

**Ultrastructural Localization and Relative Quantification of 4-Hydroxynonenal  
Modified Proteins in Tissues and Cell Compartments**

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Running head: Localization of 4HNE-modified proteins

## *Introduction*

Reactive oxygen species (ROS) are produced as byproducts of aerobic metabolism.<sup>1</sup> At high levels ROS are toxic, but recent studies have shown that, at lower concentrations, ROS regulate physiologic processes, including activities of signal transduction pathways and transcription factors.<sup>2</sup>

Measurement of ROS has been difficult, since these chemical species are so reactive that they persist in tissues for only short periods of time. One approach to this problem is to rely on biochemical assays of oxidative damage products. One major problem with such assays is that they rely on analysis of tissue homogenates, thereby precluding measurement of levels of oxidative damage products in specific cell types from complex tissues composed of many different types of cells. To circumvent this problem, my laboratory and others have developed techniques to localize and quantify oxidative damage products using specific antibodies and immunomorphologic procedures. These techniques have then applied to the study of a number of pathologic processes, including cancer,<sup>3-4</sup> neurodegenerative diseases,<sup>5-6</sup> and aging.<sup>7</sup>

Interactions of ROS with polyunsaturated fatty acids of membrane lipids results in the production of a variety of aldehydes and alkenals, including malonaldehyde and 4-hydroxy-2-nonenal (4HNE).<sup>8</sup> Lipid peroxidation products can subsequently react with other cellular constituents, including proteins.<sup>9</sup> Antibodies to 4HNE bound to specific amino acid residues have been generated and can be used to localize 4HNE-modified proteins in both physiologic and pathologic processes *in vitro* and *in vivo*. The present chapter will present methods to localize 4HNE-modified proteins *in vivo*. Methods for relative quantification of 4HNE-modified proteins in tissues and subcellular

compartments are also provided. These latter analytical methods allow one to compare levels of 4HNE-modified proteins between an experimental and a control group, but do not allow determination of absolute concentrations of 4HNE-modified proteins. Two previous studies have documented validity of the immunomorphologic approaches presented herein by documenting good agreement between biochemical and immunomorphologic analyses in an experimental model of oxidative stress (iron nitritotriacetate induced injury of rat kidney<sup>10</sup>) and analysis of oxidative damage in skeletal muscle of aging rhesus monkey<sup>7</sup>. Biochemical assays usually measure free aldehydes plus alkenals, while immunomorphologic assays specifically measure 4HNE bound to protein. The rate and efficiency of 4HNE binding to proteins is not known and may vary between proteins and cell types. Further, degradation rates of oxidatively damaged proteins may vary between cell types. Finally, cell turnover rate is unique for each cell type. Thus, while previous studies in our laboratory showed good correlation between 4HNE levels measured biochemically and levels of 4HNE-modified proteins measured with immunomorphologic techniques, the levels are not strictly comparable for the reasons mentioned above.

### *Procedures*

#### Antibody specificity

The use of antibodies with proven specificity is of utmost importance. Both rabbit polyclonal and mouse monoclonal antibodies have been used in previous studies. The rabbit polyclonal antibody used was raised to 4HNE-modified keyhole limpet hemocyanin. The specificity of this antibody has been characterized by immunochemical techniques, and it was concluded that the epitope recognized by the antibody appears to

be the hemiacetal form of the 4HNE-derived portion of protein-4HNE adducts.<sup>11-12</sup> The monoclonal antibody used was raised by immunizing mice with a 4HNE-keyhole limpet hemocyanin conjugate and was also thoroughly characterized with immunochemical techniques. These latter immunochemical studies suggest the monoclonal antibody is directed against the Michael addition-type 4HNE-histidine adduct.<sup>13</sup> Procedures described herein are for the monoclonal antibody, but we have obtained similar results with the polyclonal antibody.

