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Biotinylated Probes for Analysis of Protein Modification by Electrophiles

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Summary

The formation of covalent protein adducts by lipid electrophiles contributes to diseases and toxicities linked to oxidative stress, but analysis of the adducts presents a challenging analytical problem. We describe selective adduct capture using biotin affinity probes to enrich protein and peptide adducts for analysis by liquid chromatography-tandem mass spectrometry (LC-MS/MS). One approach employs biotinamidohexanoic acid hydrazide to covalently label residual carbonyl groups on adducts. The other employs alkynyl analogs of lipid electrophiles, which form adducts that can be post labeled with azidobiotin tags by Cu^+ -catalyzed cycloaddition (Click chemistry). To enhance the selectivity of adduct capture we use an azidobiotin reagent with a photocleavable linker, which allows recovery of adducted proteins and peptides under mild conditions. This approach allows both the identification of protein targets of lipid electrophiles and sequence mapping of the adducts.

Keywords

electrophile; Click chemistry; protein adducts; lipid oxidation; photocleavable biotin; shotgun proteomics

1. Introduction

Electrophilic lipid oxidation products initiate toxic responses by modifying proteins and triggering specific biochemical and cellular responses (1-4). The evaluation of the protein targets and the biological effects of lipid electrophiles in both their “free” and phospholipid-esterified forms will help identify new candidate biomarkers for oxidative stress at the cellular level (5,6) as well as in plasma (7,8). The problem of identifying protein targets of electrophiles is complicated in general by the diversity of targets and the low levels of modification under relevant conditions. It is even harder to map the sites of modifications on proteins for the same reason. To address these problems, we have employed a combination of mass spectrometry (MS)-based shotgun proteomics analysis and novel affinity labeling for selective capture and analysis of the modified proteins. The key to this strategy is the use of probes that enable selective capture of adducts. Two approaches have proven successful in our studies. The first employs biotinamidohexanoic acid hydrazide (biotin hydrazide) for covalent capture of carbonyl-containing adducts. The second employs alkynyl analogs of lipid electrophiles and postlabeling of the resulting adducts using “Click” chemistry with a novel, photocleavable biotinylating reagent during sample workup. Here we describe biotin probe strategies to capture both adducted proteins and to map adduct sites by selective capture of adducted peptides.

2. Materials

2.1. Cell Culture, HNE Treatment and derivatization

1. Dulbecco's Modified Eagle's Medium (DMEM) (Gibco/BRL, Bethesda, MD) supplemented with 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO).
2. Solution of trypsin (0.25%) (Gibco/BRL, Bethesda, MD).
3. The lipid electrophile 4-hydroxy-2-nonenal (HNE) (Cayman Chemical, Ann Arbor, MI) is dissolved in ethanol (64 mM), stored in aliquots at -80°C , and added to tissue culture dishes as required.
4. M-Per lysis buffer (Pierce, Rockford, IL) is stored at room temperature.
5. Dimethyl sulfoxide (DMSO).
6. Biotin hydrazide (Sigma-Aldrich, St. Louis, MO). A 50mM stock solution in DMSO is prepared fresh before every experiment. The final concentration of biotin hydrazide per sample is 5mM.
7. Sodium borohydride. A 500mM stock solution in distilled H_2O is prepared fresh before every experiment. The final concentration of sodium borohydride is 50mM.
8. Ice cold 1 x PBS, pH 7.2.
9. Protease inhibitor cocktail: 1.0 mM phenylmethylsulfonylfluoride, 1.0 mM N-ethylmaleimide, $10\text{ }\mu\text{g mL}^{-1}$ leupeptin, $10\text{ }\mu\text{g mL}^{-1}$ aprotinin, $10\text{ }\mu\text{g mL}^{-1}$ pepstatin
10. Phosphatase inhibitor cocktail: 1.0 mM sodium fluoride, 1.0 mM sodium molybdate, 1.0 mM sodium orthovanadate, 10.0 mM γ -glycerophosphate.
11. BCA Protein Assay Kit (Pierce, Rockford, IL).
12. Distilled H_2O .

2.2. Affinity Capture

1. Amicon Ultra Centrifugal Filter Devices, 10,000 Da molecular weight cutoff (Millipore, Billerica, MA).
2. Streptavidin Sepharose High Performance beads (GE Healthcare, Uppsala, Sweden).
3. 1% sodium dodecyl sulfate.
4. 4M urea. Urea is freshly prepared before every experiment.
5. 1M sodium chloride prepared in 1x PBS.
6. Dithiothreitol. A 1M stock solution is stored at -20°C . The working concentration of DTT is 50mM.
7. NuPAGE LDS Sample Buffer (4x) (Invitrogen, Carlsbad, CA).

