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Identification of the oxidative stress proteome in the brain

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ABSTRACT

The redox proteomics technique normally combines two-dimensional gel electrophoresis, mass spectrometry, and protein databases to analyze the cell proteome from various samples, thereby leading to the identification of specific targets of oxidative modification. Oxidative stress that occurs because of increased levels of reactive oxygen species and reactive nitrogen species can target most biomolecules, consequently leading to altered physiological function of the cells. Redox proteomics has identified oxidatively modified protein targets in various pathological conditions, consequently providing insight into the pathways involved in the pathogenesis of these conditions. This approach also can be used to identify possible protective mechanisms to prevent or delay these disorders.

Introduction

Proteomics is the science involved in identifying the proteome of a cell [1,2]. With advances in technology a number of approaches have been developed to assess the cell proteome. Proteomics most commonly uses two-dimensional gel electrophoresis as the major mechanism of separation, though new methods of separation have been developed, such as 2D–high-performance liquid chromatography (2D-HPLC) and isotopically coded affinity tags [3,4]. One of the oldest and most successful techniques that are employed in proteomics is 2D gel electrophoresis coupled to mass spectrometry [5]. Unlike the human genome, which consists of about 30,000 genes (http://www.ncbi.nlm.nih.gov/genome/guide/Human/), the proteome contains several hundred thousand proteins. This diversity in the number of proteins can be explained based on alternative splicing and posttranslational modifications (PTMs). A large number of PTMs, among which are reversible modifications of proteins, such as phosphorylation, carbonylation, nitration, acetylation, and glycosylation [6], have been identified. Some PTMs, such as oxidation of cysteine to sulfonic acid, cause irreversible modification of proteins. Proteomics not only identifies proteins, but also determines post-translational modifications, localization, interactions, function, and expression.

In the case of oxidatively modified proteins, redox proteomics [7] is used as a tool to identify such proteins, i.e., proteins that are modified by reactive oxygen and reactive nitrogen species (ROS/RNS). Increased levels of ROS/RNS or decreased levels of antioxidant enzymes lead to a condition called oxidative/nitrosative stress. ROS and RNS can react with biomolecules such as proteins, lipids, carbohydrates, and nucleic acids, leading to oxidative modification [8–17]. Oxidative modification of proteins, for example, leads to modification of the structure and consequently either gain or loss of function of proteins. Oxidative modification of proteins thereby plays an important role in physiological and pathological conditions [18,19]. Oxidative stress has been shown to be one of the mechanisms involved in a number of diseases, including neurodegenerative disorders, ischemia, cancer, etc.

In this paper, we discuss the approach that is most commonly used to identify the protein oxidation markers. Protein oxidation is most commonly indexed by the amount of protein carbonyls and protein-bound 3-nitrotyrosine (3-NT) [20]. Protein carbonyls are produced by backbone fragmentation, hydrogen atom abstraction at α carbons, oxidant attack on several amino acid side chains (Lys, Arg, Pro, Thr,

Abbreviations: 2D–HPLC, 2D–high-performance liquid chromatography; 2D–PAGE, two-dimensional polyacrylamide gel electrophoresis; 3-NT, 3-nitrotyrosine; AD, Alzheimer disease; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; BSA, bovine serum albumin; DNP, 2,4-dinitrophenylhydrazone; DNPH, 2,4-dinitrophenylhydrazine; DTT, dithiothreitol; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; HNE, 4-hydroxy-2-*trans*-nonenal; IA, iodoacetamide; IEF, isoelectrofocusing; MS, mass spectrometry; NBT, nitroblue tetrazolium; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; PTM, posttranslational modification; RNS, reactive nitrogen species; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.

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etc.), or formation of Michael adducts between His, Lys, and Cys residues and reactive alkenals (e.g., 4-hydroxy-2-*trans*-nonenal (HNE)) [19]. In addition, glycation/glycoxidation of Lys amino groups, forming advanced glycation end products [7,19–22], can also lead to formation of protein carbonyls. As noted, protein carbonyls are widely used as a marker to assess the extent of oxidation of proteins under both in vivo and in vitro conditions [7,20,22]. 3-NT, the ultimate product of $ONOO^-$-mediated radical formation on tyrosine residues, is another protein oxidation marker [9,23]. Protein nitration is a reversible and selective process that sometimes serves as a cellular signaling mechanism, similar to protein phosphorylation. In a neurodegenerative disease such as Alzheimer disease (AD), mitochondrial abnormalities occur [24], associated with leakage of $O_2^{\bullet-}$, which coupled to $NO^{\bullet}$ leads to increased formation of highly reactive peroxynitrite. As noted above, $ONOO^-$ in the presence of CO_2 can act on various amino acids such as cysteine, methionine, tryptophan, phenylalanine, and tyrosine, which are particularly susceptible to nitration. A number of studies support the notion that nitrosative stress also contributes to disease, for example neurodegeneration in AD [7,10,14,17,25].

