Madison, WI). Peroxynitrite (ONOO⁻) was handled as previously (23,24). Peroxynitrite concentration was determined at 302 nm in 1 M NaOH (ε = 1670 M⁻¹ cm⁻¹). GAPDH concentration was determined at 280 nm (ε = 1.46 x 10⁴ M⁻¹ cm⁻¹) (25).

GSH – nitroalkene reactions. GSH (1 mM) solvated in 50 mM sodium phosphate buffer (pH 7.4) was treated with equimolar LNO₂ or OA-NO₂ at 20°C for 30 min. The reaction mixture was analyzed by ESI-MS with and without liquid chromatography. When the adduct was analyzed by ESI-MS with and without liquid chromatography. When the adduct was analyzed by ESI-MS with and without liquid chromatography. When the adduct was analyzed by ESI-MS with and without liquid chromatography. When the adduct was analyzed by ESI-MS with and without liquid chromatography. When the adduct was analyzed by ESI-MS with and without liquid chromatography. When the adduct was analyzed by ESI-MS with and without liquid chromatography. When the adduct was analyzed by ESI-MS with and without liquid chromatography.

GAPDH activity analysis. Enzyme assay mixtures contained 100 mM sodium pyrophosphate, 0.1 mM DTPA, 0.25 mM NAD⁺, 10 mM sodium arsenite and 0.5 mM GAP, pH 7.4. GAPDH activity (0.05 µM final GAPDH concentration) was assessed at 20°C by following NAD⁺ reduction at 340 nm for the first 20 seconds after addition of GAP (Shimadzu, UV 2401-PC) (25). As a control, GAPDH was fully activated in 50 mM pyrophosphate, 0.1 mM DTPA, 20 mM DTT, pH 8.5 for 30 min at 4°C (25). Excess thiol was removed by size exclusion HPLC using a HiTrap column equilibrated with 50 mM pyrophosphate, 0.1 mM DTPA, pH 7.4.

Exposure of GAPDH to oxidants and reductants. GAPDH solvated in 100 mM sodium pyrophosphate, 0.1 mM DTPA, pH 7.4 was treated at 20°C with nitroalkenes (0-20 µM), ONOO⁻ (0-100 µM) or H₂O₂ (0-500 µM) and GAPDH activity immediately measured. Oxidant-treated GAPDH was incubated with GSH (0.01 – 20 mM), DTT (20 mM) or ascorbic acid (20 mM) for 30 min at room temperature (RT) and enzymatic activity re-determined. The pH profile of GAPDH inactivation by nitroalkenes was analyzed using GAPDH maintained for 5 min in 50 mM sodium pyrophosphate buffer adjusted from pH 5 to 10, followed by nitroalkene addition. Aliquots were then removed for GAPDH activity assay. For quantitation of GAPDH thiol content, GAPDH preparations were added to 50 mM Tris-HCl, 5 mM EDTA, 1% SDS and 1 mM 5-5'-dithiobis(2-nitrobenzoic) acid (DTNB), pH 8.0, using ε = 1.36 x 10⁴ M⁻¹ cm⁻¹ at 412 nm for the thionitrobenzoate anion (24,25).

Matrix-assisted laser desorption and ionization time of flight mass spectrometry (MALDI-TOF MS) analysis. MALDI-TOF MS was performed using a Voyager DE PRO system (Applied Biosystems, Foster City, CA), equipped with a N₂ laser source (337 nm). Mass spectra were acquired for positive ions in linear and reflector mode. For whole molecular mass determinations, native and nitroalkene-treated GAPDH (0.5 µM) was desalted by using C18-ZipTip (P10) (Millipore Corporation, Bedford, MA), following manufacturer protocols. Proteins were eluted by adding the matrix solution (sinapinic acid, 10 mg/ml in 50% acetonitrile (ACN), 0.2% TFA) and nitrogen analysis following pyrolysis, using an Antek chemiluminescent nitrogen detector (Houston, TX).
directly applied to the stainless steel sample plate. GAPDH-derived peptide masses were measured in the reflector mode with an accuracy of ~50 ppm, attained by internal mass calibration using characteristic GAPDH tryptic peptides as mass standards and α-cyano-4-hydroxycinnamic acid as the matrix. To obtain complete post-source decay (PSD) spectra, a series of reflectron mass spectral segments were acquired, each optimized to focus fragment ions within different mass/charge (m/z) ranges. Each segment was stitched together using Biospectrometry Workstation software to generate a composite PSD spectrum (26).

Peptide mapping and electrospray ionization liquid chromatography-mass spectrometry analysis (ESI-LC-MS). Native and nitroalkene-modified GAPDH (10 µM) were digested with sequencing grade trypsin in 50 mM pyrophosphate buffer, pH 7.4 at 37°C for 20 h using an enzyme:substrate ratio of 1:50 (w/w). Peptide samples were analyzed by ESI-LC-MS using a LCQ ion trap mass spectrometer (LCQ Deca, Thermo Finnigan, San Jose, CA). A reversed-phase column (5 μm, 2.1 x 150 mm, 300 Å, from Grace Vydae, Hesperia, CA) was eluted with Solvent A (0.1% formic acid) and solvent B (0.08% formic acid in acetonitrile). Peptides were eluted at 40°C with a linear gradient of solvent B (2 to 60% in 105 min) at a flow rate of 0.25 ml·min⁻¹. Electrospray voltage was 5 kV and capillary temperature was 260°C. Peptides were detected in the positive ion mode using a mass range of 100 - 2000, following mass calibration with the GS-OA-NO₂ adduct. MS/MS peptide analyses were performed by nanospray ion trap mass spectrometry (LTQ, Thermo Electron, San Jose, CA) using a BioBasic C18 picofrit column, 5μm, 75μm x 10cm (Thermo Electron) and a Zorbax 300SB-C18 trap column, 5μm, 0.3 x 50mm (Agilent Technologies, Chicago) with a flow rate of 700nl·min⁻¹ and similar gradient conditions.

GAPDH - liposome interactions. A lipid film was prepared by dissolving soybean phosphatidylcholine (200 mg) and cholesterol (50 mg) in chloroform and removal of solvent under a stream of nitrogen. Multilamellar vesicles were prepared by hydrating the lipid film with 10 ml of 10 mM pyrophosphate buffer, pH 7.4 at 25°C for 1 hr. After vortexing, the suspension was placed in a 25°C sonicating water bath for 30 min. Subsequently, control or nitroalkene treated GAPDH preparations (100 µl of 10 mg/ml protein) were incubated with liposome suspensions (1 ml) for 30 min at 25°C. Liposome-associated GAPDH was sedimented by ultracentrifugation at 100,000 x g for 30 min. Soluble GAPDH in the supernatant and sedimented liposome-associated GAPDH was analyzed by SDS-PAGE electrophoresis (4-12% BI-Tris, Criterion™ Precast Gel, Bio-Rad, Hercules, CA), stained with Coomassie Blue and quantitated by densitometry using Alphalnotech software.

Identification of endogenous nitroalkylated GSH in human red blood cells. All human studies were reviewed and approved by the UAB Institutional Review Board (Protocol #X040311001). Human red blood cells (RBCs) were obtained by centrifugation (800 x g for 10 min) of freshly drawn heparinized blood from a healthy donor. RBCs were washed twice with 0.15 M NaC and an aliquot of packed RBCs (1.5 ml) was lysed by diluting with 5 volumes of 0.1% formic acid in water, acidified to inhibit further nitroalkylation reactions during sample processing. Samples were centrifuged at 100,000 x g for 20 min at 4°C, supernatants collected and GS-[13C18]LNO₂ (10 µl, 155 nM) and GS-[13C18]OA-NO₂ (10 µl, 116 nM) added as internal standards. Samples were then loaded onto PrepSep™ C18 columns (Fisher Scientific, Pittsburgh, PA) equilibrated with 0.1% formic acid. After washing, samples were eluted with 0.1% formic acid in methanol. The eluted fractions were concentrated in vacuo and GSH-nitroalkene adducts measured by ESI-LC-MS/MS in the positive ion mode using a multiple reaction monitoring (MRM) scan mode on a 4000 Q-Trap (Applied Biosystems, Foster City, CA). The MRM transitions of m/z 635.3/506.3 (GS-OA-NO₂), 653.3/524.3 (GS-[13C18]OA-NO₂); 633.3/504.3 (GS-LNO₂); and 651.3/522.3 (GS-[13C18]LNO₂) were used, consistent with the generation of the y ion of added glutathione (27). The identity of the GSH-nitroalkene adduct was further confirmed by performing enhanced product ion (EPI) analysis and comparing fragmentation patterns with those obtained from EPI analysis of the synthetic standard GS-OA-NO₂.

