Microscopy Techniques for Immunolocalisation of Nitrated Proteins in Different Tissues

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Immunohistochemistry is an important and often used tool in the investigation of post-translational protein modifications; facilitating the identification of modified proteins at cellular level. Nitration of tyrosine residues (NO₂Tyr) has been detected in multiple species, organ systems, and cell types during both acute and chronic inflammation. This post-translational protein modification is a marker of oxidative injury that is frequently linked to altered protein function during inflammatory conditions. Methods that allow for definitive NO₂Tyr identification in tissues depend on the immunoreactivity of the NO₂Tyr adduct to antibodies raised against artificially nitrated proteins. In previous reports we have utilized both immunofluorescence and light microscopy to reveal the occurrence of protein nitration in glaucomatous retinal damage, ocular inflammation, liver and kidney damage. Elevated levels of NO₂Tyr in all of these diseases imply the formation of secondary species capable of nitration reactions.

Keywords: Immunohistochemistry, immunofluorescence microscopy, nitration, inflammation.

1. Tyrosine Nitration Mechanisms

Post-translational modification of tyrosine (Tyr) to 3-nitrotyrosine (NO₂Tyr) occurs via multiple pathways and involves a variety of reactive species. To nitrate Tyr, nitrogen dioxide (•NO₂) reacts with a tyrosyl radical (Tyr•) at diffusion-limited rates (3 x 10⁹ M⁻¹ s⁻¹) [1], initially Tyr• is generated on free or protein-bound Tyr via reaction with one electron oxidants, including the carbonate anion radical (•CO₃²⁻):

Tyr + •OH, •CO₃ or •NO₂ → Tyr• + OH⁻, CO₃²⁻ or NO₂⁻

Next the tyrosyl radical reacts with nitrogen dioxide to give NO₂Tyr:

Tyr• + •NO₂ → NO₂Tyr

The limiting step in this reaction is the formation of •NO₂, which occurs both enzymatically and nonenzymatically via three primary pathways described below, but it can be easily inferred that nitration of proteins always stems indirectly from •NO production or the presence of dietary nitrite (NO₂⁻) [2, 3]. Indeed NO₂Tyr-generating pathways invariably include Tyr•, •NO₂ and •CO₃ radicals, so for the most part, NO₂Tyr formation generally proceeds as outlined above [4]. Glutathione, cysteine, ascorbate, and other reductants exist in high concentrations intracellularly and can thus inhibit the accumulation of initiating radicals to limit NO₂Tyr formation. Spatial sequestration of key mediators of Tyr nitration into hydrophobic environments such as the interior of cell membranes and hydrophobic regions of proteins may provide the protection necessary to propagate these reactions [5].

Immunohistochemistry is an important and often used tool in the investigation of post-translational protein modification [2, 6] which is a marker of oxidative injury that is frequently linked to altered protein function during inflammatory conditions [2, 6]. Nitrotyrosine can be detected in multiple species, organ systems, tissues, and cell types during inflammation.

2. Microscopy Techniques for Visualization of Nitrated Proteins

Immunohistochemical detection of tyrosine-nitrated proteins was developed by means of anti-NO₂Tyr antibodies. Immunohistochemical protocols have been tested in many tissues that have been fixed in paraformaldehyde, cryoprotected and frozen. There are three microscopy techniques which have been utilized for visualization of nitrated proteins in tissues [7, 8].

2.1 Light Microscopy

Light microscopy can be used to reveal the occurrence of protein nitration in different tissues. Primary antibodies from rabbit or mouse are used for the qualitative identification of antigens by light microscopy [7, 9].
Processing for immunohistochemistry can be done on paraffin sections by fixing blocks of tissue (15mm x 15mm x 5mm) with 3-4% formaldehyde in Ringer’s solution and dehydrated through a graded ethanol series. Specimens are further dehydrated with 3 changes of absolute xylene for 15 minutes per change and embedded in 2 changes of liquid paraffin at 57°C. Embedded tissue is allowed to solidify into blocks and is mounted for sectioning on a microtome. Tissues from outer and inner regions of organs is sectioned at a thickness of 5µ and collected onto (+) charged slides [10].

Prior to immunostaining, paraffin sections are melted on the slides at 57°C for 1 hour and deparaffinized. Deparaffinization involves 3 changes of absolute xylene with agitation for 5 minutes per change and a reverse ethanol gradient is applied. Samples are then rehydrated in Dulbecco’s phosphate buffered saline (PBS) without Mg²⁺ or Ca²⁺, (pH 7.4) and postfixed with 3% formaldehyde for 15 minutes to promote tissue adhesion to slides [11].

