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Proteins modified by the lipid peroxidation aldehyde DODE in MCF7 breast cancer cells

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Abstract

The hydroperoxide of linoleic acid (13-HPODE) degrades to 9,12-dioxo-10(E)-dodecenoic acid (DODE) which readily modifies proteins. This study identified the major proteins in MCF7 cells modified by DODE. To reduce false positives, three methods were used to identify DODE-modified proteins. First, cells were treated with a synthetically biotinylated 13-HPODE (13-HPODE-biotin). Modified proteins were enriched by neutravidin affinity and identified by 2D-LC-MS/MS. Second, cells were treated with native 13-HPODE. Protein-carbonyls were biotinylated with an aldehyde reactive probe (ARP) and modified proteins enriched by neutravidin affinity and identified by 2D-LC-MS/MS. Third, using a newly developed DODE antibody, DODE modified proteins were located by 2D-SDS-PAGE and Western blot and identified by in-gel digestion and LC-MS/MS. Analysis of the proteins characterized by all three methods revealed a significant overlap and identified 32 primary proteins modified by DODE in MCF7 cells. These results demonstrated the feasibility for the cellular formation of DODE protein-carbonyl adducts that may be future indicators of oxidative stress.

Keywords

Protein carbonyls; lipid peroxidation; 13-HPODE; DODE; ONE; HNE; cytochrome c; ARP; MCF7

Introduction

Cellular oxidative stress can result in damage of bio-macromolecules such as proteins, DNA and polyunsaturated fatty acids (PUFA). Oxidation of PUFA results in the formation of a variety of lipid hydroperoxides occurring from reactive oxygen species (1) or the enzymatic action of cyclooxygenases (2) and lipoxygenases (3). These hydroperoxides are prone to decomposition into α,β -unsaturated aldehydes through multiple pathways (4-10). Our previous studies have demonstrated that lipid peroxidation chemistry dominates DNA adduct formation in a mouse inflammation model (11). It therefore seemed likely that lipid-derived carbonyl protein adducts are also a dominant chemistry of inflammation and would make potential biomarkers of inflammation and cellular oxidative stress.

Linoleic acid is the most abundant PUFA found in mammals and its lipid peroxidation products likely account for the majority of lipid-derived protein-carbonyls (12). The lipid

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hydroperoxide formed from the oxidation of linoleic acid, 13-hydroperoxy-9,11(Z,E)-octadecadienoic acid (13-HPODE), reductively degrades to 4-hydroperoxy-2-(E)-nonenal (HPNE) and 4,5-epoxy-2-(E)-decenal (EDE). HPNE undergoes dehydration or reduction to form the unsaturated aldehydes 4-oxo-2-(E)-nonenal (ONE) and 4-hydroxy-2-(E)-nonenal (HNE) respectively (Scheme 1A). The formation of the unsaturated aldehyde, 9,12-dioxo-10(E)-dodecenoic acid (DODE), is derived directly from the linoleic acid hydroperoxide (13-HPODE) degradation. DODE has also been shown to be a breakdown product of 9-hydroperoxy-10,12-octadecadienoic acid in lentil seeds (4). The oxidation of α -linolenic acid by lipoxygenase has been shown to produce the 13-OOH of this PUFA (13), and the possibility exists for this hydroperoxide to also degrade to DODE, but has yet to be studied. These α,β -unsaturated aldehydes have been shown to covalently modify DNA (14,16), cellular glutathione (16,17) and proteins (18-22). Protein modification occurs through conjugate addition (often Michael addition) to nucleophilic amino acid side chains (Lys, Cys and His), and HNE, ONE and DODE also form carbonyl-containing adducts and EDE a pyrrole adduct (Scheme 1B). Of the α,β -unsaturated aldehydes shown in Scheme 1A, only DODE is exclusive to linoleic acid decomposition, retaining the carboxylic acid functional group. We have recently shown that DODE is the most reactive of the protein-modifying carbonyls occurring from 13-HPODE decomposition and only DODE modifications were observed when cytochrome c was incubated with its precursor 13-HPODE (22). Therefore, due to DODE's reactivity and uniqueness to linoleic acid, the focus of this study was to identify the major cellular proteins modified by DODE in MCF7 breast cancer cells and demonstrate feasibility for the cellular formation of these potential indicators of oxidative stress.

To reduce the number of false positives and identify only DODE-modified proteins, three independent methods were used for modified protein identification. First, MCF7 cells were treated with a biotin-linked 13-HPODE (Scheme 3). The biotin group was coordinated to the carboxylic acid of the hydroperoxide. Since DODE is the only reactive carbonyl to retain the acid functional group, only proteins modified by DODE would be biotinylated using this tag. Modified proteins were avidin affinity purified and identified by 2D-LC-MS/MS. Second, MCF7 cells were treated with standard 13-HPODE. Proteins were separated by 2D chromatography and DODE modified proteins were located by Western blot using a newly-developed polyclonal DODE antibody. These proteins were identified by in-gel digestion and LC-MS/MS. Third, proteins from 13-HPODE treated cells were reacted with an aldehyde reactive probe (ARP). ARP is a biotinylated hydroxylamine that reacts preferentially with aldehyde/keto groups (23), and Maier, et al., have demonstrated the usefulness of this reagent to label and affinity purify carbonyl-modified proteins from rat heart mitochondria (24) and HNE-modified thioredoxin (25). The hydroxylamine of ARP forms either an aldoxime or ketoxime with the aldehyde or keto functionality of the lipid-derived carbonyl adduct as shown in Scheme 2. Carbonyl-containing proteins from the MCF7 cells were derivatized with ARP and selectively enriched via the biotin side-chain. Modified proteins were identified by 2D-LC-MS/MS.

Comparison of the proteins identified from the three techniques resulted in the identification of a total of 32 major DODE-modified proteins. These proteins were classified into five major functional groups: structural, regulatory, molecular chaperones, glycolysis and nucleic-acid binding. Protein location was mainly cytoplasmic, although a number of membrane-associated and nuclear proteins were also identified. These results characterized the major proteins modified by DODE in MCF7 breast cancer cells and introduced DODE-modified proteins as possible target biomarkers for oxidative stress. This study verified biotin labeling, affinity chromatography and 2D-LC-MS/MS as a successful method to identify DODE modified proteins from a complex biological system.

Materials and Methods

Materials

Aldehyde reactive probe (ARP) was purchased from Invitrogen (Eugene, OR) and biotin-PEO-LC-Amine was purchased from Pierce (Rockford, IL). Diisopropylethylamine (DIPEA), dimethylformamide (DMF), 2-(1H-7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate methanaminium (HATU), lipoxidase, cytochrome c (equine heart), ammonium bicarbonate, acetic acid, trifluoroacetic acid, and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin was purchased from Promega (Madison, WI). Linoleic acid was purchased from Cayman Chemical. Gases were supplied by AirGas (Salem, NH). DODE was a generous gift from Prof. Ian A. Blair (University of Pennsylvania).

Synthesis of 13-HPODE

13-HPODE was synthesized by the enzymatic oxidation of linoleic acid with soybean lipoxidase (26). Briefly, 50 mg of linoleic acid was oxidized by the addition of 600,000 units of soybean lipoxidase in 50 mL of 0.2 M borate buffer, pH = 9. The reaction was stirred on ice for 1.5 hours under O₂. The solution was acidified with concentrated HCl to pH 3, and 13-HPODE was extracted with 2 × 25 mL of ether. Ether extractions were washed with 50 mL of deionized H₂O, dried with sodium sulfate and filtered. Ether was evaporated and the 13-HPODE oil residue was dissolved in a minimal amount of hexane. 13-HPODE was further purified by flash chromatography using a 15 cm × 2 cm packed silica gel column with a mobile phase of 70% hexane 30% ethyl acetate at a flow rate of about 2 ml/min. The product was eluted in 5 mL fractions around fractions 6-10. The purity of 13-HPODE was checked by normal phase HPLC-DAD using a Regis Rexchrome silica column (25cm × 100 mm, 5 μm) isocratic mobile phase of 98.5% hexane, 1.5% isopropanol, 0.1% acetic acid at a flow rate 3 ml/min. Final purified 13-HPODE was dried, re-dissolved in ethanol to give a 20 mg/mL or 65 mM concentration ($\epsilon_{234\text{nm}} = 25,600 \text{ M}^{-1}\text{cm}^{-1}$, and stored at -80°C.

Synthesis of 13-HPODE-biotin

13-HPODE was synthesized and purified as described above. To allow for complete coupling to the biotin, a small excess of 13-HPODE was used to give a molar ratio of 1.1 : 1 for 13-HPODE : biotin-PEO-LC-Amine. 13-HPODE (6.5 μmoles; 20 mg/ml) and 6.5 μmoles 2-(1H-7-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) were dissolved in 100 μL of DMF. 6.0 μmoles of biotin-PEO-LC-Amine, dissolved in DMF, were added along with 32.5 μmoles (5 × molar excess) of N,N-diisopropylethylamine (DIPEA) to give a final reaction volume of ~ 200 μL. The reaction was stirred for 1 hour at room temperature, dried and re-dissolved in 100% ethanol. The purity of 13-HPODE-biotin was checked by reverse phase HPLC-ESI-TOF-MS using a Microsorb-MV 2.5 × 250 mm, 5 μm C18 column and a mobile phase gradient of 2% B to 80% B over 60 min at 0.3 ml/min; A = water/0.5% acetic acid and B = acetonitrile/0.5% acetic acid. The mass spectrum of the major product showed *m/z* 713, corresponding to [M + H]⁺ for the biotinylated 13-HPODE. A second minor signal at *m/z* 313 was also detected, corresponding to [M + H]⁺ for un-reacted 13-HPODE. From the peak heights it was determined that the sample contained about 98% 13-HPODE-biotin and 2 % 13-HPODE. No un-reacted biotin-PEO-LC-Amine was seen, and no further purification was necessary. The sample was dissolved in 100% ethanol to a concentration of about 20 mg/mL.

