An Azido-Biotin Reagent for Use in the Isolation of Protein Adducts of Lipid-derived Electrophiles by Streptavidin Catch and Photorelease

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HNE (4-hydroxynonenal), a byproduct of lipid peroxidation, reacts with nucleophilic centers on proteins. A terminal alkynyl analog of HNE (alkynyl HNE, aHNE) serves as a surrogate for HNE itself, both compounds reacting with protein amine and thiol functional groups by similar chemistry. Proteins modified with aHNE undergo reaction with a click reagent that bears azido and biotin groups separated by a photocleavable linker. Peptides and proteins modified in this way are affinity purified on streptavidin beads. Photolysis of the beads with a low intensity UV light releases bound biotinylated proteins or peptides, i.e. proteins or peptides modified by aHNE. Two strategies, (a) protein catch and photorelease and (b) peptide catch and photorelease, are employed to enrich adducted proteins or peptide mixtures highly enriched in adducts. Proteomics analysis of the streptavidin-purified peptides by LC-MS/MS permits identification of the adduction site. Identification of 30 separate peptides from human serum albumin by peptide catch and photorelease reveals 18 different aHNE adduction sites on the protein. Protein catch and photorelease shows that both HSA and ApoA1 in human plasma undergo significant modification by aHNE. *Molecular & Cellular Proteomics 8:2080–2089, 2009.

Polyunsaturated lipids in biological membranes are particularly reactive targets for oxygen radicals (1–3). Lipid peroxidation, the chain reaction of peroxyl radicals that is a consequence of oxidative stress, is thought to be involved in human diseases such as cancer, atherosclerosis, and neurodegenerative disorders (4–8). A variety of electrophilic compounds are byproducts of lipid peroxidation, 4-hydroxynon-2-enal (HNE) being a particularly toxic electrophile (9–12) that forms mutagenic DNA adducts (13–15). HNE and other lipid-derived electrophiles also form protein modifications, and some of these adducts have been characterized on a limited number of proteins and peptides by mass spectrometry (MS) and in tissues by antibody-based methods (16). Until recently, relatively little was known about the target selectivity of oxidant-derived electrophiles in proteins, the relative reactivities of different amino acid targets, and the properties of the adducts. We recently described the application of a post-labeling strategy in which biotin hydrazide was used to biotinylate carbonyl-containing adducts formed by HNE in RKO cells (17). When combined with shotgun proteome analysis of the captured proteins, this approach provided a global perspective on patterns of protein damage by a prototypical lipid electrophile. However, biotin hydrazide labels many carbonyls, thus generating a background inventory derived from endogenous carbonyls, which is difficult to characterize and may mask more subtle patterns of selectivity in protein adduction. Moreover, the biotin hydrazide approach can only capture adducts with a reactive carbonyl group.

To deal with these limitations, we have explored labeled electrophile probes and selective adduct capture chemistries (18). We recently reported that 4-hydroxynon-2-en-8-ynal, alkynyl-HNE (aHNE), can be used as an HNE surrogate in whole cells to isolate proteins that are adducted by this electrophile (19). aHNE displays similar toxicity in RKO cells as does HNE, and studies with model peptides and isolated proteins show that HNE and the alkynyl surrogate display similar chemistry in reactions with protein nucleophiles. For example, reaction of aHNE with proteins or peptides followed by sodium borohydride reduction gives Michael and imine adducts as shown in structures 1 and 2. This same chemistry is observed for HNE itself.

Reaction of cellular aHNE protein adducts with an azido-biotin reagent followed by capture of the triazole cycloadducts on streptavidin beads permitted a number of adducted proteins to be identified by shotgun proteomics (19). Thus, tryptic digestion of the proteins pulled down by means of the thin cholesterol acyl transferase; ABCA1, ATP-binding cassette transporter A1; FA, fumaric acid; RT, room temperature; LC-MS/MS, liquid chromatography mass spectrometry mass spectrometry.
alkyne affinity tag generates mixtures that include adducted peptides such as 3 as well as unmodified peptides. The chemistry associated with the alkyne electrophile works as planned, but the strategy suffers from two significant drawbacks. First, nonspecific protein binding to the streptavidin beads complicates the identification of adducted proteins and second, biotinylated peptides such as 3 generated in the sequence have MS/MS fragmentation patterns that do not permit the ready identification of the amino acid adduction site on the peptide. The biotin appendage is a major site of positive charge localization in the MS/MS experiment, and the formation of characteristic b and y ions is frequently not sufficient for peptide identification.

