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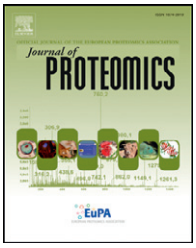

A comparative 'bottom up' proteomics strategy for the site-specific identification and quantification of protein modifications by electrophilic lipids

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ABSTRACT

We report a mass spectrometry-based comparative "bottom up" proteomics approach that combines d_0/d_4-succinic anhydride labeling with commercially available hydrazine (Hz)-functionalized beads (Affi-gel Hz beads) for detection, identification and relative quantification of site-specific oxylipid modifications in biological matrices. We evaluated and applied this robust and simple method for the quantitative analysis of oxylipid protein conjugates in cardiac mitochondrial proteome samples isolated from 3- and 24-month-old rat hearts. The use of d_0/d_4-succinic anhydride labeling, Hz-bead based affinity enrichment, nanoLC fractionation and MALDI-ToF/ToF tandem mass spectrometry yielded relative quantification of oxylipid conjugates with residue-specific modification information. Conjugation of acrolein (ACR), 4-hydroxy-2-hexenal (HHE), 4-hydroxy-2-nonenal (HNE) and 4-oxo-2-noneal (ONE) to cysteine, histidine and lysine residues were identified. HHE conjugates were the predominant subset of Michael-type adducts detected in this study. The HHE conjugates showed higher levels in mitochondrial preparations from young heart congruent with previous findings by others that the n−3/n−6 PUFA ratio is higher in young heart mitochondrial membranes. Although this study focuses on protein adducts of reactive oxylipids, the method might be equally applicable to protein carbonyl modifications caused by metal catalyzed oxidation reactions.

1. Introduction

Mass spectrometry-based proteomics enables the identification and relative quantification of hundreds of proteins in complex biological matrices. Although progress has been made in the identification of post-translationally modified proteins, the site-specific assignment of distinct protein modifications remains a major analytical challenge. Successful strategies for site-specific mapping of post-translational modifications (PTMs) combine protein chemistry, bioaffinity strategies and LC-MS/MS techniques [1–4]. Beside the classical key PTMs, phosphorylation and glycosylation, oxidative protein

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modifications have emerged as significant modulators of redox homeostasis and signaling, and are considered as emerging markers of oxidative stress insult in numerous chronic diseases and age-associated pathological conditions [5–8].

Protein carbonylation can be the result of insult by a wide variety of reactive oxygen species (ROS) and electrophilic lipids [9]. Electrophilic aldehydes, such as 2-alkenals and 4-hydroxy-2-alkenals, are derived from lipid peroxidation processes [10,11]. The best studied electrophilic keto-containing lipids are prostanoids with cyclopentenone structure [12]. These α,β-unsaturated carbonyl-containing compounds are highly reactive toward nucleophilic amino acid residues (cysteine, histidine, and lysine) and predominately form Michael-type rather than Schiff-base adducts [13–20]. The accumulation of oxidatively modified proteins in cells, tissue and bodily fluids has been discussed as a reflection of the severity of oxidative stress under normal and diseased conditions. Elevated levels of oxidatively modified proteins have been reported to be present in various tissues in the context of numerous age-associated diseases and aging [21–23]. We and others propose that oxidative protein modifications may play an important role in the molecular mechanisms that contribute to mitochondrial dysfunction and the development of age-related cardiovascular diseases [24–26].

Oxidative modifications of proteins are difficult to characterize and quantify in biological samples due to the complexity of protein extracts, the low abundance of the modified proteins, and the absence of distinguishing UV or visible spectrophotometric absorbance/fluorescence properties. Instead, detection and quantification of carbonylated proteins require the use of specific chemical probes, which include 2,4-dinitrophenylhydrazine (DNPH) [27], tritiated sodium borohydride [27–29], and fluorescence probes [30,31]. More recently, chemical proteomic approaches have emerged that combine chemoselective affinity labeling of protein carbonyls and mass spectrometry for the identification of protein targets of oxidative modifications. These approaches allow the assignment of the modification site in favorable cases. We and others have introduced strategies that employ biotin-containing probes that target the aldehyde/keto group specific to oxidatively modified proteins [32–34]. In addition, capture-release solid-phase-based approaches have been developed, in which hydrazide-functionalized glass beads were synthesized and used for the enrichment of 4-HNE conjugated peptides [35–37]. Quantification or relative assessment of levels of oxidative protein modifications is achieved by combining chemical labeling with stable-isotope labeling approaches [36,38].