Reaction of cytochrome c with 13-HPODE and 13-HPODE-biotin

Cytochrome c (1 mg/mL, 100 μl in pH 7.0 chelex-treated 100 mM HEPES buffer) was reacted with 13-HPODE or 13-HPODE-biotin (224 nmols, 10 μL ethanol) in the presence of

vitamin C (10mM) at 37°C for 24 h. The sample was filtered (regenerated cellulose 3,000 Da MWCO; Amicon,) to remove un-reacted 13-HPODE or 13-HPODE-biotin and vitamin C, and brought up in PBS to a concentration of 1 mg/mL.

ARP derivatization of DODE modified cytochrome c

Cytochrome c samples (1 mg/mL) treated with 13-HPODE were incubated with Aldehyde Reactive Probe (ARP) (10 mM) at a final volume of 1.0 mL in phosphate buffer, pH = 5-6. The reaction was stirred vigorously for 12 hours at room temperature. The samples were filtered (regenerated cellulose 3,000 Da MWCO; Amicon,) to remove unreacted ARP.

ARP derivatization MCF7 cellular protein carbonyls

Extracted cellular proteins were dialyzed (4°C) for a minimum 48 hrs into PBS (2,000 Da MWC, 3mL cassette; Pierce). To the dialyzed sample a protease inhibitor cocktail was added: 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (1 mM), E-64 (10 µM), pepstatin A (1.4 µM), EDTA (1 mM), bestatin (40 µM). None of these protease inhibitors contain ketones or aldehydes to interfere with the ARP oxime formation reaction. ARP was added to a final concentration of 3.0 mM. The pH was adjusted to 6.0 with 5% HCl and the reaction was stirred at room temperature for 12 hours. Derivatized protein was dialyzed against PBS for at least 48 hours to remove any un-reacted ARP. The total protein concentration was measured by BCA assay (Pierce) before avidin affinity chromatography.

Treatment of MCF7 cells with 13-HPODE and 13-HPODE-biotin

MCF7 cells were purchased from Invitrogen. Cells were grown in a media containing Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, MEM non-essential amino acids, sodium pyruvate, and L-glutamine. Cells were grown to 90% confluence on 10 cm plates before treatment and protein extraction.

The media from 40 plates (90% confluence) was removed, the cells washed with PBS, and 10 mL DMEM (no FBS) was added to each plate. Either 13-HPODE or 13-HPODE-biotin (10 µL of 20 mg/ml or 64 µM final concentration) was added to each plate, followed by incubation for 1/2 hour at 37°C. Medium was then removed and the cells washed with PBS. MCF7 growth medium (10 mL, containing 1.0 mM ascorbic acid) was added, and the plates incubated at 37°C for 4 hours. After incubation the medium was removed and the cells washed with PBS. Cells were lysed with 0.5 mL of nondenaturing lysis buffer (Sigma, CelLytic M Cell Lysis Reagent) containing a cocktail of protease inhibitors: AEBSF (1 mM), E-64 (10 µM), pepstatin A (1.4 µM), EDTA (1 mM), bestatin (40 µM). Lysed cells were scraped from the plates and transferred to 1.5 mL eppendorf tubes and aspirated through a 20-gauge needle 8x to assure complete cell lysis. Cell debris was pelleted, and the supernatants then removed, combined, and dialyzed (Pierce Slide-A-Lysed 3000 MWCO) into PBS for 48 hours at 4°C. Control MCF7 cells were not treated with 13-HPODE-containing media. To 40 plates of cells (90% confluence) 10 mL of growth media was added (final concentration 1.0 mM). Plates were incubated at 37°C for 4 hours. Control cells were lysed and treated as described above.

Immunostaining and imaging of MCF7 cells treated with 13-HPODE-biotin

Cells treated with 13-HPODE-biotin and untreated controls were cultured as described above, after which medium was aspirated and the cells washed with sterile PBS (x3). The cultures were then incubated with Cy3 conjugated streptavidin (Invitrogen, catalog # 43-4315; 1:100 dilution) for an hour and Hoechst 33342 (Invitrogen, catalog # H-3570; 1:5000 dilution) was added into the medium for 30 seconds before washing with PBS (x3).

Imaging was done with an epifluorescence microscope (Zeiss Axiovert-100) using Chroma Set CZ 915 and CZ 902 filters for Cy-3 and Hoechst respectively.

Neutravidin affinity chromatography of cell lysates

Immobilized Neutravidin (Pierce) was packed into a column with dimensions ID = 9 mm, OD = 7 mm, height = 60 mm. The final column volume was 2 mL. All buffers and samples were allowed to warm to room temperature. The column was washed with 4 column volumes of PBS. A protease inhibitor cocktail was added to protein samples: AEBSF (1 mM), E-64 (10 μ M), pepstatin A (1.4 μ M), EDTA (1 mM), bestatin (40 μ M). Samples (in PBS) were added directly to the neutravidin column. To remove non-biotinylated proteins, the column was washed with 10 column volumes of PBS-2% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS), 5 column volumes of PBS and 5 column volumes of deionized water. Wash fractions were analyzed by UV-Vis spectrometry for baseline absorbance (280 nm). Bound proteins were eluted by the addition of 4 column volumes of 0.4% TFA/80% acetonitrile. Elution fractions were combined, frozen, lyophilized, and stored at -20°C.

Gel electrophoresis for biotinylated and non-biotinylated proteins

100 μ g (Coomassie) or 30 μ g (Western) of sample was processed by 2-D gel electrophoresis for purposes of protein identification/Western blotting. For anti-biotin western blotting and Coomassie, proteins were focused on 11 cm 4-7 immobilized pH gradient (IPG) strips (Immobiline™ DryStrip gels, Amersham Biosciences) using an IPGphor focusing apparatus (Amersham Biosciences). For anti-DODE western blotting and Coomassie staining, proteins were focused on a 11 cm 3-10 pH gradient strip. Samples were applied by cup loading. IPG strips were then equilibrated in equilibration buffer (50 mM Tris-HCl, 6M urea, 30% glycerol, 2% SDS) supplemented with 1% dithiothreitol (DTT) to maintain the fully-reduced state of proteins, followed by 2.5% iodoacetamide to prevent re-oxidation of thiol groups during electrophoresis. Proteins were separated on 12.5% Tris-glycine gels (BioRad) using a Criterion System (BioRad). Proteins were visualized by Coomassie, SimplyBlue SafeStain™ (Invitrogen).

Development of Anti-DODE antibody

DODE was conjugated to Mariculture keyhole limpet hemocyanin (mCKLH) by incubating a 1:1 (w/w) ratio of DODE to mCKLH in 100 mM HEPES buffer pH = 7.1; reaction was stirred overnight at 37°C. Conjugated mCKLH was spin filtered (regenerated cellulose 10,000 Da MWCO; Amicon) and sent to Covance Research Products Inc for the development of an antibody to DODE in rabbits. After antibody development, the IgG DODE antibody was purified from the rabbit anti-sera using immobilized A/G protein beads (Pierce). Eluted/purified DODE antibody was buffer exchanged and concentrated to 10 mg/mL in PBS and stored at -80°C for Western blot analysis.

Anti-Biotin and Anti-DODE Western blots

Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (BioRad). Precision Plus protein standard (BioRad) was used to estimate molecular weights. Anti-biotin (Cell Signaling) 1:20,000 was used to detect biotinylated proteins. Polyclonal anti-DODE (Covance) 1:1500 was used to detect DODE modified proteins.

In-Gel Digestion

Protein spots were picked and washed in Milli-Q® water for 15 min, then washed three times in 25 mM NH₄HCO₃/50% CH₃CN for 30 min. Gel plugs were dehydrated in 100% CH₃CN for 10 min while vortex-mixing. The supernatant was removed, and gel plugs were

dried in the SpeedVac. Trypsin (1 μ g/50 μ l) (Sigma) suspended in 25 mM NH₄HCO₃ was added, and gel plugs were allowed to re-hydrate for 30 min on ice and allowed to digest overnight at 37°C. The samples were then centrifuged, and the supernatant was removed. The pellet was re-suspended in CH₃CN with 1% TFA, vortexed, and sonicated for 30 minutes to release hydrophobic peptides. The supernatant was removed and combined with the previous supernatant and stored at -20°C until ready for MS/MS analysis.

Strong cation exchange (SCX) separation for MCF7 proteins

Neutravidin elutions of proteins from cell lysates from 13-HPODE treated cells and 13-HPODE-biotin treated cells were digested in-solution with trypsin as follows: to 200 μ g of protein 4 μ g of sequencing grade trypsin (Promega) was added in ~200 μ L 25 mM ammonium bicarbonate; proteins were digested for 4 hours at 37°C. Digested proteins were desalted by SPE (Strata 50um, tri-Func, C18-E) dried and re-dissolved in 100 μ L mobile phase A (described below) for SCX separation. Purified peptides were separated by SCX liquid chromatography using an Agilent 1100 LC system with a Polysulfoethyl A 100 \times 4.6 mm, 5 μ m column and a flow rate of 0.25 ml/min. Mobile phase A was 10 mM sodium phosphate (Na₂HPO₄), 25% acetonitrile, pH = 2.8 and mobile phase B was 10 mM sodium phosphate (Na₂HPO₄), 25% acetonitrile, 0.4 M KCl, pH = 2.8. A linear gradient was used as follows: 0% B from 0-10 min, 100 % B at 70 min, 100% B at 75 min and 0% B at 80 min. Peptides were collected in 1 min fractions to give about 40-60 fractions per sample. Fractions were desalted by ZipTips® and re-dissolved in 5 μ L 0.4% acetic acid before LC-ESI-QTOF MS/MS analysis.