We report here a strategy that couples the alkyne electrophile azido-biotin capture for the isolation of adducted protein with a photochemical release of the adduct from streptavidin. This approach reduces the protein nonspecific binding problem because release from the bead requires only a photochemical event, and it permits the identification of specific nucleophilic sites on proteins that are modified by reactive electrophiles. By the application of this strategy to capture both adducted proteins and peptides, we have identified plasma protein targets of the probes and also mapped several nucleophilic sites on the plasma protein ApoA1 that are modified by aHNE.

EXPERIMENTAL PROCEDURES

Materials—Streptavidin and Alexa Fluor®-680-conjugated fluorescent secondary antibodies were obtained from Molecular Probes (Eugene, OR). Anti-human ApoA1 polyclonal antibody raised against the sequence of RLAEYHAKTEH in rabbit was purchased from Cayman Chemicals (Ann Arbor, MI). Anti-human serum albumin (HSA) was obtained from Cortex Biochem™ (Concord, MA). Secondary antibodies were obtained from Molecular Probes® (Invitrogen). Streptavidin-Sepharose™ high performance was purchased from GE Healthcare, Trypsin Gold was purchased from Promega (Madison, WI). Purified human serum albumin was purchased from Sigma. All other chemical reagents were purchased from commercial sources and were used without further purification. All solutions were prepared fresh before each use.

Plasma—Plasma was obtained from the fresh blood donated from a healthy subject following Vanderbilt IRB protocols. The fresh blood was drawn to BD Vacutainer® (BD Science, Franklin Lakes, NJ), and it was centrifuged using Heraeus® Labofuge® 400 (Thermo Electron Corporation) with 2000 rpm for 10 min. Isolated plasma was aliquoted to 1-ml Eppendorf tubes and kept at −80 °C until use.

Solid Phase Extraction (SPE)—All of the final recovered peptides were subjected to SPE using Oasis HLB® purchased from Waters Corp. (Milford, MA) (30 mg). The SPE cartridge was activated with 1 ml of 0.1% FA 80% ACN in H₂O and then equilibrated with 1 ml of 0.1% FA H₂O. The peptides were loaded onto the column and washed with 1 ml 0.1% FA H₂O. Then the peptides were eluted with 1 ml of 0.1% FA 80% ACN in H₂O. The collected fraction was dried using a Savant SpeedVac® (SPD 121P) concentrator system.

Click Chemistry of Human Serum Albumin (HSA) Adducts—1 ml of human serum albumin (2 μg/μl or 30 μg/μl to mimic plasma concentration) and aHNE (final concentration = 2 mM) were incubated in 37 °C for 1 h. The solution was reacted with NaBH₄ (10 mM) for 1 h at room temperature. The pH of the reaction mixture was adjusted to 7.0 and then filtered using an Amicon (MWCO = 50 kDa filter) in an Allegra™ 6R centrifuge with 32,000 rpm for 20 min twice. The recovered reaction mixture was adjusted to 1 ml in PBS and then sodium ascorbate (2 mM or tris-(2-carboxyethyl)phosphine), CuSO₄ (2 mM), ligand (0.2 mM), and N₃-biotin (5, 1 mM) were added, mixed, and allowed to react at room temperature for 1 h, in the dark. The reaction mixture was filtered using an Amicon (MWCO = 50 kDa) as described above. The recovered reaction mixture was adjusted to 1 ml and divided into two portions, one for study of the protein catch and photorelease route and the other for the peptide catch and photorelease route.

Protein Catch and Photorelease of HSA Adducts—A filtered reaction mixture was immobilized on a streptavidin slurry (500 μl) overnight in the cold (4 °C) in the dark. The next day, the slurry was washed stringently using 1 ml of 1× SDS, 4 μl urea, 1 μl NaCl, H₂O, PBS twice each to remove nonspecific streptavidin-bound proteins (samples were handled in the dark). The washed slurry was suspended in 1 ml of PBS and then exposed to a hand-held UV light (365 nm; Entela, Upland, CA) for 1 h at RT with stirring. The supernatant was collected, and the beads were washed with PBS twice. The combined aliquots were concentrated to 50 μl using Amicon (MWCO = 50 kDa). The photoreleased protein was resolved using
10% NuPAGE Novex BisTris® gel. One gel was stained with Simply Blue® to be excised for in-gel trypsin digestion. The other was transferred to polyvinylidene difluoride membrane to run Western blots for anti-HSA (supplemental Fig. S1). They were visualized with Alexa Fluor 680-conjugated with donkey anti-goat secondary antibody for HSA. Biotinylation was confirmed by Alexa Fluor 680-conjugated with streptavidin prior to photolysis. The excised band was trypsin-digested in gel for 4 h at 37 °C incubator with shaking (200 rpm).