Processing for immunohistochemistry can also be done on frozen sections. In this case, tissues are placed in cryomolds containing optimum cutting temperature (OCT) embedding medium. Samples are flash-frozen by immersing in a metal cup filled with 2-methyl butane prechilled in a larger metal cup containing liquid nitrogen. Frozen blocks are wrapped in aluminum foil and stored at -180ºC or sectioned immediately on a cryostat. Five micron sections are collected on (+) charged slides and are fixed, just before completely drying, in either absolute methanol or acetone at -20ºC for 10 minutes. Sections can also be fixed in 3% formaldehyde in PBS for 45 minutes at room temperature. Samples are then briefly rinsed in PBS and processed for standard immunostaining [10].

For peroxidase staining, endogenous peroxidase activity is blocked by incubating tissue sections with 3% hydrogen peroxide for 5 min prior to application of the primary antibody [12-15]. Primary antibody incubations are done via commercially available primary or secondary antibodies for NO₂-Tyr according to manufacturer’s instructions. Nitrotyrosine antibodies can either be polyclonal or monoclonal. Polyclonal antibodies are obtained from immunization of animals (rabbit, rat, goat, mouse) with peroxynitrite-treated keyhole limpet haemocyanin (KLH) and affinity purified on an immobilized NO₂-Tyr-BSA column. Polyclonal antibodies are capable of recognizing many epitopes of the same antigen, generating higher detection sensitivity. Monoclonal antibodies are developed from mouse via peroxynitrite-treated KLH. Antibodies, purified from hybrid cell culture supernatant are capable of recognizing only one antigen epitope, yielding more specific results [13, 16, 17].

Following primary antibody incubations, sections are washed and incubated with biotinylated secondary antibody according to manufacturer’s instructions. Slides are washed and incubated with streptavidin-linked Horseradish Peroxidase (HRP) [14, 15, 18, 19]. Incubation with streptavidin-HRP is usually performed for 10 (+1) minutes and slides are rinsed with substrate-chromogen solution either 3-amino-9-ethylcarbazole (AEC) [20] or 3'-diaminobenzidine (DAB) [7, 21]. Counterstaining with hematoxylin results in a palette of dark blue coloration of cell nuclei. Diaminobenzidine chromogen yields an alcohol insoluble end-product and can be used with an alcohol-based hematoxylin [7, 21]. When AEC substrate-chromogen is used, the colored end-product of the staining reaction is alcohol soluble and should only be used with aqueous-based counterstains [20]. Slides can be mounted and cover slipped with mounting media. The AEC reaction product is soluble in organic solvents and is therefore not compatible with tolueene or xylene-based, permanent mounting media. On the other hand DAB may be mounted with any permanent mounting media [20,21].

The quality control of the protocol can be checked by positive and negative control tissues. Differences in tissue processing and technical procedures in the user’s laboratory may produce significant variability in results. Positive control tissues are indicative of correctly prepared tissues and proper staining techniques. One positive control tissue for each set of test conditions should be included in each staining run. Positive control tissues should also be weakly positive for each primary antibody to detect changes in reagent sensitivity. Commercially available tissue slides or specimens processed differently from the patient sample validate reagent performance only, and don’t verify tissue preparation. Known positive control tissues should only be utilized for monitoring the correct performance of processed tissues and test reagents, not as an aid in formulating a specific diagnosis of patient samples. If the positive control tissues fail to demonstrate positive staining, results with the test specimens should be considered invalid. One negative control tissue fixed, processed and embedded in a manner identical to the patient sample should be stained with each primary antibody used in each staining run to verify the specificity of each primary antibody. This tissue should not exhibit specific staining and serves as an indicator of non-specific staining. Negative control tissues should be used as an interpretive aid to distinguish specific staining from non-specific staining results. The variety of different cell types present in most tissue sections offers internal negative control sites. If staining occurs in the negative control tissue, results with the patient specimen should be considered invalid [22-26].