One of the products of lipid peroxidation, HNE [26], can covalently modify cysteine, lysine, or histidine residues by Michael addition [19,26]. HNE causes membrane structural damage, changes conformation of proteins, produces diffusible secondary bioactive aldehydes, and induces cell death in many cell types [12,27–34]. In AD subjects, the levels of free and protein-bound HNE were found to be significantly increased in brain, plasma, cerebrospinal fluid, etc., compared with control subjects [29,35].

Our laboratory was the first to use redox proteomics to identify brain protein targets of oxidation in AD [36,37]. Using redox proteomics, our laboratory also identified changes in brain protein carbonyls, HNE adducts, glutathionylation, and the nitration of tyrosine residues in AD, mild cognitive impairment, and models of AD, Huntington disease, amyotrophic lateral sclerosis, and Parkinson disease [10,17,36–42]. Table 1 shows carbonylated, HNE-bound, and 3-NT proteins that were identified in AD brain using redox proteomics approaches [10,17,35–37,42–44]. The increase in the specific oxidation of proteins identified by proteomics agrees with previous studies that showed an increase in the total levels of oxidative stress in AD brain [19,45,46], and the use of redox proteomics showed enolase as a common target of oxidative modification among protein carbonyls, 3-NT, and protein-bound HNE in AD, suggesting that the brain shows specific patterns of protein-oxidative PTMs in AD. As seen in Table 1, redox proteomics led to the identification of a number of brain proteins that regulate glucose metabolism as being oxidatively altered in AD, consistent with results from positron emission tomography studies showing decreased glucose utilization reported in AD brain [10,16,17,35–37,42,44]. Further, redox proteomics studies in AD brain led to the identification of peptidylprolyl-*cis*/*trans*-isomerase, a protein that plays an important role in regulating the function of amyloid precursor protein and tau protein, consequently potentially contributing to AD pathology [42,47]. Other proteomics studies [21,43,48], in addition to those from our laboratory, have identified oxidatively modified proteins, consistent with reported oxidative stress in neurodegenerative diseases [14,49,50].

The combination of two-dimensional polyacrylamide gel electrophoresis (2D–PAGE), mass spectrometry (MS), and protein databases makes proteomics a powerful tool (Fig. 1). However, this technique has a number of limitations including: (a) solubilization of membrane proteins, because the ionic detergents used for solubilization of such proteins can interfere with the isoelectric focusing process; (b) the mass range and detection limits, which represent technical limitations of the method; and (c) proteins with high Lys/Arg content (which produce very low molecular weight tryptic peptides). Our laboratory and many others are trying to overcome these issues by using chaotropic agents, subcellular 2D gel electrophoresis, methods to concentrate the proteome being investigated, etc. High-throughput proteomic techniques, such as HPLC, are also available to separate proteins without 2D electrophoresis [51]. However, the application of these techniques in redox proteomics is still relatively new, and more development in these techniques is needed.

Principles

Protein carbonyls

Protein carbonyls most often are detected by two methods, i.e., derivatization with 2,4-dinitrophenylhydrazine (DNPH), followed by immunochemical detection with an antibody against the resulting protein hydrazone, or formation of the Schiff base by biotin–hydrazide followed by detection of the protein-bound imine with enzyme- or fluorophore-linked avidin or streptavidin. Here we describe the DNPH-based detection method that is routinely used for detection of carbonylated proteins. In the DNPH method, samples are derivatized with DNPH. DNPH reacts with carbonyl groups to form protein-resident 2,4-dinitrophenylhydrazone (DNP; Fig. 2), which can be detected using commercially available anti-DNP antibodies.

Table 1
Functional categorization of oxidatively proteins identified in AD

Protein function	Carbonylation[a]	HNE bound[b]	Nitration[c]
Energy dysfunction	Creatine kinase, **α-enolase**, triose phosphate isomerase, phosphoglycerate mutase 1	**α-Enolase**	**α-Enolase**, γ-enolase, lactate dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, triose phosphate isomerase
Mitochondrial dysfunction		ATP synthase, Mn superoxide dismutase	ATP synthase, voltage-dependent anion channel protein 1
Antioxidant defense/detoxification dysfunction		Peroxiredoxin VI, Mn superoxide dismutase	
Cell cycle; Aβ production; tau phosphorylation	Peptidylprolyl-*cis*/*trans*-isomerase		
Lipid abnormalities and cholinergic dysfunction	Neuropolypeptide h3		
Neuritic abnormalities and structural dysfunction	DRP2, β-actin, γ-SNAP	DRP2, α-tubulin	DRP2, β-actin
Excitotoxicity	Excitatory amino acid transporter, glutamine synthetase	Glutamine synthetase	
pH dysfunction	Carbonic anhydrase II		Carbonic anhydrase II
Protein degradation	UCHL 1, heat shock protein		

Protein in bold is a common target of the various oxidative modifications examined.
[a]For carbonylated proteins identified in AD please see Refs. [36,37,42].
[b]For these HNE-bound proteins identified in AD please see Ref. [35].
[c]For these nitrated proteins identified in AD please see Ref. [10,17].