**Peptide Catch and Photorelease of HSA Adducts**—A filtered reaction mixture was treated with dithiothreitol (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 were then digested in solution with trypsin (1:100, w/w) for 4 h at 37 °C with shaking in the dark. The digested peptides were immobilized to a streptavidin slurry (500 μl) overnight in the cold room in the dark. The next day the slurry was washed as described in the previous section. The washed slurry was resuspended in 1 ml of PBS and then exposed to UV light for 1 h in the same manner as described above. The supernatant and washed fractions were combined and dried in vacuo. The dried sample was resuspended in 0.5 ml of H2O followed by SPE as described above. The dried sample was resuspended in 50 μl of H+ /H2O for LC-MS/MS analysis.

**Click Chemistry with Healthy Human Plasma**—1 ml of human plasma was supplemented with aHNE (0.2 mM) at 1 h at 37 °C. The reaction mixture was mixed with NaBH4 (10 mM) at 1 h at RT. The pH of the reaction mixture was adjusted to pH 7 and then filtered with an Amicon 10-kDa filter with an additional 3 ml of PBS. The recovered reaction mixture was adjusted to 1 ml and divided into two portions, one for study of the protein catch and photorelease route the other for the peptide catch and photorelease route.

** Protein Catch and Photorelease of Adducted Plasma Proteins**—The aHNE-treated plasma was further treated with click reagents for 2 h at room temperature to selectively label the adducted proteins with biotin to be used in affinity purification. After filtration using Amicon filter (MWCO = 10 kDa), the proteins were immobilized on a streptavidin slurry (500 μl) overnight in the cold room in the dark. The beads were washed in the same manner as described above and were then resuspended in 1 ml of PBS and exposed to UV light for 1 h at RT with stirring. The supernatant and the washed fractions were combined and concentrated using an Amicon 10-kDa filter. The eluted proteins were resolved by SDS-PGE on a 10% BisTris gel, and then with iodoacetamide (10 mM) at RT for 10 min in the dark. The reduced, alkylated proteins were then digested in solution with trypsin as described above. The digested peptides were purified by SPE described above. The collected fraction was dried in a SpeedVac concentrator system and then resuspended in 50 μl of 0.1% FA H2O for LC-MS/MS analysis.

**Peptide Catch and Photorelease of Adducted Plasma Proteins**—An additional 0.5 ml of plasma was treated with 0.0, 0.2, 0.5, 1, and 2 mM aHNE for peptide catch route in order to obtain the maximum yield of the adduction (supplemental Fig. S3). The reaction mixture was digested by the addition of 1 ml of isopropanol and n-butanol (6:4) (20) and agitated for 30 min at room temperature. The aqueous layer was recovered and continued to carry on click chemistry. The reaction mixture was filtered with Amicon (10 kDa) filter, and then the recovered proteins were reduced, alkylated, and digested in solution with trypsin as described above. The digested peptides were immobilized on a streptavidin slurry (500 μl) overnight in the cold room in the dark. The next day the slurry was washed as described above and then resuspended in 1 ml of PBS. It was then exposed to UV light for 1 h in the same manner described above. The dried sample was resuspended in 0.5 ml of H2O and desalted using Oasis as described above. The dried sample was resuspended in 50 μl of H+ /H2O for LC-MS/MS analysis.

**LC-MS/MS Analyses**—The resulting peptides were subjected to LC-MS/MS analysis using a Thermo LTQ ion trap or LTQ-Orbitrap hybrid mass spectrometer (ThermoFisher, San Jose, CA) operated with Xcalibur 2.0.7 instrument control software and equipped with a Thermo nanoelectrospray source and either a MicroAS autosampler and Thermo Surveyor HPLC pump and/or an Eksigent nanoLC and autosampler (Dublin, CA). Peptides were resolved on 100 μm × 11 cm fused silica capillary column (Polymicro Technologies, LLC Phoenix, AZ) packed with 5 μm, 300 A Jupiter C18 (Phenomenex, Torrance, CA). Liquid chromatography was 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). Centrifuged MS/MS scans were acquired using an isolation width of 2 m/z, an activation time of 30 ms, an activation Q of 0.250, and 35% normalized collision energy using 1 microscan with a maximum ion time of 100 ms for each MS/MS scan and 1 microscan with a maximum ion time of 500 for each full scan. The mass spectrometer was tuned prior to analysis using the synthetic peptide TpepK (AVAGKAGAR) so that some parameters may have varied slightly from experiment to experiment, but typically the tune parameters were 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. A full scan obtained for eluting peptides in the range of 400–2000 amu was collected on the Orbitrap portion of the instrument at a resolution of 60,000, followed by five data-dependent MS/MS scans on the LTQ portion of the instrument with a minimum threshold of 1000 set to trigger the MS/MS spectra. MS/MS spectra were recorded using dynamic exclusion of previously analyzed precursors for 60 s with a repeat of 1 and a repeat duration of 1.