2.3. Western Blot Procedure

1. NuPage Bis-Tris 10% SDS-PAGE gels (Invitrogen, Carlsbad, CA)
2. Precision Plus Protein Standard Kaleidoscop Molecular Weight Marker (Bio-Rad Laboratories, Hercules, CA).

3. 20x-MES running buffer (Bio-Rad Laboratories, Hercules, CA).
4. 20x Tris/Glycine transfer buffer (Bio-Rad Laboratories, Hercules, CA).
5. Polyvinylidene difluoride (PVDF) membrane (Invitrogen, Carlsbad, CA).
6. Methanol.
7. 1x TBS-Tween (0.05% Tween 20).
8. Blocking Buffer for Near Infra Red Fluorescent Western Blotting (Rockland, Gilbertsville, PA).
9. Anti-HNE-Michel reduced rabbit polyclonal antibody (EMD biosciences, San Diego, CA).
10. Primary antibodies: Anti-heat shock protein 90 (HSP90) rabbit polyclonal antibody, anti-actin mouse monoclonal antibody, anti-tubulin rabbit polyclonal antibody, anti-cofilin rabbit polyclonal antibody, anti-glutathione-S transferase P (GSTP) rabbit polyclonal antibody, anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody, anti-thioredoxin reductase 1 (TrxRd1) mouse monoclonal antibody, anti-peroxiredoxin 6 (Prdx6) rabbit polyclonal antibody are purchased from Abcam (Cambridge, MA).
11. Primary antibodies: Anti-cullin3 goat polyclonal antibody and anti-bax rabbit polyclonal antibody are purchased from Santa Cruz (Santa Cruz, CA).
12. Secondary antibodies: Streptavidin and AlexaFluor®680 conjugated fluorescent secondary antibodies were obtained from Molecular Probes (Eugene, OR) and IRDye™800 conjugated fluorescent secondary antibodies are obtained from Rockland (Gilbertsville, PA).
13. Odyssey™ Infrared Imaging System and Odyssey software (Li-Cor, Lincoln, NE).

2.4. LC-MS-MS procedures

1. Trypsin Gold Mass Spectrometry Grade (Promega, Madison, WI) reconstituted in 50 mM acetic acid to a final concentration of 1 mg ml⁻¹. Aliquots are stored frozen at -20 °C. Trypsin is diluted in 25 mM ammonium bicarbonate to 0.01 µg/mL used at a ratio of 1:50 (trypsin:protein).
2. Colloidal Coomassie Blue (Invitrogen, Carlsbad, CA) is used for 1h staining followed by 1h destaining in water.
3. Iodoacetamide, DTT, and ammonium bicarbonate.
4. All reagents are prepared immediately before use.
5. LC-MS-MS analysis is performed using a LTQ ion trap mass spectrometer (Thermo Electron, San Jose, CA) equipped with an Eksigent nanoLC (Dublin, CA) and Thermo Surveyor HPLC pump, Nanospray source and Xcalibur 1.4 instrument control. Peptides are resolved on 100µm × 11 cm fused silica capillary column (Polymicro Technologies, LLC Phoenix, AZ) packed with 5 µm, 300 Å Jupiter C18 (Phenomenex, Torrance, CA).
6. Liquid chromatography is carried out at ambient temperature at a flow rate of 0.6 µL min⁻¹ using a gradient mixture of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B). Centroided MS/MS scans are acquired using an isolation width of 2 m/z, an activation time of 30 ms, an activation Q of 0.250 and 30% normalized collision energy using 1 microscan with a max ion time of 100 ms for each MS/MS scan. The mass-spectrometer is tuned

prior to analysis using the synthetic peptide TpepK (AVAGKAGAR), so that some parameters may vary slightly from experiment to experiment, but typically the tune parameters are as follows: spray voltage of 2 KV, a capillary temperature of 150°C, a capillary voltage of 50 V and tube lens of 120 V. The MS/MS spectra of the peptides are collected using data-dependent scanning in which one full MS spectrum is followed by four MS/MS spectra. MS/MS spectra are recorded using dynamic exclusion of previously analyzed precursors for 60 s.