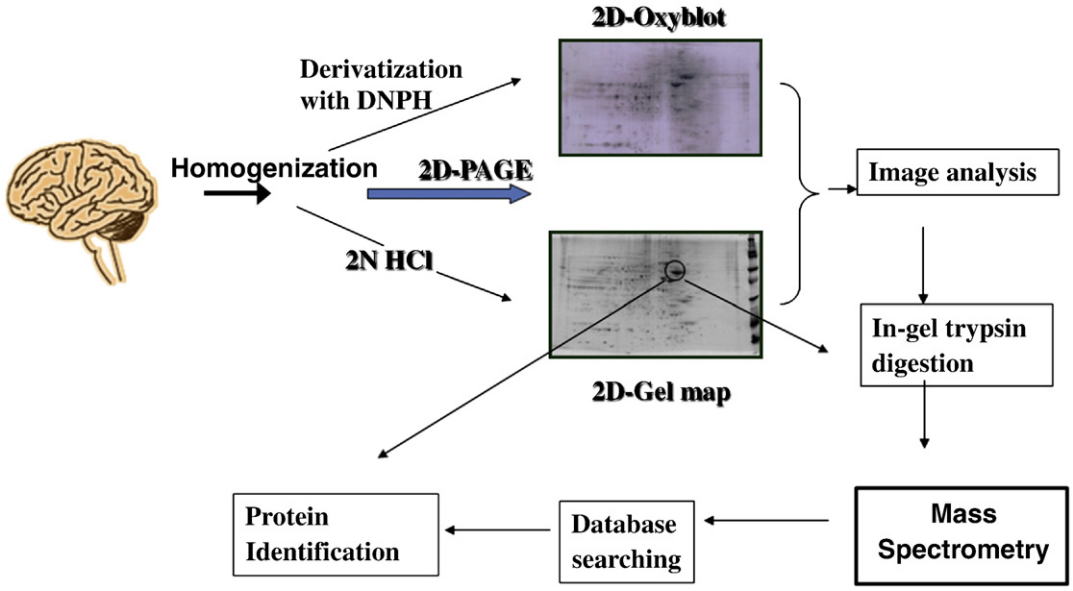

Fig. 1. Outline of redox proteomics showing the incorporation of 2D–PAGE, MS, and a protein database to identify oxidatively modified proteins.

Two-dimensional gel electrophoresis

2D gel electrophoresis separates a mixture of proteins into single detectable protein spots in most cases. The separation of proteins is usually carried out in two steps, i.e., isoelectric focusing in the first dimension and molecular migration rate in the second dimension. In isoelectric focusing, proteins are separated based on their isoelectric point, i.e., the pH at which the net charge on each protein is 0 (Fig. 3). In the second dimension, all the proteins are imparted a negative charge by addition of an anionic detergent such as sodium dodecyl sulfate (SDS) followed by separation of the proteins based on their relative mobilities (Fig. 3). Hence, on 2D gels, proteins having smaller size-to-charge ratio will move faster than the proteins having larger size-to-charge ratio. These criteria allow separation of a large number of proteins, and each spot almost always represents a single protein.

PDQuest analysis

PDQuest image analysis allows gel-to-gel or blot-to-blot matching and analysis of visualized protein spots among different gels and blots (Fig. 4). The principles of measuring intensity values by 2D analysis software are similar to those of densitometric measurement. After completion of spot matching, the normalized intensity of each protein spot from individual gels (or blots) is compared between groups using statistical analysis. Although the software uses raw image-based alignment coupled with specific landmarks and neighboring spots to improve spot matching steps with significantly reduced analysis time, investigator-initiated hands-on processing is still necessary for higher accuracy of the matching. Normally, only spots that are statistically different between control and experimental groups are subjected to in-gel trypsin digestion for subsequent analysis by mass spectrometry. Each sample is run in triplicate to reduce variability and increase confidence in the resultant protein identification. In our experience, the percentage coefficient of variation (CV) is approximately 10%. Hence, fold change in levels or oxidative modification of at least 20–30% (often much more elevated oxidative modification is observed in AD brain proteins) over controls is necessary to pursue subsequent redox proteomics identification of specific protein spots.

