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Site-specific proteomic analysis of lipoxidation adducts in cardiac mitochondria reveals chemical diversity of 2-alkenal adduction

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ABSTRACT

The modification of proteins by lipid peroxidation products has been linked to numerous diseases and age-related disorders. Here we report on the identification of endogenous protein targets of electrophilic 2-alkenals in cardiac mitochondria. An aldehyde/keto-specific chemical labeling and affinity strategy in combination with LC–MS/MS resulted in 39 unique lipoxidation sites on 27 proteins. Several of the target sites were modified by a variety of 2-alkenal products including acrolein, β-hydroxyacrolein, crotonaldehyde, 4-hydroxy-2-hexenal, 4-hydroxy-2-nonenal and 4-oxo-2-nonenal. Many of the adduction sites are implicated in the catalytic function of key mitochondrial enzymes suggesting potential impact on pathways and overall mitochondrial function.

1. Introduction

Since the 1950s when Harman's "free radical theory of aging" was introduced there have been a considerable number of studies focused on understanding the molecular mechanisms of oxidative stress and its impact on living systems [1,2]. It is now widely accepted that oxidative stress plays a role in an increasing number of human health disorders and the aging process [3–9]. Within the rapidly advancing field of proteomics, several efforts have been devoted to identifying chemical changes to the proteome in relation to oxidative stress [10–15].

Mitochondria are central to studies of oxidative stress, as they are known to be generators of reactive oxygen species (ROS) within the cell and play a pivotal role in cell death [7,8,16,17]. There are a number of ROS producing sites within the mitochondria including Complex I and Complex III of the mitochondrial electron transport chain [18,19]. Because the ROS are generated in close proximity to the mitochondrial membrane, which is rich in polyunsaturated fatty acids, a whole host of reactive lipid peroxidation products are formed. These lipid peroxidation products are relatively long lived compared to the highly reactive radical oxygen species which led to their formation, allowing them to diffuse further from their site of production to potentially react with proteins and other biomolecules located in the inner membrane space and mitochondrial matrix. Among the most abundant and studied of these lipid peroxidation products are the highly electrophilic α,β-unsaturated aldehydes, such as 4-hydroxy-2-nonenal (HNE), 4-oxo-2-nonenal (ONE), 4-hydroxy-2-hexenal (HHE), and acrolein [20,21].

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A number of techniques exist for measuring oxidative modifications to proteins and other biomolecules. Among the most widely used of these techniques are assays based on the use of either 2,4-dinitrophenylhydrazine (DNPH) or thiobarbituric acid (TBARS) derivatization for protein carbonyl determinations. While these methods are able to provide researchers with a general overview of levels of protein carbonyls present, they fail to provide specific information such as the identity of the modified proteins, and the site and chemical nature of the modification. In addition to the traditional colorimetric and immunochemical-based methods of analysis, mass spectrometry has emerged as a powerful technique for studying protein carbonylations [22–24]. Towards the goal of identifying the sites of protein carbonyls in proteins, multiple methods involving chemical derivatization followed by mass spectrometric detection have been developed. Chemical tools that have been employed to label and detect carbonyl sites in proteins include biotin-based hydrazide-functionalized reagents, Girard's P reagent, and Click probes [25–27]. We recently demonstrated the use of a hydroxylamine-functionalized biotin derivative, i.e. N′-amino-oxymethylcarbonylhydrazino-D-biotin, for selective labeling of aldehydic proteins and subsequent mass spectrometry-based profiling studies of complex biological matrices [13].

Numerous *in vitro* studies have demonstrated that α,β-unsaturated aldehydes, due to their electrophilic nature, covalently modify proteins at nucleophilic sites, in particular at cysteine, histidine and lysine residues [28–30]. Several recent studies have been devoted to identifying the protein targets of lipid-derived electrophiles, in particular targets of HNE [31–33]. While there has been much success in identifying protein targets from *in vitro* exposure studies, there has been only a few studies reported attempting the site-specific identification of endogenous oxidative modifications in complex proteome samples [24,34]. Here we report the use of a chemical aldehyde/keto-specific labeling and affinity enrichment strategy in combination with LC–MS/MS that enabled the site-specific identification of major sites and the concomitant detection of multiple endogenous 2-alkenal modifications to mitochondrial proteins.