2.5. Plasma treatment

1. Plasma is obtained from the fresh blood donated from a healthy subject following Vanderbilt IRB-approved protocols (7). The fresh blood is drawn by venipuncture into 7 ml Vacutainer tubes using EDTA as anticoagulant (BD Worldwide, Franklin Lakes, NJ) and then centrifuged using a Heraeus® Labofuge® 400 (Thermo Electron Corporation) with 2000 rpm for 10 min. Plasma is aliquoted to 1 mL Eppendorf tubes and kept at -80 °C until use.
2. Anti-human ApoA1 polyclonal antibody is purchased from Cayman Chemicals (Ann Arbor, MI). Anti-human ApoA1 monoclonal antibody is from BioDesign International (Saco, ME). Anti-human serum albumin (HSA) is from Cortex Biochem™ (Concord, MA).
3. Solid phase Extraction: OASIS HLB® (Waters Corp., Milford, MA) (30 mg) is used to desalt eluted peptides. The SPE cartridge is activated with 1 ml of solvent B (0.1% FA 80% ACN in H₂O) and then equilibrated with 1 ml of solvent A (0.1% FA H₂O). The sample is loaded and washed with 1 mL of solvent A and then peptides are eluted with 0.5 mL of solvent B.

3. Methods

The first approach to identify protein targets of lipid oxidation products is to covalently capture carbonyl-containing adducts biotin hydrazide (5,6). To generate adducts, we treat RKO cells with the prototypical lipid electrophile HNE at 50 or 100 µM HNE. HNE Michael adducts formed contain a residual carbonyl group and can be biotinylated by reaction with biotin hydrazide. Following capture with streptavidin, the biotinylated proteins are eluted and resolved by one dimensional sodium dodecyl sulfate-polyacrylamide (SDS) gel electrophoresis, digested with trypsin and the resulting peptides are analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Of the 1500+ proteins identified, 417 displayed a statistically significant increase in spectral counts with increasing HNE exposure concentration (5,6). This relationship distinguishes true adducts from proteins nonspecifically captured with streptavidin or proteins containing carbonyls not derived from HNE treatment. A subset of the identified HNE protein targets can be verified with a streptavidin capture and immunoblotting approach. The subsequent immunoblotting analysis allows for a rapid screening of individual proteins to determine if these proteins have been adducted. Proteins that have undergone modifications following the exposure to HNE are identified in the eluate fraction.

The second approach to characterize protein and peptide adducts of lipid electrophiles is to use an HNE analog bearing a terminal alkyne (aHNE) (7,8). The alkynyl-tagged protein or peptide adducts can be biotinylated with an azidobiotin probe using a Cu⁺-catalyzed cycloaddition (Click reaction). To improve selectivity for capture of aHNE-labeled proteins and peptides, we use a photocleavable azido-biotin-linker (7,8). The photocleavable linker allows selective release of adducts under very mild conditions, thus reducing the contamination of protein adducts with other proteins or peptides nonspecifically bound to the beads. The residual tag after photocleavage is small (311 mass units), which minimizes

interference with adduct analysis by LC-MS/MS. Human plasma is exposed to the aHNE and then Click reactions are performed with photocleavable azido-biotin-linker to profile both proteins and peptides modified by aHNE. Protein adducts are analyzed by affinity capture of intact proteins prior to digestion (protein catch and release) or capture of peptide adducts after digestion (peptide catch and release). The former approach identified 14 plasma proteins as plasma targets of this lipid-derived electrophile with high confidence. The latter approach dramatically enhances detection of adducted peptides and allowed identification of 50 specific adduction sites in 14 plasma proteins adducted with aHNE.

3.1. Biotinylation of HNE protein targets in cellular lysates

3.1.1. Cell culture and in vivo HNE treatment

1. RKO human colorectal carcinoma cells are grown to 80% confluence in McCoy's 5A medium supplemented with 10% fetal bovine serum, at 37 °C in an atmosphere of 95% air / 5% CO₂.
2. Treatments are carried out with varying concentrations of HNE dissolved in ethanol. Confluent cells plated in 150 mm culture dishes are washed first with 5 mL cold phosphate buffered saline and incubated with 0, 50, or 100 µM HNE delivered in 10 mL McCoy's 5A medium without fetal bovine serum. The total concentration of ethanol per culture should be 0.1% of the total medium volume.
3. Cells are exposed to electrophile for 1 h at 37 °C in an atmosphere of 95% air / 5% CO₂, then scraped off the culture dishes directly in the treatment medium with a disposable cell scraper, and centrifuged at 100g for 5 min. The treatment medium is slowly aspirated off and the cell pellets are washed twice with cold phosphate buffered saline, pH 7.4.
1. Cell pellets from each 150 mm treated plate are lysed on ice in 2 mL of cold M-PER buffer supplemented with 150 mM NaCl and the in-house made protease inhibitor and phosphatase inhibitor cocktails.
2. The lysate is cleared by centrifugation at 10,000 ×g for 10 min to remove cellular debris and the total protein concentration of the supernatant is determined using the BCA protein assay.
3. The protein concentration should be adjusted to 2 mg ml⁻¹ for each sample using lysis buffer, and the final volume of each sample should be 1mL.
4. A 50 µl sample from each sample is transferred into a new pre-labeled 1.5ml Eppendorf tube. To these sample aliquots are added DTT (50mM) and NuPage Sample buffer (4x), and the samples are heated for 10min at 95°C, and then stored at -20°C. This sample is referred to as the *Whole Cell Lysate*.