Mass spectrometry

The primary MS methods used in our laboratory are peptide mass fingerprinting (PMF) after matrix-assisted laser desorption ionization (MALDI) time-of-flight MS and electrospray ionization tandem MS

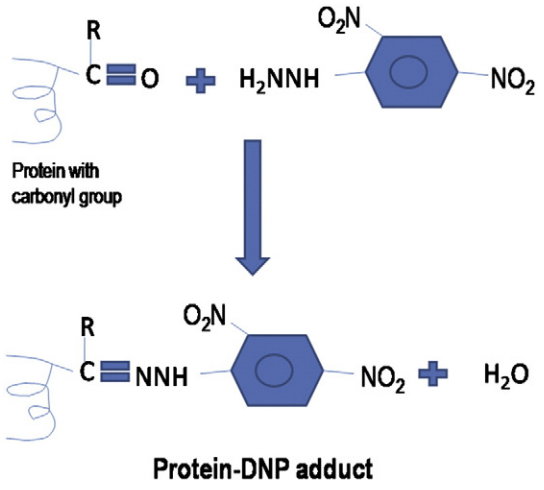

Fig. 2. Derivatization of the protein carbonyl group on a protein by 2,4-dinitrophenylhydrazine to form a Schiff base product, 2,4-dinitrophenylhydrazone.

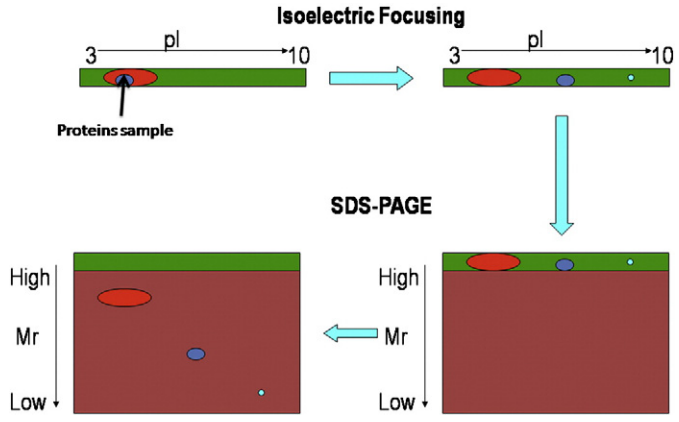

Fig. 3. Two-dimensional gel electrophoresis allows separation of proteins based on the isoelectric point (p*I*) in the first dimension and relative mobility (M_r) in the second dimension.

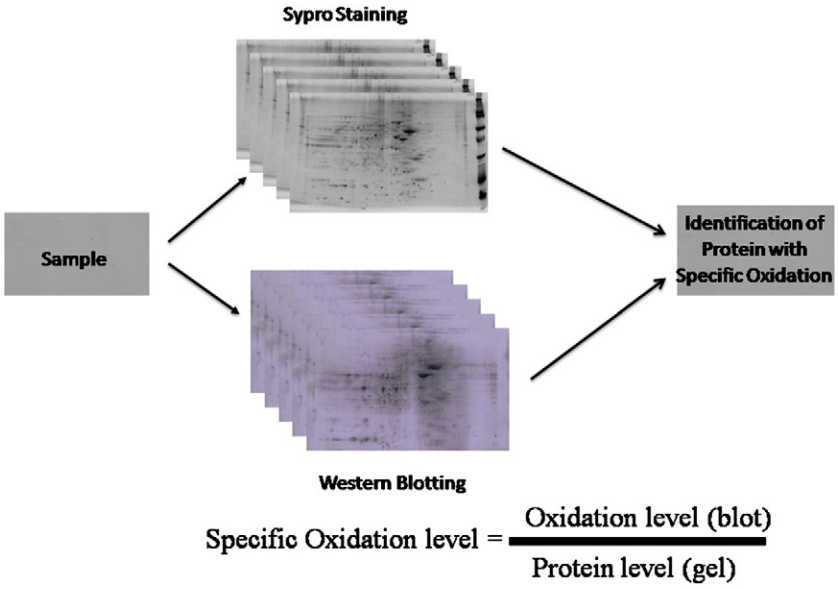

Fig. 4. Determination of oxidized proteins by PDQuest software. PDQuest image analysis allows gel-to-gel or blot-to-blot matching and provides the intensity of each of the spots present on both gels and blots.

