18

Protein Carbonyl Levels—An Assessment of Protein Oxidation

Alessandra Castegna, Jennifer Drake, Chava Pocernich, and D. Allan Butterfield

1. INTRODUCTION

Oxidative stress may be a hallmark of several neurodegenerative disorders, including Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD), as well as Creutzfeldt-Jakob disease (CJD), frontotemporal dementia, and amyotrophic lateral sclerosis (ALS) (1). Oxidative stress occurs when the formation of reactive oxygen species (ROS) increases, or when scavenging of ROS or repair of oxidatively modified molecules decreases (2,3). ROS are highly reactive, toxic oxygen moieties, such as hydroxyl radical, peroxyl radical, superoxide anion, and hydrogen peroxide. Collectively, ROS can lead to oxidation of proteins and DNA, peroxidation of lipids, and, ultimately, cell death. To counteract these damaging radicals, antioxidant systems have been developed. Among these are enzymes, such as glutathione peroxidase, glutathione reductase, superoxide dismutase (SOD), and catalase, among others; and small, nonprotein, cellular antioxidants such as, glutathione, vitamin C, vitamin E, and uric acid.

Oxidative stress has been shown to increase protein oxidation (2–6) and has been reported in AD (7), animal models of AD (8) and HD (9,10), rheumatoid arthritis (RA) (11), respiratory distress syndrome (12), PD (13), atherosclerosis (14), and accelerated aging (15). One way to measure protein oxidation is to determine levels of protein carbonyls. Protein carbonyls can result from oxidative cleavage of the protein backbone, direct oxidation of amino acids such as lysine, arginine, histidine, proline, glutamic acid, and threonine, or by the binding of aldehydes produced from lipid peroxidation

From: Methods in Pharmacology and Toxicology: Methods in Biological Oxidative Stress
Edited by: K. Hensley and R. A. Floyd © Humana Press Inc., Totowa, NJ

such as 4-hydroxynonenal (HNE) or acrolein (2,3) (Fig. 1). In addition, carbonyl groups may be introduced into proteins by reactions with reactive carbonyl derivatives generated as a consequence of the reaction of reducing sugars or their oxidation products with lysine residues of proteins, glycation, or glycoxidation reactions (16,17).

The assay of carbonyl groups in proteins provides a convenient technique for detecting and quantifying oxidative modification of proteins. 2,4-dinitrophenylhyrdazine (DNPH) reacts with protein carbonyls to produce hydrazones (Fig. 2). Hydrazones can be detected spectrophotometrically at an absorbance of 370 nm or by fluorescence (18). Western- or slot-blotting techniques are also used for sensitive and specific detection of the protein-2,4-dinitrophenyl hydrazone moiety (19).

2. MATERIALS

1. 0.2% (w/v) DNPH prepared in 2 *N* HCl.
2. 6 *M* guanidine hydrochloride dissolved in 20 m*M* sodium phosphate buffer with a pH of 6.5.
3. 100% (w/v) tricholoroacetic acid.
4. Ethanol/ethyl acetate solution (1:1 v/v).
5. 200 m*M* DNPH stock solution prepared in 100% trifluroacetic acid (TFA).
6. 12% (w/v) Sodium dodecyl sulfate (SDS).
7. Neutralization solution (2 *M* Tris/30% glycerol/ 19% 2-mercaptoethanol).
8. Primary antibody to the 2,4-dinitrophenylhydrazone protein moiety, available from Sigma (St. Louis, MO, D-8406) or Intergen (Purchase, NY, OxyBlot Kit #S7150).
9. Labeled secondary antibody to the species of the primary antibody appropriate for the method of detection (chemiluminescence, fluorescence, colorimetry).
10. Biological sample to be assayed for protein carbonyl determination. Protein concentration is determined by the Pierce BCA method.

3. METHODS

3.1. Spectrophotometric Assay

1. Sample derivatization. Two 1-mg aliquots are needed for each sample to be assayed. Samples are extracted in a final concentration of 10% (w/v) TCA. The precipitates are treated with 500 µL of 0.2% DNPH or 500 µL of 2 N HCl. Samples are incubated at room temperature for 1 h with vortexing at 5-min intervals. The proteins are then precipitated by adding 55 µ