**Database Search and Data Analysis**—The “ScanSifter” algorithm v0.1, an in-house developed software, read tandem mass spectra stored as centroided peak lists from Thermo RAW files and transcoded them to DTA files. Spectra that contained fewer than six peaks or that had less than 2e1 measured total ion current did not result in DTA. If 90% of the intensity of a tandem mass spectrum appeared at a lower m/z than the precursor ion, a single precursor charge was assumed; otherwise, the spectrum was processed under both double and triple precursor charge assumptions. Proteins were identified using the SEQUEST v.27 algorithm on a high speed, multiprocessor Linux cluster in the Advanced Computing Center for Research & Education at Vanderbilt University using the human subset of the IPI human protein database, version 333, modified 2007/10/04. To estimate false discovery rates, each sequence of the database was reversed and concatenated to the database, for a total of 135,674 entries. The database search encompassed tryptic peptides with a maximum of five missed cleavage sites for an enzyme search and with a maximum number of 10 internal cleavage sites. All cysteines were expected to undergo carboxamidomethylation and were added mass shift of 57 Da. All methionines were allowed to be mono-oxidized. In addition to that, an anticipated mass shift of 311 to histidine, cysteine, and lysine in case of derivatized aHNE Michael adduct and 295 in case of derivatized aHNE imine adduct was also searched. Precursor ions were required to fall within 1.25 m/z of the position expected from their average masses, and fragment ions were required to fall within 0.5 m/z of their mono-isotopic positions. The database searches produced raw identifications in SQT format (21).

Peptide identification, filtering, and protein assembly were done with IDPicker software. Initial filtering took place in multiple stages.
Click Biotin Photocleavable Reagent for Protein Adducts

First, IDPicker filtered raw peptide identifications to a target false discovery rate (FDR) of 5%. The peptide filtering employed reversed sequence database match information to determine thresholds that yielded an estimated 5% FDR for the identifications of each charge state by the formula 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 removed proteins supported by less than two distinct peptide identifications in the analyses. Indistinguishable proteins were recognized and grouped. Parsimony rules were applied to generate a minimal list of proteins that explained all of the peptides that passed our entry criteria.

RESULTS

Reaction of aHNE with three model peptides, 4a-c, Ac-Ala-Val-Ala-Gly-Xa-Ala-Gly-Ala-Arg a, Xa = His; b, Xb = Lys; c, Xc = Cys consumed the peptides and, after reduction with NaBH₄, gave predominantly Michael adducts for all three peptides. The aHNE adducts were used as standard alkynyl-tagged peptides for which an appropriate photoreagent was developed. We settled on the azido-biotin 5 as a photorelease reagent for these initial studies because of its ease of synthesis, as described in Supplementary Information as well as its appropriate photochemistry (22–23). The phenone 5 is stable when exposed to moderate room light for brief time periods.

Reaction of the aHNE peptide adducts of 4a-c with 5 under standard “click” conditions gave the triazole cycloadducts 6 as shown in Fig. 1. The subsequent photorelease from the biotin half of the molecule appears to be quantitative or nearly so. The photoreaction typically proceeds in less than half-an hour with a 4 watt hand-held UV lamp that is normally used for visualizing thin-layer chromatography plates. Both the click and photorelease reactions were monitored by HPLC-MS/MS, and good conversion was observed for both steps with mixtures of adducted and un-adducted tryptic peptides are isolated from adducted proteins. This allows confident identification of proteins that are modified with aHNE. In peptide catch and photorelease, tryptic digestion is followed by binding of biotinylated peptides to streptavidin and photorelease of peptide adducts. This approach is used to concentrate only adducted peptides from all adducted proteins and to map the sites of modifications.