LC-ESI-TOF/MS

Whole protein cyt c analyses were performed on an Agilent ESI-TOF mass spectrometer (Palo Alto, CA). ESI was conducted using a needle voltage of 3.5 kV. Nitrogen was used as the drying (10 l/min), and nebulizer (15 psig) gas with the heated capillary at 325°C. Chromatography was performed using an Agilent 1200 LC system. LC separations were performed using a Zorbax 300SB-C18 (2.1 \times 100mm; 3.5 μ m; Agilent Technologies Palo Alto, CA) column with a flow rate of 0.2 ml/min. Solvent A was 0.25% acetic acid in water and solvent B was 0.25% acetic acid in 90% acetonitrile. A linear gradient was performed as follows: 5% B at 0 min, 50% B at 45 min, 80% B at 50 min, 5% B at 55 min, and 5% B at 65 min. Data analysis: to evaluate modification of cyt c with either DODE or DODE-biotin, the intact-protein mass spectrum was deconvoluted using the BioConfirm function in the Agilent MassHunter Workstation software.

LC-ESI-QTOF MS/MS

Peptide analyses for protein ID were performed with either the Applied Biosystems (Framingham, MA) QSTAR Elite quadrupole-TOF mass spectrometer equipped with a nanospray source (Proxeon Biosystems, Odense, Denmark), or the Agilent 6520 Accurate-Mass Q-TOF (Palo Alto, CA).

ABS QSTAR Elite MS/MS—The electrospray interface for this instrument uses a micro-tee (Upchurch Scientific, Oak Harbor, WA) with a 1-in. piece of platinum rod, inserted into one arm of the micro-tee, to supply the electrical connection. The electrospray voltage was typically 1600-1700 V applied just upstream of the column. Data-dependent MS/MS analysis was performed on the three most intense peaks in each full-scan spectrum, using multiply-charged states (most of the non-peptide background constituents are singly charged). Accumulation time and pulsar frequency were maintained at 3 s and 6.99, respectively; the mass tolerance was 50 mmu. MS/MS was performed using nitrogen as the collision gas. Rolling collision energies were calculated (in this study) as follows: $z = 1$,

(collision energy) = $0.06 \cdot (m/z) + 8$; $z = 2$, (collision energy) = $0.05 \cdot (m/z) + 5.6$; $z = 3$, (collision energy) = $0.04 \cdot (m/z) + 6.7$; $z > 3$, (collision energy) = $0.015 \cdot (m/z) + 25$.

Chromatography was performed using an Agilent 1100 capillary LC system with fused-silica capillary columns (75 μm i.d. \times 360 μm o.d.; 14 cm length, tip 8 μm , New Objective, Woburn, MA) that were packed in-house, with 5- μm C18 reverse-phase material (Vydac, Hesperia, CA). The flow rate from the pumps was 3-5 $\mu\text{L}/\text{min}$, and flow was split before introduction of sample on the column to 100-200 nL/min. Solvent A was 0.5% acetic acid in water and solvent B was 0.5% acetic acid in acetonitrile. A linear gradient was performed as follows: 2% B at 0 min, 2% B at 3 min, 65% B at 70 min, 80% B at 80 min, 80% B at 90 min, 2% B at 130 min, and 2% B at 170 min.

Agilent QTOF MS/MS—Capillary electrospray ESI was conducted using a capillary voltage of 3000 V and fragmentor voltage of 205 V. Nitrogen was the drying (5 l/min) gas and the heated capillary was at 325°C. Multiple MS/MS experiments were carried out with increasing collision energies over 0 – 50 V to evaluate fragmentation of modified and unmodified peptides. Liquid chromatography was performed using an Agilent 1200 capillary LC system with a Zorbax 300SB-C18 5 m 0.3 \times 150 mm (Agilent Technologies) at a flow rate of 10 $\mu\text{L}/\text{min}$. Solvent A was 0.5% acetic acid in water and solvent B was 0.5% acetic acid in acetonitrile. The gradient profile was: 2% B at 0 min, 2% B at 3 min, 70% B at 20 min, 70% B at 25 min, 95% B at 30 min, 2% B at 35 min, and 2% B at 40 min.

Database searches and protein identification

Data were analyzed using Spectrum Mill proteomics software (Rev A.03.02.060; Agilent) and verified using Mascot version 2.2 (Matrix Science) (27).

Spectrum Mill identification—Raw data were extracted under default conditions and searched against human sequences in the NCBIInr database using trypsin as the protease, allowing 2 missed cleavages, and including variable modifications of oxidized methionine. Peptides were considered valid with a forward-reverse score >2 and a rank 1-rank 2 score >2 , a score threshold >7.67 , and percentage-scored peak intensity $>70\%$. Only proteins with 2 or more validated peptides and a total score >25 were considered valid for reporting. To compare identified proteins between treatment groups, the number of spectra and the summed ion intensity of peptides for each protein (total ion intensity) were used as indicators of protein amounts. The spectral intensity of each ion was calculated from the extracted ion chromatogram of each precursor ion. Total intensity was calculated as the sum of spectral intensity (Spectrum Mill default settings). Total spectral intensity values between proteins were compared only within each experiment to give a qualitative value for total protein abundance in the samples.

Mascot identification—Raw data were extracted to mzData or mgf files for Agilent or Applied Biosystems instruments respectively, using either the MicroMass or Analyst software. For protein identification, either the NCBIInr and/or the SwissProt human databases were searched. Parameters included: enzyme as trypsin; max missed cleavages = 2; fixed modifications carbamidomethyl (C) for 2-D gel analysis only; variable modifications of methionine oxidation (M); precursor tolerance was set at 0.1 Da; MS/MS fragment tolerance was set at 0.2 Da. Proteins were identified with 2 or more peptides. Significance of a protein match for Mascot was based on an expectation value of < 0.05 and a combined peptide score > 50 .

Results

ARP modification of 13-HPODE treated cytochrome c

The ability of ARP to derivatize the DODE protein-carbonyl was evaluated. DODE-modified protein was synthesized by treating purified cyt c with 13-HPODE and ascorbic acid as described above (Materials and Methods). We have previously identified DODE as the major modification of cyt c treated with 13-HPODE (22). Treated protein was then incubated with ARP and analyzed by LC-MS. The deconvoluted mass spectrum of unmodified cyt c ($m/z = 12,359$) is represented in Figure 1A. After treatment with 13-HPODE, the appearance of $m/z = 12,585$ (Figure 1B) indicated the DODE modification of cyt c with the addition of 226 Da; equivalent to the mass of DODE. The derivatization of the DODE-carbonyl modification by ARP (Figure 1C) is indicated by $m/z = 12,899$ corresponding to the addition of 314 Da; equivalent to the mass of ARP with the loss of water. Comparison of peak heights in Figure 1C, suggests >75% derivatization efficiency of ARP to the protein-carbonyl DODE.

Treatment of cytochrome c with 13-HPODE-biotin

To determine if the 13-HPODE-biotin would successfully create a DODE-biotin adduct, cytochrome c was treated with 13-HPODE-biotin and ascorbic acid as described above (Materials and Methods) and the modified cyt c analyzed by LC-MS. The deconvoluted mass spectrum of the biotin modified cyt c can be seen in Figure 2, with unmodified cyt c at $m/z = 12,359$ and the major modification at $m/z = 12,985$; a difference of 626 Da corresponding to the mass of the DODE-biotin adduct. The peak at $m/z = 13,611$ - an addition of 1252 Da - was equivalent to the addition of two DODE-biotin modifications on cyt c. This result confirmed the major protein modification product from the 13-HPODE-biotin tag to be the DODE carbonyl modification.

Fluorescent staining of MCF7 cells treated with 13-HPODE-biotin

To determine if the 13-HPODE-biotin would successfully transverse the cellular membrane, MCF7 cells were treated with 13-HPODE-biotin and ascorbic acid as described in Materials and Methods; control cells were left untreated. Plated cells were located using nuclear Hoechst staining (Figure 3A). 13-HPODE-biotin was located using Cy3 conjugated streptavidin (Figure 3B). Overlaying the Cy3 and Hoechst stains (Figure 3C) demonstrated the 13-HPODE-biotin to have been taken up by the MCF7 cells. Cy3 fluorescence was only seen in the 13-HPODE-biotin treated cells and no fluorescence was observed in the controls.

Validation of DODE antibody

To test antibody specificity for DODE modifications, cyt c was modified with each of the four different lipid peroxidation adducts HNE, ONE, EDE and DODE as previously described (22). Modified and un-modified cyt c were separated by 1D-SDS-PAGE and the modifications analyzed by Western blot, using the polyclonal anti-DODE antibody. As seen in Figure 4, the DODE antibody recognized only DODE-modified cyt c. The DODE antibody showed no cross recognition to other lipid peroxidation modifications or to un-modified cyt c. This result established the ability of the DODE antibody to recognize only DODE-modified protein.

Neutravidin affinity chromatography validation

To determine the ability of the neutravidin chromatography to enrich for only biotinylated proteins from a cellular lysate, MCF7 cells were treated with 13-HPODE and ascorbic acid as described earlier. Cells were lysed and the proteins purified (Materials and Methods). Proteins were reacted with ARP and biotinylated proteins then enriched using a neutravidin

affinity column (Materials and Methods). Enriched proteins were analyzed by 2-D SDS-PAGE using both anti-Biotin Western blot and Coomassie staining. Figure 5A shows the Coomassie stained gel and Figure 5B the Western blot. The similarity between the two gels demonstrated that the majority of the enriched proteins were biotinylated. These results verified both the neutravidin affinity technique and further validated the ARP reaction with cellular proteins.