Identification of endogenous nitroalkylated GAPDH in human red blood cells. 300 µl of freshly-obtained, packed RBCs were lysed as before and the cytosolic and membrane fractions separated by ultracentrifugation (100,000 x g for 20 min at 4°C). Membranes were resuspended in 100 µl of PBS and aliquots of both cytosolic and
membrane fractions (50 μl) resolved by electrophoresis under non-reducing and denaturing conditions (4-15% gradient gel, BioRad, Hercules, CA), then stained with Coomasie Blue. The 36 kDa band corresponding to purified GAPDH was excised and in-gel digestion with trypsin performed. Peptides were eluted and analyzed by nano LC -ESI-MS/MS ((700 nL/min; Finnigan LTQ, Thermo Electron Corporation, CA) using a Zorbax 300SB-C18, 5μm, 0.3 x 50mm column (Agilent Technologies, Chicago).

Crystal Structure of the Rabbit Muscle GAPDH. The image of GAPDH structure obtained by X-Ray diffraction methods and produced using MolScript and Raster3D was downloaded from Pubmed, Protein Data Bank (1J0X) (28).

RESULTS

The in vitro reaction of LNO2 with GSH was monitored by ESI-ion trap MS, which revealed a GS-LNO2 adduct (m/z 631.3), indicative of the nitroalkylation of GSH (Fig. 1A). Either source fragmentation of the m/z 631.3 adduct or residual LNO2 (m/z 324.2) and GSH (m/z 306.1) accounted for the other ions present in the spectrum. Collision-induced dissociation (CID) of the GS-LNO2 adduct precursor ion (m/z 631.3) yielded a main product ion with m/z of 306.1, corresponding to GSH (Fig. 1B). This was further confirmed by MS/MS/MS of the pair m/z 631.3/306.1 (Fig. 1C), which yielded the specific product ions characteristic of GSH (Fig. 1C), as previously reported (29). Similar results were obtained for OA-NO2 reaction with GSH (not shown).

The nitroalkylation adduct of OA-NO2 and GSH was also analyzed by reverse phase LC-MS and compared with the elution characteristics of GAPDH (Fig. 1D). GSH [(M+H)+ 308.3] was not retained on column and eluted with the void volume (Fig. 1D, black tracing). In contrast, the GS-OA-NO2 adduct [(M+H)+ 635.2] eluted at ~74 min, when the solvent gradient reached 38% acetonitrile (Fig. 1D, red tracing). This increased organic phase requirement for elution by reverse phase HPLC reveals that the nitroalkylation of peptides and proteins confers strong hydrophobic character. The elution profile of the GS-OA-NO2 adduct, revealing multiple resolving peaks (Fig. 1D, red tracing), suggests that different isomers are being formed by the reaction of OA-NO2 with GSH.

GAPDH, which contains a critical redox and electrophile-sensitive catalytic cysteine (Cys-149, (17)), was incubated with nitro fatty acids. There was a dose-dependent inactivation of GAPDH (0.5 μM) upon incubation of the enzyme with either LNO2 or OA-NO2 (0 – 10 μM) for 15 min (Fig. 2A). The loss of GAPDH activity as a function of nitroalkene concentration was sigmoidal. The nitroalkene concentration required to inactivate 50% of initial activity (IC50) was ~3 and ~4 μM for OA-NO2 and LNO2, respectively. Compared with other recognized biological thiol oxidants, this concentration of nitroalkenes is 30 times less than that of H2O2 and similar to that of ONOO- for inducing similar extents of GAPDH inactivation (Fig. 2B) (25,30). The time course of OA-NO2-mediated GAPDH inactivation was fast, with 50% inactivation occurring within 2 min under the conditions studied (Fig. 2C). The inactivation of GAPDH by OA-NO2 was strongly pH-dependent, with maximal inactivation obtained at alkaline pH, (Fig. 2D).

The biochemical nature of nitroalkene-GAPDH interactions was investigated in more detail. GAPDH thiol content was determined after OA-NO2-mediated enzyme inactivation (Fig. 3A). Nitroalkene-induced GAPDH thiol depletion paralleled the loss of enzyme catalytic activity, with 50% thiol depletion occurring at nitroalkene concentrations inducing a 50% loss of initial enzyme activity (Fig. 3A).

In order to gain further insight into the mechanisms by which nitroalkenes inactivate GAPDH and the oxidation state of the critical thiol, the reversibility of nitroalkene-mediated GAPDH inhibition by other thiol containing reducing agents was studied. The inhibition of GAPDH by nitroalkenes was reversed by incubation with low concentrations of DTT or GSH (Figs. 3B and 3C). Of significance, inactivation of GAPDH by ONOO- and H2O2 was not reversible by DTT or GSH, as previously (25), while both reducing agents restored ~85% of initial GAPDH activity following OA-NO2 reaction (Fig. 3B). Extremely low concentrations of thiol (10 μM GSH) reversed GAPDH nitroalkylation and restored ~50% of initial GAPDH activity, emphasizing the reversibility of protein adduction by nitroalkenes under biological conditions (Fig. 3C). In contrast, incubation of...
nitroalkene-inactivated GAPDH with high concentrations of ascorbic acid (20 mM) did not restore enzymatic activity (not shown).

MALDI-TOF MS analysis of nitroalkene-treated GAPDH showed a shift in the mass of the enzyme (Fig. 4A), revealing covalent modification by nitroalkenes. GAPDH displayed a broader mass distribution following reaction with OA-NO₂ (10 μM, OA-NO₂/GAPDH molar ratio 20/1), with an increase in mass of up to ~2.0 kDa (~36 kDa to a maximum of ~38 kDa; Fig. 4B). This profile of mass shifts indicates that GAPDH was adducted by up to ~ 7 molecules of OA-NO₂. Reduction of GAPDH-OA-NO₂ adducts with GSH (10 mM) eliminated the OA-NO₂-induced higher mass species of GAPDH and restored the precursor protein mass (Fig. 4C).

The capacity of OA-NO₂ to form covalent adducts with GAPDH was confirmed by LC-MS using a 2D-linear ion trap. The greater mass resolution of the 2D-linear ion trap resolved the expected mass of native GAPDH (Fig. 4D, black trace) and revealed multiple GAPDH-OA-NO₂ derivatives that all differed by the neutral mass of OA-NO₂ (m/z 327 Da). These nitroalkylated GAPDH derivatives had 3 to 6 OA-NO₂ adducts (Fig. 4D, red trace). A difference of 73 Da between the theoretical mass and the measured mass of GAPDH adducted with 3 OA-NO₂ was observed (Fig. 4D, red trace), suggesting that other modifications (i.e. Cys-, Met- oxidations) were formed. Indeed, Cys and Met were both detected in the native and oxidized form in the tryptic peptide mapping.