We have carried out experiments utilizing both protein and peptide catch and photorelease schemes and we illustrate below both strategies. Reaction of 0, 0.2, 0.5, 1.0, and 2.0 mM aHNE with human serum albumin (HSA) for 1 h, under conditions similar to those reported for the reaction of albumin and HNE itself (18), was followed by 10 mM sodium borohydride reduction and click dipolar cycloaddition of the modified protein for 2 h with 1 mM of azide 5. Filtration of excess reagents from the protein with a 50 molecular weight cutoff filter was followed by solution tryptic digestion. The mixture of peptides generated in this way was incubated with streptavidin beads overnight at 4 °C and the beads were washed extensively and then photolyzed at 365 nm with a 4W UV lamp for 1 h. LC-MS/MS analysis of the mixture released from the beads by photolysis unambiguously identified 30 peptides analogous to 7. The MS/MS spectra obtained were searched against HSA sequences using MonsterMod (manuscript in preparation), a program that calculates mass differences between search peptide sequences and MS/MS precursors and assigns the mass shift to
a sequence position in the peptide based on the fragment peaks in the MS/MS spectrum. Search sequences included tryptic peptides with missed lysine cleavages that would result from adduction. Mass shifts of 311 correspond to Michael adducts of an amino acid residue derivative analogous to 7 while shifts of 295 represent an imine adduct.

As the aHNE concentration is increased, the adducted HSA increases as measured by a streptavidin Western blot (supplemental Fig. S1). In concentration-dependent experiments, eight histidine Michael or imine adducts were observed at albumin residues 3, 9, 67, 105, 146, 242, 338, and 510 while nine lysine imine or Michael adducts were observed at residues 12, 137, 162, 199, 225, 378, 414, 432, and 525. The assignments are based on the criteria of an adducted peptide being found at least twice in multiple experiments with various concentrations of aHNE. Without the use of the click affinity strategy described here, a previous study of the reaction of albumin with HNE revealed only 10 adduction sites on albumin from 11 modified peptides (18). The alkynyl electrophile-click cycloaddition strategy, when coupled to a streptavidin peptide photorelease reagent, identifies 18 adduction sites from 30 peptides, an obvious advantage for the study of HNE protein adducts.

Although the HSA study described above involves peptide capture and photorelease, the alternate approach relies on binding to and photorelease of the whole protein from streptavidin. In experiments that are illustrative of this strategy, we added 0, 0.2, 0.5, 1.0, and 2 mM of aHNE to platelet-rich human plasma for 1 h and followed this with reduction of the sample by sodium borohydride (supplemental Fig. S2). Click dipolar cycloaddition of the adducted protein with azide 5 gave a mixture that was then filtered of excess reagents with a 10 kDa molecular weight cutoff filter. Gel electrophoresis of the product mixture showed that a number of important plasma proteins, including human serum albumin and the plasma protein apolipoprotein A1 (ApoA1), were extensively biotinylated and hence extensively adducted with aHNE as evidenced by immunoblotting with Alexa Fluor 680-conjugated streptavidin. Control experiments carried out in the absence of aHNE showed no evidence of protein biotinylation and hence no protein adduction. Biotinylated proteins obtained from the above protocol were captured on streptavidin beads overnight at 4 °C. The beads were washed and then photolyzed, releasing adducted plasma proteins. Among the proteins released by photolysis was ApoA1, which was detected by immunoblotting as illustrated in Fig. 4.
In Table I, the major adducted proteins identified by the protein catch and photorelease are reported. A number of proteins were detected by at least two distinct peptides within a single experiment, and 14 listed proteins were identified with high confidence (FDR < 5%). Those peptides were found more than once, as indicated by higher spectral counts in most of the cases.

The peptide catch and photorelease strategy was also applied to the plasma experiment in order to determine the adduction sites on the major plasma proteins adducted. Tryptic peptides were analyzed by LC-MS/MS following affinity purification and photocleavage. The data were searched against tryptic peptides of all the proteins that are identified in Table II using the Monster Mod program. We conclude with high confidence (see supplemental Figs. S4–S17) that more than one site was modified by aHNE in the major proteins shown in Table II. All of the modified peptide residues are unambiguously assigned on the basis of sufficient b and y ions of the modified amino acid residues, as well as measurements of peptide precursor ions within 10 ppm of predicted values with a Thermo Orbitrap.