2.2 Immunofluorescence Microscopy

Immunofluorescence microscopy can also be used to reveal the occurrence of protein nitration in different tissues such as liver, kidney and lung. Paraffin-embedded tissue sections or cryosections, prepared as described above, can also be processed for fluorescence immunostaining. Sections are permeabilized using 0.1% Triton-X-100 in phosphate-buffered saline and post-fixed with 3% formaldehyde in PBS for 15 min at 25°C to promote tissue adhesion to slides. Samples are rinsed briefly with PBS and processed for immunofluorescence by blocking in 1% BSA in PBS or 5-50% normal serum for 15-30 min at 25°C. Tween-20 (0.05 to 0.1%) can be added to blocking buffer before and during antibody incubation, as well as to PBS washes after antibody incubation. Primary antibody incubations are performed
with commercially available primary or secondary anti- \( \text{NO}_2\text{Tyr} \) antibodies. The secondary antibody is fluorophore conjugated which can have different excitation wavelengths depending on the choice of fluorophore used. Samples are washed in PBS after both primary and secondary antibody incubation. Nuclei can be counterstained with a nuclear dye. Nonspecific staining can be ruled out by control experiments as described above [27, 28].

2.3 Confocal Microscopy

The third technique for visualization of nitrated proteins in cell preparations is confocal microscopy. Samples are fixed with 4% paraformaldehyde in PBS for 10 min, permeabilized in PBS containing 0.1% Triton X-100 for 30 min and rinsed with PBS. The fixed samples are incubated with either monoclonal or polyclonal primary antibodies according to manufacturer instructions and then with fluorophore conjugated secondary antibody. Nuclear staining can be performed via a nuclear dye [8].

3. Immunolocalisation of Nitrated Proteins In Different Tissues

Nitrotyrosine can be immunolocalized in different tissues in consequence of human diseases and in some animal models of disease as further described below.

3.1 Cardiovascular System

Atherosclerosis leads to \( \text{NO}_2\text{Tyr} \) immunoreactivity in LDL particles within the aortic lesion, necrotic core of plaque, sub-intimal fatty streaks, macrophages, foam cells, smooth muscle cells, and endothelial cells [29-36]. Cardiac allograft rejection in humans also causes \( \text{NO}_2\text{Tyr} \) formation in macrophages and adjacent myocytes from endomyocardium [37]. Preeclampsia causes \( \text{NO}_2\text{Tyr} \) formation in placental villous vessels, villous stroma, cytotrophoblasts and in endothelial cells of maternal blood vessels [38-43]. Human transplant coronary artery disease leads to protein \( \text{NO}_2\text{Tyr} \) in macrophages and smooth muscle cells [44]. Angiotensin II induced vascular dysfunction in rats causes protein nitration in endothelial cells of the thoracic aorta [45]. Similarly, autoimmune myocarditis leads to protein \( \text{NO}_2\text{Tyr} \) formation in damaged myocytes and macrophages [46, 47]. Balloon injured artery in rat models is characterized by \( \text{NO}_2\text{Tyr} \) immunostaining in neo-intimal smooth muscle and endothelial cells [48]. Nitration can also be observed in the ventricular wall, necrotic myocardium, myocytes, microvascular endothelial cells in rats following myocardial ischemia reperfusion injury [49-51]. Immunocytochemical analysis of protein \( \text{NO}_2\text{Tyr} \) in descending thoracic aortic segments from LPS-treated mice is shown in figure 1.
Figure 1. Immunocytochemical analysis of protein 3-nitrotyrosine (3-NT) in LPS treated mice. Descending thoracic aortic segments from LPS-treated mice display intense immunofluorescent staining for NT (red) that is associated with the endothelium and smooth muscle cells. Nuclei were counter-stained with Hoechst in all experiments.

3.2 Central Nervous System

Nitrotyrosine is detected in frontal cortical neurons following AIDS dementia [52]. Likewise, sporadic and familial Amyotrophic Lateral Sclerosis (ALS) is associated with NO$_2$Tyr formation within the spinal cord, neurofilament aggregates, motor neurons, astrocytes and vascular wall [53-57]. Alzheimer’s disease in humans causes protein nitration in hippocampal neurons and neurofibrillary tangles [58-60]. Nitrotyrosine immunostaining can also be observed in hypertrophic astrocytes and within areas of demyelization in humans with Multiple Sclerosis [61,62]. The core of Lewy bodies can similarly be stained for NO$_2$Tyr in human Parkinson’s disease [63]. Transient spinal cord ischemia in rabbits leads to NO$_2$Tyr immunostaining in large pyramidal motor neurons [64]. Nitrotyrosine immunostaining has also been shown in both the hippocampus and thalamus of rats following cortical ischemia [65]. Pneumococcal meningitis in rats leads to protein nitration in meningeal blood vessels and inflammatory cells in the subarachnoid space [66]. Cerebral cortical neurons have also been shown to immunostain for NO$_2$Tyr in a murine model of Huntington’s disease [67].