To determine precise sites of GAPDH nitroalkylation, native and OA-NO₂-treated GAPDH were digested with trypsin and analyzed by different mass spectrometric analytical approaches including LC-MS, nanospray LC-MS/MS and MALDI-TOF. Tryptic peptide mass mapping by LC-MS analysis of native GAPDH identified peptides covering 75% of the primary sequence (Supp. Fig. 1 and Supp. Table 1). LC-MS analysis of the peptide map of OA-NO₂-treated GAPDH showed marked differences (Table 1 and Fig. 5). There were 5 peptides displaying modified MS and chromatographic behavior in OA-NO₂-treated GAPDH, while all remaining peptides had similar relative ion intensities and HPLC retention times in both native and treated GAPDH. As an example, peptides #7 and 17, which do not contain Cys or His, are shown to retain similar ion intensity and retention time in OA-NO₂-treated GAPDH (Table 1, Fig. 5A). Of the 5 uniquely-behaving peptides, 3 contained His and displayed significantly less relative ion intensity in OA-NO₂-treated GAPDH (peptides #2, 18 and 23, Table 1 and Fig. 5B). The other two modified peptides contained Cys as the nucleophilic residue and were undetectable in their non-modified form in OA-NO₂-treated GAPDH. Peptide #19 contains 2 Cys residues, the catalytic active Cys-149 and the Cys-153. The other peptide (#21) contains the Cys-244. (Table 1, Fig. 5C). Of note, the catalytic thiol-containing peptide (#19) contained an intramolecular disulfide bond between both Cys residues in control GAPDH, showing a loss of 2 Da with respect to the expected mass of the peptide (m/z = 1705.86 to 1703.86) (Table 1 and Fig. 5C). This disulfide was probably formed during trypsin digestion and/or LC-MS analysis, as previously (18).

Furthermore, relative to the theoretical calculated masses of the unmodified peptides, four of these five peptides (peptides #2, 18, 21 and 23) were detected by nanospray LC-MS/MS with an increased mass of 327.3 Da, corresponding to the addition of a single molecule of OA-NO₂. In particular, a new peptide eluting at 76 min [(M+H)+ = 1556.7 Da] was identified in the tryptic digest of OA-NO₂-treated GAPDH (Fig. 6A). The mass of this peptide, not present in native GAPDH, corresponded to the mass of peptide #18 (sequence 321-331) plus 327.3 Da, the neutral mass of OA-NO₂. Nanospray LC-MS/MS analysis revealed that His-327 was adducted to OA-NO₂ in this particular peptide (Fig. 6A and accompanying Table). The MS/MS spectrum and the [M+2H]²⁺ ion at m/z 844.34 from this OA-NO₂-modified peptide are shown (Fig. 6B and Table 2). The full series of singly-charged y and b fragments were detected, with y and b fragments corresponding to the ions that retain the charge at the carboxyl and amino terminal groups of the peptide, respectively. The mass difference between y5 and y6, and also between b6 and b7 fragments corresponded to the mass of the His residue (137.06 Da) increased by the neutral mass of OA-NO₂ (Fig. 6B and Table 2). These results demonstrate that His-327 is the modified residue in this peptide. Modified peptide #18 was also found in analysis of trypsin-digested GAPDH by MALDI-TOF MS (Fig. 6C). Interestingly, post-source decay (PSD) sequencing analysis of this
peptide showed a principal, unique product ion with a mass of 437.1 Da. This fragment corresponds to the immonium ion of His (H = 110.1 Da) adducted to OA-NO2 (327.3 Da, Figs. 6D and 6E). This adducted immonium ion was also detected by MS/MS analysis performed via nanospray LC–ion trap MS (Fig. 6B). Interestingly, the adducted immonium ion does not appear as a major fragment in this spectrum. This is likely due to differences in the way ions are generated and fragmented in the two different techniques.

The nitroalkylation of peptide #23 (sequence 116-136) by OA-NO2 was also determined by an increased mass of 327 Da (Supp. Fig. 2). This modification accounted for a 75% decrease in ion intensity of the native peptide upon LC-MS analysis (Fig. 5B). When this modified-peptide was analyzed by PSD, the immonium ion of His attached to OA-NO2 was detected, indicating that His-134 was derivatized by OA-NO2 in peptide #23 (Supp. Fig. 2). The OA-NO2-adducted immonium ion of His detected by PSD sequencing analysis was a revealing and readily-detected footprint of nitroalkene-modified His-containing peptides by MALDI-TOF MS.

The MS/MS spectrum and data for the OA-NO2-modified peptide #2 ([M+2H]2+ ion at m/z 462.06; 105Ala-Gly-Ala-His-Leu-Lys110 plus the mass addition of 327.3) are shown in Supp. Fig. 3 and the accompanying table. This modified peptide was only detectable in OA-NO2-treated GAPDH. The mass difference between the y3 and y2 fragments corresponded to the mass of His (137.06 Da) increased by the neutral mass of OA-NO2 (327.3 Da, Supp. Fig. 3 and accompanying Table), affirming that His-108 is the modified residue in this peptide.

The final peptide detected to have a single OA-NO2 adduct was peptide #21. The MS/MS spectrum and data for this peptide are shown in Supp. Fig. 4 and the accompanying table. The masses of the b9 fragment and y2* (i.e. y2-NH3), together with the mass difference between the b9 and the b8, demonstrate that Cys-244 is adducted to OA-NO2 in this peptide.

During nanospray LC-MS/MS, peptide #19, displayed an increase in mass of 654.6 Da, corresponding to the addition of two OA-NO2. The mass of the fragments b*4 (i.e. b4-NH3) and y7 identifies Cys-149 and Cys-153 as the two modification sites in this peptide (Supp. Fig. 5 and the accompanying table). Thus, by nanospray LC-MS/MS analysis we demonstrated that in vitro, Cys-149, Cys-153, Cys-244 and His-108 and His-327 are the GAPDH residues adducted to OA-NO2.

The increased hydrophobicity induced by OA-NO2 addition of GSH was also manifested by all five OA-NO2-modified GAPDH peptides (Table 2). The nitroalkylation of peptides profoundly increased the percentage of hydrophobic solvent required to elute adducted peptides from a reverse phase C18-column. Independently of the retention time of the non-modified, native form of the peptides, all nitroalkylated peptides eluted when the concentration of ACN reached ~ 40% (Table 2). These results explain why this abundant post-translational protein modification has not been appreciated sooner, since most LC-MS analyses of peptides rarely utilize ACN concentrations exceeding 30% in gradient elution schemes.

The increased hydrophobicity of nitroalkylated peptides suggests that nitroalkenes may facilitate membrane trafficking following post-translational modification of target proteins. The interaction of nitroalkylated GAPDH with membrane lipids was evaluated by treating GAPDH with increasing concentrations of OA-NO2, and then incubating the treated protein with liposomes. Residual soluble GAPDH was separated from liposome-associated GAPDH by ultracentrifugation and identified by electrophoretic analysis of the supernatant (Fig. 7A). This analysis also revealed a dose-dependent increase in OA-NO2-adducted GAPDH association with sedimented liposomes (Fig. 7B). Three of the six GAPDH residues identified to be nitroalkylated in vitro (Cys-149; His-134 and His-327) are located on the surface of the protein and exposed to the solvent (28). The other three potentially-modified residues (His-108; Cys-153 and Cys-244), are buried deeper within the protein (28), suggesting that nitroalkylation at these sites occurs provided that: 1) the initial nitroalkylation of exposed residues induces conformational changes that result in the exposure of previously hidden domains; and/or 2) nitroalkenes are able to diffuse to and react with non-solvent exposed protein residues. These data support that post-translational nitroalkylation of proteins by nitrated fatty acids will impact protein hydrophobicity, membrane interactions and consequently, subcellular distribution.
To probe whether nitroalkylation occurs in vivo, we analyzed proteins, focusing on GAPDH, from red blood cells obtained from healthy humans. After separating cytosolic and membrane-associated proteins by electrophoresis under non-reducing conditions, the 36 kDa protein band (which includes GAPDH) was excised and digested in-gel with trypsin. Peptides were eluted and analyzed by nanospray LC-MS/MS (Figs. 8A and B). The MS/MS spectrum of the human homolog of rabbit GAPDH peptide #19, encompassing the catalytically active Cys-149, showed adduction by OA-NO2 (Fig. 8A). The mass of the b fragments, particularly fragment b2, showed that Cys-149 was modified by OA-NO2 in vivo. Another His-containing GAPDH peptide was also OA-NO2 adducted (Fig. 8B). The increased mass of the peptide by 327 Da and the mass of fragment b3 identified His-303 as the modified, adducted residue. This peptide was found both in cytosolic (not shown) and membrane fractions (Fig. 8B) of red cells, affirming that GAPDH is modified by OA-NO2 in vivo (Fig. 8).