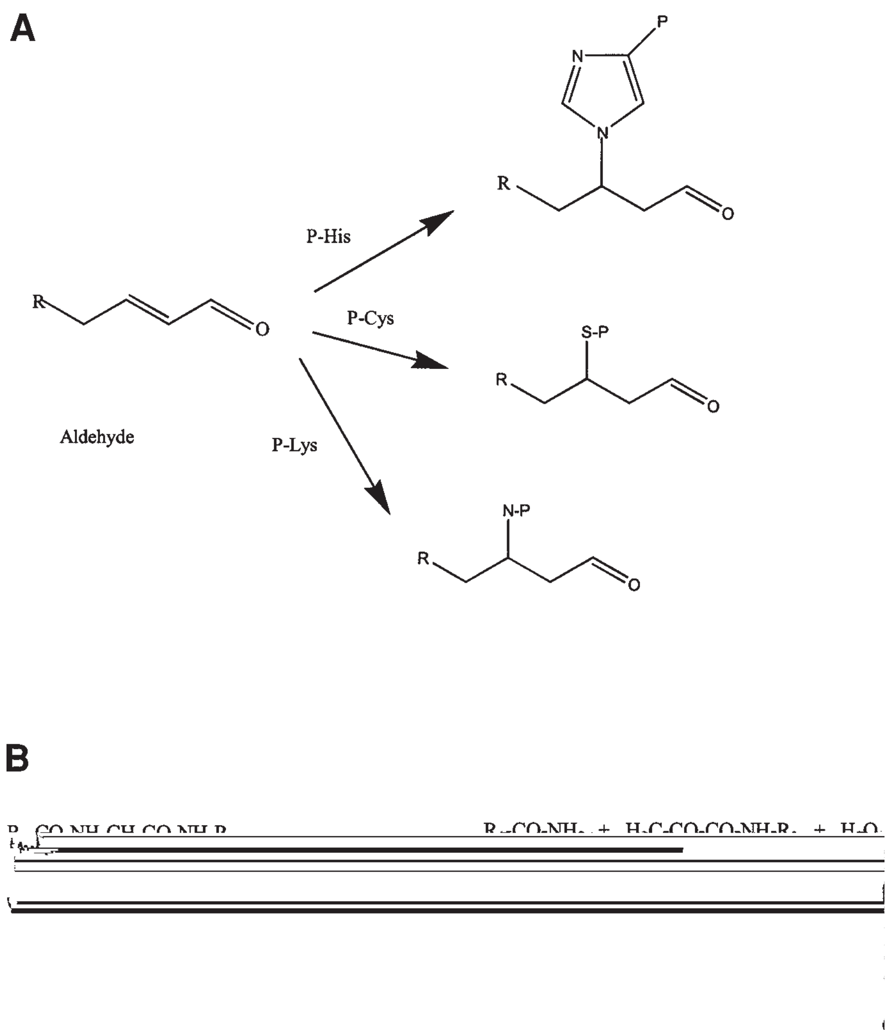

Fig. 1. (A) Michael addition of an aldehyde to protein lysine (P-lys), histidine (P-His), or cysteine (P-Cys) residues resulting in the addition of carbonyl groups to the protein. (B) Oxidation of the glutamyl side chain leads to formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative.

2. The carbonyl content is determined by reading the absorbance at the optimum wavelength (= 360…390 nm) of each sample against its appropriate blank

3.2. Immunodetection Methods

1. Sample derivatization. 200 mM DNPH stock solution is diluted 10 times with water. Five µL of sample is incubated at room temperature with 5 µL of 12%

SDS and 10μ

4. ANALYSIS

For the spectrophotometric assay, 2,4-dinitrophenylhydrazone protein adducts are calculated using the millimolar adsorptivity of $21.0\ mM^{-1}\ cm^{-1}$ for aliphatic hydrazones. Results are reported as nmol of DNPH incorporated per mg of protein. The carbonyls detected in the immunodetection methods can be reported either by comparing to a standard with an amount of 2,4-dinitrophenylhydrazone protein adducts as determined by the spectrophotometric method or by comparing the density to the control sample density employing suitable imaging software.

5. DISCUSSION

The method chosen for analysis depends on the sample and the needs of the researcher. The spectrophotometric method actually quantitates the 2,4-dinitrophenylhydrazone protein adducts and is therefore useful for comparing samples that will be collected and quantitated at different times. However, because a large amount of sample is required for analysis, it may be inappropriate for samples such as cerebrospinal fluid (CSF) where protein concentration is limited. For samples with limited protein concentra-

Fig. 3. Sample slot blot of bovine serum albumin (BSA) oxidized by hydroxyl radicals, produced by Fe^{2+} and H_2O_2, and reacted with DNPH, demonstrating sensitivity as low as picomolar levels of protein carbonyls.

