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REVIEW ARTICLE

Functional consequences of actin nitration: in vitro and in disease states

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Abstract To link the phenomena of inflammatory-induced increases in protein nitrotyrosine (NO_2Tyr) derivatives to protein dysfunction and consequent pathological conditions, the evaluation of discrete NO_2Tyr modifications on specific proteins must be undertaken. Mass spectrometric (MS) proteomics-based strategies allow for the identification of all individual proteins that are nitrated by separating tissue homogenates using 2D gel electrophoresis, detecting the nitrated proteins using an anti-NO_2Tyr antibody, and then identifying the peptides generated during an in-gel proteolytic digest using matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) MS. Actin, one of the most abundant proteins in eukaryotic cells, constitutes 5% or more of cell protein and serves with other cytoskeletal proteins as a critical target for nitration-induced functional impairment. Herein, examples of actin nitration detected under physiological conditions in various models of human disease or in clinically derived tissues are given and the impact that this post-translational protein modification can have on cell and organ function is discussed.

Keywords Nitrotyrosine · Actin · Reactive nitrogen species · Reactive oxygen species · Protein structure and function

Tyrosine nitration mechanisms

An important modification caused by reactive nitrogen species [RNS, e.g., nitrogen dioxide ($\cdot NO_2$), peroxynitrite ($ONOO^-$)] is nitration, the covalent introduction of a nitro ($-NO_2$) group to biomolecules. The covalent addition of $-NO_2$ to free and protein-bound tyrosine residues at either of the equivalent ortho positions of the aromatic ring creates the 3-nitro-L-tyrosine (NO_2Tyr) adduct (Beckman 1996). Because detection of RNS is hampered by the short half-lives of intermediates and fast reactions with other biomolecules, measurement of NO_2Tyr serves as an indirect biomarker for nitrating species derived from ·NO and is a reflection of extent of in vivo RNS production during physiological and pathological conditions (Aslan et al. 2006, 2007, 2008).

Post-translational modification of Tyr to 3-NO_2Tyr occurs via multiple pathways and involves a variety of reactive species. To ultimately nitrate Tyr, nitrogen dioxide ($\cdot NO_2$) reacts with the tyrosyl radical (Tyr·) at diffusion-limited rates ($3 \times 10^9\ M^{-1}\ s^{-1}$) (Beckman 1996) in a concerted, two-step mechanism, whereby initially Tyr· is generated on free or protein-bound Tyr via reaction with one electron oxidants, including the carbonate anion radical ($\cdot CO_3^-$):

$$\mathrm{Tyr} + \cdot\mathrm{OH},\ \cdot\mathrm{CO}_3^-\ \text{or}\ \cdot\mathrm{NO}_2 \rightarrow \mathrm{Tyr}\cdot + \mathrm{OH}^-,\ \mathrm{CO}_3^{2-}\ \text{or}\ \mathrm{NO}_2^- \quad (1)$$

Next, the tyrosyl radical reacts with nitrogen dioxide to give nitrotyrosine:

$$\mathrm{Tyr}\cdot + \cdot\mathrm{NO}_2 \rightarrow \mathrm{NO}_2\mathrm{Tyr} \quad (2)$$

An essential contribution to reaction 2 is $\cdot NO_2$, which can be formed both enzymatically and non-enzymatically 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 (NO_2^-) (Greenacre and Ischiropoulos 2001).

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Generation of peroxynitrite can lead to nitrogen dioxide formation

The most widely studied nitrating intermediate is $ONOO^-$, which is formed from the radical–radical reaction of ·NO with $O_2^{\cdot-}$. Superoxide readily undergoes a free radical termination reaction with ·NO because the rate constant for this reaction is three times faster (1.9×10^{10} M^{-1} s^{-1}) (Kissner et al. 1997) than the reaction rate of $O_2^{\cdot-}$ with SOD. This extremely fast reaction for ·NO with $O_2^{\cdot-}$ and the high concentrations of ·NO make the reaction to form $ONOO^-$, an extremely likely pathway to direct $O_2^{\cdot-}$ and ·NO toxicity (Beckman 1996). Peroxynitrite is in fast, dynamic equilibrium with its conjugated acid, peroxynitrous acid (ONOOH, pK_a 6.8), which can either rapidly undergo homolytic cleavage to yield $\cdot NO_2$ and ·OH (Schopfer et al. 2003) or isomerize to nitrate (NO_3^-). Tyrosines will then react with ·OH and $\cdot NO_2$ derived from $ONOO^-$ to form Tyr·, which recombines with $\cdot NO_2$ to produce NO_2Tyr.