Finally, due to a) the reversibility of in vitro protein nitroalkylation reactions by GSH, b) the identification of endogenous OA-NO2-adducted GAPDH in human red cells and c) the fact that GSH is present in high concentrations in red cells (~5 mM), the potential presence of nitroalkene-adducted GSH in red cells was examined (Fig. 9). Multiple reaction monitoring (MRM) analysis of partially-purified cytosolic fractions of red cells revealed endogenous species that co-chromatographed with synthetic GS-[13C18]OA-NO2 (Fig. 9A) and GS-[13C18]LNO2 (Fig. 9B) and displayed identical mass transitions (Figs. 9A and 9B, red vs. black traces). The identity of endogenous OA-NO2 and LNO2-adducted GSH was further confirmed by enhanced product ion (EPI) analysis and comparison of fragmentation patterns with those obtained from the EPI analysis of synthetic GS-OA-NO2 (Figs. 10A and 10B). Fragmentation of both the synthetic standard (Fig. 10A) and the endogenous molecules (Fig. 10B) gave the corresponding b2 (m/z 560.3), y2 (m/z 506.3), y2-HNO2 (m/z 506.3) and Cys-immonium ion (CI), all adducted with OA-NO2. To further characterize the GS-OA-NO2 adduct, the y2 fragment of the synthetic adduct was analyzed by MS3 (Fig. 10C). The CID of this product ion resulted in product ions, including those indicative of the loss of the NO2 group, supporting that the lipid backbone of the adduct was bonded to the cysteiny1-glycine portion of the molecule and not to the γ-Glu. Moreover, CID of the y2 ion also induced the formation of the cysteiny1 immonium ion (CI) adducted to the OA-NO2 (m/z 403.3). The loss of the NO2 group from this ion (HNO2; m/z 356.3) further confirmed that the nitroalkene was attached to the cysteiny1 residue of GSH. Based on this fragmentation pattern, it is not possible to determine the location of the NO2 group on the fatty acid backbone and thus to which carbon the GS is adducted.

The fragmentation profile of the GS-OA-NO2 adduct strongly depends on the MS mode used to analyze the molecule. In the negative ion mode, collision-induced dissociation (CID) of the GS-LNO2 adduct precursor ion yielded GSH and LNO2 as principal product ions (Fig. 1). Interestingly, no ions formed by the combination of GSH with the lipid were observed. In contrast, in the positive ion mode all of the principal product ions (y2, b2, CI) maintained the lipid backbone of the molecule. In aggregate, these results reveal the existence of nitroalkylated GSH in vivo. GS-OA-NO2 and GS-LNO2 were present at ~3.3 and ~1.3 nM, respectively, in healthy human red cells.

DISCUSSION

Chromatographic and mass spectrometric analyses reveal that nitrated unsaturated fatty acids are potent electrophiles that mediate reversible nitroalkylation reactions with both GSH and the Cys and His residues of proteins. This occurs both in vitro and in vivo (Scheme 1) and is viewed to transduce redox- and NO-dependent cell signaling by a covalent, thiol-reversible post-translational modification that regulates protein structure, function and subcellular distribution.

GAPDH, a tetramer consisting of identical catalytically-active subunits, is a key intermediary metabolism enzyme that reversibly catalyzes the oxidation and phosphorylation of D-glyceraldehyde 3-phosphate (GAP). Enzyme catalysis involves the formation of an hemithioacetal between GAP and the Cys and His residues of proteins. This occurs both in vitro and in vivo (Scheme 1) and is viewed to transduce redox- and NO-dependent cell signaling by a covalent, thiol-reversible post-translational modification that regulates protein structure, function and subcellular distribution.
Alkylation of Cys-149 by iodoacetate (36) or oxidation by non-physiological peroxynitrite and hydrogen peroxide concentrations induce irreversible enzyme inhibition (18,25,37). Nitroalkenes are more potent inhibitors of GAPDH than H2O2, HNE and peroxynitrite, and unlike these oxidants, nitroalkenes inactivate GAPDH within a physiological concentration range (Fig. 2B, (5,22)). Of significance, nitroalkene-induced GAPDH inhibition is reversible by low thiol concentrations, a property which is also not observed with the aforementioned oxidative byproducts. GAPDH not only is inhibited by active site thiol-directed oxidation reactions, but also by the modification of other nucleophilic amino acids (i.e., His, Lys, and non-catalytic Cys residues) (18). Thus, nitroalkene-induced inhibition of GAPDH could also be due to nitroalkylation of Cys-153, Cys-244 and His-108 and His-327. While GAPDH inactivation by nitroalkenes was paralleled by a loss of titrateable thiols, the kinetics of enzyme inactivation followed a biphasic, sigmoidal curve (Fig. 2). This supports a differential impact of the multiple documented sites of nitroalkene adduction on enzyme activity, as previously shown for HNE-induced GAPDH inhibition (18).

Recent evidence reveals that GAPDH is a multi-functional protein that displays cell signaling activities beyond its conventionally-viewed role as an intermediary metabolism enzyme. This includes an influence on DNA repair, transcriptional regulation, membrane fusion, tubulin bundling and apoptotic signaling (38). Under physiologic conditions, a significant extent of red cell GAPDH is bound to the membrane of intact cells and is catalytically inactive (39,40). The regulatory mechanisms whereby GAPDH fulfills its non-glycolytic functions and is targeted to different specific intracellular loci are unknown (41), but it is proposed that the functional diversity and differential subcellular distribution of GAPDH is mediated by post-translational modifications and protein-protein and protein-nucleic acid interactions (41). We reveal herein that nitroalkylation of GAPDH not only directs translocation to the membrane, but also inhibits catalytic activity. Thus, nitroalkylation of GAPDH provides a mechanism that can explain changes in the subcellular distribution and functional diversity of GAPDH. Future studies should reveal interesting patterns of subcellular distribution and trafficking of not only GAPDH, but also other reversibly nitroalkylated proteins.

GAPDH is a target of NO, which induces enzyme inhibition (42-46) and plays an undefined role in promoting the pro-apoptotic translocation of NO-modified GAPDH into the nucleus and its participation in nuclear events (21). In addition to the present demonstration of nitroalkylation, Cys residues of GAPDH can be S-glutathionylated (47) and S-nitrosylated (21,44,45). Appreciating that a) NO-dependent mechanisms mediate fatty acid nitration (5,48) and b) nitrated fatty acids give spurious, false-positive reactions for S-nitrosothiols (RSNO) in assays qualitatively and quantitatively identifying this species (2), the contributions of protein nitroalkene derivatives to putative S-nitrosothiol adducts and downstream signaling reactions should be addressed in more detail.

In contrast to the pro-inflammatory reactions mediated by oxidized lipids in inflammatory diseases, particularly in atherosclerosis (11,49,50), nitrated fatty acids exert potent anti-inflammatory cell signaling actions in both vascular and non-vascular tissues (2,5,7-9,51,52). These actions were initially attributed to a capacity to release NO and to activate PPAR receptors (2,5,9). The present observations reveal a new cell signaling reactivity of nitrated fatty acids – the capability to selectively induce reversible post-translational modification of proteins by nitroalkylation. Current data reveals that nitroalkenes induce pronounced effects on MAPK and JNK cell signaling cascades for reasons only now becoming evident. For example, protein tyrosine phosphatases contain an active site motif that includes an invariant Cys with a low pK_a (53), a property that promotes nucleophilic reactivity and susceptibility to nitroalkylation. Additionally, Cys residues critical for NFkB transcriptional regulation of inflammatory gene expression are potently influenced by nitroalkylation (54).