3.3 Eye

Nitrotyrosine can be detected in humans with glaucomatous optic neuropathy [68]. Rat model of glaucoma and autoimmune uveitis also show significant increase in NO$_2$Tyr immunoreactivity occurring in retinal photoreceptors, ganglion cells, nerve fibers, and in retinal blood vessels [14, 69]. Experimental uveitis in rats also leads to corneal protein nitration [15].
3.4 Gastrointestinal System

Nitrotyrosine is immunolocalized in small intestinal crypt enterocytes in humans with Celiac disease [70]. Gastric ulcer, associated with Helicobacter pylori, also leads to NO\textsubscript{2}Tyr immunostaining in active ulcer margins, epithelial cells and lamina propria in humans [71]. Likewise, Helicobacter pylori gastritis in humans cause protein nitration in epithelial cells, inflammatory cells and extracellular matrix [72]. Inflammatory bowel disease in humans is characterized by NO\textsubscript{2}Tyr immunostaining in colonic epithelium, lamina propria and tissue macrophages [73,74]. Ulcerative colitis in humans is similarly associated with NO\textsubscript{2}Tyr formation in epithelial cells and lamina propria [75]. Pancreatic islet \(\beta\)-cells and macrophages can be immunostained for NO\textsubscript{2}Tyr in a murine model of autoimmune diabetes [76].

3.5 Kidney

Human diabetic nephropathy is associated with nitrotyrosine immunoreactivity in the collecting ducts, thin limb of loop of Henle, proximal and distal tubules [77]. Renal allograft rejection in humans is also associated with protein NO\textsubscript{2}Tyr in tubular epithelial cells [78]. The proximal and distal tubules are immunoreactive for NO\textsubscript{2}Tyr in both Sickle Cell Disease (SCD) mouse and human but, unlike mice, human SCD kidneys are also immunoreactive for NO\textsubscript{2}Tyr in glomeruli [28].

3.6 Liver

Cholangiocarcinoma is associated with nitrotyrosine immunoreactivity in biliary epithelial cells [79]. Kupffer cells and hepatocytes are also immunoreactive for NO\textsubscript{2}Tyr in chronic hepatitis [80, 81], hepatic allograft rejection [82] and in haemorrhagic shock [83]. Hepatocytes surrounding the central veins similarly demonstrate NO\textsubscript{2}Tyr immunoreactivity in SCD [28]. Liver ischemia reperfusion injury in rats leads to NO\textsubscript{2}Tyr formation in pericentral hepatocytes [84].

3.7 Respiratory System

Nitrotyrosine is detected in lung interstitium, alveolar epithelial cells and alveolar exudate during acute lung injury [85]. Acute respiratory distress syndrome is also associated with NO\textsubscript{2}Tyr formation in alveolar epithelial cells, capillary endothelial cells, and in broncholavage fluid [86, 87]. Idiopathic pulmonary fibrosis also causes protein nitration in airway and alveolar epithelial cells [88]. Immunocytochemical distribution of NO\textsubscript{2}Tyr in lung tissue of rats treated with LPS is shown in figure 2.
4. Quantitation of Nitrotyrosine Immunostaining

Nitrotyrosine immunostaining can be quantified by computerized densitometry following image acquisition. Software program designed for densitometric quantitation can adjust images to appropriately remove background staining or fluorescence. Pixel intensity can be analyzed by measuring staining or fluorescence of the region of interest. Depending on the technical procedure used, results can either be defined as fluorescence intensity [27] or be expressed as the inverse log of intensity gray value per area (ILIV/AREA). ILIV/AREA results are proportional to the unweighted average optical density which is used to determine the concentration of immunoreactive antigen [89].

5. Summary

Tyrosine-nitrated proteins can be detected by means of anti-NO$_2$Tyr antibodies via immunohistochemistry. Immunohistochemical protocols have been tested in different tissues that have been fixed in paraformaldehyde, cryoprotected and frozen. Three microscopy techniques are utilized for visualization of nitrated proteins in tissues which include light microscopy, immunofluorescence microscopy and confocal microscopy. Nitrotyrosine can be immunolocalized in different tissues such as cardiovascular system, central nervous system, eye, gastrointestinal system, kidney, liver and respiratory system as a result of human diseases and animal models of disease. Nitrotyrosine
immunostaining can be quantified by computerized densitometry following image acquisition. Results can either be defined as fluorescence intensity or be expressed as the inverse log of intensity gray value per area.

References