(ESI–MS/MS). Full details of the methods of both techniques can be found elsewhere [52], and here a brief overview of each MS method will be given. In PMF, in-gel tryptic peptides of each spot of interest are mixed with an acidic solid matrix such as α-cyano-4-hydroxycinnamic acid, which provides high sensitivity and negligible matrix adduction during the laser absorption [53], and subjected to laser radiation. The peptides are incorporated into the crystal lattice of the matrix during the condensation process. When the pulsed laser is applied to the matrix/analyte mixture, the peptides along with the matrix particles absorb energy from the laser pulse and the matrix molecules containing the analyte are transferred into the gas phase. The positive ions of the peptides are formed in gas phase because of the acidic nature of the matrix in an as-yet unknown process [54]. The ions are then accelerated into the mass analyzer where, in the time-of-flight tube, they are separated based on their m/z ratio. The peaks of the resulting mass spectrum represent the masses of the peptide ions of the sample of interest. Protein databases are available for theoretical digests of all known proteins, which can be used for the identification of the protein of interest. The protein database provides information such as molecular weight, p*I,* and the probability of a random identification of a protein. The p*I* and molecular weight in the database are compared to the experimental p*I* and molecular weight as a further indication of the correctness of the identification of the unknown protein. Many search engines perform this matching process, with an output of a probability score for each theoretical digested protein indicating the correctness of the identification. The threshold score, which indicates whether the experimental mass spectrum significantly matches the theoretical digested protein spectrum, is calculated by mathematical algorithms specific to each search engine and each experimental mass spectrum. Often this is manifested as a Mowse score ($-10\log_{10}P$), where P is the probability of a random identification. Given the high Mowse score required for significance, the P value for PMF-identified proteins is often exceedingly small (often 10^{-8} or lower), giving confidence in the protein identification.

In the case of ESI–MS/MS, tryptic peptide samples are loaded into a 96-well plate rack for nanoelectrospray infusion using an Advion TriVersa Nanomate (Ithaca, NY, USA). Electrosprayed peptides are analyzed with an LTQ-Orbitrap XL (Thermo Scientific, Waltham, MA, USA) mass spectrometer. The Orbitrap normally is set to acquire a full MS scan at 60,000 resolution and in Data Dependent mode the eight most intense ions are selected for fragmentation and mass analyzed in the Orbitrap at 30,000 resolution. Conditions for fragmentation in the ion trap include a normalized collision energy of 35%, activation time of 30 ms, and selection of only +2 charge states or higher. Total acquisition time is approximately 5 min per sample. SEQUEST is used for database searching against the UniProt SwissProt database. Filter criteria of returned protein lists included protein probabilities <0.01; peptide XCorr values >1.5 (for +1 charge state), 2.0 (+2 charge state), 2.5 (+3 charge state), and 3.0 (+4 charge state); peptide ΔCN values >0.1, and at least two peptides sequenced for each protein. As with MALDI, protein MW and p*I* information is used to assess individual protein identification based on the location of the excised protein spot from the 2D gel. Only protein spots assigned to a single protein are considered further. As indicated above, the output of this MS method is the actual sequence of at least two tryptic peptides of the unknown protein, which, when searched against the SEQUEST database, gives the identity of the protein.

In both PMF and ESI–MS/MS methods of protein identification, validation by independent means (often immunoprecipitation) is performed to ensure the correctness of the MS-identified proteins.

Materials

The chemicals used for redox proteomics were purchased mostly from Sigma–Aldrich (St. Louis, MO, USA), unless stated otherwise: *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid (Hepes) buffer, sodium chloride (NaCl), potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), magnesium sulfate ($MgSO_4$), leupeptin, pepstatin, type IIS soybean trypsin inhibitor, phenylmethanesulfonyl fluoride (PMSF), DNPH, hydrochloric acid (HCl), trichloroacetic acid (TCA), ethanol, ethyl acetate, urea, thiourea, Chaps, biolytes, dithiothreitol (DTT), bromophenol blue, Tris–HCl, SDS, glycerol, iodoacetamide (IA), running buffer (Bio-Rad, Hercules, CA, USA), methanol, acetic acid, SYPRO ruby stain (Bio-Rad), phosphate-buffered saline, Tween 20, sodium azide, bovine serum albumin (BSA), anti-DNPH antibody (Chemicon International, Temecula, CA, USA), anti-rabbit antibody conjugated to alkaline phosphatase, SigmaFast tablet (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT)), Criterion precast gels (8–16%; Bio-Rad), and immobilized pH gradient (IPG) strips (pH 3–10; Bio-Rad).

Instrumentation

The IEF instrument was purchased from Bio-Rad, centrifuges were purchased from Beckman (Fullerton, CA, USA), UV transilluminator (λ_{ex} 470 nm, λ_{em} 618 nm) was from Molecular Dynamics (Sunnyvale, CA, USA), μQuant Microtiter plate reader was purchased from Bio-Tek Instruments (Winooski, VT, USA), and the vortex mixer was purchased from VWR International (Radnor, PA, USA).

Protocol

Sample preparation for detection of oxidatively modified proteins

Prepare 10% tissue homogenate in homogenization buffer (10 mM Hepes, 137 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, 0.5 μg/ml leupeptin (stored as an aliquot at −20 °C), 0.7 μg/ml pepstatin (stored as an aliquot at −20 °C), 0.5 μg/ml type II S soybean trypsin inhibitor, 40 μg/ml PMSF dissolved in deionized water stored at 4 °C). Centrifuge at 2500 *g* to remove intact cells and nuclei. Determine the protein content by using a BCA reagent kit from Pierce (Rockford, IL, USA).