There is a precedence for GSH forming adducts with modified fatty acids that confer biological activity. The cysteinyl leukotrienes LTC4, LTD4 and LTE4 (GSH S-conjugates of leukotriene A4), recognized by the seven-transmembrane-spanning G protein-coupled receptors CysLT1 and CysLT2 (55,56), are potent pro-inflammatory lipid mediators. These oxidized fatty acid-GSH adducts are generated via the 5-
lipoxigenase pathway and have been implicated in a variety of pathologic conditions (57). It is thus intriguing to speculate whether nitroalkylation of GSH induces signaling actions that parallel those already characterized for nitroalkenes (i.e., generally anti-inflammatory), and whether additional signaling events are mediated by further receptor-ligand interactions.

Nitroalkylation represents a new form of lipid-dependent protein modification, which presently includes co- or post-translational myristoylation, palmitoylation and prenylation reactions (58). The extent and nature of protein nitroalkylation will be dependent on a number of factors summarized in Scheme 2. First, NO-dependent oxidative inflammatory reactions, diet and possible enzymatic synthesis will dictate endogenous levels of free and esterified nitroalkene derivatives (5,48). Second, the regulated activation of lipases will mediate the hydrolytic release of fatty acid nitroalkene derivatives esterified to complex lipids, thus influencing net levels, anatomic distribution, aqueous vs. hydrophobic partitioning and reactivities of free nitroalkenes. Third, the local environment can lower the pKₐ and increase the nucleophilicity of critical protein thiol and histidine residues, thus rendering greater reactivity with electrophilic nitroalkenes. Susceptibility to nitroalkylation will also be governed by steric factors and solvent accessibility (59). Fourth, protein nitroalkylation is thiol-reversible, indicating that the net redox status of cells and tissues will also govern extents of biomolecule nitroalkylation. These aggregate micro-environmental and biochemical properties thus confer a) specificity to nitroalkylation of specific amino acids on particular proteins and b) an ability of cells to regulate extents and sites of protein adduction by nitroalkenes.

In summary, we have described a new reactivity of endogenously-present nitrated fatty acids. These recently-detected derivatives mediate pluripotent signaling activities by acting as a high affinity ligands for PPARγ and PPARα, activating protein kinase signaling cascades and serving as a hydrophobically-stabilized reserve for cGMP-dependent NO signaling. We now show that nitroalkenes mediate the relatively specific, reversible post-translational modification of proteins that serves to transduce redox- and NO-dependent cell signaling by regulating protein function and distribution.
REFERENCES

1. Rubbo, H., Darley-Usmar, V., and Freeman, B. A. (1996) *Chem Res Toxicol* **9**(5), 809-820
2. Schopfer, F. J., Baker, P. R., Giles, G., Chumley, P., Batthyany, C., Crawford, J., Patel, R. P., Hogg, N., Branchaud, B. P., Lancaster, J. R., Jr., and Freeman, B. A. (2005) *J Biol Chem* **280**(19), 19289-19297
3. Napolitano, A., Camera, E., Picardo, M., and d'Ischia, M. (2000) *J Org Chem* **65**(16), 4853-4860
4. O'Donnell, V. B. (2003) *Antioxid Redox Signal* **5**(2), 195-203
5. Baker, P. R., Lin, Y., Schopfer, F. J., Woodcock, S. R., Groeger, A. L., Batthyany, C., Sweeney, S., Long, M. H., Iles, K. E., Baker, L. M., Branchaud, B. P., Chen, Y. E., and Freeman, B. A. (2005) *J Biol Chem* **280**(51), 42464-42475
6. Gladwin, M. T., Schechter, A. N., Kim-Shapiro, D. B., Patel, R. P., Hogg, N., Shiva, S., Cannon III, R. O., Kelm, M., Wink, D. A., Graham Espey, M., Oldfield, E. H., Pluta, R. M., Freeman, B. A., Lancaster, J. R., Jr., Feelsich, M., and Lundberg, J. O. (2005) *Nature Chemical Biology* **1**(6), 308-314
7. Coles, B., Bloodsworth, A., Clark, S. R., Lewis, M. J., Cross, A. R., Freeman, B. A., and O'Donnell, V. B. (2002) *Circ Res* **91**(5), 375-381
8. Coles, B., Bloodsworth, A., Eiserich, J. P., Coffey, M. J., McLoughlin, R. M., Giddings, J. C., Lewis, M. J., Haslam, R. J., Freeman, B. A., and O'Donnell, V. B. (2002) *J Biol Chem* **277**(8), 5832-5840
9. Schopfer, F. J., Lin, Y., Baker, P. R., Cui, T., Garcia-Barrio, M., Zhang, J., Chen, K., Chen, Y. E., and Freeman, B. A. (2005) *Proc Natl Acad Sci U S A* **102**(7), 2340-2345
10. Ono, N. (2002) Michael Addition. In: Feuer, H. (ed). *The Nitro Group in Organic Synthesis*, John Wiley & Sons, Inc., New York
11. Ceaser, E. K., Moellering, D. R., Shiva, S., Ramachandran, A., Landar, A., Venkatraman, A., Crawford, J., Patel, R., Dickinson, D. A., Ulasova, E., Ji, S., and Darley-Usmar, V. M. (2004) *Biochem Soc Trans* **32**(Pt 1), 151-155
12. Esterbauer, H., Zollner, H., and Scholz, N. (1975) *Z Naturforsch [C]* **30**(4), 466-473
13. Uchida, K., and Stadtman, E. R. (1992) *Proc Natl Acad Sci U S A* **89**(10), 4544-4548
14. Szweda, L. I., Uchida, K., Tsai, L., and Stadtman, E. R. (1993) *J Biol Chem* **268**(5), 3342-3347
15. Crabb, J. W., O'Neil, J., Miyagi, M., West, K., and Hoff, H. F. (2002) *Protein Sci* **11**(4), 831-840
16. Levonen, A. L., Landar, A., Ramachandran, A., Ceaser, E. K., Dickinson, D. A., Zanoni, G., Morrow, J. D., and Darley-Usmar, V. M. (2004) *Biochem J* **378**(Pt 2), 373-382
17. Harris, I., Meriwether, B. P., and Park, J. H. (1963) *Nature* **198**, 154-157
18. Ishii, T., Tatsuda, E., Kumazawa, S., Nakayama, T., and Uchida, K. (2003) *Biochemistry* **42**(12), 3474-3480
19. Uchida, K., and Stadtman, E. R. (1993) *J Biol Chem* **268**(9), 6388-6393
20. Chuang, D. M., Hough, C., and Senatorov, V. V. (2005) *Annu Rev Pharmacol Toxicol* **45**, 269-290
21. Hara, M. R., Agrawal, N., Kim, S. F., Cascio, M. B., Fujimuro, M., Ozeki, Y., Takahashi, M., Cheah, J. H., Tankou, S. K., Hester, L. D., Ferris, C. D., Hayward, S. D., Snyder, S. H., and Sawa, A. (2005) Nat Cell Biol 7(7), 665-674
22. Baker, P. R., Schopfer, F. J., Sweeney, S., and Freeman, B. A. (2004) Proc Natl Acad Sci U S A 101(32), 11577-11582
23. Botti, H., Batthyany, C., Trostchansky, A., Radi, R., Freeman, B. A., and Rubbo, H. (2004) Free Radic Biol Med 36(2), 152-162
24. Radi, R., Beckman, J. S., Bush, K. M., and Freeman, B. A. (1991) J Biol Chem 266(7), 4244-4250
25. Souza, J. M., and Radi, R. (1998) Arch Biochem Biophys 360(2), 187-194
26. Spengler, B. (1997) J Mass Spectr 32, 1019-1036
27. Burford, N., Eelman, M. D., and Groom, K. (2005) J Inorg Biochem 99(10), 1992-1997
28. Cowan-Jacob, S. W., Kaufmann, M., Anselmo, A. N., Stark, W., and Grutter, M. G. (2003) Acta Crystallogr D Biol Crystallogr 59(Pt 12), 2218-2227
29. Piraud, M., Vianey-Saban, C., Petritis, K., Elfakir, C., Steghens, J. P., Morla, A., and Bouchu, D. (2003) Rapid Commun Mass Spectrom 17(12), 1297-1311
30. Little, C., and O'Brien, P. J. (1969) Eur J Biochem 10(3), 533-538
31. Racker, E., and Krimsky, I. (1952) Nature 169(4312), 1043-1045
32. Sirover, M. A. (1999) Biochim Biophys Acta 1432(2), 159-184
33. Brodie, A. E., and Reed, D. J. (1990) Arch Biochem Biophys 276(1), 212-218
34. Brodie, A. E., and Reed, D. J. (1987) Biochem Biophys Res Commun 148(1), 120-125
35. Fukuda, A., Osawa, T., Hitomi, K., and Uchida, K. (1996) Arch Biochem Biophys 333(2), 419-426
36. Harris, J. L., and Walters, M. (1976) Glyceraldehyde-3-phosphate dehydrogenase. In: Boyer, P. D. (ed). The Enzymes, Academic Press, New York
37. Hyslop, P. A., Hinshaw, D. B., Halsey, W. A., Jr., Schraufstatter, I. U., Sauerheber, R. D., Spragg, R. G., Jackson, J. H., and Cochrane, C. G. (1988) J Biol Chem 263(4), 1665-1675
38. Kim, J. W., and Dang, C. V. (2005) Trends Biochem Sci 30(3), 142-150
39. Kliman, H. J., and Steck, T. L. (1980) J Biol Chem 255(13), 6314-6321
40. Tsai, I. H., Murthy, S. N., and Steck, T. L. (1982) J Biol Chem 257(3), 1438-1442
41. Mazzola, J. L., and Sirover, M. A. (2003) Biochim Biophys Acta 1622(1), 50-56
42. McDonald, L. J., and Moss, J. (1993) Proc Natl Acad Sci U S A 90(13), 6238-6241
43. Mohr, S., Stamler, J. S., and Brune, B. (1994) FEBS Lett 348(3), 223-227
44. Mohr, S., Stamler, J. S., and Brune, B. (1996) J Biol Chem 271(8), 4209-4214
45. Molina y Vedia, L., McDonald, B., Reep, B., Brune, B., Di Silvio, M., Billiar, T. R., and Lapetina, E. G. (1992) J Biol Chem 267(35), 24929-24932
46. Stamler, J. S. (1994) Cell 78(6), 931-936
47. Ravichandran, V., Seres, T., Moriguchi, T., Thomas, J. A., and Johnston, R. B., Jr. (1994) J Biol Chem 269(40), 25010-25015
48. O'Donnell, V. B., Eisierich, J. P., Chumley, P. H., Jablonsky, M. J., Krishna, N. R., Kirk, M., Barnes, S., Darley-Usmar, V. M., and Freeman, B. A. (1999) Chem Res Toxicol 12(1), 83-92.
49. Berliner, J. A., and Heinecke, J. W. (1996) Free Radic Biol Med 20(5), 707-727
50. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witztum, J. L. (1989) N Engl J Med 320(14), 915-924
51. Cui, T., Schopfer, F. J., Zhang, J., Chen, K., Ichikawa, T., Baker, P. R., Batthyany, C.,
    Chako, B. K., Feng, X., Patel, R. P., Agarwal, A., Freeman, B. A., and Chen, Y. E.
    (2005) J Exp Med, submitted
52. Lim, D. G., Sweeney, S., Bloodsworth, A., White, C. R., Chumley, P. H., Krishna, N. R.,
    Schopfer, F., O'Donnell, V. B., Eiserich, J. P., and Freeman, B. A. (2002) Proc Natl Acad
    Sci U S A 99(25), 15941-15946
53. Barford, D., Jia, Z., and Tonks, N. K. (1995) Nat Struct Biol 2(12), 1043-1053
54. Cui, T., Schopfer, F. J., Zhang, J., Chen, K., Ichikawa, T., Baker, P. R., Batthyany, C.,
    Chako, B. K., Feng, X., Patel, R. P., Agarwal, A., Freeman, B. A., and Chen, Y. E.
    (2006) J Biol Chem, submitted (M6-03357)
55. Heise, C. E., O'Dowd, B. F., Figueroa, D. J., Sawyer, N., Nguyen, T., Im, D. S., Stocco,
    R., Bellefeuille, J. N., Abramovitz, M., Cheng, R., Williams, D. L., Jr., Zeng, Z., Liu, Q.,
    Ma, L., Clements, M. K., Coulombe, N., Liu, Y., Austin, C. P., George, S. R., O'Neill, G. P.,
    Metters, K. M., Lynch, K. R., and Evans, J. F. (2000) J Biol Chem 275(39), 30531-
    30536
56. Lynch, K. R., O'Neill, G. P., Liu, Q., Im, D. S., Sawyer, N., Metters, K. M., Coulombe,
    N., Abramovitz, M., Figueroa, D. J., Zeng, Z., Connolly, B. M., Bai, C., Austin, C. P.,
    Chateauneuf, A., Stocco, R., Greig, G. M., Kargman, S., Hooks, S. B., Hosfield, E.,
    Williams, D. L., Jr., Ford-Hutchinson, A. W., Caskey, C. T., and Evans, J. F. (1999)
    Nature 399(6738), 789-793
57. Lewis, R. A., Austen, K. F., and Soberman, R. J. (1990) N Engl J Med 323(10), 645-655
58. Smotrys, J. E., and Linder, M. E. (2004) Annu Rev Biochem 73, 559-587
59. Creighton, T. E. (1993). In: Freeman, W. H., and company (eds). Proteins: Structures
    and Molecular Properties, 2nd Ed., New York