3.1.2. Derivatization of HNE-adducted proteins and Streptavidin Affinity Capture

1. Biotinylation of the reactive carbonyl group in HNE-adducted proteins is achieved with biotin-hydrazide (Fig. 1) added to a final concentration of 5 mM to each 1 mL sample (*See Note 1*). The mixture should be incubated with gentle rotation at room temperature for 2h in the dark.
2. Hydrazone bonds formed during the reaction are reduced with sodium borohydride (100 mM) for an additional of 60 min at room temperature.

¹All chemical reagents are purchased from commercial sources and are used without further purification. All reagents should be prepared fresh before each use. Biotin hydrazide stored in solution at -20 °C loses reactivity toward carbonyls.

3. Excess reagents are removed at the end of the incubation time by filtration using 10,000 Da molecular weight cutoff Amicon Ultra Centrifugal Filter Devices. One filter device per sample is needed and each filter device is pre-equilibrated first with 1ml 1x PBS.
4. Upon termination of the 60 min incubation, each sample is added to the upper chamber of a preequilibrated filter device together with 2.5mL cold 1x PBS. Samples are centrifuged at 2500 *g* for 20 min and the filtrate is discarded. Another 2.5ml of 1x PBS is added to the filter device and the sample is centrifuged at 2500 *g* for another 20min. This process is repeated twice for a total of three washes.
5. The washed, biotinylated proteins are resuspended in lysis buffer to a total volume of 1 mL. Transfer 100µl of each sample into a new pre-labeled 1.5mL Eppendorf tube, followed by the addition of DTT (50mM) and NuPage Sample buffer (4x). The samples are heated for 10 min at 95°C and stored at –20°C. This sample is referred to as the *Input*.
6. Equilibrate Streptavidin Sepharose High Performance beads by washing a 50:50 (w/v) bead slurry three times with lysis buffer. Use 1 mL of bead slurry per sample. Each time the beads are centrifuged at 10000 *g* for two min and the supernatant is carefully discarded.
7. Biotinylated proteins are incubated with streptavidin-agarose beads (~ 2 mg protein per 1 mL bead slurry previously equilibrated with lysis buffer) for 2 h with rotation at room temperature.
8. At the end of the incubation period, samples are centrifuged at 10000 *g* for two min and 100µl of the supernatant is transferred into a new pre-labeled 1.5ml Eppendorf tube. DTT (50mM) and NuPage Sample buffer (4x) are added to each sample. Samples are heated for 10 min at 95°C and stored at –20°C. This sample is referred to as the *flow through*. The remainder of the supernatant is transferred into another 1.5ml Eppendorf tube and stored at –20°C.
9. The bound proteins are washed two times with 1% SDS solution in PBS, two times with 4 M urea solution in PBS (*See Note 4*), two times with 1 M NaCl solution in PBS, and two times with PBS 1X, pH. 7.4. Each first time wash with each of the reagents rotate the beads for 10 min at room temperature. After each wash step, the beads are centrifuged at 10,000 ×*g* for 1 min and the supernatant is discarded.
10. Proteins are eluted from the beads in 100µl NuPage Sample buffer (4x) and DTT (50mM). Heat samples for 10min at 95°C. This sample is referred to as the *eluate*.
11. Aliquots of the protein input, flow through and eluates (20 µL) are then subjected to immunoblot analyses with antibodies directed against individual proteins that had been detected by LC-MS-MS as putative HNE targets.
12. Blots were probed for heat shock protein 90 (HSP90), actin, tubulin, cofilin, glutathione-S transferase Pi (GSTP), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), thioredoxin reductase 1 (TrxRd1), peroxiredoxin 6 (Prdx6), cullin3 and bax (Fig. 2).

3.1.3. Western Blotting

1. The input, flow through and eluate samples from each experimental condition are heated for 10min at 95°C.

⁴Urea has to be freshly prepared before every experiment.