A major concern regarding $ONOO^-$ as a primary contributor to oxidative/nitrosative damage in vivo is that $ONOO^-$ generation requires the simultaneous production of $O_2^{\cdot-}$ and ·NO in the same subcellular locale, a condition that is difficult to predict or measure in a non-homogeneous, compartmentalized biological system (Grisham et al. 1999). In vitro studies show that equivalent rates of ·NO and $O_2^{\cdot-}$ production in a 1:1 ratio are optimal for $ONOO^-$ formation (Miles et al. 1996), suggesting that obtaining favorable NO_2Tyr yields might necessitate an equivalent ratio of radicals because a surplus of either ·NO or $O_2^{\cdot-}$ can react additionally with Tyr· and $\cdot NO_2$ diverting these radicals down other reaction pathways away from NO_2Tyr formation. Studies reveal that the direct reactions of $ONOO^-$ are not affected by excess ·NO or $O_2^{\cdot-}$ (Jourd'heuil et al. 1999), but NO_2Tyr formation relies on the protonation of $ONOO^-$ to ONOOH and hemolytic scission to yield ·OH and $\cdot NO_2$, which can still react with excess ·NO and $O_2^{\cdot-}$ to attenuate NO_2Tyr production.

In compartmentalized biological systems such as cells, where the reacting species have different membrane permeabilities and catalytic systems aimed to detoxify excess radicals, ·NO and $O_2^{\cdot-}$ steady-state concentrations are much lower and resistant to variations, despite large changes in $O_2^{\cdot-}$ and ·NO formation rates that may occur. Indeed, in biological systems, ·NO diffusion and $O_2^{\cdot-}$ dismutation pathways compete with peroxynitrite formation and prevent the accumulation of the radicals when their relative fluxes are different to one another. Thus, in biological systems, $ONOO^-$-mediated nitration processes have low yields but are responsive to increases in $O_2^{\cdot-}$ and ·NO production rates. In any case, $ONOO^-$-mediated (both carbon dioxide and metal-catalyzed), heme peroxidase-catalyzed, and "Fenton-like" tyrosine nitration pathways converge for the formation of tyrosyl radicals and $\cdot NO_2$ that recombine to form 3-nitrotyrosine (Quijano et al. 2005).

Formation of nitrogen dioxide via homolytic cleavage of nitrosoperoxocarbonate

In the presence of bicarbonate buffers, but not in phosphate-based buffers, an increase in $ONOO^-$-mediated luminol oxidation occurred, indicating that a more reactive oxidizing species was being created. To explain these phenomena, the formation of nitrosoperoxocarbonate ($ONOOCO_2^-$) and its decay into $\cdot CO_3^-$ was proposed (Radi et al. 1993).

Peroxynitrite reacts with CO_2 at a reaction rate of 4.6×10^4 M^{-1} s^{-1} at pH 7.4 and 37°C to produce $ONOOCO_2^-$ (Denicola et al. 1996). The putative intermediate $ONOOCO_2^-$ decays by homolysis of the peroxidic bond to form $\cdot NO_2$ and $CO_3^{\cdot-}$. The radicals are initially formed in a solvent cage. About 35% of the radicals escape the cage and are able to react with targets. The rest react inside the cage forming NO_3^- and CO_2· (Goldstein and Merényi 2008). A mechanism for Tyr nitration was advanced involving one electron oxidation by $ONOOCO_2^-$, rather than direct nitration of the Tyr aromatic ring by a $ONOOCO_2^-$ intermediate (Lymar et al. 1996). This free radical mechanism proposed that $\cdot CO_3^-$ is an extremely facile oxidant for Tyr and preferentially generates Tyr· by abstracting hydrogen atoms from the phenolic ring of Tyr in a manner more efficient than the $ONOO^-$-mediated route (Alvarez and Radi 2003); the $\cdot NO_2$ radical then adds to Tyr· to give NO_2Tyr. This mechanism was confirmed using continuous fast flow EPR to detect $\cdot CO_3^-$ formed during $ONOOCO_2^-$ decomposition (Bonini et al. 1999). Compared to the homolysis of protonated $ONOO^-$ which occurs at a slow rate (0.9 s^{-1}), it is kinetically more likely that the formation of NO_2Tyr is mediated by the faster reaction of $ONOO^-$ with CO_2. Additionally, an increase in nitration reactions in response to $ONOOCO_2^-$ can be attributed to elevated $\cdot NO_2$ generation due to rapid electron transfer between $\cdot CO_3^-$ and NO_2^- to form CO_3^{2-} and $\cdot NO_2$ ($k = 4 \times 10^6$ M^{-1} s^{-1}) (Schopfer et al. 2003). Given that CO_2 levels are present in high concentrations both intracellularly and extracellularly (>1 mM) and that the reaction of CO_2 with $ONOO^-$ is one of the fastest known reactions of $ONOO^-$ (Lymar and Hurst 1995), $ONOOCO_2^-$ may be a more physiologically relevant nitrating agent (Dalle-Donne et al. 2005).