Proteins identified by 2D SDS-PAGE and anti-DODE Western blots

MCF7 breast cancer cells were treated with 13-HPODE and ascorbic acid; control cells were treated only with ascorbic acid. Both control and treated cells were lysed and pelleted, and the protein fractions then buffer-exchanged into deionized water by spin filtration. Cellular proteins were separated by 2-D SDS-PAGE. Two gels were run in tandem; an anti-DODE Western to locate DODE-modified proteins and a Coomassie-blue-stained gel for in-gel digestion and LC-MS/MS identification of modified proteins. Western blots of control cells showed no significant DODE-modified proteins. Anti-DODE Western blots of 13-HPODE treated cells revealed a number of DODE-modified proteins (Figure 6). The corresponding spots were located on the Coomassie-blue gel, in-gel digested and analyzed by nano-LC-MS/MS. Proteins were identified by Mascot database searching. A complete list of DODE-modified proteins identified is shown in Supporting Information, Table 1. A list of each identified protein's PI, molecular weight and 2-D gel spot location can be seen in Supporting Information, Table 2.

Proteins identified by ARP derivatization and 2D-LC-MS/MS

MCF7 breast cancer cells were treated with 13-HPODE and ascorbic acid as described (materials and methods); control cells were treated only with ascorbic acid. Both control and treated cells were lysed, pelleted and the protein fraction was buffer exchanged into PBS (pH = 6) by spin filtration. Carbonyl-modified proteins in both control and treated samples were derivatized with biotin using ARP as described earlier. ARP-derivatized proteins were retained on a neutravidin column, unmodified proteins were washed from the column and ARP-derivatized/carbonyl-modified proteins eluted with 80% acetonitrile, 0.4% TFA. Eluted proteins from both control and treated samples were separated into 40-60 fractions by strong cation exchange. Fractions were desalted and analyzed by capillary-LC-MS/MS. Proteins were identified by database searching with both Mascot and Spectrum Mill. A total list of modified proteins identified in both control and treated cells can be seen in Supporting Information, Table 1.

Proteins identified by 13-HPODE-biotin treatment and 2D-LC-MS/MS

MCF7 breast cancer cells were treated with 13-HPODE-biotin and ascorbic acid as described (materials and methods). Cells were lysed, pelleted and the protein fraction was buffer exchanged into PBS (pH = 7) by spin filtration. Modified proteins were retained on a neutravidin column, unmodified proteins were washed from the column and ARP-derivatized/carbonyl-modified proteins eluted with 80% acetonitrile, 0.4% TFA. Eluted proteins were separated into 40-60 fractions by strong cation exchange. Fractions were desalted and analyzed by capillary-LC-MS/MS. Proteins were identified by database searching with both Mascot and Spectrum Mill. The 13-HPODE-biotin produces a DODE-biotin modification; the proteins identified using this tag were therefore considered to be DODE-modified. A total list of modified proteins identified using the 13-HPODE-biotin tag can be seen in Supporting Information, Table 1.

Discussion

A consequence of inflammation is the formation of reactive oxygen and nitrogen species from activated phagocytes, which are used to combat disease or infection. If inflammation is chronic, these reactive chemicals can damage surrounding host tissue, resulting in protein modification and the generation of protein-carbonyls. Proteins are considered most likely to be ubiquitously affected by disease (28), and carbonyl-modified proteins shed from affected tissue are therefore potential early disease markers. Numerous inflammatory-disease studies have measured the formation of protein-carbonyls as a consequence of heart disease, lung disease, aging, neurodegenerative disorders and inflammatory bowel disease (29-34). A major pathway for the formation of protein carbonyls comes from the oxidation of polyunsaturated fatty acids, leading to a variety of unsaturated lipid hydroperoxides. These hydroperoxides break down in the presence of reductants to generate α,β -unsaturated aldehydes, which readily modify proteins, to form lipid peroxidation protein-carbonyls. We have previously demonstrated the lipid-peroxidation aldehyde DODE to be the most reactive protein-modifying aldehyde arising from linoleic acid oxidation and degradation (22). DODE-modified proteins are also of interest since this carbonyl modification is uniquely formed from linoleic acid oxidation (8); evidence of its endogenous formation can therefore be linked back to a single pathway of lipid peroxidation. This research evaluated the ability of DODE protein modifications to occur in MCF7 breast cancer cells and identified the major proteins modified by DODE.

Identification of protein modification generally requires the characterization of the modified peptide and the site and nature of the modification, involving affinity purification of the modified peptide followed by mass spectrometry. Unfortunately, the ARP-biotin and the 13-HPODE-biotin tags created numerous fragment ions during MS/MS that hampered peptide identification by database searching and we were unsuccessful at identifying specific DODE modified peptides. The polyclonal antibody used in this study was effective in Western blots, but bound the DODE antigen too tightly to be used for purification by immunoaffinity chromatography. Therefore to avoid the necessity of identifying specific DODE-modified peptides, this study used three distinct methods to identify DODE modified proteins which effectively reduced the number of false positives and decreased the number of false negatives, eliminating the need to identify a DODE-modified peptide for each protein identified. First, cells were treated with a synthetically biotinylated hydroperoxide of linoleic acid (13-HPODE-biotin), which readily forms a biotin-DODE protein modification *in vitro* (Figure 2). This method allowed for proteins modified only by DODE to be affinity purified and identified by mass spectrometry. Second, cells were treated with the native hydroperoxide of linoleic acid (13-HPODE) to determine if the protein modification pattern differed from 13-HPODE-biotin treatment. This method used an aldehyde reactive probe (ARP) to biotinylate all carbonyl-modified proteins since proteins could potentially be modified by all the three HNE, ONE and DODE aldehydes occurring from 13-HPODE degradation (Scheme 1). This method also allowed for the identification of false positives by reacting proteins with ARP from control cells, that were not treated with 13-HPODE. Controls established a set of background proteins caused by non-specific binding to affinity columns or from carbonyl-modification not specific to the 13-HPODE treatment. Third, a polyclonal DODE antibody was used to locate, by Western blot, DODE modified proteins in cells treated with 13-HPODE. Spots from these proteins were cut from Coomassie-blue stained gels and identified by mass spectrometry.

The ability of the α,β -unsaturated aldehydes HNE, ONE, EDE and DODE (methyl ester) to induce apoptosis in endothelial cells has been shown by Jian, *et al.*, to be in the low μM concentration (16). The concentration of 13-HPODE and 13-HPODE-biotin used in this study was about a 10-fold increase over these previously established levels. As described

(Materials and Methods), cells were treated for 30 minutes with 10 mL of FBS free media containing 64 μM 13-HPODE or 13-HPODE-biotin. This concentration is high and not necessarily physiologically relevant, but treatment showed no observable effect on the cell growth and viability, suggesting that cellular DODE concentrations were below the apoptosis inducing levels of 2-5 μM , as established by Jian *et al.* (16). The kinetics for the cellular uptake and degradation of 13-HPODE is not well understood and we were therefore unable to calculate the true cellular concentration of DODE. Since we were able to identify a number of DODE modified proteins, the cellular concentration of DODE must have been either high enough to overcome any detoxification mechanisms (such as glutathione scavenging) or protein modification may have occurred during a specific aldehyde/protein interaction (as described below). Any future studies should analyze cellular lysates for glutathione-DODE adducts; this would help establish the ability of these cells to take up 13-HPODE as well as give a relative cellular DODE concentration from 13-HPODE degradation.

Using all three methods (13-HPODE ARP, 13-HPODE-biotin, anti-DODE western blot), 98 proteins were characterized as potentially modified by DODE (Supporting Information, Table 1). As represented in Figure 7, there was substantial overlap, with 18 proteins identified in all three methods and 31 proteins identified by the two biotin methods (ARP and 13-HPODE-biotin). Only six proteins were exclusively identified by the DODE antibody (Figure 7) and the majority of antibody-identified proteins overlapped with the two biotin systems, demonstrating biotin tagging as a successful technique for the enrichment of carbonyl-modified proteins. As previously stated, the 13-HPODE-biotin method was designed to identify only the DODE modification. For a number of reasons this technique identified the highest number of modified proteins (Figure 7 and Supporting Information, Table 1). The biotinylation of the carboxylic acid group of 13-HPODE likely facilitated its ability to penetrate cell walls by elimination of the charge on the carboxyl group, and a greater uptake of this species would allow for more protein modification. A recent study by Szapacs, *et al.*, identified new carboxylated electrophile adducts occurring from oxidation of a biotin-modified phospholipid containing linoleate (35). Furthermore, the carboxylic acid containing reactive aldehyde 9-hydroxy-traumatol has been identified as an enzymatic degradation product of 13-HPODE in soybean and alfalfa seeds (36). The degradation of 13-HPODE can also lead to the formation of the hydroxy version of DODE or 9-hydroxy-12-oxo-10(E)-dodecenoic acid (HODE or HODA) (35). Through keto-amide formation, HODE creates a non-carbonyl containing protein modification, which would therefore be undetectable using the ARP method, but protein modification by Michael addition of HODE retains the carbonyl functional group, allowing for derivatization/detection with ARP. Therefore in our experiments using 13-HPODE-biotin, some of the proteins we identified may have arisen from decomposition products other than DODE. Although we have not detected these adducts from 13-HPODE decomposition *in vitro*, their existence in a cell remains feasible and this type of modification may further explain the greater number of proteins identified with the 13-HPODE-biotin method.