#### Light microscopy techniques

The use of either of these antibodies with standard light microscopy immunomorphologic techniques allows one to determine which cell type(s) within a tissue contain significant levels of 4HNE-modified proteins. Immunomorphologic procedures can involve either immunogold or immunoperoxidase techniques. Techniques for light microscopy are relatively standard and procedures utilized can be found elsewhere.<sup>3-4,7</sup> Such techniques allow identification of which cell types within a tissue are positive for 4HNE-modified proteins, but do not allow identification of subcellular localization, and quantification of levels of 4HNE-modified proteins using light microscopy techniques is less sensitive and precise than immunogold ultrastructural techniques.

#### Fixation

Fixation is always a major issue when analyzing any immunomorphologic problem. For optimal studies, tissues should be perfused with the fixative of choice at physiologic pressures *in situ*, the tissues then removed, and the tissues post-fixed in the same fixative for one hour. Practically, many studies involve tissues already fixed, so

perfusion is not possible. The choice of fixative is very important, since some fixatives destroy antigenicity and others result in a high false positive background. For 4HNE-modified protein staining at the ultrastructural level, tissues are fixed for one hour in Carson-Millonig's fixative (4% formaldehyde in 0.16 M monobasic sodium phosphate buffer, pH 7.2). For relative quantification of 4HNE levels in an experimental versus a control group, it is crucial that tissues be treated in a similar manner: specifically, fixation must be performed in an identical fashion. Further, immunostaining must be performed with the same reagents with experimental and control slides being stained at the same time.

#### Immunogold electron microscopy

Tissues are cut into 1 mm<sup>3</sup> blocks and fixed in Carson-Millonig's fixative for one hour. The samples are embedded in LR White resin (Electron Microscopy Sciences, Fort Washington PA), since this resin allows preservation of antigenicity and therefore allows postembedding staining. After rinsing for 30 minutes in 0.1 M phosphate buffer, pH 7.4, the samples are dehydrated by 2 x 10 min changes each of 60% and 80% ethanol, followed by 2 x 30 min changes in 95% ethanol, then LR White resin/95% ethanol (2/1:v/v) for 1 hour. After immersion and infiltration in undiluted LR White resin overnight, the samples are washed with fresh undiluted LR White resin for 1 hour. Resin polymerization is thermally induced in sealed gelatin capsules at 50°C for 48 hours in the absence of accelerator. Ultrathin sections (70-80nm) are cut and transferred to nickel grids (G300-NI, Electron Microscopy Sciences) for postembedding immunogold procedures. These sections are incubated at room temperature with filtered Tris-buffered saline (TBS: 0.05 M Tris, 0.9% NaCl, pH 7.6) for 10 min, followed by 30 minutes in

filtered 0.5% Aurion BSA-C™ (25557, Electron Microcopy Sciences) in PBS to block nonspecific antibody binding sites. After the acetylated BSA block, the sections are rinsed for 5 min in filtered PBS at room temperature and then incubated with primary antibody (mouse anti-4HNE, 1:140) overnight at 4°C in a humidified chamber. Each new lot of primary and/or secondary antibody requires a study of optimal antibody titers to determine the amount of primary and/or secondary antibody needed for maximal immunogold labeling. On the second day, the sections are allowed to warm at room temperature for 1 hour. After washing 4 times with filtered TBS wash buffer (1:10 dilution of BSA-C Block:0.05M TBS), for 5 min each, the sections are washed in one change of filtered alkaline TBS (pH 8.2) for 10 min. The grids are incubated with diluted (1:50) gold conjugated anti-Mouse IgG (B.B.International, Cardiff, UK) for 90 min at room temperature. The sections are then washed in 2 changes of filtered TBS wash buffer for 10 min each followed by 2 changes of filtered distilled water for 5 min each. Following counterstaining with filtered 7.7% aqueous uranyl acetate for 10 min, sections are examined with a Hitachi H-600 transmission electron microscope operated at 75 kV. Using low magnifications so that beads are not discernible, 10-30 regions are randomly selected for each sample and photographed. Electron micrographs are developed and then converted to digital images using a scanner (Epson Perfection 4870 Photo Scanner) with the unsharp mask filter option selected. Negatives are scanned in grayscale at a higher resolution (600 dpi), saved as TIFF files, and then an 8x10 inch copy is printed on a laser printer (Laserjet 1000, Hewlett Packard). Areas of interest are outlined by hand on the 8x10 printout and using a photo imaging program (Adobe Photoshop Elements 2.0) the number of gold beads in each area is visually counted and recorded in Microsoft