From a clinical perspective, the treatment of acute respiratory distress syndrome involves ventilation of inflamed lungs in a manner that increases CO_2 tensions to create hypercapnic conditions (15% CO_2). This reduced ventilation rate and tidal volume is done in the hopes of reducing mechanical stress to the alveolar air–blood

barrier; however, epithelial barrier function is actually impaired in model systems due to the increase in inflammatory responses secondary to the increase in CO_2 levels. Alveolar epithelial cells under hypercapnic conditions also express elevated levels of ·NO and iNOS that is concomitant with an increase in cell NO_2Tyr content formed from presumed reactions of $ONOOCO_2^-$-derived species (Lang et al. 2000). Along the same lines, hypercapnic conditions caused an increase in lung inflammation and oxidative injury in an anesthetized, ventilated rabbit model of acute respiratory distress syndrome, which correlated with an increase in lung iNOS expression and NO_2Tyr formation (Lang et al. 2005). The effects of hypercapnia appear in these cases to be injurious in a setting of inflammation rather than protective. These studies demonstrate that cell structure and function can be negatively impacted under conditions of elevated CO_2 due to apparently more potent actions of $ONOOCO_2^-$, compared with $ONOO^-$, in oxidative and nitrosative inflammatory responses.

Formation of nitrogen dioxide by heme peroxidases

Heme peroxidases, such as MPO and eosinophil peroxidase (EPO), contribute to Tyr nitration in the presence of H_2O_2 by converting NO_2^-, the primary metabolic end product of ·NO, to $\cdot NO_2$ (van der Vliet et al. 1997; Brennan et al. 2002; Baldus et al. 2002). Like $ONOO^-$, the heme peroxidase-mediated generation of $\cdot NO_2$ leading to protein nitration relies on inflammatory and infectious conditions that promote greater ·NO production. MPO-dependent NO_2Tyr formation relies on the bioavailability of NO_2^-, which is created endogenously in the vasculature by (1) the direct reaction of ·NO with molecular oxygen, (2) the reaction of methemoglobin (Hb^{3+}) with ·NO forming a complex (Hb–NO) that releases NO_2^- through hydrolysis, (3) the decomposition of ONOOH and (4) dietary sources. Therefore, increased NO_2^- levels frequently reflect an elevation in ·NO production; however, the accrual of NO_2^- in vivo is prevented by its rapid oxidation to NO_3^- by oxymyoglobin, oxyhemoglobin or catalase.

The MPO/H_2O_2 system of oxidation employs a complex mechanism and involves three different enzyme redox states. When fully reduced, the ferric heme (Fe^{3+}) can be converted by H_2O_2 oxidation to the two electron oxidized ferryl heme [Fe(IV)=O] with an associated cation radical, a state termed Compound I, which proceeds by the following reaction:

$$\text{Peroxidase}\,(Fe^{3+}) + H_2O_2 \rightarrow \text{Compound I}\,([\text{Fe(IV)} = \text{O}] + \text{cation radical}) \quad (3)$$

The strongly oxidizing Compound I converts NO_2^- to $\cdot NO_2$ through a one-electron oxidation reaction, giving Compound II and retaining the ferryl heme according to

$$\text{Compound I} + NO_2^- \rightarrow \text{Compound II}\,[\text{Fe(IV)} = \text{O}] + \cdot NO_2 \quad (4)$$

Initial in vitro investigations of NO_2^- oxidation by the MPO/H_2O_2 system indicated that $\cdot NO_2$ was thought to be the likely intermediate product responsible for the formation of NO_2Tyr by a two-step mechanism (van der Loo et al. 2000). In the first step, Tyr is oxidized to Tyr·, and a subsequent $\cdot NO_2$ can then combine with Tyr· to form NO_2Tyr.

Other biologically relevant reactions of nitric oxide leading to nitrotyrosine formation

Aside from the addition of $\cdot NO_2$ to Tyr· to form NO_2Tyr, Tyr· can also undergo direct attack by ·NO, forming a nitroso-Tyr, which upon further oxidation results in the formation of NO_2Tyr. The direct nitration of Tyr· by ·NO in a $ONOO^-$-independent manner has been observed in prostaglandin H synthase-2 (Gunther et al. 1997) and ribonucleotide reductase (Lepoivre et al. 1994), which rely on a catalytic metal center to direct ·NO reactivity toward the Tyr·. Another NO_2Tyr pathway that is independent of $ONOO^-$ depends on the acidification of NO_2^- to nitrous acid (HNO_2) under low pH conditions (pH < 3.0), such as those found in the gastric system and phagosomes and lysosomes. Tyrosine nitration of albumin and β-casein in vitro was achieved at low pH after long incubations in high concentrations of NO_2^- (25 mM) (Natake and Ueda 1986). However, cysteine residues were predominantly converted to nitrosocysteine over other residues in the presence of acidified NO_2^- (Simon et al. 1996), and so this pathway may only result in a minor route for the slow nitration of Tyr in conditions unfavorable to $ONOO^-$ formation. Additionally, this reaction most likely leads to the formation of artifacts during chromatographic analysis of NO_2Tyr from samples extracted under acidic conditions (Yi et al. 2000).