To highlight DODE-modification, a master list of likely DODE-modified proteins was created (Figure 8). To create this list, proteins were selected only if they were identified by all three techniques (HPODE-biotin, HPODE ARP and anti-DODE Western). Proteins were also selected if they were identified by both the HPODE-biotin and HPODE ARP techniques, but were not seen in the ARP controls. A final set of 31 were identified (Figure 8) and were considered the major proteins modified by DODE in MCF7 cells treated with 13-HPODE. A greater overlap of protein identification was seen between the two biotin methods (HPODE-biotin and HPODE ARP) than with the antibody method. This is not surprising since the biotin methods used 2D-LC to identify proteins, which is generally considered a more sensitive technique than the 2D-SDS-PAGE method used with the DODE

antibody. To emphasize this point, Figure 8 shows these proteins ordered by their Spectrum Mill total spectrum intensity (Materials and Methods); generally the higher abundant proteins were more likely to be identified by all three methods and—as abundance decreased—the SDS-PAGE method appeared to be less likely to identify the protein. As described in Materials and Methods, this type of spectral counting was used only in a qualitative manner to give a general estimate of relative protein abundance.

The DODE-modified protein cellular locations are shown in Figure 9A. Proteins were mainly cytoplasmic (Figure 9A), which was expected. The number of membrane bound proteins identified was limited since the extraction methods used in this study were not optimal for the analysis of this class of proteins. Future studies to characterize proteins endogenously-modified by inflammation should probably take greater account of membrane proteins, since the nitric oxide produced during the inflammatory process has a greater solubility in the lipid membrane than in the aqueous cytoplasm (37). Surprisingly, a significant number (17%) of proteins had locations in the nucleus. Jian, *et al.*, have shown that DODE was unable to translocate across plasma membranes (16). Therefore, in this study, for proteins to be DODE-modified in the nucleus, 13-HPODE would have to remain intact long enough to cross the nuclear membrane. Two of these nuclear proteins (valosin-containing protein, and elongation factor 2) are involved in nucleotide binding and transport into the cytoplasm (39-40), and it is therefore likely that they were modified outside the nucleus in the cytoplasm. DODE modification of the nuclear proteins nucleolin, ribosomal protein L5 and proliferating cell nuclear antigen is also somewhat surprising. All three proteins are involved in cell growth and are likely abundant in MCF7 cells increasing the probability of modification even in the nucleus.

DODE-modified protein functions are shown in Figure 9B. Overall, proteins modified by DODE in this study had a limited number of functions, suggesting some protein specificity for DODE modification in cells. We expected a general non-specific protein modification, resulting largely in the identification of highly abundant structural proteins. This, however, was not the case, and DODE modification was distributed among five major protein groups: molecular chaperones, glycolysis, regulatory, nucleotide binding and structural. The lower-than-expected number of structural proteins may indicate some resistance to lipid peroxidation adduction by these proteins, but more likely DODE modification of other protein types were enhanced due to specific protein-adduct interactions, such as binding and transport. The specificity for DODE modification may be influenced by the detoxification of cellular reactive aldehydes by glutathione (GSH) or other abundant reductants. GSH has been shown to react *in vivo* with the lipid peroxidation aldehyde ONE (17) and it is likely that GSH or other cellular reductants would also react with DODE to decrease the amount of cellular DODE available, thus limiting modification primarily to proteins directly interacting with 13-HPODE. This study was designed to monitor only high molecular weight proteins, and any lower molecular weight DODE adducts (such as DODE-GSH) were removed by ultra-filtration. For a more complete picture of cellular DODE interactions, future studies should monitor the formation of a DODE-GSH species.

The structural proteins identified as DODE-modified were actin, ankyrin, spectrin alpha, tropomyosin, vitamin D-binding protein, and myristoylated alanine-rich C-kinase substrate. Their modification was a probable result of their high cellular abundance as previously mentioned. The nucleotide binding proteins involved in transport may additionally bind to or interact with lipids, which would explain the preferential DODE modification of this class of proteins. In fact, valosin-containing protein binds both nucleotides and lipids during cellular mitosis (40). The identification of a number of molecular chaperone proteins, such as heat shock proteins (HSP) 27, 70 and 90 was not surprising since these multifunctional proteins are involved in the transport of a large array of molecules. HSPs contain binding domains

that bind exposed hydrophobic residues of substrate proteins or peptides; they also bind non-protein molecules, including lipid-based TLR ligands, LPS and lipoproteins, with exposed hydrophobic residues (41-45). It is therefore likely that a specific binding between 13-HPODE and these molecular chaperones occurs, enhancing their modification by DODE. Furthermore, a recent study by Vila *et al* identified HSP 70 and 90 as two of the major protein targets of the linoleate decomposition product HNE (46) and our studies further emphasize the potential importance of these proteins with regards to lipid peroxidation modification.

Another group of proteins modified by DODE were enzymes involved in glycolysis (pyruvate dehydrogenase kinase, glyceraldehyde-3-phosphate dehydrogenase, glucosidase II, fructose-bisphosphate aldolase, glucose-6-phosphate dehydrogenase, and lactate dehydrogenase). Their enhanced modification may be specific to the MCF7 cells, since - like most cancer cells - they exhibit an increased rate of glycolysis (47,48) and DODE-specific modification may be explained by higher cellular concentrations of these enzymes. Increased expression of lactate dehydrogenase (LDH) is common in tumor cells under hypoxic conditions. The MCF7 cells used in this study likely accumulated LDH since they were grown to 90% confluence, thus creating a lowered oxygen environment. It is thought that the increased expression of LDH in tumor cells may be a critical part of the metabolic reprogramming that occurs during tumorigenesis (49) and the specific modification of LDH by reactive aldehydes may therefore be of some interest with respect to this hypothesis. Preferential DODE modification of these proteins, may involve some overlap in substrate recognition between glycolysis enzymes and fatty acid metabolism enzymes. Glucose-6-phosphate dehydrogenase (G6PD) is a key enzyme in the pentose pathway, a major producer of NADPH (50) and is also involved, in lipid and cholesterol synthesis (51). G6PD is up-regulated during oxidative stress providing cellular protection by generating NADPH to maintain reduced glutathione levels (23). G6PD is also inhibited by and presumably binds polyunsaturated fatty acids, such as linoleate, which may potentially explain a direct interaction between G6PD and 13-HPODE (53,54).

A number of “regulatory” proteins involved in cellular signaling, including Ras related protein Rab 27A, galectin and Ras-GTPase-activating protein, were modified by DODE. These regulatory proteins are membrane-associated and contain lipid anchor domains, which could explain their selective modification by DODE, but it remains unknown if DODE modification of these proteins occurs in the lipid domain. The 14-3-3 proteins theta, sigma and epsilon were identified as DODE modified. Although the 14-3-3 proteins can be considered “regulatory” they are also molecular chaperones able to interact with over 200 proteins and hundreds of small molecules (55). They are involved in numerous functions, including cell growth, migration, signaling, transcription and apoptosis (55,56). It is therefore not surprising these ubiquitous proteins may also bind and chaperone 13-HPODE, allowing for a preferential DODE modification. 14-3-3 sigma (or stratifin) is a tumor suppressor and may play a key roll in carcinogenesis. It has been shown to be down-regulated in MCF7 cells (57) and therefore identification of 14-3-3 sigma in this study further demonstrates a directed or targeted type of modification, dependent on a protein/DODE interaction. It may be possible that such preferential modification will enhance the carcinogenic roll of a protein, such as 14-3-3 sigma.

Conclusions

Three independent methods were used to identify the major proteins modified by the DODE lipid peroxidation aldehyde occurring from the oxidation and degradation of linoleic acid in MCF7 breast cancer cells. To our knowledge these are the first intracellular DODE-modified

proteins to be identified, highlighting the DODE modification as a potential protein modification during cellular oxidative stress.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

| | |
|----------|--|
| 13-HPODE | 13-hydroperoxy-9,11(Z,E)-octadecadienoic acid |
| ARP | aldehyde reactive probe (aminooxymethylcarbonylhydrazino D-biotin) |
| cyt c | cytochrome c |
| DODE | 9,12-dioxo-10(E)-dodecenoic acid |
| EDE | 4,5-epoxy-2(E)-decenal |
| HNE | 4-hydroxy-2(E)-nonenal |
| ONE | 4-oxo-2(E)-nonenal |

