Methods of Evaluating Protein Oxidation Induced by Ischemia/Reperfusion Injury

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Ischemia/reperfusion injury (IRI) of the brain is one of the most common pathophysiological processes affecting more than 500,000 people per year in the United States in the form of strokes [1,2]. IRI is known to produce oxiradicals capable of neuronal cell damage [1–4]. Although the mechanism for postischemic cell damage is not known, these oxiradicals are capable of oxidizing proteins, lipids, and nucleosides (DNA and RNA); disrupting calcium homeostasis; and promoting the accumulation of excitatory amino acids, prostaglandins, leukotrienes, and polyamines at the onset of reperfusion [1,2]. Protein oxidation by oxiradicals can lead to membrane damage and inactivation of enzymes responsible for many critical cellular functions such as ion transport and protein synthesis [1]. Protein oxidation has been evaluated by electron paramagnetic resonance (EPR) spin-labeling, glutamine synthetase (GS) activity assay, protein carbonyl content by 2,4-dinitrophenylhydrazine (2,4-DNPH) assay, and protein carbonyl content by fluorescence microscopy [1–10]. These methods of evaluating protein oxidation will be briefly discussed.

One method of evaluating oxidative modification of proteins used in our laboratory is EPR spin labeling with the protein specific spin label 2,2,6,6-tetramethyl-1-pyrene (MAL-6) [1–3]. The MAL-6 spin label covalently binds to the cysteine sulfhydryl groups of cytoskeletal and transmembrane proteins of synaptosomal membranes [1–3]. There are two environments of the protein in which the MAL-6 may bind. The first is a weakly (W) immobilized site that does not significantly restrict the motion of the spin label, thereby yielding relatively narrow EPR spectral lines [1–3]. The second environment is the strongly (S) immobilized site, such as the narrow pockets of a protein that restricts the motion of the spin label, thereby producing broadened EPR spectral lines [1–3]. The parameter used as a measure of protein oxidation is the W/S ratio of the $M_1 = +1$ low-field EPR spectral lines (Fig. 1) [1–3]. The W/S ratio is sensitive to perturbations in the normal interactions of cytoskeletal proteins and may increase or decrease upon structural changes of the protein [1–3]. Free radical oxidation of synaptosomal membrane proteins leads to a decreased W/S ratio [5]. For a 10-min transient ischemia and 1-hr reperfusion for both adult (3 months of age) and aged (15–18 months of age) male Mongolian gerbils, the W/S ratio was observed to have maximum decrease as compared to the control, with the aged gerbils experiencing a more profound effect [1,4]. A decrease in the W/S ratio is indicative of protein oxidation as a result of hydroxyl radical (•OH) attack at the onset of reperfusion following ischemia [1,2,5]. Consistent with this interpretation, neocortical synaptosomal membrane protein oxidation may be nullified by pretreatment with the free radical trapping agent N-tert-butyl-o-phenylnitroxide (PBN); in this case the W/S ratio does not decrease with respect to control [2].

The 2,4-DNPH assay for protein carbonyl content has been used as a measure of protein oxidation [3,4,6]. During protein oxidation, there are many possible amino acid modifications that may occur to form carbonyl derivatives [4,6]. These carbonyl derivatives react with 2,4-DNPH to form protein hydrazone derivatives detectable by spectrophotometry [3,6]. The oxidative damage as a result of 10 min ischemia followed by 0 to 24 hr of reperfusion shows an increase in the carbonyl content compared to control [6]. A maximum change was observed at 2 hr of reperfusion and a progressive decrease in carbonyl content followed, but remained above that seen in the control [6]. The 2,4-DNPH-reactive protein carbonyl content caused by IRI was partially prevented by pretreatment with PBN 60 min prior to the IRI [6].

Both qualitative and quantitative information on cellular protein carbonyl content can also be determined using a recently developed fluorescence microscopy technique reported by Harris et al. [7]. Protein carbonyl derivatives produced by oxidative stress react with biotin-4-aminobenzoic hydrazide to form Schiff bases [7]. Streptavidin containing a chromophore becomes fluorescent...
upon binding to biotin, which it does so with great avidity [7]. Fluorescence of the labeled complex was detected and quantified by using confocal laser microscopy, a method that is more sensitive than the 2,4-DNPH assay [7]. Using this approach, we have demonstrated significant increases in protein oxidation following both iron/peroxide (Fenton) and amyloid β-peptide (1-40) exposures (Fig. 2; see color plate) [7].

Changes in protein function due to oxidation have also been demonstrated using assays of GS as determined by the method of Rowe et al. as modified by Miller et al. [3,4,6,8-10]. These assays were corrected for nonspecific glutaminase activity by comparison in the presence and absence of ATP and arsenate [3,4,6,8]. In the brain, GS is found exclusively in astrocytes, and is responsible for production of the antioxidant glutathione and conversion of glutamate to glutamine, among other functions [3,4,6]. GS is exquisitely sensitive to site-specific oxidation, whereupon its activity is diminished [3,4,6,8]. The GS activity assay of tissue from a 10-min ischemia followed by a 2 hr reperfusion resulted in a 65% loss of GS activity, which can be partially prevented by pretreatment with PBN 60 min prior to IRI [6].

Protein oxidation results from oxyradical damage upon reperfusion following ischemia [1-2,6]. Consistent with this idea, the damage to the proteins can be partially abrogated by pretreatment with PBN [2,6]. Evaluation of the protein damage has been indexed by EPR spin-labeling, carbonyl content, and GS activity. The results of these methods provide valuable insight into the alterations of proteins following IRI and may be useful in the assessment of therapeutic treatments for stroke patients.

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References