FOOTNOTES
*This work was supported by National Institutes of Health Grants HL58115 and HL64937 (B.A.F.); F.J.S.
    was supported by a postdoctoral fellowship from the American Heart Association Southeast Affiliate.
P.R.S.B. and L.M.S.B. were supported by National Institutes of Health Cardiovascular Hypertension
    Training Grant T32HL07457.

ACKNOWLEDGMENTS.
We thank Mutende J. Sikuyayenga, Marshall Long and Phil Chumley (University of Alabama at
Birmingham) for their helpful technical assistance.

ABREVIATIONS
1The abbreviations used are: ⋅NO, nitric oxide; LNO2, nitro-linoleic acid, 10-nitro-9,12-octadecadienoic
    acid and 12-nitro-9,12-octadecadienoic acid; OA-NO2 nitro-oleic acid, 9- and 10-nitro-9-cis-
    octadecenoic acids; NO2+, nitronium ion; ⋅NO2, nitrogen dioxide; NO2-, nitrite; ACN, acetonitrile;
    ONOO-, peroxynitrite; RSNO, nitrosothiols; N2O3, dinitrogen trioxide; GAPDH glyceraldehyde-3-
    phosphate dehydrogenase; GAP, D,L-glyceraldehyde 3-phosphate dehydrogenase; HPLC,
    high pressure liquid chromatography; MS, mass spectrometry; m/z, mass/charge; MI, monoisotopic mass;
    ESI, electrospray ionization; MRM, multiple reaction monitoring;
EPI, enhanced product information; MALDI-TOF, matrix-assisted laser desorption ionization-time of flight; CID, collision induced dissociation; PSD, post-source decay.

FIGURE LEGENDS

Fig. 1. Mass spectrometric analysis of the alkylation of glutathione by nitroalkenes and its impact on GSH lipophilicity. A) ESI-ion trap MS spectrum in negative ion mode (LCQ Deca, Thermo Electron, San Jose, CA) of the reaction product generated by LNO₂ (300 μM) reaction with GSH (300 μM) in 50 mM sodium phosphate buffer pH 7.4, at 20°C for 30 min. Previous to the MS analysis the reaction mixture was diluted in methanol, 0.1% NH₄OH; B) MS/MS spectrum in negative ion mode of the GS-LNO₂ adduct (m/z = 631.3); C) MS/MS/MS spectrum of fragment ion 306 m/z from GS-LNO₂ adduct (m/z=631.3). Insert: structural scheme of the adduct showing main fragmentation sites. The mass of main fragment ions is shown in red as detected in the negative ionization mode; D) Total ion chromatogram in positive ion mode shows a significant increase in the lipophilicity of GSH following alkylation by OA-NO₂, as indicated by the change in retention times of GSH [(M + H)⁺ = 308.3] (D; black) vs. the GS-OA-NO₂ adduct [(M+H)+ = 635.3] (D; red).

