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Original Contribution

Subcellular localization of tyrosine-nitrated proteins is dictated by reactive oxygen species generating enzymes and by proximity to nitric oxide synthase

Harry F.G. Heijnen [a,b,c,*], Elly van Donselaar [b,c], Jan W. Slot [b,c], Diana M. Fries [d,e], Beatrice Blachard-Fillion [d], Roberto Hodara [d], Richard Lightfoot [d], Manuela Polydoro [d], Dave Spielberg [d], Leonor Thomson [d,f], Elizabeth A. Regan [g], James Crapo [g], Harry Ischiropoulos [d]

[a] *Thrombosis and Hemostasis Laboratory, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*
[b] *Department of Cell Biology, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands*
[c] *Institute for Biomembranes, Utrecht, The Netherlands*
[d] *Stokes Research Institute, Department of Biochemistry and Biophysics, Children's Hospital of Philadelphia and University of Pennsylvania, Philadelphia, PA 19104, USA*
[e] *Ministry of Health, Sao Paulo, SP, Brazil*
[f] *Facultad de Ciencias, Universidad de la Republica, Montevideo, Uruguay*
[g] *National Jewish Medical and Research Center, Denver, CO 80206, USA*

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Abstract

Using high-resolution immuno-electron microscopy the steady-state subcellular distribution of tyrosine-nitrated proteins in different cells and tissues was evaluated. In quiescent eosinophils and neutrophils in the bone marrow intracellular nitrated proteins were mainly restricted to the peroxidase-containing secretory granules. The inducible nitric oxide synthase (iNOS) was expressed in the same granules. Proteins nitrated on tyrosine residues were also abundant in the cytosol of circulating erythrocytes. In the vasculature, nitrated proteins were mainly located in mitochondria and endoplasmic reticulum of the endothelial cells, fibroblasts, and smooth muscle cells. Endogenous nitrated proteins were also found in chondrocytes in cartilage, where it was typically associated with the cytoplasmic interface of the endoplasmic reticulum membrane. Nitrated proteins were also prominent in the peroxisomes of liver hepatocytes and of secretory cells in the lacrimal gland. Challenge of mouse dendritic cells with lipopolysaccharide induced iNOS protein expression in cytosol and peroxisomes and was associated with an increased 3-nitrotyrosine formation in cytosol, mitochondria, and peroxisomes. These data indicate that nitric oxide-dependent protein tyrosine nitration is a physiologically relevant process localized within specific subcellular compartments in close proximity to iNOS and to enzymes capable of peroxidative chemistry and reactive oxygen species production.

Keywords: Immuno-electron microscopy; Nitric oxide; Nitrotyrosine; Superoxide; Peroxidases; Free radicals

The formation of nitric oxide (NO) in biological systems has been intimately linked with the discovery of a number of NO-dependent posttranslational protein modifications that could regulate protein function and/or be employed as transducers of nitric oxide signaling [1,2]. One of the nitric oxide-mediated protein modifications is the nitration of one of the two equivalent carbons (carbon 3) in the aromatic ring of tyrosine residues. This results in the formation of an unusual amino acid, 3-nitrotyrosine. Because the formation of 3-nitrotyrosine requires the formation of higher oxides of nitric oxide, protein nitration has been mostly associated with pathological conditions of oxidative stress [3–5]. Indeed, immunohistochemical and analytical methodologies have been employed to detect and quantify nitrated proteins in several major human diseases [3,4]. The advent of proteomics has dramatically and significantly expanded these data by identifying specific proteins in the context of disease conditions that are modified by nitration

* Corresponding author. Thrombosis and Hemostasis Laboratory, University Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. Fax: +31 30 2541797.
E-mail address: h.f.g.heijnen@azu.nl (H.F.G. Heijnen).

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[6–10]. Furthermore, electron microscopic (EM) examination of tissues has also revealed intriguing subcellular localizations of nitrated proteins [11–14]. For example in human Parkinson disease nitrated α-synuclein was found almost exclusively on Lewy bodies, the hallmark protein inclusions in Parkinson disease, and dystrophic neurons [13]. Overall these data have provided evidence for a rather selective process that modifies specific proteins in vivo.

However, with the exception of a few studies, the subcellular localization of nitrated proteins under physiological conditions has not been investigated. One study exploring the EM localization of nitrated proteins in rat brain under physiological conditions revealed localization in the outer mitochondrial membranes in dendrites, within asymmetric synapses in dendritic spines, in synaptic vesicles in axon terminals, and in astrocytic processes [11]. In aging rat vascular tissue, nitrated proteins were localized in the cytoplasm, nucleus, and mitochondria [12]. In the present study we demonstrate by immuno-electron microscopy, employing specific affinity-purified polyclonal antibodies, that NO-dependent protein nitration occurs at distinct intracellular locations under steady-state physiological conditions in several rat and human tissues. Our data support the concept that superoxide and peroxidases in addition to nitric oxide synthase (NOS) enzymes are the critical determinants that dictate the specific intracellular sites of protein nitration.