The wide spectrum of reactions that promote NO_2Tyr formation in biological tissues indicates that NO_2Tyr cannot be used simply as a footprint for $ONOO^-$. Instead, NO_2Tyr modifications are a sign that there is an amplification in ·NO production and its reactions with oxygen or ROS with the source of nitration being a specific function of the type of disease, the progression of disease, the physiological process that mediates RNS or ROS upregulation and the types and amounts of antioxidants present (Greenacre and Ischiropoulos 2001). Because of the complexity of cellular systems, the nitration of proteins is likely to be the product of the multiple $\cdot NO_2$-generating pathways that operate simultaneously. However, during conditions of chronic inflammation, such as atherosclerosis, where NO_2Tyr formation is concomitant with heme peroxidase activity and leukocyte infiltration, the MPO/H_2O_2 pathway

is most likely going to predominate (Ischiropoulos 1998). It is also important to note that in inflammation both nitric oxide and superoxide formation rates increase, opening the way to peroxynitrite-dependent nitration mechanisms as well. In summary, the identification of specific nitrating agents in clinical inflammatory processes and models of inflammatory diseases is complex and multifaceted.

Actin: structure, function and compartmentalization

Actin is a globular, roughly 42-kDa protein found in nearly all eukaryotic cells where it may be present at concentrations of over 100 μM. It is also one of the most highly conserved proteins, differing by no more than 20% in species as diverse as algae and humans. Actin participates in many important cellular processes including muscle contraction, cell motility, cell division, cytokinesis, vesicle and organelle movement, cell signaling, the establishment and maintenance of cell junctions and cell shape (Kabsch and Vandekerckhove 1992). Many of these processes are mediated by extensive and intimate interactions of actin with cellular membranes (Doherty and McMahon 2008).

In vertebrates, three main groups of actin isoforms, alpha, beta, and gamma, have been identified. Alpha-actin is found in muscle tissue and is a major constituent of the contractile apparatus. Beta- and gamma-actin co-exist in most cell types as components of the cytoskeleton, and as mediators of internal cell motility (Khaitlina 2001). Individual subunits of actin are known as globular actin (G-actin). G-actin subunits assemble into long filamentous polymers called F-actin (Fig. 1). Two parallel F-actin strands rotate 166° and layer on top of each other. This gives the appearance of a double helix and more importantly, gives rise to microfilaments of the cytoskeleton. Microfilaments measure approximately 7 nm in diameter with a loop of the helix repeating every 37 nm (Kabsch and Vandekerckhove 1992).

In muscle, actin is the major component of thin filaments, which, together with the motor protein myosin, is arranged into actomyosin myofibrils. These fibrils comprise the mechanism of muscle contraction. Using the hydrolysis of ATP for energy, myosin heads undergo a cycle during which they attach to thin filaments, exerting a tension, and then depending on the load, perform a power stroke that causes the thin filaments to slide past, shortening the muscle (Sheterline et al. 1995).

Nuclear actin is essential for transcription from RNA polymerases (Pol) I, II and III. In Pol I transcription, actin and myosin act as a molecular motor. For Pol II transcription, β-actin is needed for the formation of the pre-initiation complex. Pol III contains β-actin as a subunit. Actin can also be a component of chromatin remodeling

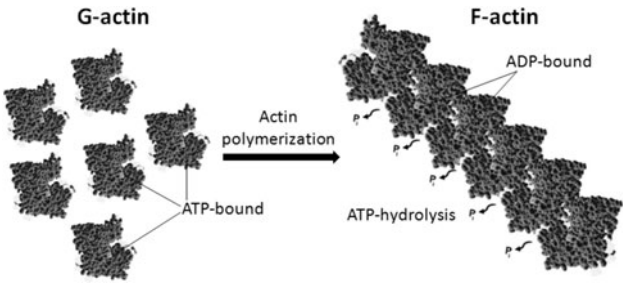

Fig. 1 Actin polymerization. Space-filled representation of actin (ref.: Otterbein et al. 2001; PDB ID: 1J6Z) produced using Rasmol version 2.7. Contact sites on actin monomers are depicted in *light grey*. During polymerization of actin monomers into microfilaments, bound ATP is hydrolyzed to ADP (which remain bound in the microfilament) and inorganic phosphate (P_i)

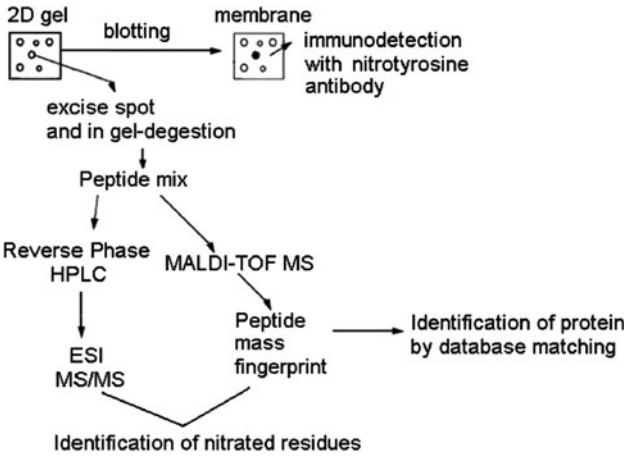

Fig. 2 Identification of nitrated proteins by mass spectrometric proteomics

complexes and is involved in nuclear export of RNAs and proteins (Zheng et al. 2009).