**DISCUSSION**

Lipid electrophiles generated during oxidative stress play an important role in biology as a consequence of the reaction

| IPI accession no. | 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 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                     | AMBP 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 o-binding protein precursor                               | 12                     | 18              |

**Fig. 4.** Western blots of plasma (500 μl) supplemented with aHNE (100 μM) 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, left top panel is anti-HSA visualized with Alexa Flur 680 donkey anti-goat. B, left bottom panel is anti-ApoA1 visualized with Alexa Flur 680 goat anti-rabbit. C, visualized by a horseradish peroxidase-modified streptavidin. 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 − aHNE can be compared in each case; photoeluted fractions are diluted compared with crude mix.
Peptides of proteins identified in Table I modified by aHNE that are detected using peptide catch and photorelease

All the observe precursor masses are within 10 ppm range of accuracy registered by a Thermo Orbitrap.

### Table II

| IPI accession no. | Protein description | Adducted peptides | Peptide precursor calculated | Peptide precursor found | z | Residue number |
|-------------------|---------------------|-------------------|-----------------------------|-------------------------|---|----------------|
| IPI00021841       | ApoA1               | TH*LAPYSDELR      | 806.9201                    | 806.9237                | 2 | 162            |
|                   |                     |                   | 538.2825                    | 538.2843                | 3 | 162            |
|                   |                     | LEALK*ENGGAR       | 490.2755                    | 490.2772                | 2 | 182            |
|                   |                     | LAEH*AK            | 571.8139                    | 571.8154                | 2 | 193            |
|                   |                     | ATEH*LSTLSEK       | 763.9067                    | 763.9084                | 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*SYPEK    | 646.6667                    | 646.666                 | 3 | 62             |
|                   |                     | SK*EQLTPLIK        | 486.9642                    | 486.964                 | 3 | 69             |
| IPI00022229       | ApoB100             | VLVDH*FGYTK        | 497.2732                    | 497.273                 | 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*INIDOFVR         | 484.9368                    | 484.936                 | 3 | 2101           |
|                   |                     | VH*ELIER           | 402.8995                    | 402.899                 | 3 | 2342           |
|                   |                     | LKOH*IEADVR        | 544.9859                    | 544.983                 | 3 | 2288           |
|                   |                     | SFDIN*IFEK         | 459.5701                    | 459.568                 | 3 | 3207           |
| IPI00021885       | Fibrinogen α-chain precursor | TVIGPDGH*K | 617.8431                    | 617.844                 | 2 | 475            |
|                   |                     | HRH*PEDAAFFDTASTGK | 733.359                     | 733.356                 | 3 | 513            |
|                   |                     | TVTK*TVIGPDGKH     | 555.3174                    | 555.319                 | 3 | 467            |
|                   |                     | ESSSH*HPGIAEPSR    | 650.3219                    | 650.326                 | 3 | 563            |
|                   |                     | DSH*SLTNTIMEILR    | 653.0068                    | 653.007                 | 2 | 103            |
|                   |                     | VQH*IQQLQK         | 473.2889                    | 473.290                 | 3 | 151            |
|                   |                     | SSSSYK*QFTSSSTYNR  | 714.3439                    | 714.346                 | 3 | 581            |
| IPI00304273       | ApoA4               | K*LVPFATELHER      | 584.3332                    | 584.332                 | 3 | 79             |
|                   |                     | LNH*QLEGLTFQM*K    | 629.3329                    | 629.336                 | 3 | 236            |
|                   |                     | LGPH*AGDVEGHLSEFK  | 706.3724                    | 706.372                 | 3 | 332            |
| IPI00032258       | Complement C4-A precursor | SH*ALQLMNR      | 455.2529                    | 455.252                 | 3 | 1342           |
|                   |                     | DK*GQAQLQR         | 428.5737                    | 428.571                 | 2 | 749            |
| IPI00553177       | Alpha-1-antitrypsin precursor | TDSH*HDQDHTPFNK   | 697.6557                    | 697.656                 | 3 | 39             |
|                   |                     | TDSH*HDQDHTPFNK    | 697.6557                    | 697.656                 | 3 | 34             |
|                   |                     | LYT*SEAFTVNFDTTEEAK| 790.3814                   | 790.377                 | 3 | 163            |
|                   |                     | LVD*K*FLEDV        | 506.2922                    | 506.294                 | 3 | 153            |
|                   |                     | LGMF*NIQHC*K       | 506.5916                    | 506.593                 | 3 | 255            |
|                   |                     | QINDYVEK*GTQGK     | 597.6479                    | 597.648                 | 3 | 187            |
|                   |                     | K*QINDYVEK         | 483.2646                    | 483.258                 | 3 | 179            |
|                   |                     | LOHLENELTH*DIITK   | 529.5361                    | 529.540                 | 4 | 293            |
|                   |                     | LOH*LENETHDIITK    | 705.7197                    | 705.722                 | 3 | 286            |
|                   |                     | K*LSSWVLML*K       | 511.3021                    | 511.301                 | 3 | 258            |
| IPI00745872       | Isoform 1 of serum albumin precursor | SLH*TLFGDK    | 443.5789                    | 443.579                 | 3 | 90             |
|                   |                     | RH*PYFYAPELFFAK    | 737.3981                    | 737.404                 | 3 | 169            |
|                   |                     | H*PYFYAPELFFAK     | 685.3644                    | 685.373                 | 3 | 169            |
|                   |                     | LK*C#ASLQK         | 629.863                     | 629.887                 | 2 | 222            |
|                   |                     | LK*C#ASLQK         | 420.2444                    | 420.245                 | 3 | 222            |
|                   |                     | LK-C#ASLQK         | 414.2409                    | 414.242                 | 3 | 222            |
|                   |                     | RH*PDYSWVLILLR     | 593.6807                    | 593.681                 | 3 | 361            |
|                   |                     | H*PDYSWVLILLR      | 541.6547                    | 541.652                 | 3 | 361            |
|                   |                     | K-QTALVELVK        | 474.6291                    | 474.628                 | 3 | 548            |
|                   |                     | NLGK*VGSK          | 557.3355                    | 557.335                 | 3 | 455            |
| IPI00478003       | α-2-macroglobulin precursor | TEH*PFTVEEFVLPK  | 662.0191                    | 662.023                 | 3 | 217            |
|                   |                     | GH*FSISIPVK        | 465.938                     | 465.937                 | 3 | 523            |
| IPI00022426       | AMBP protein precursor | H*HGPTITAK        | 424.9068                    | 424.906                 | 3 | 141            |
of these lipid remnants with nucleic acids and proteins. The protein adducts of these electrophiles are catalogued and characterized with great difficulty because the adducted species make up only a small fraction of the proteome. Indeed, there has been a clear need for strategies that lead to enrichment of adducts so that sets of proteins adducted in a proteome can be characterized, and specific information about the particular sites of modification of those proteins can be provided.