The whole procedure potentially allows automated identification of several oxidized proteins at the time, yet some problems can occur. First, reproducibility of 2D-gel analysis, which is crucial for control-sample spot comparison, might be reduced by the instability of pH-gradients in the first dimension (isoelectric focusing; IEF). To circumvent this limitation, the use of immobilized pH gradients (IpHGs) may be suitable (24). IpHGs are formed by copolymerization of buffering and titrant groups of acrylamido-derivatives into a polyacrylamide gel matrix, assuring a steady-state focusing and a consequent high reproducibility of the spot position.

In addition, oxidative modification may result in altering, even slightly, the electrophoretic properties of a protein and its migration with respect to the unmodified one. This problem can be overcome (22,25), especially with the help of specific 2D-gel softwares, which increase the resolution and the possibility of spot crossmatching.

This automated method of proteomic analysis is in its infancy with respect to neurodegenerative diseases, but is under investigation in our laboratory (26,27)

ACKNOWLEDGMENTS

This work was supported in part by grants from NIH.

REFERENCES

1. Butterfield, D. A. and Kanski, J. (2001) Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins. Mech. Ageing Dev. 122, 945–962.

2. Stadtman, E. R. (1992) Protein oxidation and aging. *Science* 257, 1220…1224.
3. Butterfield, D. A. and Stadtman, E. R. (1997) Protein oxidation processes in aging brain. *Adv. Cell Aging Gerontol.* 2, 161…191.
4. Howard, B. J., Yatin, S., Hensley, K., Allen, K. L., Kelly, J. P., Carney, J. M., and Butterfield, D. A. (1996) Prevention of hyperoxia-induced alterations in synaptosomal membrane-associated proteins by N-tert-butyl-phenyl-nitrone (PBN) and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (Tempol). *J. Neurochem.* 67, 2045…2050.
5. Aksenov, M. Y., Aksenova, M. V., Mrkesbery, W. R., and Butterfield, D. A. (1998) Amyloid -peptide (1-40)-mediated oxidative stress in cultured hippocampal neurons. *J. Mol. Neurosci.* 10, 181…192.
6. Koppal, T., Drake, J., Yatin, S., Jordan, B., Varadarajan, S., Bettenhausen, L., and Butterfield, D. A. (1999) Peroxynitrite-induced alterations in synaptosomal membrane proteins: Insight into oxidative stress in Alzheimer•s disease. *J. Neurochem.* 72, 310…317.
7. Hensley, K., Hall, N., Subramaniam, R., Cole, P., Harris, M., Aksenov, M., et al. (1995) Brain regional correspondence between Alzheimer•s disease histopathology and biomarkers of protein oxidation. *J. Neurochem.* 65, 2146…2156.
8. Yatin, S. M., Link, C. D., and Butterfield, D. A. (1999) In-vitro and in-vivo oxidative stress associated with Alzheimer•s amyloid-peptide. *Neurobiol. Aging* 20, 325…330.
9. LaFontaine, M. A., Geddies, J. W., Banks, A., and Butterfield, D. A. (2000) 3-Nitropropionic acid induced in-vivo protein oxidation in striatal and cortical synaptosomes: insights into Huntington•s disease. *Brain Res.* 858, 356…362.
10. LaFontaine, M. A., Geddies, J. W., Banks, A., and Butterfield, D. A. (2000) Effect of exogenous and endogenous antioxidants on 3-nitropropionic acid-induced in-vivo oxidative stress and striatal lesions: insights into Huntington•s disease. *J. Neurochem.* 75, 1709…1715.
11. Chapman, M. L., Rubin, B. R., and Gracy, R. W. (1989) Increased carbonyl content of proteins in synovial fluid from patients with rheumatoid arthritis. *J. Rheumatol.* 16, 15…19.
12. Gladstone, I. M. and Levine, R. L. (1994) Oxidation of proteins in neonatal lungs. *Pediatrics* 93, 764…768.
13. Yoritaka, A., Hattori, N., Uchida, K., Tanaka, M., Stadtman, E. R., and Mizuno, Y. (1996) Immunohistochemical detection of 4-hydroxynonenal protein adducts in Parkinson disease. *Proc. Natl. Acad. Sci. USA* 93, 2696…2701.
14. Uchida, K., Toyokumi, S., Kishikawa, S., Oda, H., Hiaia, H., and Stadtman, E. R. (1994) Michael addition-type 4-hydroxy-2-nonenal adducts in modified low-density lipoproteins: markers for atherosclerosis. *Biochemistry* 33, 12,487…12,347.
15. Butterfield, D. A., Howard, B. J., Yatin, S., Allen, K. L., and Carney, J. M. (1997) Free radical oxidation of brain proteins in accelerated senescence and its modulation by N-tert-butyl- -phenylnitrone. *Proc. Nat. Acad. Sci. USA* 94, 674…678.