2. Materials and methods

2.1. Chemicals

N′-aminooxymethylcarbonyl hydrazino-D-biotin (Aldehyde Reactive Probe, ARP) was purchased from Dojindo Molecular Technologies Inc. (Rockville, MD). Ultralink Monomeric Avidin, Zeba Spin Desalting columns, SuperSignal West Pico Chemiluminescent substrate were purchased from Thermo Scientific (Rockford, IL). Neutravidin-HRP was purchased from Pierce. Sequencing-grade modified trypsin was purchased from Promega (Madison, WI). Ready Gels (12% Tris–HCl) were purchased from Bio-Rad. Immobilon-P PVDF membranes were purchased from Millipore.

2.2. Preparation of rat cardiac mitochondria

The Institutional Animal Care and Use Committee (IACUC) at Oregon State University approved the experimental protocol for the use of animals in this study. Male Fischer 344 rats, obtained from the National Institute of Aging (Bethesda, MD), were housed in individual plastic cages covered with Hepa filters and allowed free access to food and water. Rats were anesthetized with diethyl ether and a midlateral incision was made in the chest to remove the heart. Subsarcolemmal mitochondria were isolated from the rat hearts by differential centrifugation according to the procedures of Palmer with minor modifications by Suh et al and stored at −80 °C until needed [35,36]. Mitochondrial samples containing approximately 1 mg total protein were washed twice with 0 °C 10 mM NaH_2PO_4, pH 7.4. The mitochondria were then re-suspended in 200 μL of 10 mM ice-cold NaH_2PO_4, pH 7.4. The mitochondria were ruptured by four rounds of freeze/thaw cycles which involved rapid freezing with liquid N_2 followed by thawing in a cold water bath with 10 min of sonication. Soluble and membrane proteins were separated by centrifugation at 14,000 *g* for 15 min. Pierce Coomassie Plus protein assay was used to determine protein concentrations.

2.3. ARP-labeling of mitochondrial proteins

Labeling of mitochondrial proteins with the ARP was accomplished by simultaneously adjusting the protein concentration to ~1 μg/μL and the ARP concentration to 5 mM in 10 mM NaH_2PO_4, pH 7.4. The mixture was then allowed to react for up to 1 h at room temperature. Excess ARP was removed using Zeba desalting spin columns with a molecular weight cut-off of 7 kDa. Protein samples were digested with a 1:50 w/w ratio of modified trypsin in 100 mM NH_4HCO_3, pH 8.3, for 6–18 h at 37 °C.

2.4. SDS-PAGE and Western blotting

For 1D SDS-PAGE analyses Bio-Rad 12% Tris–HCl 'readymade' gels were used. Mitochondrial protein samples (20 μg per well) was used and gels were run at a constant voltage of 130 V for 60 min. For visualization of the protein bands the gels were incubated with Biosafe Coomassie dye for 1 h. Western blotting was accomplished by transferring the proteins from the gel to a PVDF membrane by applying a constant current of 150 mA for 2 h. The blot was then blocked with 5% milk in TBS buffer with 0.1% Tween-20 and then washed extensively before being incubated with 40 ng/mL of HRP-NeutrAvidin for 1 h. After additional wash steps the gel was exposed to the PicoWest chemiluminescent substrate for 10 min and developed using Kodak BioMax X-ray film.

2.5. Affinity enrichment of ARP-labeled peptides

Ultralink monomeric avidin (100–300 μL) was packed into Handee Mini Spin columns which accommodate solvent addition with a Luer Lok syringe. The column was washed with 1.5 mL of 10 mM NaH_2PO_4, pH 7.4. Irreversible binding sites were blocked by washing with three column volumes of 2 mM D-biotin. To remove excess D-biotin the column was washed with five column volumes of 2 M glycine–HCl, pH 2.8. The column was then re-equilibrated by washing with 2 mL of 2× PBS (20 mM NaH_2PO_4, 300 mM NaCl). The mitochondrial peptide sample was then slowly added to the affinity column and the flow-through collected. To remove non-labeled and