Excel. When counting of gold beads is complete, the image is resized to approximately 300 dpi to allow for adequate size and display resolution when opened with image analysis software (Scion Image Beta 4.02, Scion Corporation, Frederick, MD). The original EM micrograph is calibrated to the appropriate image scale by calculating the known distance (the distance in millimeters measured between the 2 edges of shadow on the negative, multiplied by 1000, and then divided by the actual magnification of the negative). The image software then automatically calibrates the image pixel properly into the desired unit area (micrometer). Once an image is opened and calibrated, the regions corresponding to those quantitated for gold beads are circled and the calculated area ( $\mu\text{m}^2$ ) is pasted into Microsoft Excel. The number of gold beads in a selected region is divided by its corresponding area ( $\mu\text{m}^2$ ) to give the labeling density (gold beads /  $\mu\text{m}^2$  area). Mean density values are calculated from the total number of micrographs and represented graphically using Microsoft Excel. Statistical analyses, Independent Student's T-Test and/or ANOVA, are performed to calculate the standard error of mean and relative significance ( $p \leq 0.05$ ) using SPSS 10 for Windows (SPSS Inc., Chicago, IL). Statistical data are represented graphically with all results.

#### Positive controls

Positive controls should be used in any morphologic analysis. 4HNE is found in large quantities in organs with large amounts of lipofuscin, including seminal vesicle and prostate. Aging brain and heart may also be used.

#### Negative controls

Preincubation of antigen with antibody should abolish staining of tissue sections. Normal mouse serum in place of primary antibody is also always used as a negative control.

### *Conclusions*

Results to date have demonstrated that several pathologic processes studied have unique expression patterns of 4HNE-modified proteins. In ischemia-reperfusion injury, 4HNE modified proteins were observed primarily in mitochondria (unpublished observations), in human renal cancer in both mitochondria and nucleus,<sup>3</sup> and in aging skeletal muscle primarily in cytoplasm.<sup>7</sup> We have recently quantified 4HNE-modified proteins in phorbol ester treated mouse skin of nontransgenic and transgenic manganese superoxide dismutase (MnSOD)-overexpressing mouse skin, and demonstrated that the mouse skin from nontransgenic mice had two fold increase in 4HNE-modified proteins as soon as 6 hr after TPA treatment compared to MnSOD transgenic mice (unpublished observations). Further, quantitative image analysis indicated an increase in oxidative damage following TPA treatment in nuclei and mitochondria, but not in the cytoplasm. These results demonstrated that TPA caused oxidative damage and showed that overexpression of MnSOD was able to reverse this damage. These results were of special interest since the transgenic mice had two fold less papilloma formation (unpublished observations). These results illustrate the importance use of immunomorphologic techniques in quantifying oxidative damage to allow study of important biological problems.

Future studies will require knowledge of the physiologic roles and location of free 4HNE before pathologic analyses can be completely interpreted in a reliable fashion.

Further, other factors that can regulate levels of free 4HNE and 4HNE-modified proteins must be taken into consideration in analysis of data, including antioxidant defense mechanisms and protein and whole cell turnover rates. Thus, cells and protein with low turnover rates and low antioxidant defenses would be expected to have higher levels of 4HNE-modified proteins. Future studies will thus need knowledge of both 4HNE formation and protein and cell turnover rate in order to fully delineate the physiologic and pathologic roles of 4HNE.

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