An alternative to hydrazide-functionalized glass beads is commercially available Affi-Gel® hydrazide (Hz)-functionalized agarose beads. This hydrazide-functionalized resin has been used previously for the identification and quantification of N-linked glycoproteins in conjunction with isotope-coded succinylation and tandem mass spectrometry [39]. Here we describe the applicability of this strategy for the analysis of Michael-type protein adducts of α,β-unsaturated aldehydic lipids in complex mixtures. We applied this comparative profiling method to the analysis of protein–oxylipid conjugates present in mitochondria from young and old rat heart.

2. Materials and methods

2.1. Materials

4-Hydroxy-2-nonenal was purchased from Cayman Chemicals Inc., Ann Arbor, MI. Succinic-d_0 anhydride and succinic-d_4 anhydride were purchased from Sigma-Aldrich (St. Louis, MO) and ISOTEC (Miamisburg, OH), respectively. Handee™ Spin columns were obtained from Pierce Biotechnology (Rockford, IL). *Escherichia coli* thioredoxin (TRX) and sequencing grade-modified trypsin were purchased from Promega Corporation, Madison, WI. α-Cyano-4-hydroxycinnamic acid was from Sigma Chemicals (St. Louis, MO). The Affi-Gel® Hz (Hydrazide) gel (product # 1536047) was obtained from BIO-RAD (Hercules, CA). According to the manufacturer's specification the Affi-gel Hz gel loading is greater than or equal to 10 μmol of Hz per mL of Affi-gel Hydrazide gel.

2.2. HNE-modification and succinic anhydride-labeling of *E. coli* thioredoxin followed by Affi-Gel Hz enrichment

Thioredoxin (1 mg/mL in 10 mM sodium phosphate, pH 7.4) was reacted with a 10-fold molar excess of HNE for 3 h at 37 °C. Excess reagent was removed by ultrafiltration (5 kDa MWCO, Amicon Ultrafree-MC, Millipore, Billerica, MA). The modified protein reaction mixture was subsequently digested with trypsin overnight (E:S=1:50, 37 °C). The digest was passed through an ultrafiltration unit and peptides below 5 kDa were collected. Succinic anhydride in acetonitrile was added to a final concentration of 5 mg/mL and the sample was incubated at 37 °C for 2 h.

Affi-Gel® Hz beads were washed in Handee™ Spin columns with 100 mM sodium phosphate pH 4.5, and reacted with peptides for 2 h at 37 °C with gentle shaking. For these experiments, the peptide:gel ratio ranged from 1 to 4 (by weight) and the total reaction volume was 2–4 times the gel volume. The mixture was subsequently rinsed 4 times each in 100 mM NH_4HCO_3, 30% acetonitrile, and H_2O, using 2 times the gel bed volume per rinse. The mixture was subsequently incubated in 2% formic acid, 40% acetonitrile for 1 h at 37 °C to release hydrazone-linked peptides. Released peptides were collected and the elution step was repeated. These fractions were combined and lyophilized prior to MALDI-MS/MS analysis.

2.3. Detection and quantification of oxylipid conjugates in mitochondrial proteome samples

Rat heart mitochondria were isolated according to Suh et al. [40]. Cardiac mitochondrial samples from three 3-month and three 24-month old male Fischer 344 rats were mixed, and disrupted by several freeze–thaw cycles. Soluble protein fractions were obtained by centrifugation. Protein concentrations were determined by using Coomassie Plus™ protein assay reagent. Aliquots of the mitochondrial proteins (350 μg each) were digested with trypsin in 0.1 M sodium phosphate buffer (pH 8.0) at 37 °C for 16 h prior to ultrafiltration (Amicon Ultrafree-MC centrifugal filter, 10 kDa MWCO, Millipore, Billerica, MA). The d_0-and d_4-succinic anhydride solution was added respectively to the aliquots of digested peptides in a final concentration of