We have approached this problem by employing affinity-tagged substrates analogous to known lipid electrophiles that enable high affinity capture of the protein adducts of those electrophiles (19, 22, 23). One such probe that we have developed is aHNE, an affinity-tag surrogate for the important electrophile 4-HNE. aHNE represents a minimal perturbation of the HNE structure, differing from the natural electrophile by only four hydrogen atoms, see Structures 1 and 2, and it reacts with peptide and protein lysines, histidines and cysteines in a manner similar to HNE itself. Reaction of peptide adducts of aHNE with an azido-biotin give triazoline cycloadducts (24) 3 that can be concentrated by streptavidin affinity techniques (25). Unfortunately, the peptide derivatives of 3 do not consistently give good b and y ion fragmentation that is required for shotgun proteomics.

Another problem that plagues this and many other biotin affinity pull-down experiments is nonspecific binding of proteins and peptides to the streptavidin separation. After complexation of biotinylated proteins or peptides to streptavidin beads, extensive washing of the solid support is carried out to remove any non-biotinylated proteins but despite this, there is always a background of residual protein that accompanies the biotinylated compounds. Release conditions are typically harsh (detergents, heat, organic solvents) and result in release of nonspecifically bound proteins. A reduction of protein and peptide background is therefore a very desirable goal to minimize false positive identifications.

In response to this problem, the click reagent 5 that has a photocleavable linker between the azido functional group and biotin was prepared. Biotinylated protein obtained by the use of this reagent can be immobilized on a streptavidin solid support, and a photochemical reaction can then be used to serve the covalent attachment of protein to the support. The physical means of freeing the protein from the streptavidin complex is unique to those proteins having a terminal alkyne because only those adducts bearing the alkyne are linked to the photolabile group by click chemistry. The photochemical reaction applied is the benzoin photocleavage first studied by Sheehan (26) and applied to several problems by Givens (27). Benzoin esters are stable to visible light but they typically undergo photochemistry in the near UV with high quantum efficiency.