The following method is used for protein carbonyl detection. To 100–150 μg of the protein add DNPH (10 mM prepared in 2 N HCl solution, which can be stored at room temperature) four times the volume of the sample, vortex the sample, and incubate at room temperature for 20 min. Then, add a final concentration of 30% TCA and incubate the sample on ice for 10 min to precipitate the proteins. Centrifuge the sample at 10,000 *g* for 5 min. Decant the supernatant and wash the pellet with ice-cold ethanol and ethyl acetate solution (1:1 v/v). Suspend the final pellet in 200 μl of IEF rehydration buffer (8 M urea, 2 M thiourea, 2% Chaps, 0.2% biolytes, 50 mM DTT, bromophenol blue dissolved in deionized water made fresh before use). Incubate the samples in rehydration buffer for a minimum of 1 h at room temperature before loading in the IEF tray (samples can be incubated for a maximum of 2 h).

For detection of HNE-modified proteins, the sample does not require any prederivatization. Samples are precipitated by TCA as described above, and the steps described above for protein carbonyls are followed for protein-bound HNE detection.

Detection of 3-NT-modified proteins, like protein-bound HNE, does not require any prederivatization. Samples are precipitated using 75% acetone, and the steps described above are followed for sample preparation.

First dimension, or isoelectric focusing

Isoelectric focusing is performed with a Bio-Rad system using 110-mm, pH 3–10 or pH 4–7 IPG strips. The selection of the IPG strips depends on nature of the samples and interest of the researcher.

Carefully load 180 μl of the sample into the bottom of the well in the IEF tray. Samples can be applied using either a microliter syringe (washed between each sample by pipetting in deionized water) or disposable micropipette tips (discard each tip after a single use). While loading samples, care should be taken to avoid bubbles, which might interfere with current flow.

Remove the plastic sheet from the IPG strips (Bio-Rad) with forceps. Place the IPG strips on top of the sample with gel side facing down, making sure that the positive end of strip is toward the positive end of the IEF tray. This is important for the correct connections.

Remove any air bubbles trapped between the IPG strip and the tray by gently tapping on the IPG strip with a forceps.

Place the IEF tray in the IEF instrument and carry out an active rehydration overnight at 50 V (20 °C). This step will enable the gel to swell and help in the penetration of the sample into the IPG strip. Add 2 ml of mineral oil (Bio-Rad) to each well after 1 h; this will prevent both evaporation of rehydration buffer and drying of IPG strips. Carry out active rehydration step for about 16 h.

After 16 h, place wet paper wicks (Bio-Rad) on both the electrodes using a forceps to prevent burning of the IPG strips during isoelectric focusing. Carry out isoelectric focusing at 20 °C as follows: 300 V for 2-h linear gradient, 500 V for 2-h linear gradient, 1000 V for 2-h linear gradient, 8000 V for 8-h linear gradient, 8000 V for 10-h rapid gradient.

After completion of IEF the IPG strips can be used directly for second-dimension separation or stored in a −80 °C freezer until use.

Second-dimension electrophoresis

Incubate the IPG strips in 4 ml of equilibration buffer containing DTT (50 mM Tris–HCl, 6 M urea, 1% (m/v) SDS, 30% (v/v) glycerol, 0.5% DTT dissolved in deionized water made fresh before use) in a disposable equilibration tray with lid (Bio-Rad) with the gel side facing up for 10 min. Keep the equilibration tray in the dark. If the IPG strips have been stored in the −80 °C freezer, thaw the IPG strips at room temperature (thawed IPG strips change color from milky white to clear) before addition of the equilibration buffer.

Transfer the IPG strips into a new equilibration tray and add 4 ml of equilibration buffer containing IA (50 mM Tris–HCl, 6 M urea, 1% (m/v) SDS, 30% (v/v) glycerol, 4.5% IA dissolved in deionized water made fresh before use), and incubate in the dark for another 10 min.

Wash the IPG strips in 1× running buffer (Bio-Rad) (to remove excess equilibration buffer), and place the IPG strips with gel side facing up into Criterion gels (Bio-Rad).

Add warm agarose solution (Bio-Rad) into the wells of the Criterion gels and then slowly push down the IPG strip until a contact is established between the gel and the IPG strip. Remove any bubbles that are trapped in the agarose solution by gently pushing the IPG strip with a forceps.

Load 2 μl of unstained (for gel only) or stained molecular weight markers (for blot only) (Bio-Rad) into a standard well adjacent to the IPG strip. Allow the agarose to solidify for 10 min.

Place the gels in the electrophoresis tank filled with running buffer and then fill the upper tank with running buffer. Run the gels at 200 V for 65 min at room temperature, until the dye front (bromophenol blue) just runs off the gel into the lower tank.

After the run is completed, disconnect the power supply (Bio-Rad) and disassemble the 2D apparatus, and remove the gels.