2. One lane of the gel is reserved for the protein standard by adding 5 μ l of the Precision Plus Protein Standard Kaleidoscope Molecular Weight Marker.
3. Load 10 μ l of the samples into the gel. Samples are added in the following order: input, flow through and eluate. This will allow for a more efficient comparison of bands during analysis. Use NuPage Bis-Tris 10% SDS-PAGE gels and follow manufacturer's directions to run the gel.
4. Proteins are transferred electrophoretically onto a PVDF membrane by following manufacturer's directions for the used transfer apparatus. Block non-specific primary antibody binding by placing the membrane into 5 mL blocking buffer (1:1 1x TBS-Tween: Blocking Buffer) for 1 h at room temperature on a rocking platform (*See Note 2*).
5. Primary antibodies to proteins of interest are prepared according to the manufacturer's directions.
6. Incubate the membrane with primary antibody overnight at 4°C while shaking on an orbital shaker.
7. Using multiple changes of 1x TBS-Tween, wash membranes for a total of 30min before adding the secondary antibody.
8. Prepare appropriate AlexaFluor@680-labeled secondary antibodies.
9. The membrane is incubated for 1hr at room temperature while shaking. Using multiple changes of 1x TBS-Tween, the membrane is washed for a total of 30min before scanning. Immunoreactive proteins are visualized using the Odyssey™ System and software as described by the manufacturer.

3.1.4. In gel trypsin digestion and MS analysis

1. Adducted proteins purified by streptavidin capture as described above (eluate) are resolved by 10% SDS-PAGE using NuPAGE Bis-Tris gels and stained with Colloidal Coomassie Blue.
2. Desired bands corresponding to different molecular weights are excised from the gel and digested in-gel with trypsin. Each excised band is chopped into 1mm cubes, placed in 1.5 ml Eppendorf tube containing 100 μ L of 100 mM ammonium bicarbonate, pH 8.0 and incubated at room temperature for 15 min.
3. Samples are reduced with 10 μ L of 45 mM DTT for 20 min at 55°C and alkylated with 10 μ L of 100 mM iodoacetamide for 20 min at room temperature in the dark.
4. The liquid is discarded and 100 μ L acetonitrile:50 mM ammonium bicarbonate (50:50, v/v) is added to destain the samples. Incubate at room temperature for 15 min then discard the liquid. Repeat twice more.
5. The gel pieces are then dehydrated with 100 μ L of acetonitrile, incubated for 15 min at room temperature, and the supernatant liquid is discarded.
6. The rehydrated gel pieces are digested with trypsin (50 μ L of 0.01 μ g/mL Trypsin Gold in 25 mM ammonium bicarbonate) overnight at 37°C.
7. The peptides are extracted twice with 100 μ L of 60% acetonitrile and 0.1% trifluoroacetic acid, each for 15 min at room temperature; the extracts are

²Incubation with primary antibody overnight at 4 °C gives a much stronger signal for Western Blotting than two hour incubation at room temperature.

combined. The extracts are evaporated under vacuum and resuspended in 10 – 20 μ L of H₂O (0.1 %FA) for LC-MS/MS analysis.

8. The resulting peptides are subjected to LC-MS/MS analysis using an LTQ ion trap mass spectrometer.

3.1.5. Database Searching

1. The “ScanSifter” algorithm, an in-house developed software, reads MS/MS spectra stored as centroided peak lists from Thermo RAW files and transcribed them to mzData v1.05 files. Spectra that contain fewer than 6 peaks or that have less than 2e1 measured TIC do not result in mzData files. Only MS/MS scans are written to the mzData files; MS scans are excluded. If 90% of the intensity of a tandem mass spectrum appears at a lower m/z than the precursor ion, a single precursor charge is assumed; otherwise, the spectrum is processed under both double and triple precursor charge assumptions.
2. Tandem mass spectra are assigned to peptides from the IPI Human database version 3.33 (September 13, 2007; 67837 proteins) by the MyriMatch algorithm(9). To estimate false discovery rates, each sequence of the database was reversed and concatenated to the database, for a total of 135,674 entries. Candidate peptides are required to feature trypsin cleavages or protein termini at both ends, though any number of missed cleavages is permitted. All cysteines are expected to undergo carboxamidomethylation and are assigned a mass of 160 kDa. All methionines are allowed to be oxidized. Precursor ions are required to fall within 1.25 m/z of the position expected from their average masses, and fragment ions are required to fall within 0.5 m/z of their monoisotopic positions. The database searches produced raw identifications in pepXML format.
3. Peptide identification, filtering and protein assembly are done with the IDPicker algorithm. Initial filtering takes place in multiple stages(10). First, IDPicker filters raw peptide identification to a target false discovery rate (FDR) of 5%. The peptide filtering employs reversed sequence database match information to determine thresholds that yield an estimated 5% FDR for the identifications of each charge state by the formula(11) $FDR = (2R)/(R+F)$, where R is the number of passing reversed peptide identifications and F is the number of passing forward (normal orientation) peptide identifications. The second round of filtering removes proteins supported by less than two distinct peptide identifications in the analyses. Indistinguishable proteins are recognized and grouped. Parsimony rules are applied to generate a minimal list of proteins that explain all of the peptides that passes the entry criteria.