The preparation of photorelease reagent 5 as described in Supporting Information is reasonably straightforward as are the procedures for its use. It is stable to the conditions used in click dipolar cycloaddition, and its photochemistry is clean and appears to be efficient. In this regard, we find no evidence for competing photochemical processes involving the azide functional group in the photo-transformations carried out as described here. Indeed, 5 itself undergoes photolysis to give the benzofuran shown above and azidopentanoic acid in essentially quantitative yield. The phenone absorbs light above 300 nm whereas the azide does not, making phenone photochemistry dominant in the near UV.

The efficacy of the photorelease approach was demonstrated by applying the strategy both to isolated proteins and to a biological fluid (Fig. 5). Application of protein catch and photorelease permits the identification of major proteins adducted by the HNE surrogate in a biological source. This is illustrated by the plasma experiment in which a number of important proteins were identified, the major ones being presented in Table I. The proteins present in the circulating lipoproteins, high density lipoprotein (HDL) and low density lipoprotein (LDL), are among the set of proteins adducted by aHNE, ApoA1 and ApoB100 being two of the lipoprotein constituents identified. Protein catch and photorelease permitted significant enrichment of the target proteins; the tryptic

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**Click Biotin Photocleavable Reagent for Protein Adducts**

| IPI accession no. | Protein description       | Adducted peptides                                                                 | Peptide precursor calculated | Peptide precursor found | z | Residue number |
|-----------------|--------------------------|----------------------------------------------------------------------------------|-----------------------------|------------------------|---|----------------|
| IPI00164623     | Complement C3            | AAVYH*FISDSGV                   | 594.981                    | 594.9813               | 3 | 918            |
|                 |                          | AAVYH*FISDSGV                   | 594.981                    | 594.9809               | 3 | 918            |
| IPI00022488     | Hemopexin                | GH*GHR                           | 437.7357                   | 437.736                | 2 | 238            |
| IPI00022463     | Serotransferrin          | DQAGDVAFVKH*STIFENLANK           | 636.8328                   | 636.8326               | 4 | 226            |
| IPI00555812     | Vitamin D-binding protein precursor | H*LSSLTTLSNR                     | 522.6385                   | 522.6435               | 3 | 208            |
digests of the photoreleased proteins contained sufficient unmodified peptides to identify constituent proteins with high confidence.

One notable observation is that ApoA1 is highly modified with lipid peroxidation products including HNE (Fig. 4). ApoA1, a principal constituent of HDL, plays an important role in the transport of cholesterol from peripheral tissues to the liver and recent studies have provided evidence that modification of ApoA1 by electrophilic species leads to loss of HDL function (28–31). HDL functional impairment also occurs in cardiovascular disease and for that reason we chose to inspect HNE adducted ApoA1 in greater detail.

In order to evaluate the biological impact resulting from protein modification, it is important to know the sites of modification on the protein. Application of the protein catch and release strategy identifies the proteins that are adducted, but does not address the challenging task of mapping the sites of modification on those proteins. Peptide catch and photorelease allow for further enrichment of the adducts at the peptide level, leading to enhanced sensitivity in LC-MS/MS analysis.

In the plasma experiment, a number of peptides were modified by HNE, and these adducts are summarized in Table I. ApoA1 HNE adducts have been previously detected in reactions of purified HDL with HNE and the four of the six ApoA1 peptides identified in Table II were found earlier in those experiments (24). The peptide catch and photorelease strategy not only finds additional peptides, but also permits isolation and identification of specific protein modifications in a complex mixture of proteins, of which ApoA1 is only a part, i.e. in native plasma.

Modification of ApoA1 as noted in Table II at histidine residues 162, 182, 193, 199, 208, and 239 could well have a significant impact on the function of the protein. Histidine 162 is found in an exposed region in the LCAT activating loop corresponding to residues 159–180 whereas histidines 193 and 199 are located in a recognition region of the protein for the receptor ABCA1, present in peripheral tissues. Of particular note is the fact that His-162 functions in a critical salt-bridge in the LCAT activation loop (30) and among the dozens of reported missense mutations of human apoA1, many of the mutations are clustered in this group.

The approach presented in these studies provides a solution to the problem of protein adduct enrichment that provides not only information about the set of proteins adducted in a proteome, but also the particular sites of modification of those proteins. This affinity purification-photorelease strategy may be extended to any alkynyl probes that covalently modify proteins and thus may prove to be generally useful in a variety of proteomic analysis applications.

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□ The on-line version of this article (available at http://www.mcponline.org) contains supplemental material.
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