Protein staining

Fix the gels containing nonderivatized proteins with unstained marker in 50 ml of fixative solution (10% (v/v) methanol and 7% (v/v) acetic acid) for 1 h at room temperature with gentle agitation [7,42,55]. Add 50 ml of SYPRO ruby gel stain (Bio-Rad) and incubate overnight at room temperature on a rocking platform.

OxyBlot (for detection of oxidatively modified proteins)

Transfer gels containing DNPH-derivatized proteins/nonderivatized proteins to nitrocellulose membranes (Bio-Rad) using a semidry transfer unit (Bio-Rad) for detection of protein carbonyls and HNE/3-NT-modified proteins, respectively. Prepare a transfer sandwich in the following order: place one soaked filter paper on the transfer unit platform first, followed by the nitrocellulose membrane, gel, and one more wet filter paper.

Transfer the proteins at 15 V for 2 h at room temperature. After transfer, block the nonspecific sites on the nitrocellulose membrane by incubating the membrane with 25 ml of blocking buffer (3% BSA in Wash Blot: 0.01% (w/v) sodium azide and 0.2% (v/v) Tween 20 dissolved in phosphate-buffered saline (PBS) and stored at room temperature) for 1 h at room temperature on a rocking platform.

After 1 h add 25 ml of the blocking buffer (3% BSA in PBS–Tween 20 made fresh before use) containing (as appropriate for the analysis desired) anti-DNPH or anti-HNE or anti-3-NT antibody (1:100/1:1000/1:1000; Chemicon International) and incubate for 1 h at room temperature on a rocking platform.

Wash three times for 5 min each with 50 ml of Wash Blot.

Incubate the membrane in the secondary antibody (anti-rabbit conjugated to alkaline phosphatase; 1:3000 in Wash Blot) for 1 h at room temperature on a rocking platform.

Wash three times for 5 min each with 50 ml of Wash Blot.

Develop the blot using SigmaFast tablets (BCIP/NBT; dissolved in 10 ml deionized water, prepared fresh). After color development, drain out the developer and wash the membrane with deionized water and dry between filter papers.

Calculations and expected results

Scan the SYPRO ruby-stained and OxyBlot blots using a UV transilluminator (λ_{ex} 470 nm, λ_{em} 618 nm; Molecular Dynamics, Sunnyvale, CA, USA) and Adobe Photoshop on a Microtek Scanmaker 4900 (Cerritos, CA, USA), respectively, and save as TIFF files.

Perform image analysis of OxyBlots and 2D gel maps using PDQuest image analysis software (Bio-Rad). This software gives the option of normalization of the actual protein content as measured by the intensity of a protein stain such as SYPRO ruby (Bio-Rad and Molecular Probes, Eugene, OR, USA).

To determine the levels of specific oxidation divide the intensity of the spot on the blot by the intensity of spot on the gel (Fig. 4). A minimum of $n=6$ independent samples (in triplicate) is run per group of samples. One of the best gels and one of the best blots showing the best resolution of spots is selected as master gel or blot to begin the analysis. PDQuest software allows the analysis of multiple gels and blots using powerful algorithms that accurately and quickly match protein spots in the master gel to the master blot. After completion of spot matching, to determine the levels of specific oxidation divide the intensity of the spot on the blot by the intensity of spot on the gel (Fig. 4). Protein spots are considered significant if their P value is less than 0.05.

Excise the spots showing a significant increase in specific oxidation compared to the control gel and perform an in gel-digestion with trypsin (Fig. 1). However, first give consecutive treatments with 0.1 M ammonium bicarbonate (NH_4HCO_3), acetonitrile, 20 mM DTT in 0.1 M NH_4HCO_3, 55 mM IA in 0.1 M NH_4HCO_3, 50 mM NH_4HCO_3, and acetonitrile. Then rehydrate the gel pieces with 20 ng/μl modified trypsin (Promega, Madison, WI, USA) in 50 mM NH_4HCO_3 overnight at 37 °C [56].

Salts and contaminants must be removed from tryptic peptide solutions using a C18 ZipTip into a fresh tube. Step 1: to the remaining gel piece add ~20 μl of buffer A (5% acetonitrile, 0.1% formic acid), and sonicate it in a water bath at 37 °C for 15 min. Add ~30 μl of buffer B (95% acetonitrile, 0.1% formic acid, 1 mM NH_4HCO_3) and sonicate it in the water bath at 37 °C for 15 min. Step 2: transfer the supernatant obtained from step 1 to the tryptic peptide solution aliquotted before. Concentrate the samples to ~10 μl using a SpeedVac. Step 3: wash the ZipTip by aspirating 10 μl of solvent C (100% acetonitrile) five times through the same ZipTip, followed by equilibration of the ZipTip with 10 μl of buffer A. Step 4: aspirate the samples four or five times followed by washing of the ZipTip with 10 μl of buffer A. Step 5: elute the sample in 10 μl of buffer D (50% acetonitrile, 0.1% formic acid). Submit the cleaned eluate obtained in step 5 for analysis by mass spectrometry, the output of which is used for protein identification.