Fig. 2. The inhibition of GAPDH following alkylation by nitrolinoleate, nitro-oleate, peroxynitrite and hydrogen peroxide. A) GAPDH (0.5 μM) was incubated with increasing concentrations of LNO₂ (0 – 10 μM) (□) or OA-NO₂ (■) in 100 mM sodium pyrophosphate, 100 μM DTPA, pH 7.4, at 20°C for 15 min. Aliquots were removed and GAPDH activity was determined. B) The relative inactivation of GAPDH by LNO₂ (●), OA-NO₂ (■), ONOO⁻ (0 – 50 μM, ▲) and H₂O₂ (0 – 100 μM, ▼). C) Time course of OA-NO₂-mediated GAPDH inhibition. GAPDH (0.5 μM) was incubated with OA-NO₂ (10 μM) in 0.1 M pyrophosphate 0.1 mM DTPA, pH 7.4, at 25°C. At the indicated time points aliquots were removed and enzyme activity determined. D) pH-profile of the inhibition of GAPDH by OA-NO₂. After pre-incubation of GAPDH (0.5 μM) for 5 min in 50 mM sodium pyrophosphate buffer adjusted to pH 5.5 – 10, at 20°C, OA-NO₂ (7.5 μM) was added and after 15 min GAPDH activity was assessed as before. The percentage of control GAPDH activity at each pH was determined.

Fig. 3. OA-NO₂-induced thiol oxidation in GAPDH and its reversibility by thiol reagents. A) GAPDH (2 μM) was incubated for 15 min in 0.1 M pyrophosphate, 0.1 mM DTPA, pH 7.4, at 25°C with OA-NO₂ (0-50 μM). Aliquots were removed and GAPDH activity determined (●). Reduced thiol content was determined by DTNB reaction of GAPDH denatured in 1% SDS (●). B) GAPDH (0.5 μM) was incubated with H₂O₂ (250 μM), ONOO⁻ (50 μM), OA-NO₂ (10 μM) or LNO₂ (10 μM) in 0.1 M pyrophosphate, 0.1 mM DTPA, pH 7.4 at 25°C for 15 min and GAPDH activity determined before (open bars) and after (solid black bars) treatment of the samples with DTT (10 mM) for 30 min at 25°C. As a control, GAPDH was pretreated with DTT and activity was measured. C) OA-NO₂-inactivated GAPDH was treated with increasing concentrations of GSH (0 – 10 mM) and enzyme activity determined.

Fig. 4. Mass spectrometric analysis of GAPDH alkylation by OA-NO₂ and its reversal by GSH. GAPDH (0.5 μM) was incubated with OA-NO₂ (10 μM) in 0.1 M pyrophosphate, 0.1 mM DTPA, pH 7.4 at 25°C for 15 min. GSH (10 mM) was then added for 15 min. After desalting, aliquots were analyzed by MALDI-TOF MS (A-C; Voyager DE PRO, Applied Biosystems, Foster City, CA) or by LC-ESI-2D-linear ion trap MS (D; LTQ, Thermo Electron, San Jose, CA). A) Spectrum of native GAPDH; B) OA-NO₂-treated GAPDH before and C) after addition of GSH; D) Native GAPDH (black trace) and OA-NO₂-treated GAPDH (red trace).

Fig. 5. ESI-HPLC-MS analysis of tryptic peptides from native and OA-NO₂-alkylated GAPDH. Selective ion chromatograms from native or OA-NO₂-treated GAPDH digested with sequence-grade trypsin and analyzed by ESI-LC-MS (LCQ-Deca, Thermo Electron, San Jose, CA). Non-alkylated peptides (A) and peptides alkylated by OA-NO₂ (B, C) are numbered as in online supplementary data Fig.
Similar relative ion intensities of non-nucleophilic peptides #7 and 17 (m/z 657.3 and 1369.7) were generated by both control and OA-NO2-nitroalkylated GAPDH; Peptides #2, 18 and 23 containing the nucleophilic, OA-NO2-reactive amino acid His were present at lower ion intensities in the tryptic digest of OA-NO2-treated GAPDH; and Peptides #19 and 21, containing OA-NO2-reactive nucleophilic amino acid Cys, were absent in tryptic digests of OA-NO2-treated GAPDH.

Fig. 6. Mass spectrometric analysis of nitroalkylation patterns following in vitro treatment of purified GAPDH with OA-NO2. A) Selective ion chromatograms from LC-ESI-MS analysis of OA-NO2-modified peptide #18 (LCQ Deca, Thermo Electron, San Jose, CA). The nitroalkylation of peptide #18 (native peptide (M+H) + = 1229.6, RT 27.9 min) by OA-NO2 increased the retention time to 76 min and the mass of the OA-NO2-modified peptide #18 was increased by 327 Da, equivalent to the neutral ion mass of OA-NO2, becoming m/z 1556.7; B) LC-nanospray-MS/MS spectrum of the nitroalkylated peptide #18 (LTQ, Thermo Electron, San Jose, CA). MS/MS spectrum of the doubly-charged ion at m/z 844.34. Colors are annotated in the corresponding table. The y0 and b0 nomenclature indicates the corresponding y-H2O and b-H2O fragments, respectively. Inset: Amino acid sequence of peptide #18 indicating major C- and N-terminal fragment ions detected by full-scan MS/MS; C) MALDI-TOF mass spectrum of the tryptic digest of OA-NO2-treated GAPDH (Voyager DE Pro, Applied Biosystem, Foster City, CA), focusing on nitroalkylated-peptide #18 [(M+H)+ = 1556.9]. D) Post source decay (PSD) MALDI-TOF-MS analysis of modified-peptide #18 gives a main product ion at m/z 437.1, corresponding to the immonium ion of the histidine (H1)-OA-NO2 adduct. E) Structure and fragmentation pattern of the His-OA-NO2 adduct, showing the immonium adduct fragment (H-OA-NO2). Table. List of MS/MS fragment ions m/z from peptide #18. Ions that are detected are highlighted in color (Fig. 6B).

Fig. 7. Increased membrane association of GAPDH following nitroalkylation by OA-NO2. Control and OA-NO2-treated GAPDH were incubated with liposomes for 30 min at 25°C. Liposomes were sedimented by ultracentrifugation and the translocation of soluble GAPDH in the supernatant (A) to a liposome membrane-associated state (B) was determined as a function of OA-NO2 treatment concentration by SDS-PAGE electrophoresis.

Fig. 8. Mass spectrometric detection of endogenous nitroalkylated GAPDH in red blood cells obtained from healthy humans. The cytosolic (A) and membrane-associated (B) protein fractions from lysed red cells were separated by SDS-PAGE using non-reducing, denaturing conditions (4-15% gradient gel). The 36 kDa Coomassie dye-binding band corresponding to the Rf of GAPDH was excised and digested in gel with sequencing grade trypsin. Peptides were extracted and separated and analyzed by LC-nanospray-linear ion trap MS/MS (LTQ, Thermo Electron, San Jose, CA). A) MS/MS spectrum of the doubly-charged ion at m/z 759.96 corresponding to the human homolog of rabbit nitroalkylated peptide #19. Inset: Amino acid sequence indicating major C- and N-terminal fragment ions detected by full-scan MS/MS; B) MS/MS spectrum of triply charged human GAPDH ion at m/z 882.71 corresponding to the human peptide sequence 305-323. Inset: Amino acid sequence indicating major C- and N-terminal fragment ions detected by full-scan MS/MS.