Detecting actin nitration

Mass spectrometric (MS) proteomics-based strategies allow for the identification of all individual proteins that are nitrated by separating a tissue homogenate using 2D gel electrophoresis, detecting the nitrated proteins using an anti-NO_2Tyr antibody, and then identifying the peptides generated during an in-gel proteolytic digest using matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) MS. Masses of peptides detected by MALDI-TOF MS can then be compared to database sequences for identification of the nitrated protein (Fig. 2) (Aslan et al. 2003). Nanoelectrospray ionization-tandem mass spectrometry can also be applied to identify post-translational nitration of proteins (Sharov et al. 2006). Indeed, sequential solution

isoelectric focusing, SDS-PAGE and nanoelectrospray ionization-tandem mass spectrometry have been applied for in vivo proteomic characterization of nitrated gamma-actin sequences from the skeletal muscle of 34-month-old Fisher 344/Brown Norway F1 hybrid rats, a well-accepted animal model for biological aging (Kanski et al. 2005).

In order to fully understand the functional alterations induced by NO_2Tyr, identification of specific sites of NO_2Tyr modifications, rather than overall levels of NO_2Tyr, provides powerful insight into the implications of protein nitration. Affinity purification of the nitrated actin protein and subsequent tandem MS analysis to identify nitration sites gave a clearer picture of the specificity of nitration (Aslan et al. 2003). To determine the location of exclusively nitrated Tyr, modified peptides from tryptic digests were analyzed in the positive ion mode using MALDI-TOF MS where the expected molecular ion for the side chain of NO_2Tyr is observed in addition to decomposition products involving the loss of one and two oxygens (−16 and −32 Da, respectively).

Alternatively, electrospray ionization (ESI)-MS can be used to analyze nitration sites on actin using a similar strategy where NO_2Tyr containing peptides could be identified by the mass difference of NO_2Tyr or its decomposition products. Precursor ion scanning combined with ESI-MS/MS is an established tandem MS method for the detection of post-translational protein nitration. Precursor ion scanning requires a fragment ion highly characteristic for the modified amino acid that is stable enough to survive low energy collision-induced dissociation (CID). Precursor ion scanning has been used for the identification of nitrated peptides utilizing the characteristic nitrotyrosine immonium ion at m/z 181.06. This method has been extended to the application of the selective detection of protein nitration in unseparated peptide mixtures (Petersson et al. 2001).

To detect biological NO_2Tyr levels in nitrated actin, isotope dilution gas chromatography (GC) joined with MS detection methods on chemically hydrolyzed actin samples has been employed (Aslan et al. 2003). Typically, after the introduction of isotope-labeled internal standards to a delipidated sample, tissue purified actin is hydrolyzed via alkaline hydrolysis as previously described (Frost et al. 2000). Measurement of chromatographic peak areas of select product ions taken from the full scan total ion chromatogram routinely allows the detection of NO_2Tyr at levels of 10 pmol/μg actin (Aslan et al. 2003).

Mechanistic consequences of actin nitration

The impact of the nitration of a single Tyr residue often has broad implications on the activity of biologically critical proteins, which has become increasingly related to pathological conditions. NO_2Tyr-induced mechanistic changes alter protein structure and function due to the creation of a bulky, anionic adduct on Tyr, which triggers changes in local protein conformations and electrostatic environments. Depending on the local environment, the addition of $–NO_2$ to Tyr can lower the pK_a of the phenolic –OH by 2–3 units from 10.1 to 7.2, which in return imparts a net negative charge to half of the nitrated residues at physiological pH (Schopfer et al. 2003). What is found most commonly, however, is that Tyr nitration is associated with the reduction or loss of essential enzyme activity, leading to cellular dysfunction and disease progression.

A dynamic network of cytoskeletal actin is required for cell function by compartmentalizing metabolic pathways (Hennessey et al. 1993), promoting intracellular motility (Weeds et al. 1991) and maintaining a dynamic cytoskeleton (Way and Weeds 1990). Organization of actin filaments is also necessary for a direct physical link between the extracellular matrix and the cytoskeleton (Maniotis et al. 1997). Importantly, multiple stimuli for actin filament depolymerization will induce apoptosis (Martin and Leder 2001; Re et al. 1994; Suarez-Huerta et al. 2000).