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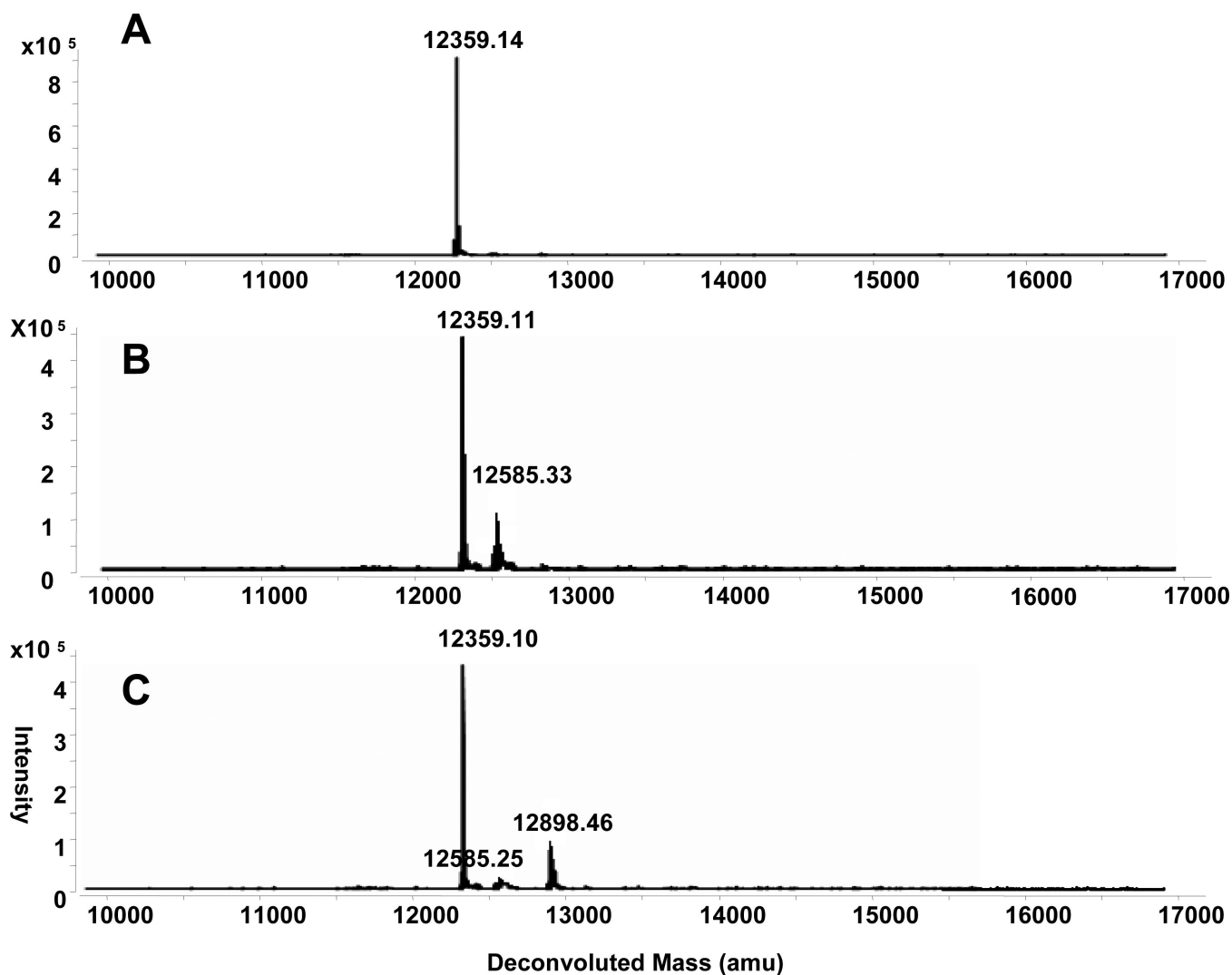


Figure 1.
ARP derivatization of DODE modified cyt c. A: Deconvoluted mass spectrum of unmodified cyt c. B: Deconvoluted mass spectrum of DODE-modified cyt c. C: Deconvoluted mass spectrum of DODE-modified cyt c after derivatization with ARP.

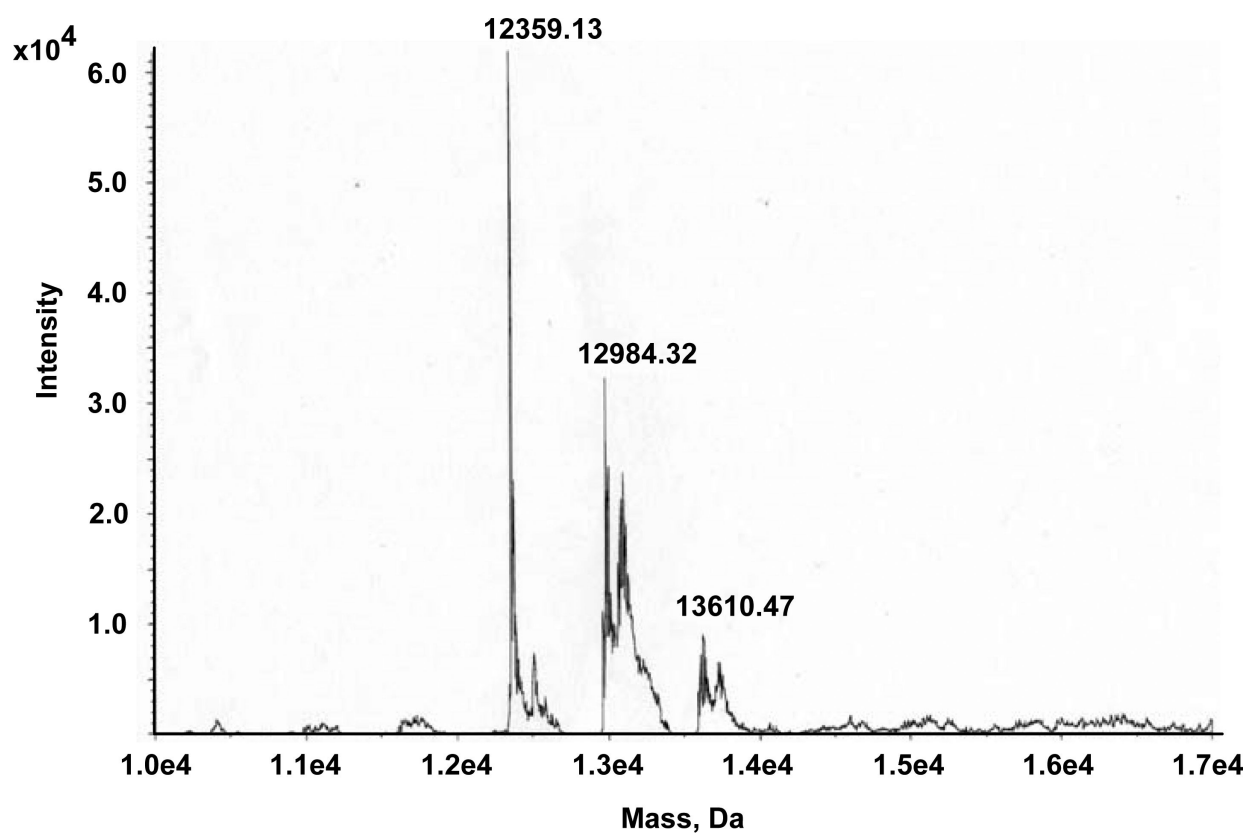


Figure 2.
Deconvoluted mass spectrum of cytochrome c modified by 13-HPODE-biotin.

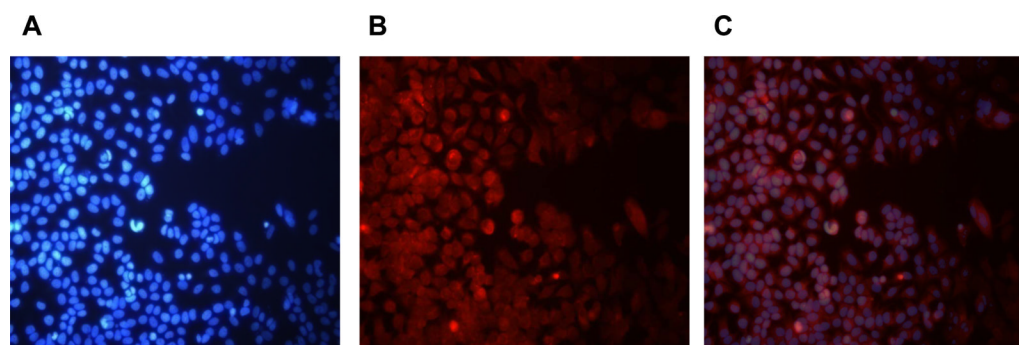


Figure 3. Immunostaining and imaging of MCF7 cells treated with 13-HPODE-biotin. A: Hoechst nuclear staining. B: Cy3 conjugated streptavidin staining. C: Merged picture of A and B.

Modified cytochrome c

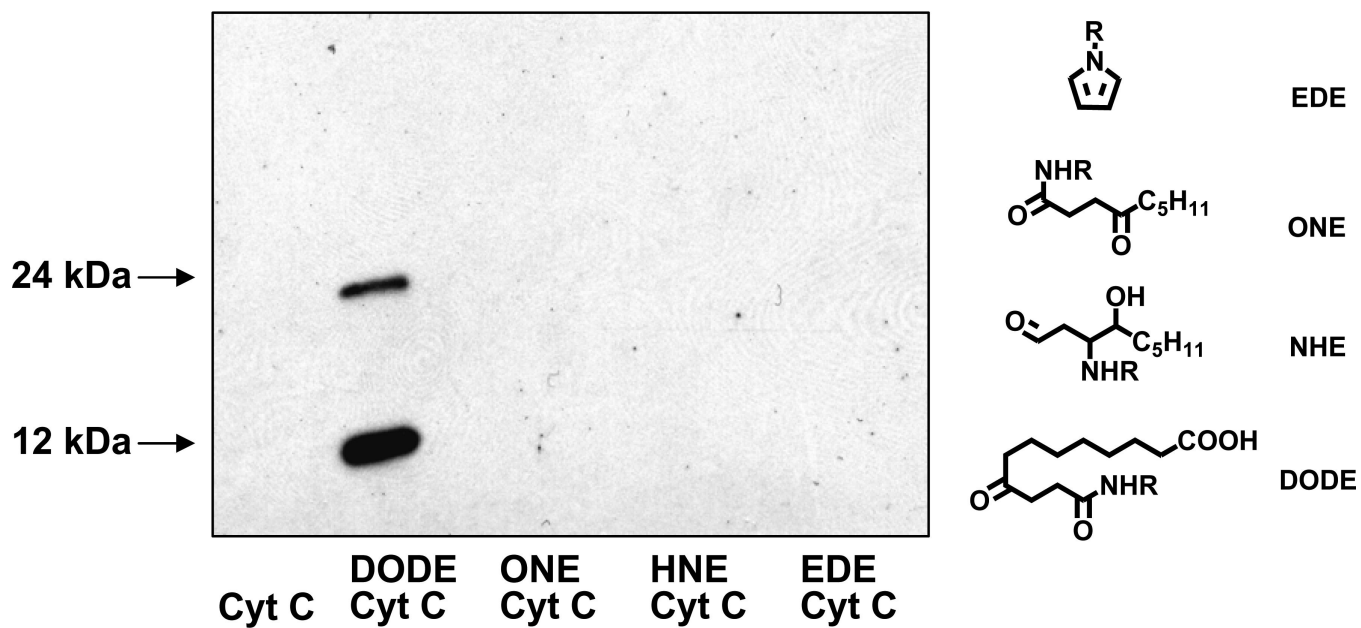


Figure 4. Western blot using a DODE antibody. The antibody was tested against unmodified cyt c and DODE, ONE HNE, and EDE modified cyt c. Only DODE-modified cyt c was recognized.