3.2. Biotinylation of HNE protein targets in plasma using Click chemistry

3.2.1. aHNE treatment and biotinylation

1. aHNE is dissolved in DMSO and added to 0.5 mL human plasma (final concentration 100 μ M, <0.3% v) and stirred gently for 1.5 h at 37 °C. This incubation forms aHNE plasma protein adducts.
2. NaBH₄ (final concentration 10 mM) is added and the tube is rotated for 1 h at room temperature and then pH is adjusted to 5.
3. The plasma is filtered with 10,000 Da molecular weight cutoff filter to remove excess reagents. Two additional washes are done with 1 volume each of 1X PBS.

4. CuSO₄, ligand, ascorbic acid (or TCEP), and azido-biotin are added to the plasma to final concentrations 1 mM, 0.2 mM, 1 mM, and 0.6 mM respectively. The reaction mixture is rotated for 2 h at room temperature in the dark (*See Note 5*).
5. The solution is filtered with a 10,000 Da molecular weight cutoff filter as described in step 3 above.
6. Protein G Agarose bead slurry (50 µL) is added and the tube is rotated for 30 min (2 µL should be saved for western blot analysis with Streptavidin).
7. Half of the collected supernatant is mixed with 0.5 mL of Streptavidin beads and the suspension is then mixed gently overnight at 4°C in the dark. (The other half of the supernatant can be processed by “peptide catch and release” to afford a sample enriched in adducted peptides—see below.)
8. The beads are washed with 1 mL each of 1% SDS (twice), 4 M urea (twice), 1 M NaCl (twice), H₂O (twice), and ammonium bicarbonate (once) sequentially in the dark.

3.2.2. Photorelease and analysis of adducted plasma proteins (Protein catch and release)

1. The washed beads are resuspended in PBS then irradiated with a hand held UV light (365 nm) for 1 h with stirring. This cleaves the linker and releases the adducted proteins from the Streptavidin beads.
2. The supernatant is collected and concentrated to 50 µL using a 10,000 Da molecular weight cutoff filter.
3. A sample of the supernatant (10 µL) is subjected to western blot analysis for human serum albumin and ApoA1 using the anti-HSA and anti-ApoA1 antibodies respectively. These analyses confirm that these abundant plasma proteins were adducted, captured and then photoreleased.
4. The remaining supernatant is resolved by SDS-PAGE on a NuPage Bis-Tris® gel. After electrophoresis, gel lanes are cut into 10 MW fractions and subjected to in-gel trypsin digestion as described above.
5. Extracted peptides are redissolved in 0.1% formic acid and analyzed by LC-MS/MS as described above.

3.2.3. Photorelease of adducted plasma peptides (Peptide catch and release)

1. The remaining half of the Protein G Agarose supernatant from step 7 of section 3.2.1 (see above) is agitated with two volumes of isopropylether/n-butanol (6:4, v/v) for 30 min to extract lipid. The aqueous phase reaction mixture is treated with DTT (5 mM) at 50 °C for 10 min and then with iodoacetamide (10 mM) at RT for 10 min in the dark. The reduced, alkylated proteins are then digested in solution with trypsin (1:100, w/w) for 4 h at 37 °C with shaking in the dark (*See Note 3*).
2. The digested peptides are immobilized on a Streptavidin slurry (500 µL) overnight at 4°C in the dark. The beads are then washed sequentially with SDS, urea, NaCl, H₂O and ammonium bicarbonate as described above. The washed slurry is

⁵Azido biotin, aHNE, and ligand (TBTA, Tris[(1 - benzyl - 1H - 1,2,3 - triazol - 4 - yl)methyl] amine) were synthesized in house (Mol Cell Proteomics. 2009 Sep;8(9):2080-9.). TBTA can be purchased from AnaSpec (Fremont, CA)

³We found that delipidation of plasma significantly increased efficiency of capturing biotinylated peptides using streptavidin.

resuspended in 1 ml of PBS and then exposed to UV light for 1 h as described above. This releases the peptide adducts.

3. The supernatant containing the peptide adducts is evaporated under vacuum and the mixture is redissolved in 0.5 ml of H₂O.
4. The solution containing the peptides is subjected to SPE using OASIS HLB[®] cartridges (30 mg). The SPE cartridge is activated with 1 ml of 0.1% formic acid in acetonitrile/water (4:1, v/v) and then equilibrated with 1 ml of 0.1% aqueous formic acid. The peptides are loaded onto the cartridge, which is then washed with 1 ml 0.1% aqueous formic acid. The peptides are then eluted with 1 ml of 0.1% formic acid in acetonitrile/water (4:1, v/v). The eluted peptide solution is evaporated under vacuum and the peptides are then redissolved in 0.1% formic acid and analyzed by LC-MS/MS as described above.