As described above, the results from the mass spectrometric analysis are searched against protein databases using automated search algorithms [57,58]. An example of the results produced is shown in Fig. 5.

Caveats

To minimize artifactual oxidation, brain samples should be obtained from subjects (or animals) at extremely short postmortem intervals (typically, in our hands, <3–4 h for human specimens; 1 min or less for animal specimens), and all the processing of the samples should be done under identical conditions. If the sample handling

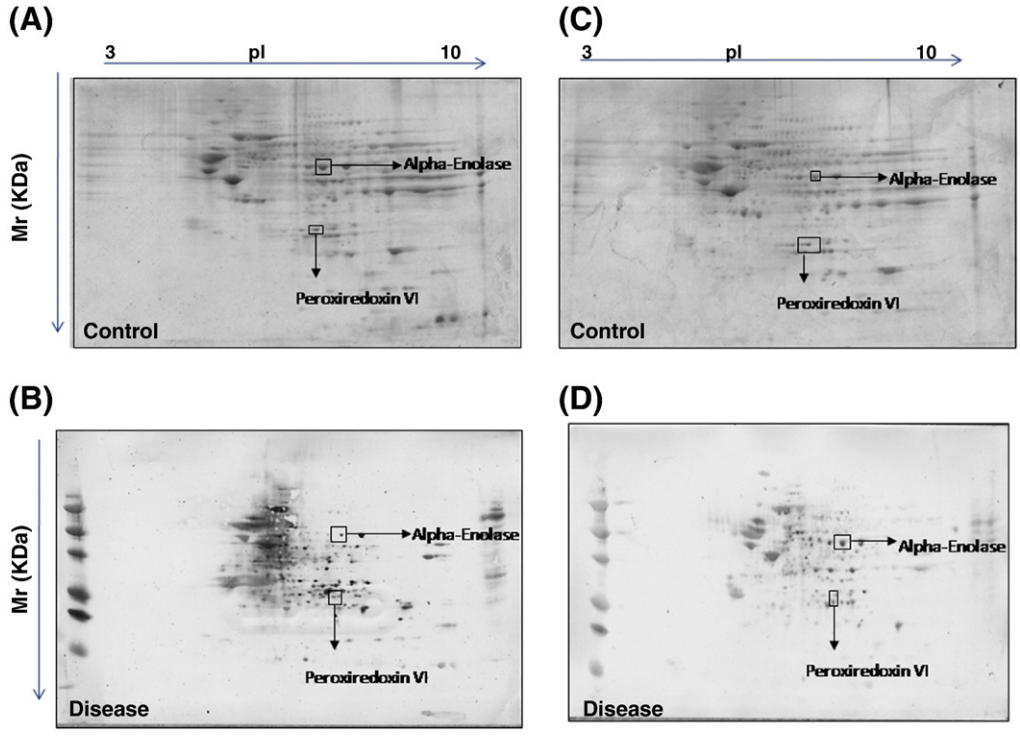

Fig. 5. An example of gels from (A) control and (B) disease samples and their representative blots from (C) control and (D) disease samples.

leads to any oxidation this would be presumed to affect both the control and the subject samples similarly.

It is important to include protease inhibitors in the sample homogenization buffer, because improper care of samples may lead to protein degradation that can have an effect on what is visualized and inaccurately reflect what is happening in the cell [59].

Proper washing of the pellet after derivatization is crucial to reduce the background signal on the blot due to excess DNPH, which might interfere with image analysis, in addition to removal of lipids. Further, it is important to ensure that ethanol and ethyl acetate solution are removed completely before adding rehydration buffer. The sample after derivatization usually has a high concentration of ions because an acidic buffer is used to optimize the reaction. The high level of ions in the buffer can cause variations in voltage and current during IEF, thereby preventing successful isoelectric separation. This phenomenon usually is manifested by horizontal smearing of the protein spot.

The sample should be incubated with IEF rehydration buffer for a minimum of 1 h to allow an efficient, quantitative solubilization of cellular proteins.

Too much glycerol addition or too little DTT in the equilibration buffer may lead to smearing and streaking of the spots, which might interfere with the spot matching and final analysis.

When developing blots it is critical to maintain the same development time for all the blots, to prevent possible artifactual data generation. BCIP/NBT substrates generate an intense black purple precipitate at the site of enzyme binding. The reaction proceeds at a steady rate, thus allowing accurate control of the development of the reaction. This allows the relative sensitivity to be controlled by the length of incubation.

In almost all cases, 2D gel separation methods lead to one protein per spot. However, if a single protein spot from the 2D gel shows the presence of more than one protein in the same spot by MS, then immunoprecipitation techniques should be employed to identify the target of oxidation.

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