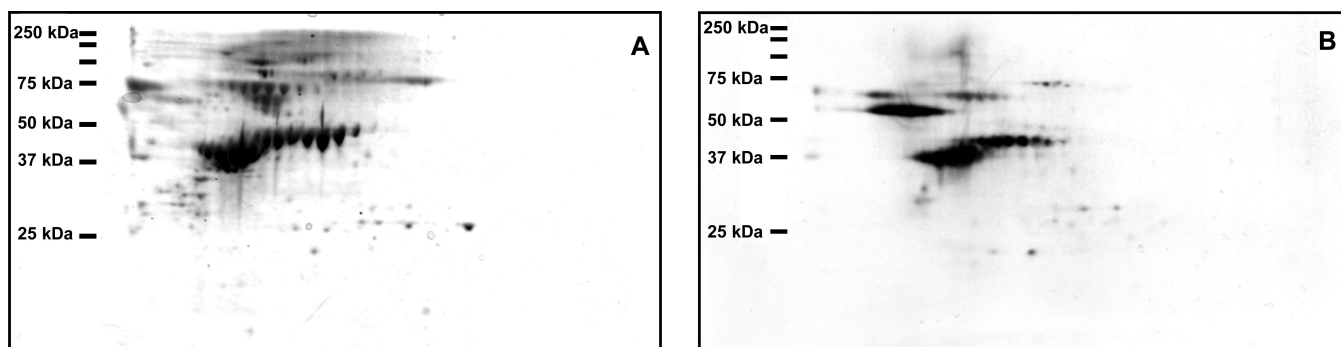


Figure 5. 2D-SDS-PAGE MCF7 proteins treated with ARP and affinity purified by neutravidin. A: Coomassie-blue stained gel. B: Anti-biotin Western blot.

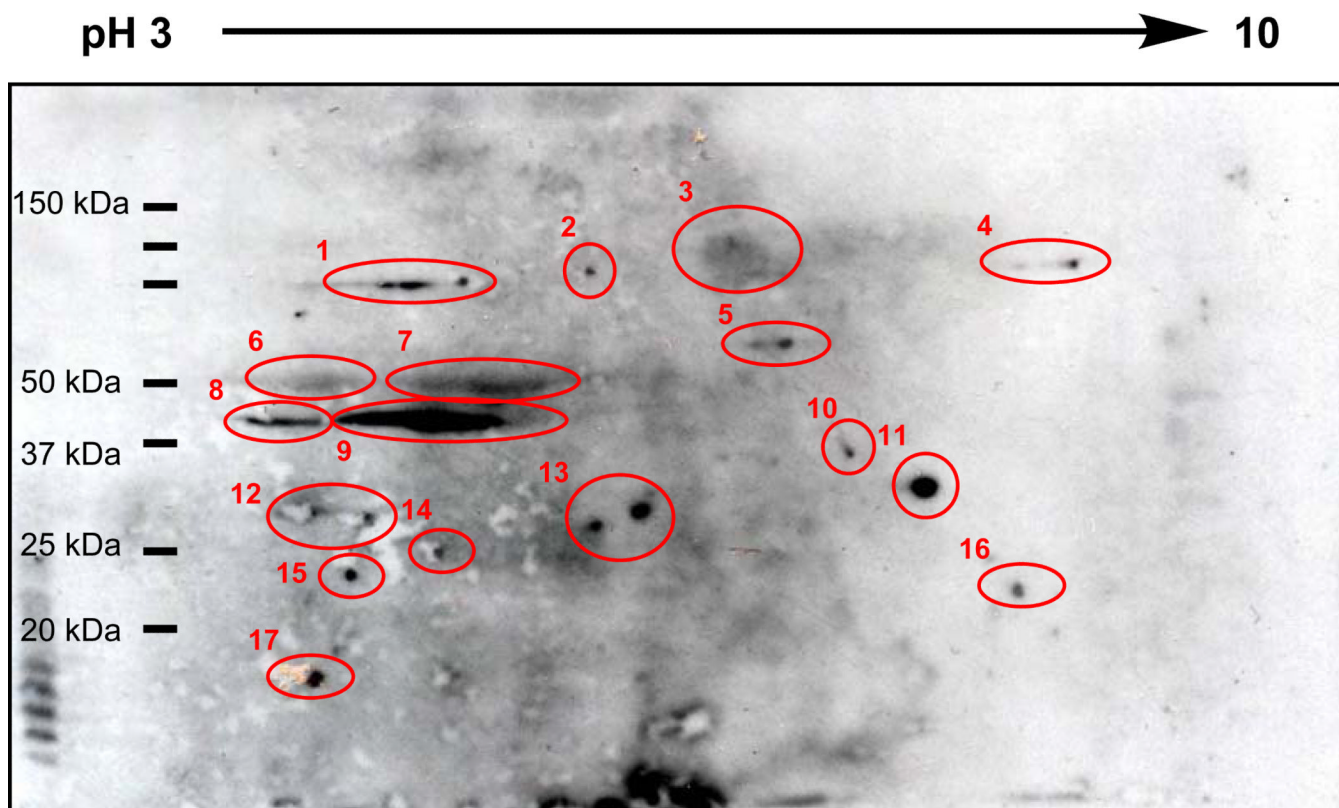


Figure 6. 2D-SDS-PAGE of MCF7 cells treated with 13-HPODE. Anti-DODE Western blot showing the presence of DODE-modified proteins; circled proteins (red) were picked for mass spectrometry identification.

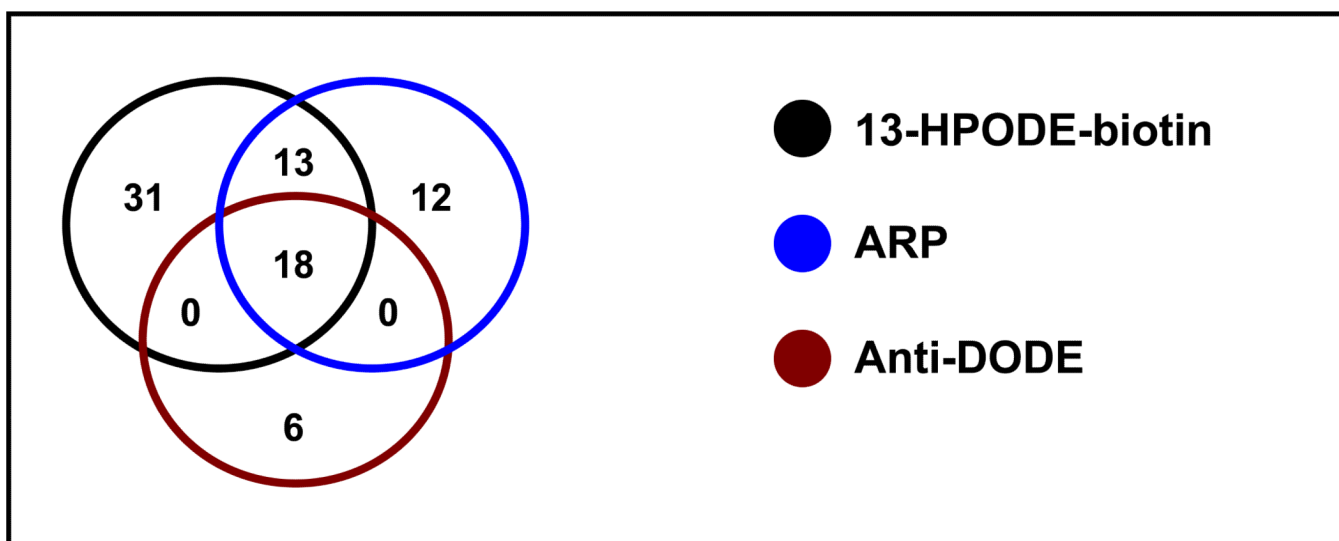


Figure 7.
Venn diagram representing the number of overlapping proteins identified by all three methods.