Fig. 9. Mass spectrometric detection of endogenous nitroalkylated GSH in healthy human red blood cells. Red cells were lysed, membranes sedimented by centrifugation and the soluble fraction was supplemented with the internal standards GS-[13C18]OA-NO2 and GS-[13C18]LNO2 before purification by reverse phase chromatography using a preparative C18 column. The eluted fraction was concentrated and analyzed by LC-ESI-MS/MS (Q-Trap 4000, Applied Biosystem, Foster City, CA). A) Mass spectra of the eluent produced by monitoring the MRM transition 635.3/506.3 (corresponding to the generation of the y2-adducted fragment) for endogenous GS-OA-NO2 (red trace) and 653.3/524.3 for the added internal standard GS-[13C18]OA-NO2 (MRM 653.3/524.3 (black trace); B) Similar to A), but monitoring the transitions for GS-LNO2.

Fig. 10. Mass spectrometric characterization of nitroalkylated GSH. A) Enhanced product ion (EPI) analysis (e.g., MS/MS fragmentation pattern) of the synthetic standard GS-OA-NO2 showing major C-
and N-terminal fragment ions detected by full-scan MS/MS (y and b fragments, respectively). Inset: scheme showing the structure and principal EPI fragments of GS-OA-NO₂; B) EPI analysis of the endogenous RBC cytosolic GS-OA-NO₂ adduct, displaying a fragmentation pattern identical to that of synthetic GS-OA-NO₂; C) MS³ spectrum of the fragment ion y2 (m/z 506.3) from GS-OA-NO2 adduct (m/z=635.2). Table. List and structural interpretation of fragment ions generated.

**Scheme 1.** Michael addition reaction of fatty acid nitroalkene derivatives with (A) thiols and (B) amino groups.

**Scheme 2.** Nitroalkene mediated post-translational modification of GAPDH and other proteins will influence protein structure, function and subcellular distribution in a GSH-reversible manner. The modified sites of the protein were randomly assigned.
| Peak/Pept. # | RT (min) | Sequence | Mol. Mass (MI, Da) | Theoretical m/z | Measured m/z | Amount Detected |
|-------------|----------|----------|-------------------|-----------------|--------------|----------------|
|            |          |          |                   |                 |              | Control        |
| # 2        | 2.06     | AGA**H**<sup>108</sup>LK | 595.35 1 | 596.35 | 596.3 | ✓ |
| # 18       | 27.86    | VVDLMV**H**<sup>327</sup>MASK | 1228.64 1 | 1229.64 | 1229.5 | ✓ |
| # 19       | 28.02    | IVSNASC<sup>149</sup>TTC<sup>153</sup>L | 1704.86 1 | 1705.86 | 1703.8 | ✓ |
|            |          | APLAK    |                   |                 |              |                |
| # 21       | 36.36    | VPTPNSVVDLT**C**<sup>244</sup>R | 1498.79 1 | 1499.79 | 1499.7 | ✓ |
| # 23       | 39.39    | VIISAPSADAPMFV**G**MV**H**<sup>134</sup>EK | 2212.11 2 | 1107.05 | 1107.1 | ✓ |
|            |          |          |                   |                 |              | OA-NO<sub>2</sub> Treated |
| Peptide | Sequence                  | Native- |          | OA-NO₂- |          |
|---------|---------------------------|---------|----------|---------|----------|
|         |                           | RT (min)| ACN (%)  | RT (min)| ACN (%)  |
| # GSH   | γ-ECG                     | 1.6     | 2.0      | 74.4    | 38.2     |
| # 2     | AGAHLK                    | 2.1     | 2.0      | 65.6    | 33.3     |
| # 18    | VVDLMVHMASK               | 27.9    | 12.6     | 76.0    | 39.1     |
| # 19    | IVSNASCTTNCLAPLAK         | 28.0    | 12.7     | 83.6    | 43.2     |
| # 21    | VPTPNVSVDLTCR             | 36.4    | 17.3     | 83.1    | 43.0     |
| # 23    | VIISAPSADAPMFVMGNHEK      | 39.4    | 18.9     | 76.6    | 39.4     |
Fig 1
Batthyany et al.
Fig 2
Batthyany et al.
Fig 3
Batthyany et al.
Fig 4
Batthyany et al.
Fig 5
Batthyany et al.
Table. MS-MS data of OA-NO$_2$-modified peptide #18

| AA | a   | b   | y   |
|----|-----|-----|-----|
| 1  | V   | 72.08 | 100.08 | - 12 |
| 2  | V   | 171.15 | 199.14 | 1587.01 | 11 |
| 3  | D   | 286.18 | 314.17 | 1487.94 | 10 |
| 4  | L   | 399.26 | 427.26 | 1372.92 | 9 |
| 5  | M   | 530.30 | 558.30 | 1259.83 | 8 |
| 6  | V   | 629.37 | 657.36 | 1128.79 | 7 |
| 7  | H*  | 1093.83 | 1121.82 | 1029.72 | 6 |
| 8  | M   | 1224.87 | 1252.86 | 565.27 | 5 |
| 9  | A   | 1295.91 | 1323.90 | 434.22 | 4 |
| 10 | S   | 1382.94 | 1410.93 | 363.19 | 3 |
| 11 | K   | 1511.03 | 1539.03 | 276.16 | 2 |
| 12 | E   | 148.06 | 1 |
Fig 7
Batthyany et al.
Human Homolog of rabbit peptide #19-OA-NO$_3$

\[
\begin{align*}
\text{S} & \quad \text{C}^* \quad \text{T} \quad \text{A} \quad \text{N} \quad \text{C} \quad \text{L} \quad \text{A} \quad \text{L} \quad \text{P} \quad \text{K} \\
& \quad \text{b}_5 \quad \text{b}_6 \quad \text{b}_7 \quad \text{b}_8 \quad \text{b}_9 \quad \text{b}_{10} \quad \text{b}_{11} \\
^* & = + 327.4; \text{ m/z 759.96; } z = 2
\end{align*}
\]

**B)**

\[
\begin{align*}
\text{D} & \quad \text{H}^* \quad \text{F} \quad \text{V} \quad \text{K} \quad \text{L} \quad \text{I} \quad \text{S} \quad \text{L} \quad \text{I} \quad \text{Y} \quad \text{D} \quad \text{N} \quad \text{E} \quad \text{F} \quad \text{G} \quad \text{Y} \quad \text{S} \quad \text{N} \quad \text{R} \\
& \quad \text{y}_5 \quad \text{y}_6 \quad \text{y}_7 \quad \text{y}_8 \quad \text{y}_9 \quad \text{y}_{10} \quad \text{y}_{11} \quad \text{y}_{12} \quad \text{y}_{13} \\
^* & = + 327.4; \text{ m/z 882.71; } z = 3
\end{align*}
\]

**Fig 8**

Batthyany et al.
**Fig 9**
Batthyany et al.
A) 

![Diagram of molecular structure with m/z values and intensities](image)

B) 

![Mass spectrum with m/z values and intensities](image)

C) 

| Fragment | m/z  | Structural Interpretation |
|----------|------|----------------------------|
| y2       | 506.3| M[+H]⁺                      |
|          | 489.3| M[-NH₃+H]⁺                  |
|          | 471.2| M[-NH₃+H₂O+H]⁺              |
|          | 459.3| M[-HNO₂+H]⁺                 |
|          | 442.3| M[-HNO₂-NH₃+H]⁺             |
|          | 424.3| M[-HNO₂-NH₂O+H]⁺             |
|          | 413.3| M[-HNO₂-CH₂O₂+H]⁺            |
| C1       | 403.3| [-H₂O]⁺                     |
|          | 385.3| C1[-H₂O]⁺                   |
|          | 356.3| C1[-HNO₂]⁺                   |
|          | 385.2| M[-OA-NO₂]+H]⁺               |
|          | 162.1| M[-OA-NO₂-NH₃+H]⁺            |

Relative Intensity (%) for various m/z values.

Fig 10
Bathyany et al.
Scheme 1
Batthyany et al.
Scheme 2
Batthyany et al.
Access the most updated version of this article at doi: 10.1074/jbc.M602814200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2006/05/10/M602814200.DC1