Acknowledgments

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Fig. 1.
(A) Biotinylation of protein reactive carbonyls with biotin hydrazide and (B) biotinylation by click chemistry using a photocleavable probe.

Fig. 2. Overlap of adducted proteins identified from RKO cells treated with 50 or 100 μ M HNE or vehicle control. The numbers represent total proteins identified in triplicate analyses of each experimental condition. Overlaps between treatments are indicated by the numbers in the corresponding segments. A total of 561 proteins were common to all groups. (Reproduced from *Mol Cell Proteomics*. 2009 Apr; 8(4):670-80 with permission from ASBMB.)

Fig. 3.

Immunoblotting validation of ten individual protein targets from RKO cellular extracts treated with increasing concentrations of HNE. The presence of the proteins was confirmed in the input (I), flow-through (F) and elution (E) fractions (red arrows), which contain adducted proteins. (Reproduced from *Mol Cell Proteomics*. 2009 Apr; 8(4):670-80 with permission from ASBMB.)

Fig. 4.

Immunoblots of plasma supplemented with *α*HNE followed by 1, 3 cycloaddition with azido-biotin then released by photolysis. The final concentrations of reagents, biotin, TCEP (or ascorbate), CuSO₄, and ligand were 1 mM, 2 mM, 2 mM, and 0.2 mM, respectively. (A) anti-HSA visualized with Alexa Fluor 680 donkey anti-goat (B) anti-ApoA1 visualized with Alexa Fluor 680 goat anti-rabbit (C) visualized by Alexa Fluor 680-conjugated with streptavidin: residual biotin is probed even after the photolysis due to multiple sites of adduction. The whole plasma proteins are shown in the crude mix on the *left* and proteins that are eluted from photolysis of beads on the *right*. Intensities of + or - *α*HNE can be compared in each case; photoeluted fractions are diluted compared with crude mix. (Reproduced from Mol Cell Proteomics. 2009 Sep; 8(9):2080-9 with permission from ASBMB.)

Table 1

Plasma proteins modified by *a*HNE as identified by *protein catch* and *photorelease*.

IPI Accession	UniProtKB/SwissProt Entry	Protein Description	unique peptide counts	spectral counts
IPI00021841	P02647	Apolipoprotein A-I precursor, reverse transport of cholesterol	13	41
IPI00021854	P02652	Apolipoprotein A-II precursor, May stabilize HDL (high density lipoprotein) structure	6	14
IPI00022229	P04114	Apolipoprotein B-100 precursor, major protein constituent of LDL and VLDL	38	44
IPI00021885	P02671	Isoform 1 of Fibrinogen alpha chain precursor, a cofactor in platelet aggregation	7	10
IPI00304273	P06727	Apolipoprotein A-IV precursor, VLDL secretion and catabolism	4	5
IPI00032258	P0C0L4	Complement C4-A precursor, activation of the classical pathway of the complement system	3	4
IPI00553177	P01009	Alpha-1-antitrypsin precursor	5	6
IPI00745872	P02768	Isoform 1 of Serum albumin precursor	140	623
IPI00164623	P01024	Complement C3 precursor, activation of the complement system, Pyogenic infection	17	25
IPI00022488	P02790	Hemopexin precursor, Binds heme and transports it to the liver for breakdown and iron recovery	9	15
IPI00022463	P02787	Serotransferrin precursor, iron binding transport proteins, stimulating cell proliferation	23	34
IPI00022426	P02760	AMBIP protein precursor, inhibits trypsin, plasmin, and lysosomal granulocytic elastase, and calcium oxalate crystallization	6	16
IPI00478003	Q9BQ22	Alpha-2-macroglobulin precursor, inhibit all four classes of proteinases by a unique 'trapping' mechanism	25	35
IPI00555812	P02774	Vitamin D-binding protein precursor	12	18

Table 2

Peptides from proteins identified in Table 1 modified by *a*HNE as detected using *peptide catch and photorelease*.