| Spectral intensity | Biotin affinity 2D-LC identification | Anti-DODE 2-D Western identification | Function |
|-------------------------|--|--|---------------|
| | Actin | Actin | Structural |
| | Ras related protein Rab 27A | Ras related protein Rab 27A | Regulatory |
| | Pyruvate dehydrogenase kinase isoform 3 | Pyruvate dehydrogenase kinase isoform 3 | Glycolysis |
| | Heat shock 27 | Heat Shock 27 | Chaperone |
| | Glyceraldehyde-3-phosphate dehydrogenase | Glyceraldehyde-3-phosphate dehydrogenase | Glycolysis |
| | Heat shock 70 | Heat Shock 70 | Chaperone |
| | Heat shock 90 | Heat Shock 90 | Chaperone |
| | 14-3-3-protein, theta | 14-3-3 protein, theta | Regulatory |
| | 14-3-3 protein, epsilon | 14-3-3 protein, epsilon | Regulatory |
| | 14-3-3 protein, sigma | 14-3-3 protein, sigma | Regulatory |
| | Glucosidase II subunit alpha | | Glycolysis |
| | Elongation factor 2 | Elongation factor 2 | Nuc. binding |
| | Valosin-containing protein | | Nuc. binding |
| | Serine/threonine-protein phosphatase 2A regulatory subunit | | Regulatory |
| | Fructose-bisphosphate aldolase C | Fructose-bisphosphate aldolase C | Glycolysis |
| | Spectrin alpha | | Structural |
| | Proteasome alpha 5 subunit | | Protease |
| | Glucose-6-phosphate dehydrogenase | Glucose-6-phosphate dehydrogenase | Glycolysis |
| | Galectin 1 | | Regulatory |
| | Lactate dehydrogenase A | Lactate dehydrogenase A | Glycolysis |
| | Ribosomal protein L5 | | Nuc. binding |
| | Dermcidin | Dermcidin | Antibacterial |
| | SET protein | SET protein | Regulatory |
| | Tropomyosin 4 | | Structural |
| | Vitamin D-binding protein (DBP) | | Structural |
| | Proliferating cell nuclear antigen | Proliferating cell nuclear antigen | Nuc. binding |
| | Ras-GTPase-activating protein | | Regulatory |
| | Nucleolin | | Nuc. binding |
| | Myristoylated alanine-rich C-kinase substrate | | Structural |
| | Cathepsin D | | Protease |
| Solute carrier family 9 | | Chaperone | |

Figure 8.
Major proteins modified by DODE in MCF7 cells treated with 13-HPODE

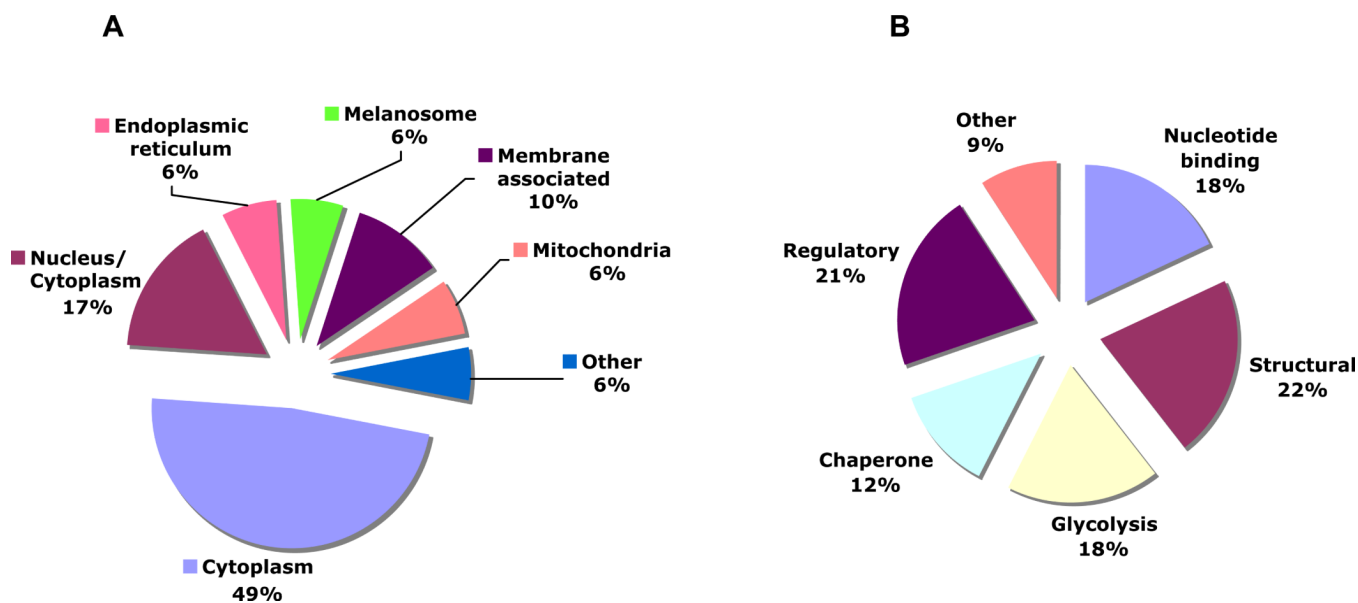
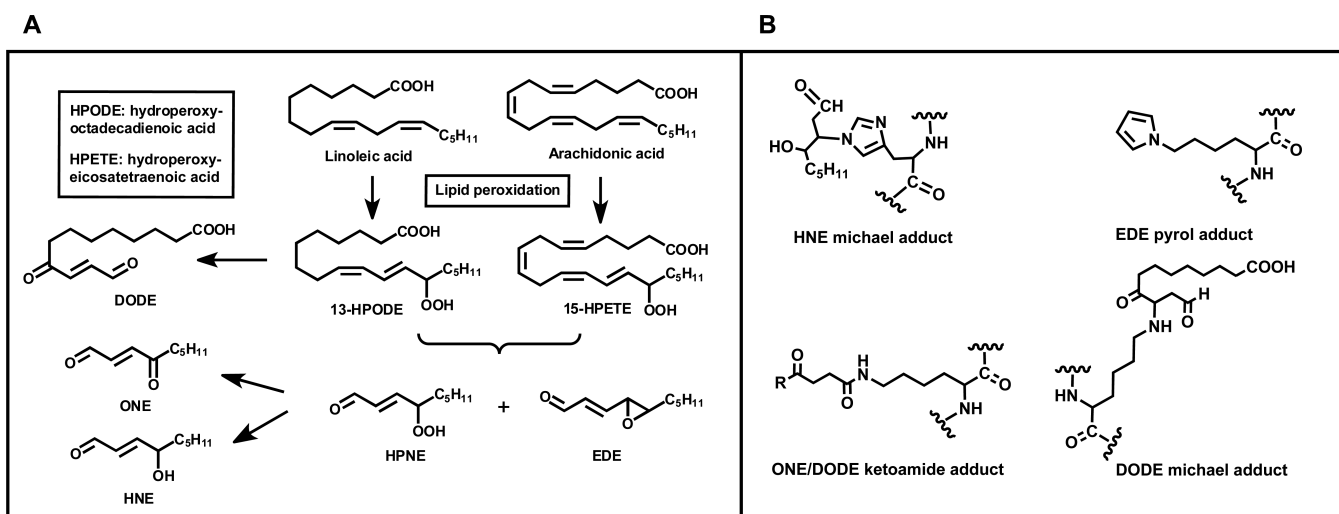
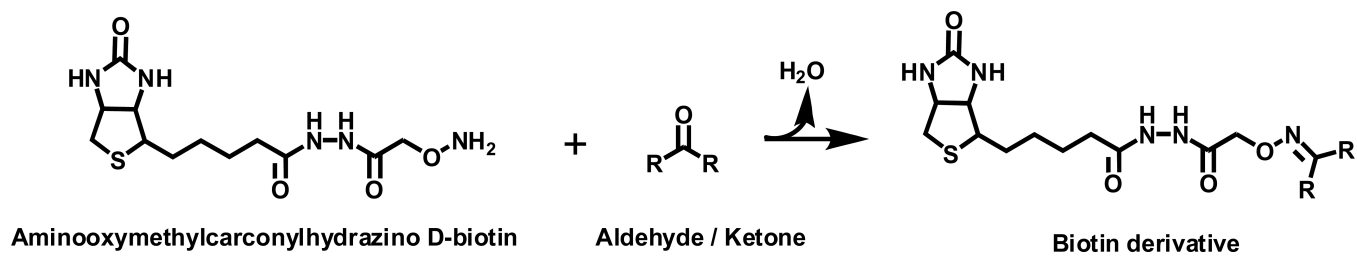


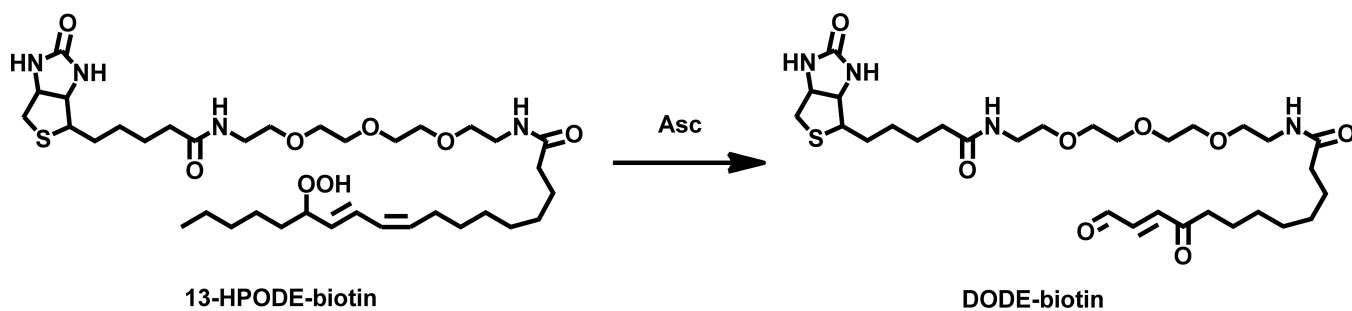
Figure 9. Location and function of major proteins modified by DODE in MCF7 cells treated with 13-HPODE. A: Protein location. B: Protein function.



Scheme 1.
 Decomposition of 13-HPODE.



Scheme 2.
Reaction of aldehyde reactive probe with carbonyls.

**13-HPODE-biotin****DODE-biotin**

Scheme 3.
Biotin-linked 13-HPODE.