The analysis of the structure of actin indicates that 15 tyrosine residues are present in mouse actin and that some tyrosines are located in key functional regions. For example, Tyr53 plays a role in stabilizing the DNase I-binding loop (His40–His50) within actin subdomain 2, and fluorescein labeling of Tyr53 has been shown to block actin polymerization. Tyr69 is also located in actin subdomain 2. This subdomain is involved in intermonomer interactions within the actin filament (Holmes et al. 1990) and is the region that undergoes a conformational change during ATP hydrolysis (Otterbein et al. 2001). Tyr143 is located in the hydrophobic pocket of actin subdomain 1, a region involved in profilin binding. The importance of Tyr143 in actin polymerization is illustrated by studies carried out with tetramethylrhodamine-5-maleimide, which covalently binds Cys374, resulting in displacement of Tyr143 within the structure, which in turn disrupts actin polymerization (Otterbein et al. 2001). Tyr306 is located in actin subdomain 3, forms part of the nucleotide-binding pocket, and has been shown to be close to the adenine base (Kabsch et al. 1990). Thus, it is possible that nitration of only one or two key tyrosine residues in actin might significantly alter actin function to a significant degree.

In vitro studies of actin nitration

Effect of actin nitration on cardiac contractile dysfunction

The effects of peroxynitrite ($ONOO^-$) were determined on cardiac actin filaments in order to more clearly understand

the impact of this reactive compound in ischemia/reperfusion injury and heart failure. Actin filaments were exposed to $ONOO^-$ in the presence of 2 mM bicarbonate. $ONOO^-$ concentrations $\geq$10 μM significantly reduced the velocities of actin filaments over cardiac myosin. These functional deficits were linearly related to the degree of tyrosine nitration. The obtained data suggested that nitration of myofibrillar actin could contribute to cardiac contractile dysfunction in pathologic states in which $ONOO^-$ is liberated (Snook et al. 2008).

Effect of actin nitration on neutrophil functional responses

Peroxynitrite and the peroxynitrite generator (SIN-1) modify actin in a concentration-dependent manner, resulting in an inhibition of globular actin polymerization and filamentous-actin depolymerization in vitro (Clements et al. 2003). The effect of peroxynitrite treatment on a number of actin-dependent neutrophil processes was analyzed. Neutrophil actin polymerization, migration, phagocytosis, and respiratory burst activity were all inhibited by SIN-1 treatment in a concentration-dependent manner. These results suggest that the ability of peroxynitrite to inhibit actin dynamics has a significant effect on actin-dependent, cellular processes in phagocytic cells and may modulate their host defense function (Clements et al. 2003).

Actin nitration in vivo and in disease states

Actin nitration in sickle cell disease (SCD)

Increased plasma and tissue protein NO_2Tyr derivatives in an animal model of SCD and clinical samples obtained from SCD patients (Aslan et al. 2003) reinforce that oxidative inflammatory pathways are operative and are mediating pathogenic tissue responses that lead to the post-translational nitration of structurally and functionally important target molecules. The occurrence of xanthine oxidase-derived $O_2^{\cdot-}$ production in SCD (Aslan et al. 2000, 2001) and elevated expression of iNOS in kidney and liver of SCD mice and humans (Aslan and Freeman 2004, 2007) leads to enhanced production of $ONOO^-$. Similarly, increased tissue levels of CO_2 that arises as a consequence of impaired vascular function occur in SCD (Maitre et al. 2000) and create a setting for increased formation of the secondary nitrating species, $ONOOCO_2^-$ (Radi et al. 1999). Additionally, MPO and other heme proteins abundantly present in SCD can oxidize NO_2^- (Eiserich et al. 1998, 2002), a ·NO metabolite shown to be elevated in SCD (Rees et al. 1995; Aslan and Canatan 2008) that serves as a precursor for the nitrating species $\cdot NO_2$. Finally, the acidotic conditions present in poorly perfused tissue compartments may promote protonation of NO_2^-, conferring a chemistry that can also result in HNO_2-mediated Tyr nitration (Knowles et al. 1974).

Immunoprecipitation and MALDI-TOF MS-assisted identification of actin as the predominant nitrated protein in the liver and kidney of SCD mouse provided critical insight into pathogenic events to be expected from this inflammatory milieu. Actin, one of the most abundant proteins in eukaryotic cells, constitutes 5% or more of cell protein (Sheterline and Sparrow 1994) and serves with other cytoskeletal proteins such as tubulin (Eiserich et al. 1999) as a critical target for nitration-induced functional impairment. MALDI-TOF MS and MS/MS analysis of NO_2Tyr-enriched actin fractions from SCD mouse liver and kidney homogenates revealed nitration of Tyr91, Tyr198 and Tyr240 (Aslan et al. 2003). As shown in Fig. 3, the MS/MS spectrum of the peptide corresponding to actin residues 85–95 (^{85}IWHHTFYNELR^{95}) reflected a mass increase of +45 in y10, y9, y8, y7 and y6 daughter ions indicative of Tyr91 nitration. Likewise, the MS/MS spectrum of the peptide corresponding to residues 197–206 (^{197}GYSFTTTAER^{206}) showed a b2 ion with an increase of +45 mass units, identifying Tyr198 as the nitrated residue. The MS/MS spectrum of the tryptic fragment ^{239}SYELPDGQVITIGNER^{254} also revealed a b2 ion that shifted +45 mass units, indicative of Tyr240 nitration.

