JOURNAL OF PROTEOMICS 74 (2011) 2395–2416

available at www.sciencedirect.com

www.elsevier.com/locate/jprot

Oxidative stress induced carbonylation in human plasma

*Ashraf G. Madian, Naomi Diaz-Maldonado, Qiang Gao, Fred E. Regnier**

Department of Chemistry, Purdue University, West Lafayette, IN 47907, USA

ARTICLE INFO

Article history:
Received 25 March 2011
Accepted 13 July 2011
Available online 30 July 2011

Keywords:
Oxidative stress
Carbonylation
Breast cancer
Biotin hydrazide
iTRAQ™
Knowledge assembly

ABSTRACT

The focus of this study was on the assessment of technology that might be of clinical utility in identification, quantification, characterization of carbonylation in human plasma proteins. Carbonylation is widely associated with oxidative stress diseases. Breast cancer patient samples were chosen as a stress positive case based on the fact that oxidative stress has been reported to be elevated in this disease. Measurements of 8-isoprostane in plasma confirmed that breast cancer patients in this study were indeed experiencing significant oxidative stress. Carbonyl groups in proteins from freshly drawn blood were derivatized with biotin hydrazide after which the samples were dialyzed and the biotinylated proteins subsequently selected, digested and labeled with iTRAQ™ heavy isotope coding reagent(s). Four hundred sixty proteins were identified and quantified, 95 of which changed 1.5 fold or more in concentration. Beyond confirming the utility of the analytical method, association of protein carbonylation was examined as well. Nearly one fourth of the selected proteins were of cytoplasmic, nuclear, or membrane origin. Analysis of the data by unbiased knowledge assembly methods indicated the most likely disease associated with the proteins was breast neoplasm. Pathway analysis showed the proteins which changed in carbonylation were strongly associated with Brca1, the breast cancer type-1 susceptibility protein. Pathway analysis indicated the major molecular functions of these proteins are defense, immunity and nucleic acid binding.

1. Introduction

Pathological levels of oxidative stress (OS) have been implicated in a plethora of diseases ranging from *diabetes mellitus*[1] and neurodegenerative diseases (Alzheimer's disease [2], Parkinson's disease[3], and amyotrophic lateral sclerosis[4]) on to inflammatory diseases (atherosclerosis[5] and chronic lung disease[6]), cancer, and aging[7–9]. At the protein level, excessive OS leads to the oxidation of proteins in 35 or more ways, one of the more prominent being carbonylation [9,10]. Carbonyl groups can be introduced into proteins either by i) direct oxidation of Pro, Arg, Lys, Thr, Glu, or Asp side chains or oxidative cleavage of the protein backbone, ii) introduction of 4-hydroxy-2-nonenal (HNE), 2-propenal or malondialdehyde from lipid peroxidation to a Cys, His or Lys residue, or iii) by formation of advanced glycation end-product adducts.

Given the prominent status of OS in so many diseases it would seem there should be numerous reports of increases in oxidized plasma proteins with disease progression. Surprisingly only alterations in isoforms of fibrinogen γ-chain precursor protein and of α-1-antitrypsin precursor were reported in studies related to heart failure [11] and Alzheimer's disease [12]. Perhaps the small number of proteins observed in these studies is due to lack of detection sensitivity. After derivatization of carbonylated proteins with 2,4-dinitrophenylhydrazine (DNP), samples were separated by two dimensional gel electrophoresis (2-DE) and the oxidized proteins detected by Western blotting using an anti-DNP anti-

* *Corresponding author at: WTHR 155/BIND 112.* Tel.: +1 765 494 3878.
E-mail address: fregnier@purdue.edu (F.E. Regnier).

1874-3919/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.jprot.2011.07.014

body. The problem with this approach is that 2-DE suffers from low reproducibility, poor quantification, and limited dynamic range[13].

Preliminary studies with 32–36 year old human male subjects have shown that numerous oxidized proteins from a wide variety of cell types and organs are shed or released into plasma [10]. The objective of the work reported here was to explore the possibility that methods could be developed that measure the levels of oxidative stress induced post-translational modifications (OSi~PTMs) in blood proteins and identify the molecular function of these proteins or the biological processes with which they are associated. This was achieved at the analytical level by adapting highly selective proteomics methods that have been used with yeast [14], normal rat plasma [15], and normal human plasma [10] for the identification of carbonylated proteins [16-18].