IPI Accession	Protein	Adducted Peptides	Peptide Precursor	Peptide Precursor	z	Residue
			Calculated	Found		Number
IPI00021841	ApoA1	TH * LAPYSELR	806.9201	806.9237	2	162
			538.2825	538.2843	3	162
		LEALK * ENGGAR	490.2755	490.2772	2	182
		LAEYH * AK	571.8139	571.8154	2	193
		ATEH * LSTLSEK	763.9067	763.9084	2	199
			509.6069	509.6078	3	199
		AK * PALEDLR	441.9259	441.9278	3	208
		K * LNTQ	457.7689	457.7712	2	239
IPI00021854	ApoA2	VK * SPELQAEAK	755.9274	755.9376	2	53
		SPELQAEAK * SYFEK	646.6667	646.666	3	62
		SK * EQLTPLIK	486.9642	486.964	3	69
IPI00022229	ApoB100	VLVDH * FGYTEK	497.2732	497.2733	3	737
		DDKH * EQDMVNGIMLSVEK	608.5425	608.5426	4	746
		LLDH * R	482.7424	482.742	2	1214
		H * VGSK	419.7427	419.7425	2	1229
		H * INIDQFVR	484.9368	484.9363	3	2101
		VH * ELIER	402.8995	402.8992	3	2342
		LKQH * IEAIDVR	544.9859	544.983	3	2288
		SFDRH * FEK	459.5701	459.5689	3	3207
IPI00021885	Fibrinogen alpha chain precursor	TVIGPDGH * K	617.8431	617.8444	2	475
		HRH * PDEAAFFDTASTGK	733.359	733.3651	3	513
		TVTK * TVIGPDGCHK	555.3174	555.3192	3	467
		ESSSH * HPGIAEFPSR	650.3219	650.326	3	563
		DSH * SLTTNIM ^ EILR	653.0068	653.0074	2	103
		VQH * IQLLQK	473.289	473.2905	3	151
		SSSYSK * QFTSSTSYNR	714.343	714.3467	3	581
	ApoA4	K * LVPFATELHER	584.3332	584.3328	3	79

IPI Accession	Protein	Adducted Peptides	Peptide Precursor	Peptide Precursor	z	Residue
		LNH [*] QLEGLTFQM [^] K	629.3329	629.3336	3	236
		LGPH [*] AGDVEGHLSFLEK	706.3724	706.3724	3	332
IPI00032258	Complement C4-A precursor	SH [*] ALQLNNR	455.2529	455.2526	2	1342
		DK [*] GQAGLQR	428.5737	428.571	2	749
	Alpha-1-antitrypsin precursor	TDTSHHDQDH [*] PTFNK	697.6557	697.6569	3	39
		TDTSH [*] HDQDHPTFNK	697.6557	697.6566	3	34
		LYH [*] SEAFTVNFVDTEAK	790.3814	790.3777	3	163
		LVDK [*] FLEDVK	506.2922	506.2941	3	153
		LGM [^] FNQHC [*] K	506.5916	506.5936	3	255
		QINDYVEK [*] GTQ GK	597.6476	597.6481	3	187
		K [*] QINDYVEK	483.2646	483.2589	3	179
		LQHLENELTH [*] DIITK	529.5361	529.5406	4	293
		LQH [*] LENELTHDIITK	705.7197	705.7221	3	286
		K [*] LSSWVLLM [^] K	511.3021	511.3018	3	258
IPI00745872	Isoform 1 of Serum albumin	SLH [*] TLFGDK	443.5785	443.5793	3	90
	precursor	RH [*] PYFYAPELLFFAK	737.3981	737.4041	3	169
		H [*] PYFYAPELLFFAK	685.3644	685.3738	3	169
		LK [*] C [#] ASLQK	629.863	629.8877	2	222
		LK [*] C [#] ASLQK	420.2444	420.2453	3	222
		LK [~] C [#] ASLQK	414.2409	414.2426	3	222
		RH [*] PDYSVVLRL	593.6807	593.681	3	361
		H [*] PDYSVVLRL	541.647	541.6522	3	361
		K [~] QTALVELVK	474.6291	474.6292	3	548
		NLGK [*] VGSK	557.335	557.3357	2	455
		SEVAH [*] R	505.2749	505.2757	2	32
IPI00478003	Alpha-2-macroglobulin precursor	TEH [*] PFTVEEFVLPK	662.0191	662.0232	3	217
		GH [*] FSISIPVK	465.938	465.937	3	523
IPI00022426	AMBP protein precursor	H [*] HGPTITAK	424.9068	424.9061	3	141
IPI00164623	Complement C3	AAVYHH [*] FISDGVR	594.981	594.9813	3	918
		AAVYH [*] HFISDGVR	594.981	594.9809	3	918

IPI Accession	Protein	Adducted Peptides	Peptide Precursor	Peptide Precursor	z	Residue
	Hemopexin	GH [*] GHR	437.7357	437.736	2	238
	Serotransferrin	DGAGDVAFVKH [*] STIFENLANK	636.8328	636.8326	4	226
IPI00022463		EFQLFSSPH [*] GK	529.9473	529.9433	3	308
IPI00555812	Vitamin D-binding protein precursor	H [*] LSLLTTLNLR	522.6385	522.6435	3	208

^{*} Michael adduct

~ Imine

S-carboxyamido

^ methionine sulfoxide