Confocal microscopy images of tissue actin distribution and morphology strongly affirm the influence of Tyr nitration on actin polymerization properties by reflecting a disorganized actin assembly in regions of both mouse and human SCD kidney where NO_2Tyr-containing actin is localized (Aslan et al. 2003). Indeed, owing to the cooperative nature of actin subunit assembly (Erickson 1989), the functional consequences of Tyr nitration on actin dynamics are profound. The introduction of a net negative charge to nitrated residues Tyr198 and Tyr240, located at the "pointed" end of actin, leads to the formation of ionic bonds with cationic residues located at the barbed end of a growing filament. This interaction stabilizes both actin nucleus and filament formation, as evidenced by the shortened lag phase and accelerated filament elongation. Depolymerization of the actin filament is twofold slower for nitrated actin as compared to native actin which is consistent with the higher affinity of nitrated monomeric-actin for the actin filament (Aslan et al. 2003). The ability of actin Tyr nitration to alter actin polymerization thus also links actin nitration with enhanced apoptosis observed in regions of NO_2Tyr immunoreactivity in the liver and kidney of SCD mouse and human (Aslan et al. 2003).

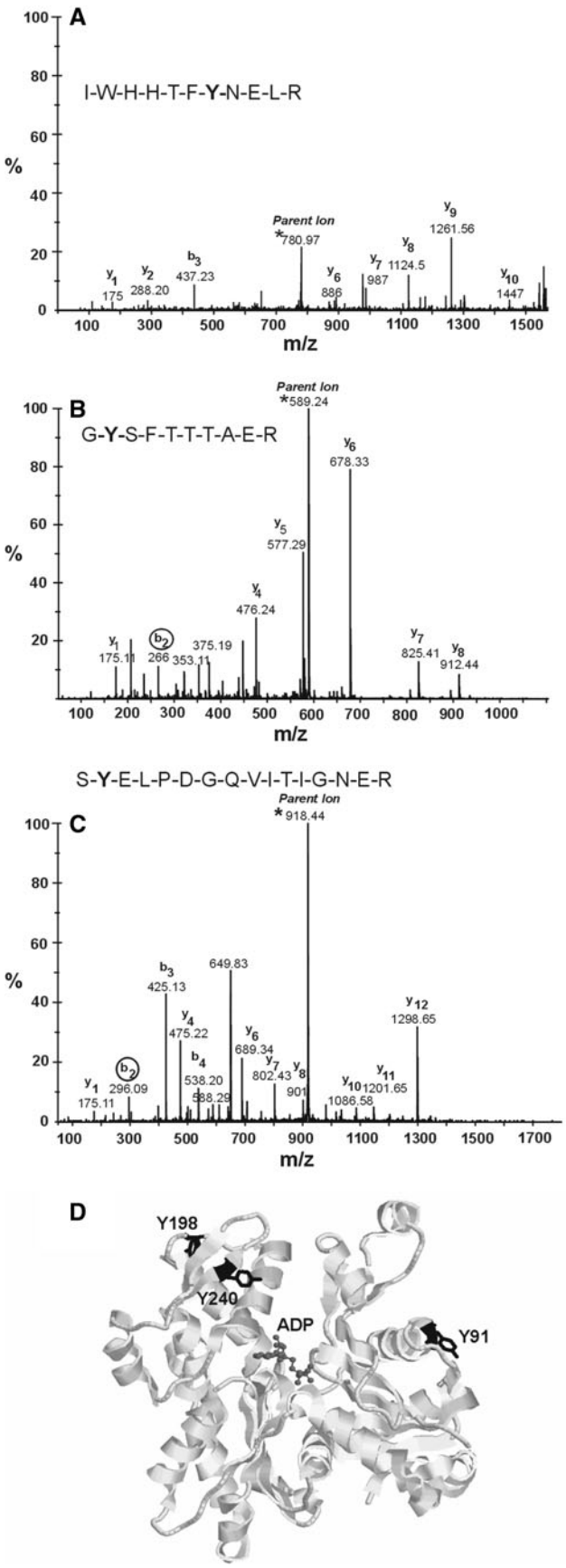

Fig. 3 MS/MS identification and representation of in vivo nitrated actin residues. **a** MS/MS spectrum of the tryptic fragment ^{85}IWHHTFYNELR^{95} $[M+2H]^{2+}$ (*m/z* 781). **b** MS/MS spectrum of the tryptic fragment ^{197}GYSFTTTAER^{206} $[M+2H]^{2+}$ (*m/z* 589). **c** MS/MS spectrum of the tryptic fragment ^{239}SYELPDGQVITIG-NER^{254} $[M+2H]^{2+}$ (*m/z* 918). **d** Ribbon representation of actin (ref.: Otterbein et al. 2001; PDB ID: 1J6Z) produced using Rasmol version 2.7. Nitrated tyrosine residues are illustrated as *black sticks* and are labeled with the *three-letter amino acid code* (modified from Aslan et al. 2003 by permission of the American Society for Biochemistry and Molecular Biology)