Breast cancer was chosen as the source of oxidative stress for this study based on strong evidence that reactive oxygen species (ROS) play an important role in tumorigensis [19,20]. Overproduction of ROS and the associated OS have been reported to occur in several ways. One is by enhanced expression of enzymes such as thymidine phosphorylase and lactoperoxidase that elevate ROS production from within the tumor [21]. Another is by extracellular production of ROS through macrophage recruitment. Additionally, extracellular ROS entering tumor cells [21] are known to oxidatively damage mitochondria, nuclear DNA, ribosomal RNA, intercellular proteins, and lipids [22]. This damage further stimulates uncontrolled growth, ischemia, and glucose deprivation followed by a reduction in neovascularization and the production of even more OS [22]. Two recent studies have shown that the total concentration of carbonylated plasma proteins is strongly connected to breast cancer risk [23,24].

Breast cancer patients and cancer free subjects in this study were compared to determine whether the elevated levels of OS occurring in the tumor impacted levels of oxidized plasma proteins. Qualitative and quantitative differences in putatively oxidized proteins found in the plasma of six breast cancer patients and matched controls were examined. This was achieved using a protocol recently described in OS studies of human plasma [10]. Biotin hydrazide (BH) was added to freshly prepared plasma samples derived from research subjects to derivatize carbonyl groups in oxidized proteins. The resulting Schiff bases were reduced with sodium cyanoborohydride and the samples were dialyzed to remove free BH. Biotinylated proteins were selected from plasma samples by avidin affinity chromatography and then trypsin digested. This proteolytic digest was then further fractionated by reversed phase chromatography and the peptides identified and quantified by tandem mass spectrometry.

2. Materials and methods

2.1. Materials

Sodium cyanoborohydride, biotin hydrazide (BH), ultralinked immobilized monomeric avidin, D-biotin, protein A/G chromatography cartridges, UltraLink Biosupport™, Slide-A-Lyzer™ dialysis cassettes, biotinylated alkaline phosphatase, biotinylated horseradish peroxidase, biotinylated protein A and biotinylated protein G were purchased from Pierce (Rockford, IL, USA). Iodoacetamide, dithiothreitol (DTT), glycine, α-cyano-4-hydroxy-cinnamic acid (CHCA), proteomics grade N-p-tosyl-phenylalanine chloromethyl ketone (TPCK)-treated trypsin, ammonium bicarbonate, guanidine, and L-cysteine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Complete, Mini, EDTA-free, protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Indianapolis, IN, USA). The ABI 4700 Proteomics Analyzer Calibration Mixture (4700 Cal Mix, bradykinin, angiotensin I, glu1-fibrinopeptide B, ACTH fragment 1–17, ACTH fragment 18–39, and ACTH fragment 7–38) and iTRAQ™ reagent multiplex kit were purchased from Applied Biosystems (ABI, Foster City, CA). Trifluoroacetic acid (TFA), and HPLC grade acetonitrile were purchased from Mallinckrodt Chemicals (Phillipsburg, NJ). OxiSelect™ 8-iso-Prostaglandin F2a Activity Assay Kit was purchased from Cell Biolabs Inc. (San Diego, CA, USA). BD Vacutainer™ venous blood collection tubes with EDTA were purchased from Fisher Scientific (Hanover Park, IL, USA). Sodium phosphate, sodium chloride and formic acid 88% were purchased from Mallinckrodt (St. Louis, MO, USA). Amicon Ultra-4™ centrifugal filter devices were purchased from Millipore (Billerica, MA, USA).

2.2. Plasma samples

Blood sample collection was carried out as part of the program on Clinical Proteomics Technology Assessment for Cancer (CPTAC) sponsored by NCI using protocols approved by Institutional Review Boards at UCSF and Purdue University. Samples were collected and derivatized at the UCSF. Breast cancer patient plasma samples were derived from newly diagnosed female subjects (five stage I and one stage II subjects) before any type of therapeutic intervention. The blood was also withdrawn before any breast biopsies performed to the donors. Six breast cancer patients and six normal matched controls donated blood (Table 1).

2.3. Biotinylation of plasma samples

Freshly drawn blood samples were collected in BD Vacutainer™ venous blood collection tubes coated with EDTA (Fisher Scientific, Hanover Park, IL, USA). A mixture of inhibitors for cysteine and serine proteases was added to inhibit intrinsic endoproteases as has been recently reported [25]. Generally, each tablet of the protease inhibitor was dissolved in 1 ml of distilled water. The protease inhibitor solution was mixed with plasma in a 1:10 ratio (v/v) respectively. Because plasma samples were maintained at neutral pH during biotinylation and affinity selection there was no need to inhibit aspartate protease along with proteases that are only active at acidic pH. Carbonylated proteins form Schiff bases with lysine residues on other proteins during storage, even at −80 °C. This means that carbonyl content will decline during storage and blood samples must be derivatized with biotin hydrazide (BH) before storage or transport. BH was added to plasma samples immediately after initial plasma preparation. Therefore, after centrifugation at 1500 ×*g* for 15 min, the supernatant was removed, and then centrifuged a second time at 2000 ×*g* for 15 min. Immediately