Materials and methods

Cell culture and tissue preparation

The procedures involving the use of animals followed the *Guidelines for Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). Rats (~150 g body wt) were anesthetized and perfusion-fixed through the left ventricle of the heart with a mixture of 2% paraformaldehyde (PFA) and 0.2% glutaraldehyde (GA) in 0.1 M sodium phosphate buffer (pH 7.4). After perfusion, the tissues of interest (i.e., aorta, pancreas, liver, testis, lacrimal gland) were excised, immediately postfixed for 2 h at room temperature in the same fixative, and stored in 1% PFA at 4°C until use. Human cartilage tissue was obtained from subjects undergoing knee surgery for arthritis and ligament reconstruction. Slabs of 0.2 mm thickness were taken from the medial femoral condyle at the margin and immediately fixed with a mixture of 2% PFA and 0.2% GA in 0.1 M sodium phosphate buffer (pH 7.4). The cartilage research was approved by the Institutional Review Board of Exampla, St Joseph Hospital (Denver, CO, USA).

Bone marrow samples were obtained from rat femur, after flushing with 2% PFA and 0.2% GA in 0.1 M sodium phosphate buffer (pH 7.4). The samples were immersion fixed in the same fixative. After being washed, samples were embedded in 10% gelatin, cooled in ice, and cut into 1-mm^3 blocks in the cold room. The blocks were infused with 2.3 M sucrose at 4°C for 24 h, frozen in liquid nitrogen, and stored until cryo-ultramicrotomy.

Development and characterization of the anti-3-nitrotyrosine antibodies

Polyclonal anti-nitrotyrosine antibodies NT432 and NT609 were raised against the octapeptide Cys-Gly-NO_2Tyr-Gly-Gly-Gly-NO_2Tyr-Gly. The antibodies were affinity purified, and the purity was confirmed by nonreduced SDS electrophoresis (Fig. 1A). The specificity of the antibodies was determined by competition enzyme-linked immunosorbent assay (ELISA) and Western blotting (Figs. 1B and 1C). No cross-reactivity was found against tyrosine, phosphotyrosine, chlorotyrosine, methyltyrosine, or dopamine. Furthermore, preadsorption with 3-nitrotyrosine in a concentration-dependent manner inhibited binding of the antibody to nitrated proteins (Fig. 1C). For Western blotting different proteins were nitrated by peroxynitrite in the presence of CO_2 as described before [9] and the nitrated proteins were transferred to PVDF membranes overnight at 20 V. Membranes were blocked with 10% milk in TBS with 0.5% Tween (TBS-t) for 2 h at room temperature. The primary antibodies were incubated in 3% milk TBS-t at room temperature for 1 h (166 and 432 ng/ml for NT609 and NT432 antibodies, respectively) followed by 10 min × 2 and 5 min × 2 washes with TBS-t. The secondary antibody (goat anti-rabbit IgG–HRP conjugate from Bio-Rad) was incubated in 1% milk TBS-t for 1 h at room temperature followed by the same washes as the primary antibody.

A mixture of 3-nitrated and nonnitrated proteins was also separated by nonreducing PAGE and transferred to PVDF by standard methods and probed with anti-nitrotyrosine NT609 (200 ng/ml) and pAb432 (500 ng/ml). Primary antibody binding was detected by goat anti-rabbit antibodies labeled with AlexaFluor 680 (200 ng/ml; Molecular Probes, Eugene, OR, USA), followed by signal detection with the Odyssey system (Li-Cor, Inc., Lincoln, NE, USA).

For affinity capturing of nitrated proteins, NT2 cells were treated with 3-nitrotyrosine, which results in the specific incorporation of 3-nitrotyorosine into the carboxy terminus of α-tubulin [17]. Cells were washed with PBS, solubilized in 100 μl ice-cold lysis buffer containing (in mM) 20 Hepes, 150 NaCl, 1 EGTA, and 1.5 $MgCl_2$; 10% glycerol; 1% Triton X-100; protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 10 mM sodium pyrophosphate, 50 mM sodium fluoride); 2 mM sodium orthovanadate; 1 μM lactacystin. Lysates of control and nitrotyrosine-treated cells were loaded on a 12% SDS–PAGE gel (50 μg of protein/lane) for Western blotting or onto anti-nitrotyrosine affinity column. The column was made by cross-linking with dimethylpimelimidate the affinity-purified anti-nitrotyrosine antibodies to a solid matrix using the ImmunoPure Protein A IgG Orientation Kit from Pierce (Cat. No. 44898), as described before [9,10]. The column was then equilibrated at room temperature and washed with 10 ml of PBS (0.15 M NaCl, 0.1 M phosphate, pH 7.2). The cellular extract was allowed to bind overnight at 4°C. The unbound fraction was eluted with 20 ml of PBS followed by a wash with 10 ml of 0.5 M NaCl. The specifically bound fraction was eluted with 10 ml of 5 mM 3-nitrotyrosine in 0.5 M