Actin nitration in chronic obstructive pulmonary disease

Exercise performance in patients with severe chronic obstructive pulmonary disease (COPD) is associated with increased quadriceps oxidative and nitrosative stress. 3-Nitrotyrosine levels were determined in the quadriceps (pre- and post-exercise) of 15 patients with severe COPD and seven healthy controls using immunoblotting (one- and two-dimensional electrophoresis) and mass spectrometry. Chronic endurance exercise induced tyrosine nitration of muscle alpha-1 actin in patients exhibiting muscle loss which may lead to altered contractile function in patients exhibiting systemic effects of the disease (Barreiro et al. 2009).

Actin nitration in Chagas' disease

Experimental animals infected by *Trypanosoma cruzi* showed an early rise in myocardial and peripheral protein NO_2Tyr that persisted during the chronic stage of disease. Protein NO_2Tyr formation in *T. cruzi*-infected animals was associated with enhanced nitric oxide expression (inferred by nitrite/nitrate levels) and myeloperoxidase activity, suggesting that both peroxynitrite- and myeloperoxidase-mediated pathways contribute to increased protein nitration in Chagas' disease. One- and two-dimensional gel electrophoresis and Western blot analysis were used to identify disease-specific plasma proteins that were NO_2Tyr-modified in *T. cruzi*-infected animals. Nitrated protein spots (56 in total) were sequenced by MALDI-TOF MS and liquid chromatography–tandem mass spectrometry and identified by a homology search of public databases. Clustering of 3NT-modified proteins according to their functional characteristics revealed that nitrated peptides derived from alpha-actin were released into the plasma of patients with Chagas' disease. Such modified proteins may thus be useful biomarkers of Chagas' disease (Dhiman et al. 2008).

Actin nitration in diabetic cardiomyopathy

Cardiac protein nitration was found to be significantly increased in a mouse model of streptozotocin-induced

diabetes. Double staining for cardiomyocytes with alpha sarcomeric actin and NO_2Tyr confirmed the cardiomyocyte-specific effects. These obtained results suggest that cardiac actin nitration may be involved in the development of diabetic cardiomyopathy (Cai et al. 2006).

Actin nitration in familial amyotrophic lateral sclerosis

An increased level of NO_2Tyr immunoreactivity was observed in spinal cord protein extracts of a transgenic mouse model of familial ALS (FALS) at a presymptomatic stage of the disease compared with age-matched controls. NO_2Tyr immunoreactivity was increased in the soluble fraction of spinal cord homogenates and was found as a punctate staining in motor neuron perikarya of presymptomatic FALS mice. Using a proteome-based strategy, nitrated proteins in vivo were identified. Actin was found to be overnitrated in presymptomatic FALS mice. In conclusion, it was proposed that protein nitration may have a role in ALS pathogenesis, acting directly by inhibiting the function of specific proteins and indirectly interfering with protein degradation pathways and phosphorylation cascades (Casoni et al. 2005).

Actin nitration in the human pituitary

Two-dimensional gel electrophoresis-based Western blotting was used to detect, and liquid chromatography (LC)–tandem mass spectrometry (MS/MS) to determine the amino acid sequence of, several different nitrated proteins in the human pituitary. Proteins from several 2D gel spots, which corresponded to the strongly positive anti-nitrotyrosine Western blot spots, were subjected to in-gel trypsin-digestion and LC–MS/MS analysis. MS/MS, SEQUEST analysis, and de novo sequencing were used to determine the nitration site of each nitrated peptide. A total of four different nitrated peptides were characterized, one of which was actin (Zhan and Desiderio 2004).

Actin nitration in patients with inflammatory bowel disease (IBD)

Nitrotyrosine formation was measured in cytoskeletal proteins from colonic mucosa of IBD patients (ulcerative colitis, Crohn's disease, specific colitis) and controls. Outcomes were correlated with IBD severity score. Actin was markedly (>50%) nitrated in inflamed tissues of active IBD, as assessed by immunoblotting for both actin and nitrotyrosine. Disruption of the actin cytoarchitecture was primarily within the epithelial cells and paracellular area. Marked actin nitration (>50%) was only seen in inflamed mucosa, suggesting that oxidant-induced cytoskeletal disruption is required for tissue injury, mucosal disruption, and IBD flare up (Keshavarzian et al. 2003).

Summary

The selectivity of actin Tyr nitration and the fact that endogenous levels of Tyr nitration are sufficient to frequently alter actin polymerization and structural protein function indicate that this process is biologically significant at least during inflammatory events. Given these considerations, however, the relevance of NO_2Tyr modifications still needs to be better defined in the context of other biomolecular alterations caused by RNS, such as oxidation of protein thiol and methionine residues, damage to iron–sulfur clusters and oxidation of transition metal centers, processes which may also disturb normal physiological processes dependent on actin.

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